

CELLS EXPRESSING ANTI-CD19 CHIMERIC ANTIGEN RECEPTORS AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of priority to U.S. Provisional
5 Application No. 63/659,501, filed on June 13, 2024; and U.S. Provisional Application No.
63/594,261, filed on October 30, 2023, the contents of both of which are hereby
incorporated by reference in their entireties.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted
10 electronically in XML file format and is hereby incorporated by reference in its
entirety. Said XML copy, created on October 18, 2024, is named AT-058_03WO_SL.xml
and is 112,901 bytes in size.

FIELD

[0003] The present disclosure relates generally to engineered immune cells (e.g., CAR T
15 cells) and uses thereof in therapeutic applications.

BACKGROUND

[0004] Adoptive transfer of engineered immune cells, such as chimeric antigen receptor
(CAR) T cells, has shown great promises in treating cancers, especially in treating
20 hematological malignancies, as well as other autoimmune disorders. CD19 is commonly
expressed on hematological cancer cells, such as non-Hodgkin's lymphoma. Today, several
autologous CAR T cell therapies targeting CD19 have been approved for treating
hematological cancers. Autologous CD19 CAR T cell therapies have also shown promises
for treating CD19-associated autoimmune diseases. Certain pathogenic B cells have been
25 shown to also express CD70, and both B cells and T cells have been implicated in
autoimmune diseases (see e.g., Ulutekin et al., Cell Reports Medicine, 2024 Jan
16;5(1):101351. doi: 10.1016/j.xcrm.2023.101351. Epub 2023 Dec 21; Han et al., Seminars
in Arthritis and Rheumatism, 2016, 45:496-501).

[0005] Allogeneic CAR T cells in which CAR T cells are produced from healthy donor
30 cells, instead of patients' own cells, can be prepared and used as off-the-shelf products. It
holds the promise of reducing manufacturing costs, improving drug product consistency,

decreasing patient wait time, and increasing accessibility of CAR T therapies to more patients. But allogeneic CAR T cells may be recognized by the host as foreign and rejected by the patient immune system, thereby limiting expansion and persistence of the infused allogeneic CAR T cells. In addition, although CD19 is an effective target for, e.g., non-
5 Hodgkin's lymphoma, a propensity of pathogenic target cells such as tumor cells to down-modulate CD19 post CAR T cell treatment has been observed in preclinical and clinical studies (see Spiegel *et al.*, 2021, Nature Medicine 27:1419-1431).

[0006] Thus, there is a need for improved anti-CD19 CAR T cells with greater potency, increased persistence, and/or enhanced functionality.

10 SUMMARY

[0007] Provided herein are engineered immune cells, e.g., engineered T cells, comprising and/or expressing an anti-CD19, or CD19-specific, chimeric antigen receptor (CAR) and a CD70-binding protein and/or a chimeric cytokine receptor (CCR). Further provided herein are engineered immune cells comprising one or more polynucleotides that encode one or
15 more recombinant proteins. In some embodiments, the one or more polynucleotides comprise one or more, at least two, or two or more coding sequences encoding the CD19-specific CAR, the CD70-binding protein, the CCR, and/or other recombinant proteins.

[0008] In some embodiments, the engineered immune cells comprise or express a CD19-specific CAR and a CD70-binding protein, or a CD70-specific CAR. In some embodiments,
20 the CD19-specific CAR comprises the amino acid sequence of SEQ ID NO: 2, 3, 4 or 5, with or without the signal peptide, and the CD70-specific CAR comprises the amino acid sequence of SEQ ID NO: 16, 17, 18, or 19, with or without the signal peptide.

[0009] In some embodiments, the engineered immune cells comprise a polynucleotide that comprises a coding sequence encoding a CD19-specific CAR and a coding sequence
25 encoding a CD70-specific CAR. In some embodiments the coding sequence encoding the CD19-specific CAR is linked to the coding sequence encoding the CD70-specific CAR by a nucleotide sequence encoding a P2A peptide.

[0010] Also provided herein are polynucleotides comprising a first coding sequence that encodes a CD19-specific CAR and a second coding sequence that encodes a CD170-
30 specific CAR. In some embodiments the coding sequence encoding the CD19-specific CAR is linked to the coding sequence encoding the CD70-specific CAR by a nucleotide sequence

encoding a P2A peptide. In some embodiments, the CD19-specific CAR comprises the amino acid sequence of SEQ ID NO: 2, 3, 4 or 5, with or without the signal peptide, and the CD70-specific CAR comprises the amino acid sequence of SEQ ID NO: 16, 17, 18, or 19, with or without the signal peptide.

5 [0011] In some embodiments, the polynucleotide comprises a truncated PGK promoter, a first coding sequence and a second coding sequence, wherein the truncated PGK promoter comprises at least 50 nucleotides deletion from the 5' end of a PGK promoter, or a wildtype or long PGK promoter having the nucleotide sequence of SEQ ID NO: 34, wherein the truncated PGK promoter is operably linked to the first coding sequence and the second
10 coding sequence, and wherein either the first or the second coding sequence encodes an anti-CD19 chimeric antigen receptor (CAR). In some embodiments, the polynucleotide comprises, from 5' to 3', a truncated PGK promoter, a first coding sequence and a second coding sequence, wherein the truncated PGK promoter comprises at least 50 nucleotides deletion from the 5' end of the long PGK promoter having the nucleotide sequence of SEQ
15 ID NO: 34, wherein the truncated PGK promoter is operably linked to the first coding sequence and the second coding sequence, and wherein either the first or the second coding sequence encodes an anti-CD19 chimeric antigen receptor (CAR). In some embodiments, the first and second coding sequences are linked by a P2A peptide.

[0012] In some embodiments, the anti-CD19 CAR comprises the amino acid sequence of
20 SEQ ID NO: 4. In some embodiments, the anti-CD19 CAR further comprises a CD8 hinge and transmembrane domains. In some embodiments, the CD8 hinge domain comprises the amino acid sequence of SEQ ID NO: 9. In some embodiments, the CD8 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 10 or 99. In some embodiments, the anti-CD19 CAR further comprises a CD3z signaling domain and/or a 4-1BB
25 costimulatory domain. In some embodiments, the CD3z signaling domain comprises the amino acid sequence of SEQ ID NO: 11. In some embodiments, the 4-1BB costimulatory domain comprises the amino acid sequence of SEQ ID NO: 12. In some embodiments, the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 5 or 7, with or without a signal peptide. In some embodiments, the anti-CD19 CAR comprises the amino acid
30 sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 5 or 7, with or without a signal peptide. In some embodiments, the coding sequence that encodes the anti-CD19 CAR comprises a nucleotide

sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 6 or 8.

[0013] In some embodiments, the truncated PGK promoter comprises at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides, or at least 350 nucleotides deletion from the 5' end of the long PGK promoter having the nucleotide sequence of SEQ ID NO: 34. In some embodiments, the truncated PGK promoter comprises a nucleotide sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, or SEQ ID NO: 38. In some embodiments, the truncated PGK promoter comprises a nucleotide sequence that comprises (a) about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, or about 390 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35; (b) about 210, about 220, about 230 about 240, about 250, about 260, about 270, about 280, or about 290 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 36; (c) about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, or about 190 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 37; or (d) about 50, about 60, about 70, about 80, about 90 or about 100 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 38. In some embodiments, the truncated PGK promoter consists essentially of or consists of a nucleotide sequence of SEQ ID NO: 35, 36, 37 or 38. In some embodiments, the truncated PGK promoter consists essentially of or consists of a nucleotide sequence having at least 85%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity of SEQ ID NO: 35, 36, 37 or 38.

[0014] In some embodiments, the first coding sequence encodes the anti-CD19 CAR and the second coding sequence encodes a CD70-binding protein. In some embodiments, the first coding sequence encodes the CD70-binding protein and the second coding sequence

encodes the anti-CD19 CAR. In certain embodiments, the CD-70 binding protein is or comprises an anti-CD70 CAR.

[0015] In some embodiments, the first coding sequence encodes the anti-CD19 CAR and the second coding sequence encodes a chimeric cytokine receptor (CCR). In some
5 embodiments, the first coding sequence encodes the CCR and the second coding sequence encodes the anti-CD19 CAR.

[0016] In some embodiments, the CD70-binding protein is a recombinant CD70-binding protein. In some embodiment, the CD70-binding protein comprises an anti-CD70 antibody or an antigen-binding fragment thereof, and a transmembrane domain. In some

10 embodiments, the anti-CD70 antibody comprises the amino acid sequence of SEQ ID NOs: 16 and/or 17. In some embodiments, the anti-CD70 antibody comprises the amino acid sequence of SEQ ID NO: 18. In some embodiments, the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 10. In some embodiments, the CD70-binding protein further comprises a hinge domain. In certain embodiments, the hinge domain and
15 the transmembrane domain comprise a CD8 hinge and a CD8 transmembrane domain. In some embodiments, the CD8 hinge domain comprises the amino acid sequence of SEQ ID NO: 9. In some embodiments, the CD8 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 10 or 99. In other embodiments, the hinge domain and the transmembrane domain comprise a CD28 hinge and a CD28 transmembrane domain. In
20 some embodiments, the CD70-binding protein further comprises a CD3 signaling domain. In some embodiments, the CD70-binding protein does not comprise a costimulatory domain. In some embodiments, the CD70-binding protein does not comprise a 4-1BB costimulatory domain. In some embodiments, the CD70-binding protein comprises a CD3 signaling domain comprising the amino acid sequence of SEQ ID NO: 11. In some
25 embodiments, the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 19 or 21, or comprises an amino acid sequence having at least about 85%, at least about 90%, at least about 95% or at least about 99% sequence identity to SEQ ID NO: 19 or 21, with or without a signal peptide. In some embodiments, the coding sequence that encodes the CD70-binding protein comprises a nucleotide sequence having at least 85%, at
30 least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 20 or 22.

[0017] In some embodiments, the CCR comprises a thrombopoietin receptor/
myeloproliferative leukemia protein receptor (TPOR/MPLR) transmembrane and JAK
binding domain and an intracellular recruiting domain. In some embodiments, the
TPOR/MPLR transmembrane and JAK binding domain comprises the amino acid sequence
5 of SEQ ID NO: 25 or 51. In some embodiments, the intracellular recruiting domain
comprises the amino acid sequence of SEQ ID NO: 26. In some embodiments, the CCR
comprises the amino acid sequence of SEQ ID NO: 27 or 29, or an amino acid sequence
having at least about 85%, at least about 90%, at least about 95%, at least about 99%
sequence identity to SEQ ID NO: 27 or 29, with or without the signal peptide. In some
10 embodiments, the coding sequence that encodes a CCR comprises the nucleotide sequence
having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%
sequence identity to SEQ ID NO: 28 or 30.

[0018] In some embodiments, the polynucleotides further comprise a 5' homology arm
and a 3' homology arm. In some embodiments, the 5' and 3' homology arms are each at
15 least about 100, at least about 150, at least about 200, at least about 250, or at least about
300 nucleotides in length or more. In some embodiments, the 5' homology arm and the 3'
homology arm each comprises a nucleotide sequence homologous to a nucleotide sequence
of a T cell receptor alpha constant region (TRAC) gene.

[0019] In related aspects, provided herein are vectors comprising the polynucleotides, and
20 engineered immune cells (e.g., engineered T cells or CAR T cells) comprising the vectors or
the polynucleotides. In some embodiments, the vector is an adeno-associated virus (AAV)
vector or a lentiviral vector.

[0020] In some embodiments, the immune cells comprise the polynucleotides integrated
into the TRAC gene. In some embodiments, the integration of the polynucleotide into the
25 human TRAC gene is mediated by an endonuclease. In some embodiments, the
endonuclease is a CRISPR endonuclease. In some embodiments, the endonuclease is not
TALEN. In some embodiments, the integration of the polynucleotide into the human TRAC
gene is mediated by a CRISPR endonuclease and the integration prevents or reduces the
expression or activities of the TRAC gene. In some embodiments, the CRISPR
30 endonuclease is Cas 9 or Cas12, e.g., Cas12i. In some embodiments, the CRISPR
endonuclease makes a double strand break to create an integration site in a target sequence
within the TRAC gene and the polynucleotide is integrated into the integration site by

AAV-mediated site-specific integration, optionally wherein the integration site is in the first, second or third exon of the TRAC gene, and further optionally wherein the target sequence comprises the nucleic acid sequence of GACCCTGCC or SEQ ID NO: 92.

[0021] In some embodiments, the immune cells further comprise an additional
5 polynucleotide that comprises a promoter operably linked to one or more coding sequences, wherein the one or more coding sequences expresses either a CCR or a CD70-binding protein, as described herein. In some embodiments, the one or more coding sequences of the additional polynucleotide further express a PD-1 dominant negative receptor. In some
10 embodiments, the additional polynucleotide further comprises a 5' homology arm and a 3' homology arm. In some embodiments, the 5' and 3' homology arms each are at least 100 nucleotides in length. In some embodiments, the 5' and 3' homology arms each comprises a nucleotide sequence homologous to a nucleotide sequence of a CD52 gene. In some
15 embodiments, the promoter comprises an EFS promoter or a truncated PGK promoter described herein. In some embodiments, the additional polynucleotide is integrated into the CD52 gene. In some embodiments, the integration of the additional polynucleotide into the human CD52 gene is mediated by a CRISPR endonuclease. In some embodiments, the
20 CRISPR endonuclease is Cas 9 or Cas12, e.g., Cas12i, and the integration prevents or reduces the expression or activities of the CD52 gene. In some embodiments, the CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the CD52 gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration.

[0022] In some aspect, the disclosure provides an immune cell, or a population of immune cells, comprising a polynucleotide integrated into a human TRAC gene, wherein the polynucleotide comprises a nucleic acid sequence comprising a 5' region of the human
25 TRAC gene, the nucleic acid sequence encoding a CD19-specific CAR and a CD70-binding protein or a CD70-specific CAR, and a 3' region of the human TRAC gene, wherein the integrated polynucleotide prevents or reduces the expression or activities of the human TRAC gene. In some embodiments, the anti-CD19 CAR comprises the amino acid of SEQ ID NO: 5 or 7, and the CD70-binding protein (or the CD70-specific CAR) comprises the
30 amino acid of SEQ ID NO: 19 or 21. In some embodiments, the polynucleotide encodes the CD19-specific CAR and the CD70-specific CAR linked by a P2A sequence. In some embodiments, the integration of the polynucleotide at the human TRAC gene is mediated a CRISPR endonuclease, for example, Cas 9 or Cas12 or Cas12i. In certain embodiments, the

CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the TRAC gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration. In some embodiments, the integration site is in the first, second or third exon of the TRAC gene. In some embodiments, the integration site is in the first exon of the TRAC gene. In some embodiments, the target sequence comprises the nucleic acid sequence of GACCCTGCC or SEQ ID NO: 92.

[0023] In another aspect, the disclosure provides an immune cell comprising a polynucleotide encoding an anti-CD19 CAR and a CD70-binding protein, wherein the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 5 or 7, and the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 19 or 21. In certain embodiments, the polynucleotide encodes a polypeptide of CD19-specific CAR and CD70-specific CAR connected by a P2A peptide that comprises an amino acid sequence that is at least 85%, 88%, 90%, 95%, 98%, or 99% or 100% identical to the polypeptide sequence of SEQ ID NO: 97. In some embodiments, the polynucleotide comprises a nucleic acid sequence that is at least 85%, 88%, 90%, 95%, 98%, or 99% or 100% identical to the polynucleotide sequence of SEQ ID NO: 96. In certain embodiments, the polynucleotide is integrated into a human TRAC gene by an endonuclease. In some embodiments, the endonuclease is a CRISPR endonuclease. In some embodiments, the endonuclease is not a TALEN endonuclease. In certain embodiments, the polynucleotide is integrated into a human TRAC gene by a CRISPR endonuclease, wherein the CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the TRAC gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration. In some embodiments, the integrated polynucleotide prevents or reduces the expression or activities of the human TRAC gene. In some embodiments, the integration site is in the first, second or third exon of the TRAC gene. In some embodiments, the integration site is in the first exon of the TRAC gene. In some embodiments, the target sequence comprises the nucleic acid sequence of GACCCTGCC or SEQ ID NO: 92.

[0024] In some embodiments, the polynucleotide encoding the CD19 CAR and the CD70-binding protein or anti-CD70 CAR is operably linked to a truncated PGK promoter disclosed herein.

[0025] In some embodiments, the immune cell is a T cell, an NK cell, an NK-T cell, a tumor infiltrating lymphocyte (TIL), or a T cell of NK cell derived from an iPSC.

[0026] In related aspects, provided are immune cells comprising a recombinant polynucleotide sequence integrated at a human TRAC gene, wherein the recombinant polynucleotide sequence comprises a 5' region of the human TRAC gene, the polynucleotide as described herein, and a 3' region of the human TRAC gene, wherein the integrated recombinant polynucleotide sequence prevents expression of the human TRAC gene. In some embodiments, the immune cells further comprise an additional recombinant polynucleotide sequence integrated at a human CD52 gene, wherein the additional recombinant polynucleotide sequence comprises a 5' region of the human CD52 gene, one or more coding sequences, and a 3' region of the human CD52 gene.

[0027] In yet related aspects, populations of immune cells and pharmaceutical compositions of immune cells or populations of immune cells are provided. In a further aspect, methods of treating a disease condition in a subject are provided comprising administering to the subject an immune cell, a population of immune cells and a pharmaceutical composition comprising the immune cell, an effective amount of the immune cells, an effective amount of the population of immune cells, or an effective amount of the pharmaceutical compositions, as described throughout. In some embodiments, the subject is a human.

[0028] In some embodiments, the disease condition is cancer, including without limitation, non-Hodgkin's lymphoma, large B cell lymphoma (LBCL), follicular lymphoma (FL), T cell lymphoma (TCL), B cell acute lymphoblastic leukemia (BALL), T cell acute lymphoblastic leukemia (TALL), primary central nervous system lymphoma (PCNSL), Mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), and peripheral T cell lymphoma (PTCL). In some embodiments, the immune cell is autologous with respect to the subject. In some embodiments, the immune cell is allogeneic with respect to the subject.

[0029] In some embodiments, the disease condition is an autoimmune disease or disorder, including without limitation, lupus, systemic lupus erythematosus (SLE), lupus nephritis, rheumatoid arthritis, systemic sclerosis, scleroderma, multiple sclerosis (MS), relapse remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS), neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis, Sjogren's syndrome, stiff person syndrome, graft v. host

disease (GvHD), autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), and myositis. In some embodiments, the immune cells are capable of reducing or depleting B cells, pathogenic B cells, autoreactive B cells, pathogenic T cells, autoreactive T cells, CD70+ T cells, or alloreactive T cells. In some embodiments, the immune cell is autologous with respect to the subject. In other embodiments, the immune cell is allogeneic with respect to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0030] **FIGs. 1A-1C** show results of in vitro long-term killing assays of the anti-CD19 CAR T cells with or without co-expressing a chimeric cytokine receptor (CCR).
- 10 [0031] **FIGs. 2A-2B** show results of in vivo cytotoxicity assay of the anti-CD19 CAR T cells with or without co-expressing the CCR (CD19CAR/CCR).
- [0032] **FIG. 3A** depicts data showing reduced in vivo anti-tumor activity of the anti-CD19 CAR T cells co-expressing a CCR (CD19 CAR/CCR T cells) generated by site-specific integration as compared to CD19 CAR/CCR T cells generated by LVV
- 15 transduction. **FIG. 3B** shows higher in vivo number of, *i.e.*, higher persistence of, CAR+ T cells generated by LVV transduction as compared to CAR T cells generated by site-specific integration.
- [0033] **FIGs. 4A-4D** show results of experiments investigating the effects of different promoters driving the expression of the CCR and the CAR in CAR T cells generated by site-specific integration. **FIG. 4A** shows the median fluorescence intensity (MFI) of the
- 20 CD19 CAR/CCR T cells at the end of CAR T cell production. **FIG. 4B** shows the results of CAR T cell killing of target Raji cells in a flow-based serial restimulation assay. **FIG. 4C** shows the CAR MFI, and **FIG. 4D** shows the CAR+ T cell counts, during the flow-based serial restimulation assay.
- 25 [0034] **FIGs. 5A-5C** compare the efficacy of CAR/CCR T cells generated by site-specific integration driven by either the EFS promoter or the PGK SSI-1 promoter, with the CD19 CAR/CCR T cells generated by LVV transduction, in a long-term killing assay (**FIG. 5A**), or in a single-stimulation flow-based potency assay (**FIGs. 5B-5C**) against the target Raji cells.
- 30 [0035] **FIGs. 6A-6B** compare the in vivo efficacy of CD19 CAR/CCR T cells generated by site-specific integration driven by the EFS promoter, the CypA300 promoter, or the PGK

SSI-1 promoter, with the CD19 CAR/CCR T cells generated by LVV, using two different CAR T cell doses 2×10^6 cells (**FIG. 6A**) or 4×10^6 cells (**FIG. 6B**).

[0036] **FIGs. 7A-7H** illustrate the interaction between allogeneic CAR T cells, alloreactive host T cells and tumor cells in which the allogeneic CAR T cells either express a CD19CAR/CCR and a CD70-binding protein (CD70-BP, or a CD70 CAR comprising CD70 binding domain and intracellularly only a CD3z signaling domain, CD70z) (CD70z.CD19CAR/CCR) (**FIGs. 7E-7H**) or do not express a CD70-binding protein (CD19CAR/CCR) (**FIGs. 7A-7D**). The CD19 CAR/CCR T cells expressing the CD70-binding protein (**FIG. 7E**) effectively reduced host T cells (**FIG. 7F**), killed tumor cells (**FIG. 7G**) and showed greater expansion of CAR T cells (**FIG. 7H**) than the CD19 CAR/CCR T cells without expressing the CD70-binding protein.

[0037] **FIGs. 8A-8D** demonstrate that CD19 CAR/CCR T cells co-expressing a CD70-binding protein (CD70z.CD19CAR/CCR) prevented CD19 antigen escape in Raji cells. **FIG. 8B** shows downregulation of CD19 expression in Raji cells after being cultured with CAR T cells for four days and the elimination of CD19 negative/CD70 wild type Raji cells by CD19 CAR/CCR T cells co-expressing the CD70-binding protein. The absolute numbers of total Raji cells, either CD70 wild type or CD70 knockout (KO) and the absolute numbers of CD19 negative Raji cells, either CD70 wild type or CD70 KO are shown in **FIG. 8C** and **FIG. 8D**, respectively. The *in vivo* data are shown in **FIGs. 8E-8F**.

[0038] **FIG. 9A** illustrates the interaction of CD19 CAR/CCR T cells that co-express the CD70-binding protein with cells that either express the CD19 CAR/CCR alone or the CD70-binding protein alone. **FIG. 9B** presents flow cytometry data showing likely fratricide of CD19 CAR/CCR singly positive T cells by the CD19 CAR/CCR T cells co-expressing the CD70-binding protein, and the protection of CD70-binding protein singly positive T cells from fratricide by the binding of the CD70-binding protein to the surface CD70 protein in cis (cis-masking), from Day 9 to Day 14 of the production process.

[0039] **FIG. 10A** describes the designs of different CD19 CAR constructs. **FIGs. 10B-10I** show the results of production and characterization of the CAR T cells.

[0040] **FIGs. 11A-11B** show the results of CAR T cells graft survival against alloreactive T cells in a mixed lymphocyte reaction (MLR) assay.

[0041] **FIGs. 12A-12D** depict data of cytotoxicity of CAR T cells against CD70 wild type Raji cells (**FIG. 12A**) or CD70 KO Raji cells (**FIG. 12C**), and data of CAR T cells expansion (**FIG. 12B and FIG. 12D**, respectively).

[0042] **FIGs. 13A-13B** show data of long-term killing assay of CAR T cells against CD70 KO Raji cells.

[0043] **FIGs. 14A-14B** show averaged data demonstrating *in vivo* anti-tumor activities of CAR T cells of different designs at two different CAR T cell doses. **FIGs. 14C-14G** show data of individual mice administered with 5×10^6 CAR T cells of different CAR designs.

[0044] **FIGs. 15A-15G** show data of production and characterization of the CD19/CD70 dual CAR T cells or PGK SSI CD19 CAR T cells engineered using the CRISPR gene editing reagents. NTD: non-transduced control T cells. **FIGs. 15H-15K** show data of T cell phenotype in the CAR+ T cell population (PGK SSI CD19/CD70 dual, PGK SSI CD19) or the non-transduced T cell population (NTD), data gated on CAR+ cells.

[0045] **FIGs. 16A-16B** show cytotoxicity data of the CD19/CD70 dual CAR T cells engineered by CRISPR reagents in cells from two different donors against CD19+ Raji cells. The cytotoxic activities of the CAR T cells against each target CD19 and CD70 independently were tested in a short-term killing assay (**FIG. 16C**), demonstrated using either the wild-type Raji RGL cells (RGL) or CD19KO or CD70KO Raji RGL cells (**FIG. 16D**). The cytotoxic activities of the CAR T cells against each target CD19 and CD70 were tested in a mixed tumor model of CD19KO RGL and CD70KO RGL cells (**FIG. 16E**) and the results in **FIG. 16F** show that CD19 negative RGL cells were only able to expand when treated with the control CD19 CAR T cells, but unable to expand when treated with the CD19/CD70 dual CAR T cells. **FIG. 16G and FIG. 16H** show results of *in vivo* killing of CD19+ tumor cells by the CD19/CD70 dual CAR T cells and *in vivo* expansion of the dual CAR T cells in the animal model. **FIG. 16I** depicts that the CD19/CD70 dual CAR T cells did not expand in cell culture in the absence of IL-2.

[0046] The data in **FIGs. 17A-17B** show that the CD19/CD70 dual CAR T cells effectively targeted primary CD19+ B cells from PBMCs from a healthy donor or PBMCs from an SLE patient.

[0047] **FIGs. 18A-18B** show data demonstrating that the CD19/CD70 dual CAR T cells effectively targeted alloreactive CD70+ primary T cells either from healthy or SLE donors in mixed lymphocyte reactions (MLRs).

[0048] **FIG. 19A** depicts the experiment protocol for establishing an *in vivo* PBMC humanized mouse model under different conditions. Evidence of successful engraftment of human B cells and T cells is shown in **FIG. 19B** and CD70 expression levels on engrafted cells are shown in **FIG. 19C**.

[0049] **FIG. 20A** shows experiment protocol for establishing the PBMC humanized mouse model under the irradiation condition and for kinetic analysis of B cell reconstitution and differentiation. **FIGs. 20B-20D** show data demonstrating proliferation and differentiation of engrafted B cells in the mouse model. **FIGs. 20E-20F** show data demonstrating PBMC-dose dependent production of human IgG and IgM.

[0050] **FIG. 21A** shows the experiment protocol for testing *in vivo* efficacy of the CD19/CD70 dual CAR in the PBMC humanized mouse model. **FIGs. 21B-21C** show data of depletion of host CD19+ B cells (**FIG. 21B** and top panel of **FIG. 21C**) and reduction of host antibody production (bottom panel of **FIG. 21C**), and **FIG. 21D** shows reduction of host T cells in the spleen of the mice, all after 7 days of CAR T treatment (i.e., 10 days post transfer of human PBMCs). **FIG. 21E** shows elimination of CD70+ T cells in the spleen of the mice after 7 days of CAR T treatment, and **FIG. 21F** shows reduction of CD70+ T cells in the spleen of mice after 0, 4, and 7 days post CAR T treatment, i.e., at Day 3, Day 7 and Day 10, respectively. **FIGs. 21G-21H** show dose-dependent expansion of the CD19/CD70 dual CAR T cells in the spleen (**FIG. 21G**, and **FIG. 21H**, left panel) or the blood (**FIG. 21H**, right panel) of the mice.

[0051] **FIG. 22A** depicts the experiment protocol of the CD34+ humanized NSG mouse model. **FIG. 22B** and the top panel of **FIG. 22C** show that the CD19/CD70 dual CAR T treatment eliminated human B cells in the CD34+ humanized mice, and **FIG. 22C**, bottom panel shows that the CAR T treatment had minimal effects on human T cells in the mice, on Day 7 post CAR T treatment. **FIG. 22D** shows data of CAR T expansion on Day 7 post treatment, and **FIG. 22E** shows the kinetics of CAR T expansion.

[0052] **FIG. 23A** is a diagram illustrating the virus-specific T cell assay. **FIGs. 23B-23C** show that the CD19/CD70 dual T cells did not affect the elimination of viral peptide

presenting antigen-presenting cells by virus-specific T (VST) cells and did not affect the VST cell counts.

[0053] **FIGs. 24A-B** show that ruxolitinib suppressed the cytotoxic activity (FIG. 24A) and the proliferation (FIG. 24B) of the CD19/CD70 dual CAR T cells against the target
 5 CD19+ CD70 KO Raji cells.

DETAILED DESCRIPTION

[0054] The practice of the instant disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the
 10 art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P.
 15 Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D.M. Weir and C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel
 20 et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J.E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C.A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean,
 25 eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J.D. Capra, eds., Harwood Academic Publishers, 1995). Gene editing techniques using TALENs, CRISPR/Cas9, Cas12, and megaTAL nucleases, for example, are within the skill of the art and explained fully in the literature, such as T. Gaj *et al.*, Genome-Editing
 30 Technologies: Principles and Applications, *Cold Spring Harb Perspect Biol* 2016;8:a023754 and citations therein.

[0055] As used herein, the terms “a” and “an” are used to mean one or more. For example, a reference to “a cell,” “an immune cell” or “an antibody” means “one or more cells,” “one or more immune cells” or “one or more antibodies.”

5 [0056] As used herein “autologous” means that cells, a cell line, or population of cells used for treating a subject that are obtained from said subject.

[0057] As used herein “allogeneic” means that cells or population of cells used for treating a subject that are not obtained from said subject, but instead from a donor.

[0058] As used herein, the term “endogenous” refers to any material from or produced inside an organism, cell, tissue or system.

10 [0059] As used herein, the term “exogenous” refers to any material introduced from or produced outside an organism, cell, tissue or system. In some embodiments, an exogenous or recombinant sequence or exogenous or recombinant protein is not a naturally occurring sequence or protein and not endogenous or natural to the cell, tissue or organism.

15 [0060] As used herein, “immune cell” refers to a cell of hematopoietic origin functionally involved in the initiation and/or execution of innate and/or adaptive immune response. Examples of immune cells include T cells, e.g., alpha/beta T cells and gamma/delta T cells, regulatory T (Treg) cells, B cells, natural killer (NK) cells, natural killer T (NKT) cells, mast cells, and myeloid-derived phagocytes.

20 [0061] As used herein, the term “expression” refers to the transcription and/or translation of a particular nucleotide sequence driven by a promoter.

[0062] As used herein, “expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operably linked to a nucleotide sequence to be expressed. Expression vectors include all those known in the art, including, without limitation, cosmids, plasmids (e.g., naked or contained in liposomes) and viruses
25 (e.g., lentiviruses, retroviruses, adenoviruses, and adeno- associated viruses) that incorporate the recombinant polynucleotide.

[0063] As used herein, “expression cassette” refers to an expression unit(s) of one or more coding sequences operably linked to a promoter. In some embodiments, the expression of multiple coding sequences can be driven by one promoter and the more than
30 one coding sequences are linked by the sequence encoding a 2A peptide, e.g., P2A or T2A. In some embodiments, the expression of multiple coding sequences occurs by ribosome

skipping. As an example, a bicistronic expression cassette allows expression of two proteins from the same RNA transcript driven by one promoter. In some embodiments, the expression of the multiple coding sequences can be driven by multiple promoters, each promoter operably linked to a coding sequence.

5 [0064] As used herein, to “functionally express” a gene means that a gene is expressed and that expression yields a functioning gene end product. For example, if a gene encodes a protein, then a cell functionally expresses the gene if expression of the gene ultimately produces a properly functioning protein. Thus, if a gene is not transcribed, or expression of the gene ultimately produces an RNA that is not translated or translation yields only a non-
10 functioning protein, e.g., the protein does not fold correctly or is not transported to its site of action (e.g. membrane, for membrane-bound proteins), for example, then the gene is not functionally expressed. Functional expression can be measured directly (e.g. by assaying for the gene product itself) or indirectly (e.g. by assaying for the effects of the gene product).

15 [0065] As used herein, “operably linked” refers to the association of nucleic acid sequences on a single nucleic acid fragment or molecule so that the function of one is affected by the other. For example, a promoter is operably linked to a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). A first sequence element may
20 be operably linked to a second sequence element in a contiguous or non-contiguous manner. Where two nucleic acid elements are operably linked, the coding region thereof remain in the same, correct reading frame.

[0066] As used herein, “expression control sequence” means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter,
25 such as a constitutive or an inducible promoter, or an enhancer or any other transcriptional regulatory elements. The expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

[0067] “Promoter” and “promoter sequence” are used interchangeably and refer to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. A
30 functional RNA can be an mRNA transcript that encodes a protein or a non-mRNA, e.g., miRNA, shRNA, or other types of interfering RNAs. In general, a coding sequence is located 3' to a promoter sequence. It is understood by those skilled in the art that different

promoters can direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions.

[0068] As used herein, the terms “DNA sequences,” “nucleic acid sequences,”

5 “nucleotide sequences,” and “polynucleotide sequences” are used interchangeably. As used herein, the terms “polynucleotides,” “DNA molecules,” and “nucleic acid molecules” are used interchangeably.

[0069] The term “extracellular ligand-binding domain” as used herein refers to an oligo- or polypeptide that is capable of binding to a ligand. Preferably, the domain will be capable

10 of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain can be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. The term “stalk domain” or “hinge domain” is used herein to refer to any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, stalk domains or hinge
15 domains are used to provide more flexibility and accessibility for the extracellular ligand-binding domain.

[0070] The term “intracellular signaling domain” refers to the portion of a protein which transduces the effector signal function signal and directs the cell to perform a specialized function.

20 [0071] Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to plus or minus 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10% that value or parameter per se. For example, description referring to “about X” includes description of “X.” Numeric ranges are inclusive of the numbers defining the range.

[0072] It is understood that wherever embodiments are described herein with the language
25 “comprising,” otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided.

[0073] Provided herein are engineered immune cells, e.g., engineered T cells, comprising and/or expressing an anti-CD19, or CD19-specific, chimeric antigen receptor (CAR) and a CD70-binding protein, and optionally comprising and/or expressing a chimeric cytokine
30 receptor (CCR). Also provided herein are engineered immune cells, e.g., engineered T cells, comprising and/or expressing an anti-CD19, or CD19-specific, chimeric antigen receptor

(CAR) and a chimeric cytokine receptor (CCR), and optionally comprising and/or expressing a CD70-binding protein.

[0074] Further provided herein are polynucleotides comprising one or more coding sequences encoding the CD19-specific CAR, and the CD70-binding protein and/or the CCR. In certain embodiments, the CD19-specific CAR and the CD70-binding protein or CCR are expressed from a bicistronic expression cassette and are linked by a P2A or T2A self-cleaving peptide. In certain aspects, the CD19-specific immune cells, e.g., CD19-specific CAR T cells, exhibit enhanced cytotoxicity and potency against CD19 positive and/or CD19 negative/CD70 positive hematologic tumor cells or other pathogenic cells, reduced rejection by the host or patient alloreactive immune cells, and/or increased persistence and cell expansion as compared to the same CD19-specific immune cells, e.g., CD19-specific CAR T cells, without expressing the CD70-binding protein and/or the CCR. In certain embodiments, the expression of the one or more coding sequences or the bicistronic expression cassette is driven by a PGK promoter, especially a truncated PGK promoter. In certain embodiments, the CD19-specific immune cells, e.g., CD19-specific CAR T cells, exhibit enhanced cytotoxicity and potency against CD19 positive pathological or pathogenic cells associated with an autoimmune indication.

[0075] In certain aspects, the polynucleotides provided herein are cloned into a lentiviral vector (LVV) and introduced into the engineered immune cells, e.g., peripheral blood mononuclear cells (PBMCs) or T cells, by lentiviral transduction. Transduction of LVV constructs into, e.g., PBMCs or T cells results in engineered cells with randomly integrated transgene(s) in the host cell genome. Alternatively, the transgene(s) can also be introduced into cells by site-specific integration (SSI) into one or more predetermined genetic loci. Site-specific integration has the added benefit of ensuring the uniformity of the insertion site of the transgene(s) in the genome and reduced number of integration events as compared to LVV-directed random integration. Thus, in certain aspects, the polynucleotides provided herein are integrated into a predetermined locus in the genome of the engineered immune cells, e.g., CD19-specific CAR T cells, CD19-specific CAR T cells expressing a CD70-binding protein or a CD70 CAR (or CD19/CD70 dual CAR T cells). In certain embodiments, the predetermined genetic locus is the T cell receptor alpha chain constant region (TRAC) locus. In certain embodiments, the predetermined genetic locus is the CD52 gene locus.

[0076] It was surprisingly demonstrated that a PGK promoter, or a truncated PGK promoter described herein (e.g., the PGK SSI promoters) can convey benefits to engineered immune cells, for example, CAR T cells, e.g., providing enhanced CAR T cell expansion and/or enhanced *in vitro* or *in vivo* cytotoxicity against target cells, including target tumor cells, when the transgene(s) is introduced by site-specific integration. Thus, in certain embodiments, the expression of the transgene(s), e.g., the one or more coding sequences, is driven by a PGK promoter, especially a truncated PGK promoter described herein. In some embodiments, the one or more coding sequences are present in a bi-cistronic or multi-cistronic expression cassette. In some embodiments, the transgene comprises, e.g., a CAR, a CCR, a CD70-binding protein, a dominant negative receptor or other sequences or sequences encoding other proteins or recombinant proteins.

[0077] In some embodiments, engineered immune cells generated by site-specific integration demonstrated surprisingly improved *in vivo* cytotoxic activity against target pathogenic cells, including tumor cells, when the expression of the transgene(s), e.g., CD19-specific CAR, is driven by the PGK promoters, especially truncated PGK promoters, e.g., the PGK SSI promoters described herein, as compared to, e.g., the EF1 α short (EFS) promoter. In some embodiments, engineered immune cells generated by site-specific integration demonstrated surprisingly improved *in vivo* cytotoxic activity against target pathogenic cells, including tumor cells, when the expression of the transgene(s), e.g., CD19-specific CAR and the CCR, is driven by the PGK promoters, e.g., the PGK SSI promoters described herein, as compared to the EFS promoter. In some embodiments, engineered immune cells generated by site-specific integration demonstrated surprisingly improved *in vivo* anti-tumor activity when the expression of the transgene(s), e.g., CD19-specific CAR and the CD70-binding protein or CD70-specific CAR, is driven by the PGK promoters, e.g., the PGK SSI promoters described herein, as compared to the EFS promoter. In some embodiments, engineered immune cells generated by site-specific integration demonstrated surprisingly improved *in vivo* cytotoxic activity when the expression of the transgene(s), e.g., CD19-specific CAR and the CCR and/or the CD70-binding protein or CD70-specific CAR, is driven by the PGK promoters, e.g., the PGK SSI promoters described herein, as compared to the EFS promoter. In certain embodiments, the polynucleotides were integrated into the TRAC locus. In some embodiments, the polynucleotides were integrated into the CD52 locus.

[0078] PGK Promoters

- [0079] In one aspect, the present invention provides polynucleotides or nucleic acid constructs or molecules that comprise one or more coding sequences or transgenes. In some embodiments, the one or more coding sequences or transgenes encode polypeptides, e.g., CAR or other recombinant proteins, and/or nucleic acid inhibitory agents, e.g., RNA interference agents. In some embodiments, the one or more coding sequences or transgenes are driven by a truncated PGK promoter to control expression of the one or more coding sequences or transgenes. In other embodiments, the truncated PGK promoter comprises one or more deletions in a promoter sequence from a corresponding promoter sequence of a genomic region that comprises a PGK gene, e.g., a human PGK gene. Table 1 provides exemplary PGK promoter sequences.

Table 1 – Promoters (**Bolded, underlined text** indicates transcription start site)

		SEQ ID NO:
Long PGK promoter region	GGACCTGGGCCTCTTCCAACCTCTGAGAGGTCTCTATTACTAAGTAAGCCTT AAGAAGCAGAATTCCATGAAGGGAGCTAGGAAACCAGGATTTTCCAAAAGGA GGTGGCATTTCGATTGATCCTGGTAGGGCAGCCTCGAATTCACGGGGTTGG GGTTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCGGCTGCTC TGGGCGTGGTTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGCACATTCT TTCACGTCCGTTGCGAGCGTCACCCGGATCTTCGCCGCTACCCCTGTGGGCC CCCCGGCGACGCTTCTGCTCCGCCCTAAGTCGGGAAGGTTCCCTGCGGTT CGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTACCCTC GCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCGATGGGCTG TGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGGCCGGGAAGG GGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCTGCCC GCGCGGT <u>GTTCCGCATT</u> CTGCAAGCCTCCGGAGCGCACGTGCGCAGTCGGCT CCCTCGTTGACCGAATCACCGACCTCTCTCCCAGCTGTATT	34
PGK SSI-1 promoter	TCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGC CGACCGCGATGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGA GCAGCGGCCGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTG GGCCCTGTTCTGCCCAGCGCGGT <u>GTTCCGCATT</u> CTGCAAGCCTCCGGAGCGC ACGTGCGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCAGC GTATT	35
PGK SSI-2 promoter	CCCTTGTGGGCCCCCGGCGACGCTTCTGCTCCGCCCTAAGTCGGGAAGG TTCCTTGCGGTTGCGGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTC ACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGAC CGCGATGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAG CGGCCGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCC CTGTTCTGCCCAGCGCGGT <u>GTTCCGCATT</u> CTGCAAGCCTCCGGAGCGCACGT CGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCAGCTGTA TT	36
PGK SSI-3 promoter	GGCTGCTCTGGGCGTGGTTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTC GCACATTCTTCACGTCCGTTGCGAGCGTCACCCGGATCTTCGCCGCTACCCCT TGTGGGCCCCCGGCGACGCTTCTGCTCCGCCCTAAGTCGGGAAGGTTCC TTGCGGTTGCGGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTA GTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCG ATGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGGC CGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGT TCTGCCCAGCGCGGT <u>GTTCCGCATT</u> CTGCAAGCCTCCGGAGCGCACGTGCGC AGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCAGCTGTATT	37
PGK SSI-4	AGGAGGTGGCATTTCGATTGATCCTGGTAGGGCAGCCTCGAATTCACGGGG TTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCGGCT	38

promoter	GCTCTGGGCGTGGTTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGCAC ATTCTTCACGTCCGTTTCGCAGCGTCACCCGGATCTTCGCCGCTACCCCTGTG GGCCCCCGGCGACGCTTCTGCTCCGCCCTAAGTCGGGAAGGTTCTTGC GGTTCGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTAC CCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCATGG GCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGGCCGGG AAGGGGCGGTGCGGGAGGCGGGGTGTGGGCGGTAGTGTGGGCCCTGTTCTT GCCCCGCGCGGT GTTCCGCAT TCTGCAAGCCTCCGGAGCGCACGTCCGCAGTC GGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCAGCTGTATT	
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[0080] In one embodiment, the PGK promoter is derived from the genomic region comprising the PGK gene. For instance, the PGK promoter can be derived from the genomic region comprising the PGK gene in homo sapiens chromosome X, GRCh38.p14 Primary Assembly (NCBI Reference Sequence: NC_000023.11). In another embodiment, the PGK promoter is derived from a polynucleotide sequence corresponding to nucleotide positions of about 78103669 to 78104334 of the genomic region comprising the PGK gene in homo sapiens chromosome X, GRCh38.p14 Primary Assembly (NCBI Reference Sequence: NC_000023.11).

[0081] In an additional aspect, the truncated PGK promoters described herein comprise one or more nucleic acid sequences that bind to or are predicted to bind to one or more transcription factors. Alternatively, the truncated PGK promoters described herein may a) lack one or more nucleic acid sequences that bind to or are predicted to bind to one or more transcription factors or b) be characterized by the absence of one or more nucleic acid sequences that bind to or are predicted to bind to one or more transcription factors. The promoter sequences described herein can be analyzed using a transcription factor binding site prediction database known as JASPAR. JASPAR is an open-access database storing manually curated transcription factor (TF) binding profiles as position frequency matrices (PFMs), which summarize occurrences of each nucleotide at each position in a set of observed TF-DNA interactions. PFMs can be used to scan any DNA sequence to predict TF binding sites.

[0082] In one embodiment, the truncated PGK promoter comprises a nucleic acid sequence that binds to or is predicted to bind to a transcription factor selected from the group consisting of ZNF692, ZNF257, YY1, TEAD4, TEAD1, TCF4, TCF3, TBX3, SP1, SOX18, RFX7, MYOG, MYF5, KLF9, KLF6, KLF4, KLF10, HIF1, FIGLA, DUXA, DUX4, CTCFL, BHLHE22, BHLHA15, ASCL1, and ARNT::HIF1A. In an additional embodiment, the truncated PGK promoter comprises the nucleotide sequence of SEQ ID NO: 35 or having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 35.

[0083] In another embodiment, the truncated PGK promoter is characterized by the absence of a nucleic acid sequence that binds to or is predicted to bind to a transcription factor selected from the group consisting of ZNF281, ZNF148, VEZF1, TLX2, TFAP2E, TFAP2C, TFAP2B, TFAP2A, SP8, SOX15, RELB, RBPJ, PRDM1, PITX2, NKX3-2, NKX2-8, NKX2-3, NFKB1, NFATC2, MSX2, MEIS1, KLF16, KLF15, KLF11, ISL2, HOXD4, HOXD12, HOXD11, HOXD10, HOXC9, HOXC4, HOXC12, HOXC11, HOXC10, HOXB9, HOXB7, HOXB4, HOXA9, HOXA4, HOXA1, GSX2, FOXD2, FOXC1, EN2, EGR1, EBF1, E2F8, E2F6, E2F4, E2F1, DRGX, CEBPE, CEBPD, CEBPB, BARHL2, and BARHL1. In an additional embodiment, the truncated PGK promoter comprises the nucleotide sequence of SEQ ID NO: 35 or having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 35.

[0084] In one embodiment, the truncated PGK promoter comprises a nucleic acid sequence that binds to or is predicted to bind to a transcription factor selected from the group consisting of ZNF692, ZNF257, YY1, TEAD4, TEAD1, TCF4, TCF3, TBX3, SPI1, SP1, SOX18, SOX10, RUNX2, RFX7, PLAGL2, OSR2, OSR1, MYOG, MYF5, KLF9, KLF6, KLF4, KLF10, IKZF1, HSF4, HIF1A, FLI1, FIGLA, FEV, ETV5, ETV4, ETS2, ERG, ELK4, ELK1, ELF5, ELF1, DUXA, DUX4, CTCFL, BHLHE22, BHLHA15, ASCL1, and ARNT::HIF1A. In an additional embodiment, the truncated PGK promoter comprises the nucleotide sequence of SEQ ID NO: 36 or having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 36.

[0085] In another embodiment, the truncated PGK promoter is characterized by the absence of a nucleic acid sequence that binds to or is predicted to bind to a transcription factor selected from the group consisting of ZNF281, ZNF148, VEZF1, TLX2, TFAP2C, TFAP2B, TFAP2A, SP8, SOX15, RELB, RBPJ, PRDM1, PITX2, NKX3-2, NKX2-8, NKX2-3, NFKB1, NFATC2, MSX2, KLF16, KLF15, KLF11, ISL2, HOXD4, HOXD12, HOXD11, HOXD10, HOXC9, HOXC4, HOXC12, HOXC11, HOXC10, HOXB9, HOXB7, HOXB4, HOXA9, HOXA4, HOXA1, GSX2, FOXD2, FOXC1, EN2, EGR1, EBF1, E2F8, E2F6, E2F4, E2F1, DRGX, CEBPE, CEBPD, CEBPB, BARHL2, and BARHL1. In an additional embodiment, the truncated PGK promoter comprises the nucleotide sequence of SEQ ID NO: 36 or having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 36.

[0086] In one embodiment, the truncated PGK promoter comprises a nucleic acid sequence that binds to or is predicted to bind to a transcription factor selected from the group consisting of ZNF692, ZNF257, YY1, TEAD4, TEAD1, TCF4, TCF3, TBX3, STAT3, STAT1, SPI1, SPDEF, SP1, SOX18, SOX10, RUNX2, RHOXF1, RFX7, 5 PLAGL2, OSR2, OSR1, MYOG, MYF5, KLF9, KLF6, KLF4, KLF3, KLF10, IKZF1, HSF4, HIF1A, FLI1, FIGLA, FEV, ETV5, ETV4, ETS2, ERG, ELK4, ELK1, ELF5, ELF1, DUXA, DUX4, CTCFL, CREB1, BHLHE22, BHLHA15, ASCL1, and ARNT::HIF1A. In an additional embodiment, the truncated PGK promoter comprises the nucleotide sequence of SEQ ID NO: 37 or having at least 80%, at least 85%, at least 90%, at least 95%, at least 10 98%, at least 99% or 100% sequence identity to SEQ ID NO: 37.

[0087] In another embodiment, the truncated PGK promoter is characterized by the absence of a nucleic acid sequence that binds to or is predicted to bind to a transcription factor selected from the group consisting of ZNF281, ZNF148, VEZF1, TLX2, TFAP2C, TFAP2B, TFAP2A, SOX15, RELB, RBPJ, PRDM1, PITX2, NKX3-2, NKX2-8, NKX2-3, 15 NFKB1, NFATC2, MSX2, KLF16, KLF15, ISL2, HOXD4, HOXD12, HOXD11, HOXD10, HOXC9, HOXC4, HOXC12, HOXC11, HOXC10, HOXB9, HOXB7, HOXB4, HOXA9, HOXA4, HOXA1, GSX2, FOXD2, FOXC1, EN2, EGR1, EBF1, E2F8, E2F6, E2F4, E2F1, DRGX, CEBPE, CEBPD, CEBPB, BARHL2, and BARHL1. In an additional embodiment, the truncated PGK promoter comprises the nucleotide sequence of SEQ ID 20 NO: 37 or having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 37.

[0088] In one embodiment, the truncated PGK promoter comprises one or more deletions, insertions or substitutions in a PGK promoter shown as SEQ ID NO: 34. In some embodiments, the one or more deletions in the truncated PGK promoter comprise one or 25 more 5' region deletions and/or one or more 3' region deletions. In other embodiments, the 5' region deletion comprises a deletion of about 300, about 301, about 302, about 303, about 304, about 305, about 306, about 307, about 308, about 309, about 310, about 311, about 312, about 313, about 314, about 315, about 316, about 317, about 318, about 319, about 320, about 321, about 322, about 323, about 324, about 325, about 326, about 327, about 30 328, about 329, about 330, about 331, about 332, about 333, about 334, about 335, about 336, about 337, about 338, about 339, about 340, about 341, about 342, about 343, about 344, about 345, about 346, about 347, about 348, about 349, about 350, about 351, about 352, about 353, about 354, about 355, about 356, about 357, about 358, about 359, about

360, about 361, about 362, about 363, about 364, about 365, about 366, about 367, about 368, about 369, about 370, about 371, about 372, about 373, about 374, about 375, about 376, about 377, about 378, about 379, about 380, about 381, about 382, about 383, about 384, about 385, about 386, about 387, about 388, about 389, or about 390 nucleotides

- 5 deletion from the 5' end of SEQ ID NO: 34, and having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35. In some embodiments, the truncated PGK promoter comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO: 35.

- 10 [0089] In other embodiments, the 5' region deletion comprises a deletion of about 210, about 211, about 212, about 213, about 214, about 215, about 216, about 217, about 218, about 219, about 220, about 221, about 222, about 223, about 224, about 225, about 226, about 227, about 228, about 229, about 230, about 231, about 232, about 233, about 234, about 235, about 236, about 237, about 238, about 239, about 240, about 241, about 242,
15 about 243, about 244, about 245, about 246, about 247, about 248, about 249, about 250, about 251, about 252, about 253, about 254, about 255, about 256, about 257, about 258, about 259, about 260, about 261, about 262, about 263, about 264, about 265, about 266, about 267, about 268, about 269, about 270, about 271, about 272, about 273, about 274, about 275, about 276, about 277, about 278, about 279, about 280, about 281, about 282,
20 about 283, about 284, about 285, about 286, about 287, about 288, about 289, or about 290, nucleotides deletion from the 5' end of SEQ ID NO: 34, and having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 36. In some embodiments, the truncated PGK promoter comprises, consists
25 essentially of, or consists of the nucleotide sequence of SEQ ID NO: 36.

- [0090] In other embodiments, the 5' region deletion comprises a deletion of about 110, about 111, about 112, about 113, about 114, about 115, about 116, about 117, about 118, about 119, about 120, about 121, about 122, about 123, about 124, about 125, about 126, about 127, about 128, about 129, about 130, about 131, about 132, about 133, about 134,
30 about 135, about 136, about 137, about 138, about 139, about 140, about 141, about 142, about 143, about 144, about 145, about 146, about 147, about 148, about 149, about 150, about 151, about 152, about 153, about 154, about 155, about 156, about 157, about 158, about 159, about 160, about 161, about 162, about 163, about 164, about 165, about 166,

about 167, about 168, about 169, about 170, about 171, about 172, about 173, about 174, about 175, about 176, about 177, about 178, about 179, about 180, about 181, about 182, about 183, about 184, about 185, about 186, about 187, about 188, about 189, about 190, about 191, about 192, about 193, about 194, about 195, about 196, about 197, about 198, about 199, or about 200 nucleotides deletion from the 5' end of SEQ ID NO: 34, and having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 37. In some embodiments, the truncated PGK promoter comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO: 37.

[0091] In other embodiments, the 5' region deletion comprises a deletion of about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, or about 100 nucleotides deletion from the 5' end of SEQ ID NO: 34, and having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 38. In some embodiments, the truncated PGK promoter comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO: 38.

[0092] In some embodiments, the truncated PGK promoter consists essentially of or consists of a nucleotide sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to the nucleotide sequence of SEQ ID NO: 35, 36, 37 or 38.

[0093] In one other aspect, the polynucleotides, nucleic acid constructs or molecules described herein comprise a donor template. The donor template can comprise one or more transgenes or coding sequences. In other embodiments, the donor template comprises a promoter, such as a truncated PGK promoter, to control the expression of the one or more transgenes or coding sequences.

[0094] In other embodiments, the polynucleotides, nucleic acid constructs or molecules comprise a donor template that can integrate into the host cell genome via homologous recombination at the site of a double strand break of the genome. In one embodiment, the donor template comprises one or more, at least two, or two or more coding sequences or transgenes. In one embodiment, the expression of the one or more, at least two, or two or more coding sequences or transgenes is under the control of a PGK promoter, e.g., a truncated PGK promoter.

[0095] In other embodiments, the polynucleotides, nucleic acid constructs or molecules further comprise one or more homology sequences that are homologous to a portion of an integration site in the genome of the engineered immune cell (for site-specific integration). In one embodiment, the integration site is a gene (target site or target gene) that is involved in T cell receptor (TCR) $\alpha\beta$ function or activity. In an additional embodiment, the gene is the TCR alpha gene constant region (TRAC) gene. In another embodiment, the integration site is in TRAC, a component of the TCR, an HLA, TCR α , TCR β , β 2-microglobulin (" β 2m"), CD52, GR, deoxycytidine kinase (DCK), PD-1, and CTLA-4 gene.

[0096] In other embodiments, the polynucleotide, nucleic acid construct or molecule comprises a 5' homology arm having a sequence homologous to a sequence that is positioned 5' to a double strand break at the integration site of the genome. In one other embodiment, the polynucleotide, nucleic acid construct or molecule further comprises a 3' homology arm having a sequence homologous to a sequence that is positioned 3' to the double strand break of the integration site of the host genome. Nucleotide sequence homologies are present in regions flanking upstream and downstream the site of the double-strand break and the site of genome integration, and the nucleic acid sequence to be introduced, e.g., one or more transgenes, is located between the two homology arms. In some embodiments, in the context of site-specific integration, the terms 5' homology arm and 3' homology arm and the terms regions flanking upstream and downstream are used to refer to the orientation of the target site or target gene for genome integration.

[0097] In some embodiments, the double strand break can be made by a rare-cutting endonuclease, for example without limitation, a zinc finger endonuclease, TALE (Transcription Activator-like Effector)-nuclease (TALEN), or CRISPR. The polynucleotide, nucleic acid construct or molecule can be integrated into the desired integration site of the genome by homologous recombination between the 5' homology and

the 3' homology arms and the homologous sequences at the integration site. Exemplary 5' and 3' homologous sequences are provided in Table 3, e.g., SEQ ID NOs: 42-45.

[0098] In one embodiment, the polynucleotide, nucleic acid construct or molecule comprises a donor template comprising a 5' homology arm, a 3' homology arm, a transgene, and a promoter, such as a truncated PGK promoter. In other embodiments, the polynucleotide, nucleic acid construct or molecule comprises a 5' homology arm, one or more coding sequences or transgenes, a promoter, such as a truncated PGK promoter, and a 3' homology arm.

[0099] In some embodiments, the polynucleotide, nucleic acid construct or molecule comprises a donor template comprising a 5' homology arm, a 3' homology arm, one or more coding sequences or transgenes, and a promoter, such as a truncated PGK promoter.

[0100] In some embodiments, the orientation of the one or more coding sequences or transgenes is the same orientation as the target site for genome integration. In some embodiments, the polynucleotide, nucleic acid construct or molecule comprises a donor template comprising a 5' homology arm, a promoter, one or more coding sequences or transgenes, and a 3' homology arm. In some embodiments, the promoter comprises a truncated PGK promoter, e.g., the PGK SSI-1, PGK SSI-2 or PGK SSI-3 promoter as described herein.

[0101] In some embodiments, the orientation of the one or more coding sequences or transgenes is the opposite orientation to the target site for genome integration. In some embodiments, the polynucleotide, nucleic acid construct or molecule comprises a donor template comprising a 5' homology arm, one or more coding sequences or transgenes, a promoter, and a 3' homology arm. In some embodiments, the promoter comprises a truncated PGK promoter, e.g., the PGK SSI-1, PGK SSI-2 or PGK SSI-3 promoter as described herein.

[0102] Polynucleotides can comprise a native sequence (e.g., an endogenous sequence that encodes an antibody or a portion thereof or an endogenous promoter sequence) or can comprise a variant of such a sequence. Polynucleotide variants contain one or more substitutions, additions, deletions and/or insertions such that the activity of the encoded polypeptide is not diminished, relative to a native molecule. The effect on the immunoreactivity of the encoded polypeptide can generally be assessed as described herein. Variants preferably exhibit at least about 70% identity, more preferably, at least about 80%

identity, yet more preferably, at least about 90% identity, at least about 91% identity, at least about 92% identity, at least about 93% identity, at least about 94%, and most preferably, at least about 95%, at least about 96% identity, at least about 97% identity, at least about 98% identity or about 99% identity to a polynucleotide sequence.

5 [0103] Two polynucleotide or polypeptide sequences are said to be "identical" if the sequence of nucleotides or amino acids in the two sequences is the same when aligned for maximum correspondence as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window," as used herein,
10 refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, or 40 to about 50, in which a sequence can be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

[0104] Optimal alignment of sequences for comparison can be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc.,
15 Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O., 1978, A model of evolutionary change in proteins - Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J., 1990, Unified Approach to
20 Alignment and Phylogenies pp. 626-645 Methods in Enzymology vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., 1989, CABIOS 5:151 -153; Myers, E.W. and Muller W., 1988, CABIOS 4:1 1 -17; Robinson, E.D., 1971, Comb. Theor. 1 1 :105; Santou, N., Nes, M., 1987, Mol. Biol. Evol. 4:406-425; Sneath, P.H.A. and Sokal, R.R., 1973, Numerical Taxonomy the Principles and Practice of Numerical Taxonomy,
25 Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J., 1983, Proc. Natl. Acad. Sci. USA 80:726-730.

[0105] In some embodiments, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide or polypeptide sequence in the
30 comparison window can comprise additions or deletions (i.e., gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The

percentage is calculated by determining the number of positions at which the identical nucleic acid bases or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (i.e. the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

[0106] Variants can also, or alternatively, be substantially homologous to a native gene, or a portion or complement thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a native antibody (or a complementary sequence).

[0107] Suitable "moderately stringent conditions" include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1 % SDS.

[0108] As used herein, "highly stringent conditions" or "high stringency conditions" are those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1 % sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1 % bovine serum albumin/0.1 % Ficoll/0.1 % polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1 % sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/m), 0.1 % SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[0109] It will be appreciated by those of ordinary skill in the art that, because of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the instant disclosure. Further,

alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the instant disclosure. Alleles are endogenous genes that are altered because of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein can, but need not, have an altered structure or function. Alleles
5 can be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

[0110] The polynucleotides of the instant disclosure can be obtained using chemical synthesis, recombinant methods, or PCR. Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can
10 use the sequences provided herein and a commercial DNA synthesizer to produce a desired DNA sequence.

[0111] For preparing polynucleotides using recombinant methods, a polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification, as further
15 described herein. Polynucleotides can be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, F-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. The polynucleotide so amplified can be
20 isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al., 1989.

[0112] Alternatively, PCR allows reproduction of DNA sequences. PCR technology is well known in the art and is described in, e.g., US Patent Nos. 4,683,195, 4,800,159, 4,754,065 and 4,683,202, as well as PCR: The Polymerase Chain Reaction, Mullis et al.
25 eds., Birkauser Press, Boston, 1994.

[0113] Accordingly, provided herein are polynucleotides comprising a promoter and at least two coding sequences, wherein one of the coding sequences encodes an anti-CD19 CAR. In some embodiments, another coding sequence encodes a CD70-binding protein or an anti-CD70 CAR.

30 [0114] Chimeric antigen Receptor (CAR)

[0115] As used herein, chimeric antigen receptors (CARs) are proteins that specifically recognize target antigens (e.g., target antigens on cancer cells). When bound to the target antigen, the CAR may activate the immune cell to attack and destroy the cell bearing that antigen (e.g., the cancer cell). CARs may also incorporate costimulatory or signaling domains to increase their potency. See Krause *et al.*, *J. Exp. Med.*, Volume 188, No. 4, 1998 (619–626); Finney *et al.*, *Journal of Immunology*, 1998, 161: 2791–2797, Song *et al.*, *Blood* 119:696-706 (2012); Kalos *et al.*, *Sci. Transl. Med.* 3:95 (2011); Porter *et al.*, *N. Engl. J. Med.* 365:725-33 (2011), and Gross *et al.*, *Annu. Rev. Pharmacol. Toxicol.* 56:59–83 (2016); U.S. Patent Nos. 7,741,465, and 6,319,494.

[0116] Using anti-CD19 CAR as an example, chimeric antigen receptors described herein comprise an extracellular domain, a transmembrane domain, and an intracellular domain, wherein the extracellular domain comprises a CD19 antigen binding domain that specifically binds to CD19. In some embodiments, the CD19 specific CAR comprises the following elements from the N- to the C- terminus: a cleavable signal sequence, a CD19 antigen binding domain (e.g., an anti-CD19 scFv), a hinge and transmembrane domain, and one or more successive signaling domains. In certain embodiments, the CD19-specific CAR disclosed herein comprises the following elements from the N- to the C-terminus: a CD8 α signal sequence, a CD19 scFv described herein, a CD8 α hinge and transmembrane region, a 41BB cytoplasmic signaling domain, and a CD3 ζ cytoplasmic signaling domain. Exemplary sequences are shown in Table 3.

a. Antigen Binding Domain

[0117] As discussed above, the CD19 CARs described herein comprise an antigen binding domain. An “antigen binding domain” as used herein means any polypeptide that binds a specified target antigen, for example the specified target antigen can be the CD19 protein or fragment thereof (referred to interchangeably herein as a “CD19 antigen”, “CD19 target antigen”, or “CD19 target”). In some embodiments, the antigen binding domain binds to a CD19 antigen on a tumor cell. In some embodiments, the antigen binding domain binds to a CD19 antigen on a cell involved in a hyperproliferative disease or a hematological cancer.

[0118] In some embodiments, the antigen binding domain comprises a variable heavy chain, variable light chain, and/or one or more CDRs described herein. In some embodiments, the antigen binding domain is a single chain variable fragment (scFv),

comprising light chain CDRs CDR1, CDR2 and CDR3, and heavy chain CDRs CDR1, CDR2 and CDR3. Exemplary anti-CD19 antibody CDR sequences are shown in Table 3.

[0119] Variants of the antigen binding domains (e.g., variants of the CDRs, VH and/or VL) are also within the scope of the disclosure, e.g., variable light and/or variable heavy chains that each have at least 70-80%, 80-85%, 85-90%, 90-95%, 95-97%, 97-99%, or above 99% identity to the amino acid sequences of the antigen binding domain sequences described herein. In some instances, such molecules include at least one heavy chain and one light chain, whereas in other instances the variant forms contain two variable light chains and two variable heavy chains (or subparts thereof). A skilled artisan will be able to determine suitable variants of the antigen binding domains as set forth herein using well-known techniques. In certain embodiments, one skilled in the art can identify suitable areas of the molecule that may be changed without destroying activity by targeting regions not believed to be important for activity.

[0120] In certain embodiments, the polypeptide structure of the antigen binding domains is based on antibodies, including, but not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic antibodies (sometimes referred to herein as “antibody mimetics”), chimeric antibodies, humanized antibodies, human antibodies, antibody fusions (sometimes referred to herein as “antibody conjugates”), and fragments thereof, respectively. In some embodiments, the antigen binding domain comprises or consists of avimers.

[0121] A CD19 antigen binding domain is said to be “selective” when it binds to CD19 more tightly than it binds to another target.

[0122] In some embodiments, the CD19 antigen binding domain is a scFv. In some embodiments, the CD19 specific CAR comprises an scFv comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, the CD19 specific CAR comprises the anti-CD19 antibody CDR sequences of SEQ ID NOs: 65-70 and comprises at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to the amino acid sequence of SEQ ID NO: 4.

[0123] In some embodiments, the CD19 specific CAR comprises a leader or signal peptide; in some embodiments the leader peptide comprises an amino acid sequence that is at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or

100% identical to the amino acid sequence MALPVTALLLPLALLLHAARP (SEQ ID NO: 1). In some embodiments, the leader (signal) peptide comprises the amino acid sequence of SEQ ID NO: 1. In some embodiments, the leader (signal) peptide is encoded by a nucleic acid sequence comprising:

- 5 ATGGCACTCCCCGTAAGTCTGCTGCTGCCGTTGGCATTGCTCCTGCACGCCG CACGCCCCG (SEQ ID NO: 48).

b. Hinge domain

[0124] The extracellular domain of the CARs of the disclosure may comprise a “hinge” domain (or hinge region). The term generally refers to any polypeptide that functions to link
10 the transmembrane domain in a CAR to the extracellular antigen binding domain in a CAR. In particular, hinge domains can be used to provide more flexibility and accessibility for the extracellular antigen binding domain.

[0125] A hinge domain may comprise up to 300 amino acids—in some embodiments 10 to 100 amino acids or in some embodiments 25 to 50 amino acids. The hinge domain may
15 be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4, CD28, 4-1BB, or IgG (in particular, the hinge region of an IgG; it will be appreciated that the hinge region may contain some or all of a member of the immunoglobulin family such as IgG1, IgG2, IgG3, IgG4, IgA, IgD, IgE, IgM, or fragment thereof), or from all or part of an antibody heavy-chain constant region.
20 Alternatively, the hinge domain may be a synthetic sequence that corresponds to a naturally occurring hinge sequence or may be an entirely synthetic hinge sequence. In some embodiments said hinge domain is a part of human CD8 α chain (e.g., NP_001139345.1). In another particular embodiment, said hinge and transmembrane domains comprise a part of human CD8 α chain. In some embodiments, the hinge domain of CARs described herein
25 comprises a subsequence of CD8 α , CD28, an IgG1, IgG4, PD-1 or an Fc γ RIII α , in particular the hinge region of any of an CD8 α , CD28, an IgG1, IgG4, PD-1 or an Fc γ RIII α . In some embodiments, the hinge domain comprises a human CD8 α hinge, a human IgG1 hinge, a human IgG4, a human PD-1 or a human Fc γ RIII α hinge. In some embodiments the CARs disclosed herein comprise a scFv, CD8 α human hinge and transmembrane domains,
30 the CD3 ζ signaling domain, and 4-1BB signaling domain. Table 3 provides amino acid sequences for exemplary hinge domains provided herein.

[0126] In certain embodiments, the hinge region comprises an amino acid sequence that is at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to the extracellular domain amino acid sequences set forth herein in SEQ ID

5 NO: 9.

c. Transmembrane Domain

[0127] The CARs of the disclosure are designed with a transmembrane domain that is fused to the extracellular domain of the CAR. It can similarly be fused to the intracellular domain of the CAR. In some instances, the transmembrane domain can be selected or
10 modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. In some embodiments, short linkers may form linkages between any or some of the extracellular, transmembrane, and intracellular domains of the CAR.

15 [0128] Suitable transmembrane domains for a CAR disclosed herein have the ability to (a) be expressed at the surface an immune cell such as, for example without limitation, a lymphocyte cell, such as a T helper (T_h) cell, cytotoxic T (T_c) cell, T regulatory (T_{reg}) cell, or Natural killer (NK) cells, and/or (b) interact with the extracellular antigen binding domain and intracellular signaling domain for directing the cellular response of an immune
20 cell against a target cell.

[0129] The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein.

[0130] Transmembrane regions of particular use in this disclosure may be derived from
25 (comprise, or correspond to) CD28, OX-40, 4-1BB/CD137, CD2, CD7, CD27, CD30, CD40, programmed death-1 (PD-1), inducible T cell costimulator (ICOS), lymphocyte function-associated antigen-1 (LFA-1, CD1-1a/CD18), CD3 gamma, CD3 delta, CD3 epsilon, CD247, CD276 (B7-H3), LIGHT, (TNFSF14), NKG2C, Ig alpha (CD79a), DAP-10, Fc gamma receptor, MHC class 1 molecule, TNF receptor proteins, an Immunoglobulin
30 protein, cytokine receptor, integrins, Signaling Lymphocytic Activation Molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptors, ICAM-1, B7-H3, CDS, ICAM-1, GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80

- (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL-2R beta, IL-2R gamma, IL-7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD1 1d, ITGAE, CD103, ITGAL, CD1 1a, LFA-1, ITGAM, CD1 1b, ITGAX, CD1 1c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, TNFR2,
- 5 TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, a ligand that specifically binds with CD83, or any combination thereof.
- 10 [0131] As non-limiting examples, the transmembrane region can be a derived from, or be a portion of a T cell receptor such as α , β , γ or δ , polypeptide constituting CD3 complex, IL-2 receptor p55 (a chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fc γ receptor III or CD proteins. Alternatively, the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.
- 15 In some embodiments, said transmembrane domain is derived from the human CD8 α chain (*e.g.*, NP_001139345.1).

[0132] In some embodiments, the transmembrane domain in the CAR of the disclosure is a CD8 α transmembrane domain. In some embodiments, the transmembrane domain in the CAR of the disclosure is a CD8 α transmembrane domain comprising the amino acid

20 sequence IYIWAPLAGTCGVLLSLVITLYC (SEQ ID NO: 10) or the amino acid sequence of SEQ ID NO: 99. In some embodiments, the hinge and transmembrane domain in the CAR of the disclosure is a CD8 α hinge and CD8 α transmembrane domain.

[0133] In some embodiments, the transmembrane domain in the CAR of the disclosure is a CD28 transmembrane domain. In some embodiments, the transmembrane domain in the

25 CAR of the disclosure is a CD28 transmembrane domain comprising the amino acid sequence of FWVLVVGGVLACYLLVTVAFIIFWV (SEQ ID NO: 56). In some embodiments, the hinge and transmembrane domain in the CAR of the disclosure is a CD28 hinge and CD28 transmembrane domain.

d. Intracellular Domain

- 30 [0134] The intracellular (cytoplasmic) domain of the CARs of the disclosure can provide activation of at least one of the normal effector functions of the immune cell comprising the CAR, *e.g.*, Signal 1/activation and/or Signal 2/costimulation. Effector function of a T cell,

for example, may refer to cytolytic activity or helper activity, including the secretion of cytokines.

[0135] In some embodiments, an activating intracellular signaling domain for use in a CAR can be the cytoplasmic sequences of, for example without limitation, the T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any synthetic sequence that has the same functional capability.

[0136] It will be appreciated that suitable (e.g., activating) intracellular domains include, but are not limited to signaling domains derived from (or corresponding to) CD3 zeta, CD28, OX-40, 4-1BB/CD137, CD2, CD7, CD27, CD30, CD40, programmed death-1 (PD-1), inducible T cell costimulator (ICOS), lymphocyte function-associated antigen-1 (LFA-1, CD1-1a/CD18), CD3 gamma, CD3 delta, CD3 epsilon, CD247, CD276 (B7-H3), LIGHT, (TNFSF14), NKG2C, Ig alpha (CD79a), DAP-10, Fc gamma receptor, MHC class 1 molecule, TNF receptor proteins, an Immunoglobulin protein, cytokine receptor, integrins, Signaling Lymphocytic Activation Molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptors, ICAM-1, B7-H3, CDS, ICAM-1, GITR, BAFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL-2R beta, IL-2R gamma, IL-7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD1 1d, ITGAE, CD103, ITGAL, CD1 1a, LFA-1, ITGAM, CD1 1b, ITGAX, CD1 1c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, a ligand that specifically binds with CD83, or any combination thereof.

[0137] The intracellular domains of the CARs of the disclosure may incorporate, in addition to the activating domains described above, costimulatory signaling domains (interchangeably referred to herein as costimulatory molecules or costimulatory domains) to increase their potency. Costimulatory domains can provide a signal in addition to the primary signal provided by an activating molecule as described herein.

[0138] A “co-stimulatory molecule” as used herein refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell, such as, but not limited to proliferation. Co-stimulatory molecules include, but are not limited to, an MHC class I molecule, BTLA and Toll ligand receptor. Examples of costimulatory molecules include CD27, CD28, CD8, 4-1BB (CD137), OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

[0139] It will be appreciated that suitable costimulatory domains within the scope of the disclosure can be derived from (or correspond to) for example, CD28, OX40, 4-1BB/CD137, CD2, CD3 (alpha, beta, delta, epsilon, gamma, zeta), CD4, CD5, CD7, CD9, CD16, CD22, CD27, CD30, CD 33, CD37, CD40, CD 45, CD64, CD80, CD86, CD134, CD137, CD154, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1 (CD11a/CD18), CD247, CD276 (B7-H3), LIGHT (tumor necrosis factor superfamily member 14; TNFSF14), NKG2C, Ig alpha (CD79a), DAP-10, Fc gamma receptor, MHC class I molecule, TNFR, integrin, signaling lymphocytic activation molecule, BTLA, Toll ligand receptors, ICAM-1, B7-H3, CDS, ICAM-1, GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL-2R beta, IL-2R gamma, IL-7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, CD83 ligand, or fragments or combinations thereof. It will be appreciated that additional costimulatory molecules, or fragments thereof, not listed above are within the scope of the disclosure.

[0140] In some embodiments, the intracellular/cytoplasmic domain of the CAR can be designed to comprise the 41BB/CD137 domain by itself or combined with any other desired intracellular domain(s) useful in the context of the CAR of the disclosure. The complete native amino acid sequence of 41BB/CD137 is described in NCBI Reference Sequence:

NP_001552.2. The complete native 41BB/CD137 nucleic acid sequence is described in NCBI Reference Sequence: NM_001561.5.

[0141] In some embodiments, the intracellular/cytoplasmic domain of the CAR can be designed to comprise the CD28 domain by itself or combined with any other desired intracellular domain(s) useful in the context of the CAR of the disclosure. The complete native amino acid sequence of CD28 is described in NCBI Reference Sequence:

NP_006130.1. The complete native CD28 nucleic acid sequence is described in NCBI Reference Sequence: NM_006139.1.

[0142] In some embodiments, the intracellular/cytoplasmic domain of the CAR can be designed to comprise the CD3 zeta domain by itself or combined with any other desired intracellular domain(s) useful in the context of the CAR of the disclosure. In some embodiments, the intracellular signaling domain of the CAR can comprise the CD3 ζ signaling domain which has amino acid sequence with at least about 70%, at least 80%, at least 90%, 95%, 97%, or 99% sequence identity with an amino acid sequence shown in SEQ ID NO: 11 or 91 (see Table 3). For example, the intracellular domain of the CAR can comprise a CD3 zeta chain portion and a portion of a costimulatory signaling molecule. The intracellular signaling sequences within the intracellular signaling portion of the CAR of the disclosure may be linked to each other in a random or specified order. In some embodiments, the intracellular domain is designed to comprise the activating domain of CD3 zeta and a signaling domain of CD28. In some embodiments, the intracellular domain is designed to comprise the activating domain of CD3 zeta and a costimulatory/signaling domain of 4-1BB.

[0143] In some embodiments, the 4-1BB (intracellular domain) comprises the amino acid sequence KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO: 12). In some embodiments, the 4-1BB (intracellular domain) is encoded by the nucleic acid sequence:

AAGCGCGGCAGGAAGAAGCTCCTCTACATTTTAAAGCAGCCTTTTATGAGGCCC
GTACAGACAACACAGGAGGAAGATGGCTGTAGCTGCAGATTTCCCGAGGAGGA
GGAAGGTGGGTGCGAGCTG (SEQ ID NO: 57).

[0144] In some embodiments, the intracellular domain in the CAR is designed to comprise a portion of CD28 and CD3 zeta, wherein the intracellular CD28 comprises the

amino acid sequence of SEQ ID NO: 13, and is encoded by the nucleic acid sequence set forth in SEQ ID NO: 58.

[0145] In some embodiments, the CD3 zeta amino acid sequence may comprise SEQ ID NO: 11 and the nucleic acid sequence that encodes the CD3 zeta amino acid sequence may comprise SEQ ID NO: 59:

[0146] AGGGTGAAGTTTTCCAGATCTGCAGATGCACCAGCGTATCAGCAG
GGCCAGAACCAACTGTATAACGAGCTCAACCTGGGACGCAGGGAAGAGTATGA
CGTTTTGGACAAGCGCAGAGGACGGGACCCCTGAGATGGGTGGCAAACCAAGAC
GAAAAAACCCCCAGGAGGGTCTCTATAATGAGCTGCAGAAGGATAAGATGGCT
10 GAAGCCTATTCTGAAATAGGCATGAAAGGAGAGCGGAGAAGGGGAAAAGGGC
ACGACGGTTTGTACCAGGGACTCAGCACTGCTACGAAGGATACTTATGACGCTC
TCCACATGCAAGCCCTGCCACCTAGG (SEQ ID NO: 59).

[0147] In some embodiments the intracellular signaling domain of the CAR of the disclosure comprises a domain of a co-stimulatory molecule. In some embodiments, the intracellular signaling domain of a CAR of the disclosure comprises a part of co-stimulatory molecule selected from the group consisting of fragment of 4-1BB (GenBank: AAA53133.) and CD28 (NP_006130.1). In some embodiments, the intracellular signaling domain of the CAR of the present disclosure comprises an amino acid sequence which comprises at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 12. In some embodiments, the intracellular signaling domain of the CAR of the disclosure comprises amino acid sequence which comprises at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity with an amino acid sequence shown in SEQ ID NO: 13.

[0148] In exemplary embodiments, a CAR of the disclosure comprises, from the N-terminus to the C-terminus: a (cleavable) CD8 α signal sequence, an anti-CD19 scFv, a CD8 α hinge and transmembrane region, a 4-1BB cytoplasmic (costimulatory) signaling domain, and a CD3 ζ cytoplasmic signaling domain. In some embodiments, the CD19-specific CAR comprises the amino acid sequence of SEQ ID NOs: 4, 9, 10, 11 and 12. In some embodiments, the CD19-specific CAR comprises the amino acid sequence of SEQ ID NO: 4, 9, 10, 11, and 12 and having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity

to SEQ ID NO: 5 or 7. In some embodiments, the CD19-specific CAR comprises the amino acid sequence having one or more conservative amino acid substitutions of the amino acid sequence of SEQ ID NO: 5 or 7. In some embodiments, the CD19-specific CAR comprises the amino acid sequence of SEQ ID NO: 5 or 7.

- 5 [0149] In certain aspects, provided herein are engineered immune cells comprising and/or expressing a CD19-specific CAR, and a CD70-binding protein and/or a chimeric cytokine receptor (CCR).

[0150] CD70-binding protein

- [0151] In a related aspect, the current disclosure provides a CD70-binding proteins as
10 described herein, wherein the CD70-binding proteins comprise an extracellular ligand or antigen binding domain that binds to CD70 (or a CD70-binding domain) and a transmembrane domain. The CD70-binding proteins can comprise none to one or more intracellular signaling domains as described herein. In some embodiments, the CD70-binding protein is a non-naturally occurring, or recombinant CD70-binding protein.

- 15 [0152] In some embodiments, the CD70-binding proteins provided herein comprise an extracellular domain that binds to CD70 (*e.g.*, an anti-CD70 single chain variable fragment (scFv)) and a transmembrane domain. In some embodiments, the CD70-binding protein is a CD70 CAR. In some embodiments, the CD70-binding proteins or CD70 CARs provided herein comprise an extracellular ligand-binding domain (*e.g.*, scFv), a transmembrane
20 domain, and an intracellular signaling domain. In some embodiments, the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 16, 17 or 18. In some embodiments, the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 16 and 17 and having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about
25 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO: 18. In some embodiments, the anti-CD70 scFv comprises the amino acid sequence having one or more conservative amino acid substitutions of the amino acid sequence of SEQ ID NO: 18. In some embodiments, the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 18.

- 30 [0153] In some embodiments, the CD70-binding protein comprises a transmembrane domain comprises the amino acid sequence of SEQ ID NO: 10 or 56.

[0154] In some embodiments, the CD70-binding protein comprises one or more intracellular signaling domains selected from the group consisting of a CD3 ζ signaling domain, a CD3 δ signaling domain, a CD3 γ signaling domain, a CD3 ϵ signaling domain, a CD28 signaling domain, a CD2 signaling domain, an OX40 signaling domain, and a 4-1BB signaling domain, or a variant thereof. In some embodiments, the intracellular signaling domain comprises the amino acid sequence of one or more of SEQ ID NOs: 11, 12, 13, 82, 83, 84, 85, 86, 87, 88, 89, 90, or 91. In some embodiments, the intracellular signaling domain comprises the amino acid sequence of one or more of SEQ ID NO: 11, 88, 89, or 90. In some embodiments, the CD70-binding protein comprises a CD3 ζ signaling domain, or a variant thereof, and does not comprise a costimulatory domain, for example, a 4-1BB costimulatory domain. In some embodiments, the CD70-binding protein or CD70-specific CAR comprises a CD3 ζ intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 11 and does not comprise a costimulatory domain. In some embodiments, the CD70-binding protein comprises a 4-1BB signaling domain, or a variant thereof, and does not comprise a CD3 signaling domain. In some embodiments, the CD70-binding protein comprises a 4-1BB signaling domain and a CD3 ζ signaling domain. In some embodiments, the CD70-binding protein does not comprise an intracellular signaling domain. Different intracellular signaling domain or combination thereof can confer different signaling strength that can contribute to T cell proliferation, potency, survival, persistence, and/or resistant to host immune cell rejection. Described herein are CD70-binding proteins comprising no to one or more intracellular signaling domains. In some embodiments, the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 18, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 and having an amino acid sequence having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO:19 or 21. In some embodiments, the CD70-binding protein comprises the amino acid sequence having one or more conservative amino acid substitutions of the amino acid sequence of SEQ ID NO: 19 or 21. In some embodiments, the CD70-binding protein comprises the amino acid sequence of SEQ ID NO:19 or 21.

[0155] CD70 is expressed on T cells, especially activated T cells, including host alloreactive T cells. In some embodiments, engineered immune cells comprising the CD70-binding proteins described herein can exhibit different levels of persistence and/or

resistance to rejection by host immune cells and can be suitable for use in lymphodepletion in vivo when administered to a patient. In some embodiments, engineered immune cells comprising the CD70-binding proteins described herein can inhibit proliferation and/or activities of host immune cells to different degrees that can allow for fine-tuning of the depth of lymphodepletion in vivo when administered to a patient. For example, engineered immune cells comprising a CD70-binding protein that demonstrate extended expansion and/or inhibition of host immune cells proliferation or activities, in, e.g., an MLR assay, can be used for an extended lymphodepletion. In contrast, engineered immune cells comprising a CD70-binding protein that demonstrate less extended expansion and/or inhibition of host immune cells in the same or similar assay can be used when a less complete or a less thorough lymphodepletion is desired. In some embodiments, the host immune cells are T cells or NK cells.

[0156] In certain aspects, the engineered immune cells (e.g., CAR T cells) that comprise and/or express a CD19-specific CAR and a CD70-binding protein or CD70-specific CAR described herein can target CD19 positive as well as CD19 negative/CD70 positive tumor cells or other pathogenic T cells or alloreactive T cells. The CD19-specific CAR T cells co-expressing the CD70-binding protein (may be referred to as the CD19/CD70 dual CAR T cells) advantageously prevent CD19 antigen escape of tumor cells or pathogenic target cells. In certain aspect, the CD19-specific CAR T cells co-expressing the CD70-binding protein and/or the chimeric cytokine receptor (CCR) demonstrate further enhanced activities.

[0157] Chimeric Cytokine Receptor (CCR)

[0158] The CCRs of the disclosure comprise transmembrane domains. The transmembrane domains of the disclosure contain sequences such that they allow for constitutive dimerization of two monomers, thus allowing constitutive JAK activation on the intracellular portion, and constitutive recruitment and phosphorylation of, for example, STAT on the cytoplasmic region of the receptor.

[0159] The transmembrane domains are on the N-terminus and are coupled to intracellular/cytoplasmic domains on the C-terminus. In some embodiments, the coupling is achieved optionally through a linker.

[0160] As used herein, the transmembrane domains are capable of insertion into the membrane of a cell in which it is expressed. In some embodiments, the CCRs of the disclosure comprise a transmembrane domain spanning a cellular membrane, and an

extracellular portion, and/or an intracellular portion. In some embodiments, the CCRs of the disclosure comprise a transmembrane domain spanning a cellular membrane, and an extracellular ligand binding domain, and/or an intracellular signaling domain. In some embodiments, the CCRs of the disclosure comprise a transmembrane domain spanning a cellular membrane, and further comprise an intracellular domain, and do not comprise an extracellular ligand binding domain. In some embodiments, the CCR comprises the amino acid sequence of SEQ ID NO: 27 or 29.

[0161] In some embodiments, the transmembrane domains of the disclosure are engineered (synthetic) and do not resemble any naturally occurring transmembrane domain, e.g. they are non-naturally occurring.

[0162] In other embodiments, the transmembrane domains of the disclosure are derived from naturally occurring receptors.

[0163] In some embodiments, the transmembrane domains and/or JAK-binding/activating domains (or simply referred to as JAK-binding domains) of the disclosure are derived from, for example, one or more of the following receptors: erythropoietin receptor (EpoR), Interleukin 6 signal transducer (GP130 or IL6ST), prolactin receptor (PrlR), growth hormone receptor (GHR), granulocyte colony-stimulating factor receptor (GCSFR), and thrombopoietin receptor/ myeloproliferative leukemia protein receptor (TPOR/MPLR). When derived from naturally occurring receptors, the entire receptor, or the entire transmembrane sequence of the receptor may not be necessary to effectuate constitutive activation and constitutive JAK binding/activation on the intracellular portion. In some embodiments, the transmembrane domain of the disclosure is derived from a truncated version of the naturally occurring TPOR/MPLR (myeloproliferative leukemia protein) receptor as shown in SEQ ID NO: 51. Accordingly fragments of naturally occurring receptors may be utilized. Furthermore, certain mutations may be introduced into the transmembrane domains derived from naturally occurring receptors, to further tune the downstream signaling. In some embodiments, the transmembrane domain of the disclosure is derived from TPOR/MPLR with the H499L/S505N/W515K substitutions as shown in SEQ ID NO: 25.

[0164] The CCRs of the disclosure comprise cytoplasmic recruiting domains. The recruiting domain can be a STAT-recruiting domain, an AP1-recruiting domain, a Myc/Max-recruiting domain; or an NFkB-recruiting domain. In some embodiments, the

recruiting domain is a Signal Transducer and Activator of Transcription (STAT)-recruiting (STAT-activating) domains, e.g. from receptor tails (cytotails) or from cytokine receptor tails. These intracellular recruiting domains of the CCRs of the disclosure allow for the propagation of Signal 3 in an immune cell comprising a CAR and a chimeric cytokine receptor (e.g. a CAR-T-cell with a chimeric cytokine receptor of the disclosure). Cytokine signaling propagated through the Stat-recruiting domain allows for the cytokine-based immune potentiation of the cell. In some embodiments, the immune-potentiation is homeostatic, e.g. signaling gives rise to increase in immune cells bearing the CAR. In some embodiments, the immune-potentiation is inflammatory, e.g. signaling gives rise to increase in the potency of the immune cells bearing the CAR. In some embodiments, the immune-potentiation prevents exhaustion, e.g. signaling maintains the long-term functionality of immune cells bearing the CAR.

[0165] In some embodiments, the recruiting domains of the disclosure are synthetic, and do not resemble any naturally occurring receptor fragment. In some embodiments, the immune-potentiation prevents exhaustion, e.g. signaling maintains the long-term functionality of immune cells bearing the CAR. In some embodiments, the Stat-recruiting domains of the disclosure are synthetic, and do not resemble any naturally occurring receptor fragment.

[0166] In other embodiments, the Stat-recruiting domains of the disclosure are derived from cytoplasmic tails of naturally occurring receptors, e.g. derived from naturally occurring cytokine receptors. These cytoplasmic tails of naturally occurring receptors may be the regions downstream of the JAK-binding domains of the transmembrane domain of the receptor. The Stat-recruiting domains of the chimeric cytokine receptors comprise at least one STAT-recruiting domain from at least one receptor. In some embodiments, the Stat-recruiting domain comprises at least one STAT1-recruiting domain. In some embodiments, the Stat-recruiting domain comprises at least one STAT2-recruiting domain. In some embodiments, the Stat-recruiting domain comprises at least one STAT3-recruiting domain. In some embodiments, the Stat-recruiting domain comprises at least one STAT4-recruiting domain. In some embodiments, the Stat-recruiting domain comprises at least one STAT5-recruiting domain. In some embodiments, the Stat-recruiting domain comprises at least one STAT6-recruiting domain. In some embodiments, the Stat-recruiting domain comprises at least one STAT7-recruiting domain.

[0167] In some embodiments, the naturally occurring receptor from which the Stat-recruiting domain is derived, is not a cytokine receptor.

[0168] In some embodiments, the naturally occurring receptor from which the Stat-recruiting domain is derived, is a cytokine receptor. Exemplary cytokine receptors through which T-cell-immune potentiating cytokines signal include, but are not limited to IL-2 receptor, IL-7 receptor, IL-15 receptor and IL-21 receptor. In alternative embodiments, the receptor from which the Stat-recruiting domain is derived, is not a cytokine receptor. By choosing the Stat-recruiting domain of the CCR, the receptor can be redirected to signaling of choice. In some embodiments, the recruiting domain comprises the amino acid sequence of SEQ ID NO: 26.

[0169] In some embodiments, the CCR of the disclosure comprises a recruiting domain connected to the C-terminus of the transmembrane/JAK2 binding domain, with or without a linker. In some embodiments, the linker comprises one or more amino acid residues. In some embodiments, the CCR comprises the amino acid sequence of SEQ ID NO: 25 and 26. In some embodiments, the CCR comprises the amino acid sequence of SEQ ID NO: 25 and 26 and having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO: 27 or 29. In some embodiments, the CCR comprises the amino acid sequence having one or more conservative amino acid substitutions of the amino acid sequence of SEQ ID NO: 27 or 29. In some embodiments, the CCR comprises the amino acid sequence of SEQ ID NO: 27 or 29.

[0170] In some embodiments, the CCR of the disclosure comprises an extracellular ligand binding domain. In some embodiments, the extracellular ligand binding domain binds to an immune inhibitory molecule, such as PDL1. In some embodiments, the extracellular ligand binding domain of the CCR comprises an antibody that binds to PDL-1. In some embodiments, the extracellular ligand binding domain of the CCR comprises an ectodomain of PD-1 (PD-1 CCR). In some embodiments, binding to PDL-1 by the PD-1 ectodomain of the PD-1 CCR can turn the inhibitory signal of PDL-1 into a stimulatory signal through the signal transduction of the intracellular cytoplasmic recruiting domain. In some embodiments, the PD-1 CCR comprises the TPOR/MPLR transmembrane/JAK binding domain comprising the amino acid sequence of SEQ ID NO: 50 and an intracellular

recruiting domain of SEQ ID NO: 26. In some embodiments, the PD-1 ectodomain comprises a wild-type PD-1 ectodomain and comprises the amino acid sequence of SEQ ID NO: 52.

[0171] In some embodiments, the PD-1 ectodomain comprises mutations to the wild-type PD-1 ectodomain sequence. In some embodiments, the PD-1 ectodomain sequence is a high affinity PD-1 ectodomain. In some embodiments, the PD-1 ectodomain sequence is a high affinity PD-1 ectodomain and comprises the amino acid sequence of SEQ ID NO: 53. In certain embodiments, the PD-1 ectodomain comprises one or more tandem repeat of the wild-type or high affinity PD-1 ectodomains.

[0172] Dominant negative receptor

[0173] In a further aspect, provided herein are PD-1 dominant negative receptor that comprises a PD-1 ectodomain and a transmembrane domain, without a functional intracellular signaling domain. In some embodiments, the PD-1 ectodomain comprises the wild-type PD-1 ectodomain. In some embodiments, the PD-1 ectodomain comprises a mutant PD-1 ectodomain. In some embodiments, the PD-1 ectodomain comprises a high-affinity PD-1 ectodomain. In some embodiments, the PD-1 ectodomain comprises the amino acid sequences of SEQ ID NO: 53.

[0174] In further embodiments, the transmembrane domain comprises a PD-1 transmembrane domain. In some embodiments, the transmembrane domain comprises a CD8a transmembrane domain. In some embodiments, the transmembrane domain comprises a CD28 transmembrane domain. In some embodiments, the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 10, 56, or 60. And conservative amino acid substitutions of the polypeptides disclosed herein are contemplated.

[0175] In some embodiments, the PD-1 dominant negative receptor comprises the amino acid sequence of SEQ ID NO: 52 or 53. In some embodiments, the PD-1 dominant negative receptor comprises the amino acid sequence of SEQ ID NO: 52 or 53 and having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO: 54 or 55. In some embodiments, the PD-1 dominant negative receptor comprises the amino acid sequence having one or more conservative amino acid substitutions of the amino acid sequence of

SEQ ID NO: 54 or 55. In some embodiments, the PD-1 dominant negative receptor comprises the amino acid sequence of SEQ ID NO: 54 or 55.

[0176] The disclosure encompasses modifications to the polypeptides disclosed herein, e.g., a CAR, e.g., CD19-specific CAR, a CD70-binding protein, CCR, and dominant negative receptor, etc., which do not significantly affect their properties and variants which have enhanced or decreased activity and/or affinity as desired. Modification of polypeptides is routine practice in the art and need not be described in detail herein. Examples of modified polypeptides include polypeptides with conservative substitutions of amino acid residues, one or more deletions or additions of amino acids which do not significantly deleteriously change the functional activity, or which mature (enhance) the affinity of the polypeptide for its ligand, or use of chemical analogs.

[0177] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody of an enzyme or a polypeptide which increases the half-life of the antibody in the blood circulation.

[0178] Substitution variants have at least one amino acid residue in the antigen binding domain removed and a different residue inserted in its place. In some embodiments, sites of interest for substitutional mutagenesis include the hypervariable regions/CDRs, but FR alterations are also contemplated. Conservative substitutions are shown in Table 2 under the heading of "conservative substitutions." If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 2, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 2: Amino Acid Substitutions

Original Residue (naturally occurring amino acid)	Conservative Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn; Ala
Asn (N)	Gln	Gln; His; Asp, Lys; Arg; Ala

Original Residue (naturally occurring amino acid)	Conservative Substitutions	Exemplary Substitutions
Asp (D)	Glu	Glu; Asn; Ala
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn; Glu; Ala
Glu (E)	Asp	Asp; Gln; Ala
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg; Ala
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine; Ala
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn; Ala
Met (M)	Leu	Leu; Phe; Ile; Ala
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr; Ala
Thr (T)	Ser	Ser; Ala
Trp (W)	Tyr	Tyr; Phe; Ala
Tyr (Y)	Phe	Trp; Phe; Thr; Ser; Ala
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

[0179] In certain aspects, the disclosure provides immune cells or engineered immune cells comprising the polynucleotides that comprise more than one, or a first and a second, coding sequences, wherein either the first or second coding sequence encodes an anti-CD19 CAR. In some embodiments, the engineered immune cells further comprise an additional
5 polynucleotide comprising one or more coding sequences.

[0180] In some embodiments, the one or more coding sequences of the additional polynucleotide comprise a coding sequence that encodes a PD-1 dominant negative receptor. In some embodiments, the PD-1 dominant negative receptor comprises a wild-type PD-1 ectodomain. In some embodiments, the PD-1 dominant negative receptor comprises a
10 high affinity PD-1 ectodomain. In some embodiments, the PD-1 dominant negative receptor does not comprise a functional intracellular signaling domain. In some embodiments, the PD-1 dominant negative receptor comprises the amino acid sequence of SE ID NO: 54 or 55.

[0181] In some embodiments, the one or more coding sequences of the additional polynucleotide encode a CCR. In some embodiments, the CCR comprises the amino acid sequence of SEQ ID NO: 27 or 29. In some embodiments, the one or more coding sequence encodes a CD70-binding protein. In some embodiments, the CD70-binding protein
5 comprises the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 21. In some embodiments, the one or more coding sequences encode a PD-1 CCR. In some embodiments, the PD-1 CCR comprises a PD-1 ectodomain comprising the amino acid sequence of SEQ ID NO: 54 or SEC ID NO: 55, a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 50, and an intracellular signaling domain comprising
10 the amino acid sequence of SEQ ID NO: 26. In some embodiments, the one or more coding sequences encode a PD-1 dominant negative receptor. In some embodiments, the PD-1 dominant negative receptor comprises the amino acid sequence of SEQ ID NO: 54 or SEQ ID NO: 55.

[0182] In some embodiments, the additional polynucleotide comprises, from 5' to 3', a
15 promoter operably linked to a third coding sequence and a fourth coding sequence. In some embodiments, the third coding sequence encodes a CCR or a CD70-binding protein. In some embodiments, the fourth coding sequence encodes a PD-1 CCR or a PD-1 dominant negative receptor. In some embodiments, the third coding sequence encodes a PD-1 CCR or a PD-1 dominant negative receptor. In some embodiments, the fourth coding sequence
20 encodes a CCR or a CD70-binding protein.

[0183] In further aspects, provided herein are engineered immune cells, e.g., CAR T cells that express a CD19-specific CAR and a CD70-binding protein and/or a CCR. In some embodiments, the engineered immune cells, e.g., CD19 CAR T cells, further express a PD-1 dominant negative receptor.

[0184] In some embodiments, the engineered immune cells comprise a polynucleotide comprising, from 5' to 3', a first and a second coding sequences driven by a truncated PGK promoter, wherein the first coding sequence encodes a CD19-specific CAR and the second coding sequence encodes a CD70-binding protein, as described herein, or wherein the first coding sequence encodes a CD70-binding protein, and the second coding sequence encodes
30 a CD19-specific CAR, as described herein. In some embodiments, the engineered immune cells further comprise an additional polynucleotide comprising one or more coding sequences, wherein the one or more coding sequences encode a CCR, and optionally a PD-1

dominant negative receptor, as described herein. In some embodiments, the additional polynucleotide comprises, from 5' to 3', a third and a fourth coding sequences, wherein the third coding sequence encodes a CCR and the fourth coding sequence encodes a PD-1 dominant negative receptor, as described herein, or wherein the third coding sequence encodes a PD-1 dominant negative receptor and the fourth coding sequence encodes a CCR, as described herein.

[0185] In some embodiments, the engineered immune cells comprise a polynucleotide comprising, from 5' to 3', a first and a second coding sequences driven by a truncated PGK promoter, wherein the first coding sequence encodes a CCR and the second coding sequence encodes a CD19-specific CAR, as described herein, or wherein the first coding sequence encodes a CD19-specific CAR and the fourth coding sequence encodes a CCR, as described herein. In some embodiments, the engineered immune cells further comprise an additional polynucleotide comprising one or more coding sequences, wherein the one or more coding sequences encode a CD70-binding protein, and optionally a PD-1 dominant negative receptor, as described herein. In some embodiments, the additional polynucleotide comprises, from 5' to 3', a third and a fourth coding sequences, wherein the third coding sequence encodes a CD70-binding protein and the fourth coding sequence encodes a PD-1 dominant negative receptor, as described herein, or wherein the third coding sequence encodes a PD-1 dominant negative receptor and the fourth coding sequence encodes a CD70-binding protein, as described herein.

[0186] Also provided are populations of engineered immune cells, e.g., CAR T cells. In some embodiments, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the population of engineered immune cells express the CD19 CAR described herein. In some embodiments, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the population of engineered immune cells express the CD19 CAR and the CD70-binding protein described herein. In some embodiments, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the population of engineered immune cells express the CD19 CAR and the CCR described herein. In some embodiments, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the population of engineered immune cells express the CD19 CAR, the CD70-binding protein and the CCR described herein.

[0187] Engineered Immune Cells

[0188] Provided herein are engineered immune cells expressing the CARs of the present disclosure (e.g., CAR-T cells). The engineered immune cells can be allogeneic or autologous with respect to the subject to which the engineered immune cells are administered.

[0189] In some embodiments, the engineered immune cell is a T cell (e.g., inflammatory T lymphocyte, cytotoxic T lymphocyte, regulatory T lymphocyte (Treg), helper T lymphocyte, tumor infiltrating lymphocyte (TIL)), natural killer (NK) cell, natural killer T cell (NKT), TCR-expressing cell, dendritic cell, killer dendritic cell, a mast cell, or a B-cell.

In some embodiments, the cell can be derived from the group consisting of CD4⁺ T-lymphocytes and CD8⁺ T-lymphocytes. In some exemplary embodiments, the engineered immune cell is a T cell. In some exemplary embodiments, the engineered immune cell is a gamma delta T cell. In some exemplary embodiments, the engineered immune cell is a macrophage. In some exemplary embodiments, the engineered immune cell is a natural killer (NK) cell.

[0190] In some embodiments, the engineered immune cell can be derived from, for example without limitation, a stem cell. The stem cells can be adult stem cells, non-human embryonic stem cells, more particularly non-human stem cells, cord blood stem cells, progenitor cells, bone marrow stem cells, induced pluripotent stem cells (iPSCs), totipotent stem cells or hematopoietic stem cells.

[0191] In some embodiments, the cell is obtained or prepared from peripheral blood. In some embodiments, the cell is obtained or prepared from peripheral blood mononuclear cells (PBMCs). In some embodiments, the cell is obtained or prepared from bone marrow. In some embodiments, the cell is obtained or prepared from umbilical cord blood. In some embodiments, the cell is a human cell.

[0192] In some embodiments, the cell is transfected or transduced by the nucleic acid vector using a method selected from the group consisting of electroporation, sonoporation, biolistics (e.g., Gene Gun), lipid transfection, polymer transfection, nanoparticles, viral transfection or transduction (e.g., retrovirus, lentivirus, AAV) or polyplexes.

[0193] In some embodiments, the engineered immune cells expressing at their cell surface membrane a CD19-specific CAR, and a CD70-binding protein and/or a CCR of the

disclosure comprise a percentage of stem cell memory and central memory cells greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. In some embodiments, the engineered immune cells expressing at their cell surface membrane a CD19-specific CAR, and a CD70-binding protein and/or a CCR of the disclosure comprise a percentage of

5 stem cell memory and central memory cells of about 10% to about 100%, about 10% to about 90%, about 10% to about 80%, about 10% to about 70%, about 10% to about 60%, about 10% to about 50%, about 10% to about 40%, about 10% to about 30%, about 10% to about 20%, about 15% to about 100%, about 15% to about 90%, about 15% to about 80%, about 15% to about 70%, about 15% to about 60%, about 15% to about 50%, about 15% to

10 about 40%, about 15% to about 30%, about 20% to about 100%, about 20% to about 90%, about 20% to about 80%, about 20% to about 70%, about 20% to about 60%, about 20% to about 50%, about 20% to about 40%, about 20% to about 30%, about 30% to about 100%, about 30% to about 90%, about 30% to about 80%, about 30% to about 70%, about 30% to about 60%, about 30% to about 50%, about 30% to about 40%, about 40% to about 100%,

15 about 40% to about 90%, about 40% to about 80%, about 40% to about 70%, about 40% to about 60%, about 40% to about 50%, about 50% to about 100%, about 50% to about 90%, about 50% to about 80%, about 50% to about 70%, about 50% to about 60%, about 60% to about 100%, about 60% to about 90%, about 60% to about 80%, about 60% to about 70%, about 70% to about 90%, about 70% to about 80%, about 80% to about 100%, about 80% to

20 about 90%, about 90% to about 100%, about 25% to about 50%, about 75% to about 100%, or about 50% to about 75%.

[0194] In some embodiments, the immune cell is an inflammatory T-lymphocyte that expresses the CD19-specific CAR and the CD70-binding protein described herein. In some embodiments, the immune cell is a cytotoxic T-lymphocyte that expresses any one of the

25 CD19-specific CARs and the CD70-binding protein described herein. In some embodiments, the immune cell is a regulatory T-lymphocyte that expresses any one of the CD19-specific CARs and the CD70-binding protein described herein. In some embodiments, the immune cell is a helper T-lymphocyte that expresses any one of the CARs described herein.

30 [0195] Prior to expansion and genetic modification, a source of cells can be obtained from a donor or a subject through a variety of non-limiting methods. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection,

ascites, pleural effusion, spleen tissue, and tumors. In some embodiments, any number of T cell lines available and known to those skilled in the art, may be used. In some embodiments, cells can be derived from a healthy donor or from a patient e.g. a patient diagnosed with cancer or from a patient diagnosed with an infection. In some embodiments, 5 cells can be part of a mixed population of cells which present different phenotypic characteristics.

[0196] Also provided herein are cell lines obtained from a transformed immune cell (e.g., T-cell) according to any of the above-described methods. Also provided herein are modified cells resistant to an immunosuppressive treatment. In some embodiments, an isolated cell 10 according to the disclosure comprises a polynucleotide encoding a CAR.

[0197] The immune cells of the disclosure can be activated and expanded, either prior to or after genetic modification of the immune cells, using methods as generally known. Generally, the engineered immune cells of the disclosure can be expanded, for example, by contacting with an agent that stimulates a CD3 TCR complex and a costimulatory molecule 15 on the surface of the T-cells to create an activation signal for the T cell. For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T cell.

[0198] In some embodiments, T cell populations may be stimulated in vitro by contact 20 with, for example, an anti-CD3 antibody such as an OKT3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an 25 anti-CD3 antibody (e.g., an OKT3 antibody) and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. The anti-CD3 antibody and an anti-CD28 antibody can be disposed on a bead, such as a plastic or magnetic bead, or plate or other substrate. Conditions appropriate for T cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain 30 factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-2, IL-15, TGF β , and TNF, or any other additives for the growth of cells known to the skilled

artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl- cysteine and 2-mercaptoethanol. Media can include RPMI 1640, A1M-V, DMEM, MEM, α - MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or

5 supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells (*e.g.*, IL-7 and/or IL-15). Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example,

10 an appropriate temperature (*e.g.*, 37° C) and atmosphere (*e.g.*, air plus 5% CO₂). T cells that have been exposed to varied stimulation times may exhibit different characteristics. In some embodiments, the cells of the disclosure can be expanded by co-culturing with tissue or cells. The cells can also be expanded *in vivo*, for example in the subject's blood after administering the cell into the subject.

15 [0199] In some embodiments, an engineered immune cell according to the present disclosure may comprise one or more disrupted or inactivated genes. In some embodiments, an engineered immune cell according to the present disclosure comprises one disrupted or inactivated gene selected from the group consisting of CD52, GR, PD-1, CD70, CTLA-4, LAG3, TIM3, BTLA, BY55, TIGIT, B7H5, LAIR1, SIGLEC10, 2B4, HLA,

20 TCR α and TCR β and/or expresses a CAR, a multi-chain CAR and/or a pT α transgene. In some embodiments, an isolated cell comprises polynucleotides encoding polypeptides comprising a multi-chain CAR. In some embodiments, the isolated cell according to the present disclosure comprises two disrupted or inactivated genes selected from the group consisting of: CD52 and GR, CD52 and TCR α , CD52 and TCR β , GR and TCR α , GR and

25 TCR β , TCR α and TCR β , PD-1 and TCR α , PD-1 and TCR β , CTLA-4 and TCR α , CTLA-4 and TCR β , LAG3 and TCR α , LAG3 and TCR β , TIM3 and TCR α , TIM3 and TCR β , BTLA and TCR α , BTLA and TCR β , BY55 and TCR α , BY55 and TCR β , TIGIT and TCR α , TIGIT and TCR β , B7H5 and TCR α , B7H5 and TCR β , LAIR1 and TCR α , LAIR1 and TCR β , SIGLEC10 and TCR α , SIGLEC10 and TCR β , 2B4 and TCR α , 2B4 and TCR β and/or

30 expresses a CAR, a multi-chain CAR and a pT α transgene. In some embodiments the method comprises disrupting or inactivating one or more genes by introducing into the cells an endonuclease able to selectively inactivate a gene by selective DNA cleavage. In some embodiments the endonuclease can be, for example, a zinc finger nuclease (ZFN),

megaTAL nuclease, meganuclease, transcription activator-like effector nuclease (TALE-nuclease/TALEN), or CRISPR (e.g., Cas9, Cas12, Cas12a, Cas12b or Cas12i) endonuclease or CRISPR reagents or CRISPR system. See, e.g., US11,168,324, US20230332119, US20230193243, and US20230235305.

5 [0200] In some embodiments, TCR is rendered not functional in the cells according to the disclosure by disrupting or inactivating TCR α gene and/or TCR β gene(s). Modified cells, which can proliferate independently of the TCR α signaling pathway are encompassed in the scope of the present disclosure. In some embodiments, a method to obtain modified cells derived from an individual is provided, wherein the cells can proliferate independently of
10 the major histocompatibility complex (MHC) signaling pathway. Modified cells, which can proliferate independently of the MHC signaling pathway, susceptible to be obtained by this method are encompassed in the scope of the present disclosure. Modified cells disclosed herein can be used for treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD); therefore in the scope of the present
15 disclosure is a method of treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD) comprising treating said patient by administering to said patient an effective amount of modified cells comprising disrupted or inactivated TCR α and/or TCR β genes.

[0201] In some embodiments, the immune cells are engineered to be resistant to one or
20 more chemotherapy drugs. The chemotherapy drug can be, for example, a purine nucleotide analogue (PNA), thus making the immune cell suitable for cancer treatment combining adoptive immunotherapy and chemotherapy. Exemplary PNAs include, for example, clofarabine, fludarabine, cyclophosphamide, and cytarabine, alone or in combination. PNAs are metabolized by deoxycytidine kinase (dCK) into mono-, di-, and tri-
25 phosphate PNA. Their tri-phosphate forms compete with ATP for DNA synthesis, act as pro-apoptotic agents, and are potent inhibitors of ribonucleotide reductase (RNR), which is involved in trinucleotide production. Provided herein are CD19-specific CAR-T cells comprising a disrupted or inactivated dCK gene. In some embodiments, the dCK knockout cells are made by transfection of T cells using polynucleotides encoding specific TAL-
30 nuclease directed against dCK genes by, for example, electroporation of mRNA encoding the specific TAL-nuclease. The dCK knockout CD19-specific CAR-T cells are resistant to PNAs, including for example clorofarabine and/or fludarabine, and maintain T cell cytotoxic activity toward CD19-expressing cells.

[0202] In some embodiments, isolated cells or cell lines of the disclosure can comprise a pTα or a functional variant thereof. In some embodiments, an isolated cell or cell line can be further genetically modified by disrupting or inactivating the TCRα gene.

[0203] The disclosure also provides engineered immune cells comprising any of the CAR
5 polynucleotides described herein. In some embodiments, a CAR can be introduced into an immune cell as a transgene via a plasmid vector. In some embodiments, the plasmid vector can also contain, for example, a selection marker which provides for identification and/or selection of cells which received the vector.

[0204] CAR polypeptides may be synthesized in situ in the cell after introduction of
10 polynucleotides encoding the CAR polypeptides into the cell. Alternatively, CAR polypeptides may be produced outside of cells, and then introduced into cells. Methods for introducing a polynucleotide construct into cells are known in the art. In some embodiments, stable transformation methods (e.g., using a lentiviral vector) can be used to integrate the polynucleotide construct into the genome of the cell. In other embodiments,
15 transient transformation methods can be used to transiently express the polynucleotide construct, and the polynucleotide construct not integrated into the genome of the cell. In other embodiments, virus-mediated methods can be used. The polynucleotides may be introduced into a cell by any suitable means such as for example, recombinant viral vectors (e.g., retroviruses, adenoviruses), liposomes, and the like. Transient transformation methods
20 include, for example without limitation, microinjection, electroporation or particle bombardment. Polynucleotides may be included in vectors, such as for example plasmid vectors or viral vectors.

[0205] In some embodiments, the nucleic acid construct is contained within a viral vector. In some embodiments, the viral vector is selected from the group consisting of retroviral
25 vectors, murine leukemia virus vectors, SFG vectors, adenoviral vectors, lentiviral vectors, adeno-associated virus (AAV) vectors, Herpes virus vectors, and vaccinia virus vectors. In some embodiments, the nucleic acid is contained within a plasmid

[0206] Methods of making engineering immune cells

[0207] Provided herein are methods of making the CARs and the CAR-containing
30 immune cells of the disclosure.

[0208] A variety of known techniques can be utilized in making the polynucleotides, polypeptides, vectors, antigen binding domains, immune cells, compositions, and the like according to the disclosure. Prior to the *in vitro* manipulation or genetic modification of the immune cells described herein, the cells may be obtained from a subject (e.g., a patient) or
 5 from a healthy donor.

[0209] a. Source Material

[0210] In some embodiments, the immune cells comprise T cells. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells (PBMCs), bone marrow, lymph nodes tissue, cord blood, thymus tissue, tissue from a site of infection,
 10 ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from the subject using any number of techniques known to the skilled person, such as FICOLL™ separation.

[0211] Cells may be obtained from the circulating blood of an individual by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes,
 15 granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In certain embodiments, the cells collected by apheresis may be washed to remove the plasma fraction, and placed in an appropriate buffer or media for subsequent processing.

[0212] In certain embodiments, T cells are isolated from PBMCs by lysing the red blood cells and depleting the monocytes, for example, using centrifugation through a
 20 PERCOLL™ gradient. A specific subpopulation of T cells, (e.g., CD28+, CD4+, CDS+, CD45RA-, CD45RO+, CDS+, CD62-, CD95-, CD95+, IL2Rβ+, IL2Rβ-, CCR7+, CCR7-, CDL-, CD62L+ and combinations thereof) can be further isolated by positive or negative selection techniques known in the art. In one example the subpopulation of T cells is CD45RA+, CD95-, IL-2Rβ-, CCR7+, CD62L+. In one example the subpopulation of T
 25 cells is CD45RA+, CD95+, IL-2Rβ+, CCR7+, CD62L+. In one example the subpopulation of T cells is CD45RO+, CD95+, IL-2Rβ+, CCR7+, CD62L+. In one example the subpopulation of T cells is CD45RO+, CD95+, IL-2Rβ+, CCR7-, CD62L-. In one example the subpopulation of T cells is CD45RA+, CD95+, IL-2Rβ+, CCR7-, CD62L-. For
 30 example, enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method for use herein is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed

to cell surface markers present on the cells negatively selected. For example, to enrich for CD4⁺ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. Flow cytometry and cell sorting may also be used to isolate cell populations of interest for use in the present disclosure.

[0213] PBMCs may be used directly for genetic modification with the immune cells (such as CARs or TCRs) using methods as described herein. In certain embodiments, after isolating the PBMCs, T lymphocytes can be further isolated and both cytotoxic and helper T lymphocytes can be sorted into naive, memory, and effector T cell subpopulations either before or after genetic modification and/or expansion.

[0214] In some embodiments, CD8⁺ cells are further sorted into naive, stem cell memory, central memory, and effector cells by identifying characteristic cell surface antigens that are associated with each of these types of CD8⁺ cells. In some embodiments, the expression of phenotypic markers of central memory T cells include CD45RO, CD62L, CCR7, CD28, CD3, and CD127 and are negative for granzyme B. In some embodiments, stem cell memory T cells are CD45RO⁻, CD62L⁺, CD8⁺ T cells. In some embodiments, central memory T cells are CD45RO⁺, CD62L⁺, CD8⁺ T cells. In some embodiments, effector T cells are negative for CD62L, CCR7, CD28, and CD127, and positive for granzyme B and perforin.

[0215] In certain embodiments, CD4⁺ T cells are further sorted into subpopulations. For example, CD4⁺ T helper cells can be sorted into naive, central memory, and effector cells by identifying cell populations that have characteristic cell surface antigens.

[0216] b. Stem Cell-derived Immune Cells

[0217] In some embodiments, the immune cells may be derived from stem cells, such as a progenitor cell, a bone marrow stem cell, an inducible pluripotent stem cell, an iPSC, a hematopoietic stem cell, and a mesenchymal stem cell. iPS cells and other types of stem cells may be cultivated immortal cell lines or isolated directly from a patient. Various methods for isolating, developing, and/or cultivating stem cells are known in the art and may be used to practice the present invention.

[0218] In some embodiments, the immune cell is an induced pluripotent stem cell (iPSC) derived from a reprogrammed T-cell. In some embodiments, the source material may be an

induced pluripotent stem cell (iPSC) derived from a T cell or non-T cell. The source material may alternatively be a B cell, or any other cell from peripheral blood mononuclear cell isolates, hematopoietic progenitor, hematopoietic stem cell, mesenchymal stem cell, adipose stem cell, or any other somatic cell type.

5 [0219] c. Genetic Modification of Isolated Cells

[0220] Methods of preparing the engineered cells, e.g., engineered immune cells, for use in immunotherapy are provided herein. In some embodiments, the methods comprise obtaining cells from a donor, e.g., donor immune cells, introducing the polypeptides described herein into the donor cells, and expanding the cells.

10 [0221] The immune cells, such as T cells, can be genetically modified following isolation using known methods, or the immune cells can be activated and expanded (or differentiated in the case of progenitors) *in vitro* prior to being genetically modified. In some embodiments, the isolated immune cells are genetically modified to reduce or eliminate expression of endogenous TCR α and/or CD52. In some embodiments, the cells are
15 genetically modified using gene editing technology (e.g., CRISPR/Cas9, CRISPR/CAS12, a zinc finger nuclease (ZFN), a TALEN or TALE-nuclease, a MegaTAL, a meganuclease) to reduce or eliminate expression of endogenous proteins (e.g., TCR α and/or CD52). In another embodiment, the immune cells, such as T cells, are optionally further genetically modified with the chimeric antigen receptors described herein (e.g., transduced with a viral
20 vector comprising one or more nucleotide sequences encoding a CAR) and then are activated and/or expanded *in vitro*.

[0222] Methods for activating and expanding T cells are known in the art and are described, for example, in U.S. Pat. No. 6,905,874; U.S. Pat. No. 6,867,041; U.S. Pat. No. 6,797,514; and PCT WO2012/079000, the contents of which are hereby incorporated by
25 reference in their entirety. Generally, such methods include contacting PBMCs or isolated T cells with a stimulatory molecule and a costimulatory molecule, such as anti-CD3 and anti-CD28 antibodies, generally attached to a plastic or magnetic bead or other surface, in a culture medium with appropriate cytokines, such as IL-2. Anti-CD3 and anti-CD28 antibodies attached to the same bead serve as a “surrogate” antigen presenting cell (APC).
30 One example is the Dynabeads® system, which is a CD3/CD28 activator/stimulator system for physiological activation of human T cells. In other embodiments, the T cells may be activated and stimulated to proliferate with feeder cells and appropriate antibodies and

cytokines using methods such as those described in U.S. Pat. No. 6,040,177; U.S. Pat. No. 5,827,642; and WO2012129514, the contents of which are hereby incorporated by reference in their entirety.

[0223] Certain methods for making the constructs and engineered immune cells of the disclosure are described in PCT application PCT/US15/14520, the contents of which are hereby incorporated by reference in their entirety.

[0224] It will be appreciated that PBMCs can further include other cytotoxic lymphocytes such as NK cells or NKT cells. An expression vector carrying the coding sequence of a chimeric receptor as disclosed herein can be introduced into a population of human donor T cells, NK cells or NKT cells. Successfully transduced T cells that carry the expression vector can be sorted using flow cytometry to isolate CD3 positive T cells and then further propagated to increase the number of these CAR expressing T cells in addition to cell activation using anti-CD3 antibodies and IL-2 or other methods known in the art as described elsewhere herein. Standard procedures are used for cryopreservation of T cells expressing the CAR for storage and/or preparation for use in a human subject. In one embodiment, the in vitro transduction, culture and/or expansion of T cells are performed in the absence of non-human animal derived products such as fetal calf serum and fetal bovine serum. In an embodiment, cryopreservation can comprise freezing in a suitable medium, such as CryoStor® CS10, CryoStor® CS2 or CryoStor® CS5 (BioLife Solutions).

[0225] For cloning of polynucleotides, the vector may be introduced into a host cell (an isolated host cell) to allow replication of the vector itself and thereby amplify the copies of the polynucleotide contained therein. The cloning vectors may contain sequence components generally include, without limitation, an origin of replication, promoter sequences, transcription initiation sequences, enhancer sequences, and selectable markers. These elements may be selected as appropriate by a person of ordinary skill in the art. For example, the origin of replication may be selected to promote autonomous replication of the vector in the host cell.

[0226] In certain embodiments, the present disclosure provides isolated host cells containing the vector provided herein. The host cells containing the vector may be useful in expression or cloning of the polynucleotide contained in the vector. Suitable host cells can include, without limitation, prokaryotic cells, fungal cells, yeast cells, or higher eukaryotic cells such as mammalian cells, and more specifically human cells.

[0227] The vector can be introduced to the host cell using any suitable methods known in the art, including, without limitation, DEAE-dextran mediated delivery, calcium phosphate precipitate method, cationic lipids mediated delivery, liposome mediated transfection, electroporation, microprojectile bombardment, receptor-mediated gene delivery, delivery mediated by polylysine, histone, chitosan, and peptides. Standard methods for viral transfection, transduction and/or transformation of cells for expression of a vector of interest are well known in the art. In a further embodiment, a mixture of different expression vectors can be used in genetically modifying a donor population of immune effector cells wherein each vector encodes a different CAR or different recombinant protein as disclosed herein.

5 mediated by polylysine, histone, chitosan, and peptides. Standard methods for viral transfection, transduction and/or transformation of cells for expression of a vector of interest are well known in the art. In a further embodiment, a mixture of different expression vectors can be used in genetically modifying a donor population of immune effector cells wherein each vector encodes a different CAR or different recombinant protein as disclosed herein.

10 The resulting transduced immune effector cells form a mixed population of engineered cells, with a proportion of the engineered cells expressing more than one different CARs or recombinant proteins.

[0228] In one embodiment, the disclosure provides a method of storing genetically engineered cells expressing CARs which target a CD19 protein. In an embodiment this involves cryopreserving the immune cells such that the cells remain viable upon thawing. In an embodiment, cryopreservation can comprise freezing in a suitable medium, such as CryoStor® CS10, CryoStor® CS2 or CryoStor® CS5 (BioLife Solutions). A fraction of the immune cells expressing the CARs can be cryopreserved by methods known in the art to provide a permanent source of such cells for the future treatment of patients afflicted with a malignancy. When needed, the cryopreserved transformed immune cells can be thawed, grown and expanded for more such cells.

15 involves cryopreserving the immune cells such that the cells remain viable upon thawing. In an embodiment, cryopreservation can comprise freezing in a suitable medium, such as CryoStor® CS10, CryoStor® CS2 or CryoStor® CS5 (BioLife Solutions). A fraction of the immune cells expressing the CARs can be cryopreserved by methods known in the art to provide a permanent source of such cells for the future treatment of patients afflicted with a malignancy. When needed, the cryopreserved transformed immune cells can be thawed, grown and expanded for more such cells.

20 malignancy. When needed, the cryopreserved transformed immune cells can be thawed, grown and expanded for more such cells.

[0229] In some embodiments, the cells are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a “pharmaceutically acceptable” carrier) in a treatment-effective amount. Suitable infusion media can be any isotonic medium formulation, typically normal saline, Normosol™ R (Abbott) or Plasma-Lyte™ A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumin.

25 effective amount. Suitable infusion media can be any isotonic medium formulation, typically normal saline, Normosol™ R (Abbott) or Plasma-Lyte™ A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumin.

[0230] The present invention provides methods for generating populations of engineered cells, e.g., engineered immune cells, that comprise and/or express the polynucleotides described herein. In one embodiment, the polynucleotides can be introduced into cells using a targeted approach such that the polynucleotide or transgene is inserted or integrated

30 cells, e.g., engineered immune cells, that comprise and/or express the polynucleotides described herein. In one embodiment, the polynucleotides can be introduced into cells using a targeted approach such that the polynucleotide or transgene is inserted or integrated

in a target gene of interest, at a specific location in the genome. In one embodiment, the targeted approach, or site-specific integration, comprises the use of a nuclease, such as a rare-cutting endonuclease, that can cleave at a specific site within the target gene to allow insertion of the polynucleotide or nucleic acid construct comprising the transgene, at the specific cleavage site. In one embodiment, the expression of the one or more coding sequences in the polynucleotide or nucleic acid construct is under the control of a PGK promoter, e.g., a truncated PGK promoter described herein.

[0231] Prior to engineering of cells, a source of cells can be obtained from a subject through a variety of non-limiting methods. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In some embodiments, any number of T cell lines available and known to those skilled in the art, may be used. In some embodiments, cells can be derived from a healthy donor. In some embodiments, cells can be part of a mixed population of cells which present different phenotypic characteristics.

[0232] In one aspect, the invention provides methods for making genetically modified immune cells that comprise one or more genetic modifications to one or more target genes that are involved in T cell receptor (TCR) $\alpha\beta$ function or activity. In one embodiment, the target gene is TRAC. In another embodiment, the target gene is CD52. The genetic modifications are made such that the expression and/or activities of the one or more target genes are disrupted or inactivated. By disruption or inactivating a target gene, it is intended that the target gene is not expressed in a functional protein form and the activity of the gene product is impaired.

[0233] In some embodiments, the method comprises inactivating one or more target genes by introducing into the cells a rare-cutting endonuclease capable of selectively inactivating the target gene by introducing a specific double strand break at the target sequence within the target gene. In other embodiments the method comprises inactivating or reducing the expression level of one or more genes by introducing into the cells a rare-cutting endonuclease capable of selectively inactivate a gene by selective DNA cleavage. In some embodiments the rare-cutting endonuclease can be, for example, a transcription activator-like effector nuclease (TALE-nuclease or TALEN), a megaTAL endonuclease, a zinc finger endonuclease, a meganuclease, or a CRISPR endonuclease (e.g., a Cas9, Cas12, Cas12a or

Cas12i). In one embodiment, the rare-cutting endonuclease is a CRISPR endonuclease. In one embodiment, the rare-cutting endonuclease is not a TALE-nuclease.

[0234] In one aspect, the inactivation of a target gene is via the use of a CRISPR endonuclease. A CRISPR endonuclease refers to a clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease, such as Cas9 or Cas12. Such endonucleases associate with a guide RNA (gRNA) that can direct cleavage by the endonuclease at a specific site through hybridization at a target region. In this case, one or more gRNA molecules can be designed to hybridize to one or more specific sites within one or more target genes to direct cleavage by at least one CRISPR endonuclease. Exemplary gRNAs targeting the TRAC gene and CD52 gene are provided in Table 3 as SEQ ID NOs: 46 and 47, respectively.

[0235] In another aspect, the rare-cutting endonuclease may be introduced to the cells before, simultaneously or after genetic modification of the cells, e.g., immune cells. In one embodiment, a polynucleotide encoding or expressing the rare-cutting endonuclease is introduced into the cells by a suitable method including, without limitation, transfection, lipofection, transduction, and electroporation. In some embodiments, the polynucleotides encoding the rare-cutting endonuclease according to the present disclosure can be mRNA which is introduced directly into the cells, for example by electroporation. In certain embodiments, the cells are introduced with a preformed gRNA and CRISPR endonuclease complex by a suitable method, e.g., transfection or electroporation. In some embodiments, cytoPulse technology can be used to transiently permeabilize living cells for delivery of material into the cells. Parameters can be modified to determine conditions for high transfection efficiency with minimal mortality.

[0236] Also provided herein are methods of transfecting a cell, such as an immune cell e.g., a T cell. In some embodiments, the method comprises: contacting a cell with a nucleic acid, e.g., an RNA, and applying to the cell an agile pulse sequence consisting of: (a) an electrical pulse with a voltage range from about 2250 to 3000 V per centimeter; (b) a pulse width of 0.1 ms; (c) a pulse interval of about 0.2 to 10 ms between the electrical pulses of step (a) and (b); (d) an electrical pulse with a voltage range from about 2250 to 3000 V per centimeter with a pulse width of about 100 ms and a pulse interval of about 100 ms between the electrical pulse of step (b) and the first electrical pulse of step (c); and (e) four electrical

pulses with a voltage of about 325 V with a pulse width of about 0.2 ms and a pulse interval of 2 ms between each of 4 electrical pulses.

[0237] In some embodiments, a method of transfecting a cell, such as an immune cell, e.g., a T cell, comprises contacting the cell with RNA and applying to the cell an agile pulse sequence comprising: (a) an electrical pulse with a voltage of about 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2400, 2450, 2500, 2600, 2700, 2800, 2900 or 3000V per centimeter; (b) a pulse width of 0.1 ms; (c) and a pulse interval of about 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 ms between the electrical pulses of step (a) and (b); (d) one electrical pulse with a voltage range from about 2250 to 3000 V per centimeter, e.g. of 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2400, 2450, 2500, 2600, 2700, 2800, 2900 or 3000V per centimeter with a pulse width of 100 ms and a pulse interval of 100 ms between the electrical pulse of step (b) and the first electrical pulse of step (c); and (e) 4 electrical pulses with a voltage of about 325 V with a pulse width of about 0.2 ms and a pulse interval of about 2 ms between each of 4 electrical pulses. Any values included in the value range described above are disclosed in the present application. Electroporation medium can be any suitable medium known in the art. In some embodiments, the electroporation medium has conductivity in a range spanning about 0.01 to about 1.0 milliSiemens.

[0238] In other embodiments, the rare-cutting endonuclease has specificity for a nucleic acid recognition sequence within the target gene. In another embodiment, the endonuclease is expressed in the cell following introduction and generates a double-stranded break or cleavage site at the nucleic acid recognition sequence within the target gene. The presence of the double-stranded break or cleavage site allows for a site-specific integration of a transgene via the recombinant vector, as further described herein.

[0239] An engineered cell, e.g., an engineered immune cell, can be genetically engineered to express one or more transgenes. The transgene can be introduced into the genome of a cell by a variety of methods, including retroviral or lentiviral transduction. The transgene delivered to the cell in a retroviral or lentiviral vector becomes randomly integrated into the genome of the cell after viral transduction. Random integration of the transgene may negatively or undesirably affect the activities of the engineered cells. Thus, in some embodiments, the one or more transgenes are introduced into a predetermined locus in the engineered immune cell by site-specific integration.

[0240] In another aspect, the site-specific integration methods for making genetically modified cells, e.g., genetically modified immune cells, comprising the use of homology directed repair (HDR) is provided. In one embodiment, the HDR comprises homologous recombination (HR). In another embodiment, the method comprises use of a recombinant
5 vector configured to use HDR, e.g., HR, to facilitate integration of a transgene into a cell, e.g., an immune cell. In another embodiment, the methods comprise the use of a polynucleotide that contains one or more sequences with homology to sequences of a predetermined integration site, e.g., a target gene. In one other embodiment, the methods do not comprise the use of non-homologous end joining (NHEJ) and/or are not performed
10 without the use of a polynucleotide that contains one or more sequences with homology to sequences of a predetermined integration site, e.g., a target gene.

[0241] In one aspect, the site-specific integration method comprises the introduction of a recombinant viral vector, e.g., an AAV vector, to a population of cells, e.g., immune cells, wherein the recombinant viral vector is targeted to the double-stranded break or cleavage
15 site in the target sequence of a target gene. In certain embodiments, the AAV vector is an AAV6 vector. The introduction of the recombinant viral vector to the cell can be before, simultaneously or after introduction of a rare-cutting endonuclease to the cell, as further described herein. In one embodiment, the method comprises providing a recombinant viral vector comprising a donor template. The donor template comprises one or more coding
20 sequences or transgenes. In other embodiments, the donor template further comprises flanking homology arms. In one other embodiment, the flanking homology arms comprise a 5' homology arm and a 3' homology arm. In another embodiment, the 5' homology arm comprises sequences homologous to sequences 5' upstream of the double stranded break or cleavage site in the target sequence, and the 3' homology arm comprises sequences
25 homologous to sequences 3' downstream of the double stranded break or cleavage site in the target sequence. In another embodiment, the donor template is integrated into the genomes of a population of cells, e.g., a population of immune cells, at the double-stranded break or cleavage site. In one additional embodiment, the method comprises culturing the cell following introduction of the recombinant viral vector under conditions sufficient to
30 allow integration of the transgene. In one embodiment, the expression of the receptor polypeptide and/or the nucleic acid inhibitory agent is under the control of a PGK promoter, e.g., a truncated PGK promoter.

[0242] In some embodiments, the methods comprise introducing a donor template comprising a transgene into cells, e.g., immune cells, and expanding the cells. In some embodiments, the disclosure relates to a method of engineering a cell, e.g., an immune cell, the method comprising the step of providing a cell, e.g., an immune cell, and expressing one or more transgenes. In some embodiments, the method comprises: introducing into the cell (such as by transfecting the cell) with at least one polynucleotide described herein and expressing the one or more coding sequences or transgenes comprised in the polypeptide. In one embodiment, the expression of the one or more coding sequences or transgenes is under the control of a PGK promoter, e.g., a truncated PGK promoter.

[0243] In some embodiments, the cells, e.g., engineered immune cells, generated using a site-specific integration (SSI) method express a higher level of the one or more transgenes introduced as compared to cells where transgenes were introduced by a non-SSI method, e.g., a retroviral or lentiviral transduction method. In some embodiments, the engineered cells, e.g., engineered immune cells, generated by an SSI method exhibit improved activities of the one or more transgenes introduced as compared to cells where transgenes were introduced by a non-SSI method, e.g., a retroviral or lentiviral transduction method. In some embodiments, the engineered cells, e.g., engineered immune cells, generated using an SSI method show less genome translocation of the one or more transgenes as compared to cells wherein transgenes were introduced by a non-SSI method, e.g., a retroviral or lentiviral transduction method.

[0244] In some embodiments, the one or more transgenes are introduced into the target gene locus by site-specific integration. In some embodiments, the engineered cell, e.g., engineered immune cell, comprises additional genetic modifications, including without limitation, knock-out or knock-down of endogenous gene(s), enhancement of endogenous gene(s), and integration of exogenous gene(s). In some embodiments, the engineered immune comprises a transgene integrated into a first genetic locus and further comprises one or more genetic modifications at a second genetic locus. In some embodiments, the one or more genetic modifications at the first and/or second genetic locus comprises integration of a transgene at the first and/or second genetic locus. In one embodiment, the expression of the coding sequences from the transgene (e.g., coding sequences for a receptor polypeptide and/or a nucleic acid inhibitory agent) is under the control of a PGK promoter, e.g., a truncated PGK promoter.

[0245] In some embodiments, the method comprises a step of introducing into the cells a polynucleotide, nucleic acid construct or molecule, wherein the polynucleotide, nucleic construct or molecule comprises a polynucleotide template for homologous recombination. In other embodiments, the polynucleotide template comprises at least a sequence

- 5 homologous to a portion of a nucleic acid sequence in a target gene, such that homologous recombination occurs between the target gene nucleic acid sequence and the polynucleotide template. In some embodiments, said polynucleotide template comprises a 5' homology arm homologous to a sequence 5' to the double strand break of the target sequence in the target gene, a sequence encoding a transgene, and a 3' homology arm homologous to a
- 10 sequence 3' to the double strand break of the target sequence in the target gene. Following cleavage of the target nucleic acid sequence, a homologous recombination event occurs between the target nucleic acid sequence and the polynucleotide template.

[0246] d. Allogeneic CAR T cells

[0247] In brief, the process for manufacturing allogeneic CAR T therapy involves

- 15 harvesting healthy, selected, screened and tested PBMCs or T cells from healthy donors. Allogeneic T cells are gene editing to reduce the risk of graft versus host disease (GvHD) and to prevent allogeneic rejection. A selected T cell receptor gene (e.g., TCR α , TCR β) is knocked out to avoid GvHD. The CD52 gene can also be knocked out to render the CAR T product resistant to anti-CD52 antibody treatment. Anti-CD52 antibody treatment can
- 20 therefore be used to lymphodeplete the host immune system and allow the CAR T cells to stay engrafted to achieve full therapeutic impact. Exemplary anti-CD52 antibody can be, e.g., alemtuzumab (SEQ ID NOs: 67-74). Next, the T cells are engineered to express CARs, which recognize certain cell surface proteins (e.g., CD19) that are expressed in, e.g., hematologic tumors. The engineered T cells then undergo a purification step and are
- 25 ultimately cryopreserved in vials for delivery to patients.

[0248] e. Autologous CAR T cells

[0249] Autologous chimeric antigen receptor (CAR) T cell therapy involves collecting a patient's own cells (e.g., white blood cells, including T cells) and genetically engineering the T cells to express CARs that recognize a target antigen expressed on the cell surface of

30 one or more specific cancer cells and kill cancer cells. The engineered cells are then cryopreserved and subsequently administered to the patient from which the cells were removed for engineering.

[0250] Methods of Treatment

[0251] The disclosure comprises methods for treating or preventing a condition associated with CD19 and/or CD70 or an undesired and/or elevated levels of CD19 and/or CD70 in a patient, comprising administering to a patient in need thereof an effective amount of at least one CAR containing immune cell, or immune-cell comprising a CAR disclosed herein. In some embodiments, the CD70-binding protein or CD70 CAR targets CD70+ alloreactive T cells in a patient and allows greater persistence and expansion of the engineered immune cell. In some embodiments, the engineered immune cell comprises a chimeric antigen receptor (CAR).

[0252] Methods are provided for treating diseases or disorders, including cancer and autoimmune diseases. In some embodiments, the disclosure relates to creating a T cell-mediated immune response in a subject, comprising administering an effective amount of the engineered immune cells disclosed herein to the subject. In some embodiments, the T cell-mediated immune response is directed against a target cell or cells.

[0253] The CAR containing immune cells of the disclosure can be used to treat a CD19-associated disease or disorder and/or a CD70-associated disease or disorder. In some embodiments, the disease or disorder is CD19-associated and/or CD70-associated autoimmune disease or disorder. CD19 is known to be expressed in plasma cells producing autoreactive antibodies. And CD70 expression is associated with acute or chronic immune activation, including diseases such as rheumatoid arthritis and systemic lupus erythematosus, and autoreactive B cells also express CD70. It has also been shown that CD70 is involved in the differentiation of proinflammatory pathogenic lymphocytes with the ability to infiltrate the CNS.

[0254] Accordingly, in some embodiments, the disease or disorder can be an autoimmune disease, including, without limitation, lupus, systemic lupus erythematosus (SLE), lupus nephritis, rheumatoid arthritis, systemic sclerosis, scleroderma, multiple sclerosis (MS), relapse remitting multiple sclerosis (RRMS), secondary progressive (SPMS), primary progressive (PPMS), neuromyelitis optica spectrum disorder (NMOSD), Sjogren's syndrome, stiff person syndrome, graft v. host disease (GvHD), autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), and myositis. In some embodiments, the autoimmune disease or disorder is B cell-associated and/or T cell-associated autoimmune disease or disorder. In some embodiments, the autoimmune disease or disorder is associated

with CD19+ and/or CD70+ B cells, and/or CD19+ and/or CD70+ T cells. In some embodiments, the autoimmune disease or disorder is associated with CD19+ B cells, and/or CD70+ T cells. In some embodiments, the autoimmune disease or disorder is associated with CD19+, CD70+ B cells, and/or CD70+ T cells. In some embodiments, the autoimmune disease or disorder is associated with CD19+ B cells, and/or CD19+, CD70+ T cells. In some embodiments, the autoimmune disease or disorder is associated with CD19+, CD70+ B cells, and/or CD19+, CD70+ T cells. In some embodiments, the CAR containing immune cells of the disclosure can be used to deplete pathogenic B cells and T cells in autoimmune patients.

- 10 [0255] In certain embodiments, the pathogenic B cells and/or T cells are activated B cells and/or activated T cells. In certain embodiments, the pathogenic B cells and/or T cells are autoreactive B cells and/or autoreactive T cells. In some embodiments, the activated B cells and activated T cells express CD19 and/or CD70. The engineered immune cells disclosed herein deplete or are capable of depleting activated B cells, short- and long-lived plasma
15 cells, and/or activated T cells without affecting naïve B cells and/or non-activated T cells.

- [0256] In some embodiments, the target cell is a tumor cell. In some aspects the disclosure comprises a method for treating or preventing a malignancy, said method comprising administering to a subject in need thereof an effective amount of at least one isolated antigen binding domain described herein. In some aspects, the disclosure comprises
20 a method for treating or preventing a malignancy, said method comprising administering to a subject in need thereof an effective amount of an immune cell, wherein the immune cell comprises at least one chimeric antigen receptor, and a CD70-binding protein and/or CCR as described herein. In some embodiments, the method for treating or preventing a malignancy comprises administering to a subject in need thereof an effective amount of a
25 population of immune cells, wherein the immune cells comprise at least one chimeric antigen receptor, and a CD70-binding protein and/or CCR as described herein. The CAR containing immune cells of the disclosure can be used to treat malignancies associated with CD19 or involving aberrant expression of CD19. In some embodiments, CAR containing immune cells of the disclosure can be used to treat such malignancies as non-Hodgkin's
30 lymphoma (NHL), refractory and/or relapse NHL, large B cell lymphoma (LBCL), follicular lymphoma (FL), T cell lymphoma (TCL), B cell acute lymphoblastic leukemia (BALL), T cell acute lymphoblastic leukemia (TALL), primary central nervous system lymphoma (PCNSL), Mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL),

peripheral T cell lymphoma (PTCL). In exemplary embodiments, the CAR-containing immune cells, e.g., the anti-CD19 CAR-T cells of the disclosure, are used to treat LBCL.

[0257] Also provided are methods for reducing the size of a tumor in a subject, comprising administering to the subject an engineered cell of the present disclosure to the
5 subject, wherein the cell comprises a chimeric antigen receptor comprising a CD19 antigen binding domain and binds to a CD19 antigen on the tumor.

[0258] In some embodiments, the subject has a solid tumor, or a blood malignancy such as lymphoma or leukemia. In some embodiments, the engineered cell is delivered to a tumor bed, such as a tumor bed found in small cell lung cancer. In some embodiments, the cancer
10 is present in the bone marrow of the subject. In some embodiments, the engineered cells are autologous immune cells, e.g., autologous T cells. In some embodiments, the engineered cells are allogeneic immune cells, e.g., allogeneic T cells. In some embodiments, the engineered cells are heterologous immune cells, e.g., heterologous T cells. In some
15 embodiments, the engineered cells are transfected or transduced ex vivo. As used herein, the term “in vitro cell” refers to any cell that is cultured ex vivo.

[0259] An “effective amount” is any amount that, when used alone or in combination with another agent, provides desired or beneficial results. A “therapeutically effective amount,” “effective dose,” or “therapeutically effective dosage” of a therapeutic agent, e.g.,
20 engineered CAR T cells, is any amount that, when used alone or in combination with another therapeutic agent, protects a subject against the onset of a disease or promotes disease regression evidenced by a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. The ability of a therapeutic agent to promote disease
25 regression can be evaluated using a variety of methods known to the skilled practitioner (e.g., a physician or clinician), such as in human subjects during clinical trials, in animal model systems predictive of efficacy in humans, or by assaying the activity of the agent in in vitro assays.

[0260] The terms “patient” and “subject” are used interchangeably and include human and non-human animal subjects as well as those with formally diagnosed disorders, those
30 without formally recognized disorders, those receiving medical attention, those at risk of developing the disorders, etc.

[0261] The term “treat” and “treatment” includes therapeutic treatments, prophylactic treatments, and applications in which one reduces the risk that a subject will develop a disorder or other risk factor. Treatment does not require the complete curing of a disorder and encompasses embodiments in which one reduces symptoms or underlying risk factors.

- 5 The term “prevent” does not require the 100% elimination of the possibility of an event. Rather, it denotes that the likelihood of the occurrence of the event has been reduced in the presence of the compound, immune cells, therapeutic agent or method.

- [0262] Desired treatment total amounts of cells in the composition comprise at least 2 cells (for example, at least one CD8⁺ T cell and at least one CD4⁺ T cell, or two CD8⁺ T cells, or two CD4⁺ T cells) or is more typically greater than 10^2 cells, and up to 10^6 , up to and including 10^8 or 10^9 cells and can be 10^{10} or 10^{12} or more cells. The number of cells will depend upon the desired use for which the composition is intended, and the type of cells included therein. The density of the desired cells is typically greater than 10^6 cells/ml and generally is greater than 10^7 cells/ml, generally 10^8 cells/ml or greater. The clinically
15 relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , or 10^{12} cells. In some aspects of the present disclosure, particularly since all the infused cells will be redirected to a particular target antigen (e.g., CD19), lower numbers of cells, in the range of 10^6 /kilogram (10^6 - 10^{11} per patient) may be administered. CAR treatments may be administered multiple
20 times at dosages within these ranges. The cells may be autologous, allogeneic, or heterologous with respect to the patient undergoing therapy.

- [0263] In some embodiments, the therapeutically effective amount of the CAR T cells is about 1×10^5 cells/kg, about 2×10^5 cells/kg, about 3×10^5 cells/kg, about 4×10^5 cells/kg, about 5×10^5 cells/kg, about 6×10^5 cells/kg, about 7×10^5 cells/kg, about 8×10^5 cells/kg, about 9×10^5 cells/kg, 2×10^6 cells/kg, about 3×10^6 cells/kg, about 4×10^6 cells/kg, about 5×10^6 cells/kg, about 6×10^6 cells/kg, about 7×10^6 cells/kg, about 8×10^6 cells/kg, about 9×10^6 cells/kg, about 1×10^7 cells/kg, about 2×10^7 cells/kg, about 3×10^7 cells/kg, about 4×10^7 cells/kg, about 5×10^7 cells/kg, about 6×10^7 cells/kg, about 7×10^7 cells/kg, about 8×10^7 cells/kg, or about 9×10^7 cells/kg.

- 30 [0264] In some embodiments, target doses for CAR⁺/CAR-T⁺ cells range from about 1×10^6 to about 1×10^{10} cells/kg, for example about 1×10^6 cells/kg, about 1×10^7 cells/kg, about 1×10^8 cells/kg, about 1×10^9 cells/kg or about 1×10^{10} cells/kg. It will be appreciated

that doses above and below this range may be appropriate for certain subjects, and appropriate dose levels can be determined by the healthcare provider as needed. Additionally, multiple doses of cells can be provided in accordance with the disclosure.

[0265] In some aspects the disclosure comprises a pharmaceutical composition comprising at least one CD19-specific CAR as described herein and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition further comprises an additional active agent.

[0266] The CAR expressing cell populations of the present disclosure may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations. Pharmaceutical compositions of the present disclosure may comprise a CAR expressing cell population, such as T cells, as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present disclosure may be formulated for intravenous administration.

[0267] The pharmaceutical compositions (solutions, suspensions or the like), may include one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono- or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. For therapeutic applications, an injectable pharmaceutical composition is preferably sterile.

[0268] In some embodiments, upon administration to a patient, engineered immune cells expressing at their cell surface any of the CD19-specific CARs described herein may reduce, kill or lyse endogenous CD19-expressing cells of the patient. In one embodiment, a

percentage reduction or lysis of CD19-expressing endogenous cells or cells of a cell line expressing CD19 by engineered immune cells expressing any one of the CD19-specific CARs described herein is at least about or greater than 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%. In one

- 5 embodiment, a percentage reduction or lysis of CD19-expressing endogenous cells or cells of a cell line expressing CD19 by engineered immune cells expressing any one of the CD19-specific CARs described herein is about 5% to about 95%, about 10% to about 95%, about 10% to about 90%, about 10% to about 80%, about 10% to about 70%, about 10% to about 60%, about 10% to about 50%, about 10% to about 40%, about 20% to about 90%,
10 about 20% to about 80%, about 20% to about 70%, about 20% to about 60%, about 20% to about 50%, about 25% to about 75%, or about 25% to about 60%. In one embodiment, the endogenous CD19-expressing cells are endogenous CD19-expressing bone marrow cells.

- [0269] In one embodiment, the percent reduction or lysis of target cells, *e.g.*, a cell line expressing CD19, by engineered immune cells expressing at their cell surface membrane a
15 CD19-specific CAR of the disclosure can be measured using the assay disclosed herein.

- [0270] The methods can further comprise administering one or more chemotherapeutic agents to a patient prior to administering the engineered cells provided herein. In certain embodiments, the chemotherapeutic agent is a lymphodepleting (preconditioning) chemotherapeutic. For example, methods of conditioning a patient in need of a T cell
20 therapy comprising administering to the patient specified beneficial doses of cyclophosphamide (between 200 mg/m²/day and 2000 mg/m²/day, about 100 mg/m²/day and about 2000 mg/m²/day; *e.g.*, about 100 mg/m²/day, about 200 mg/m²/day, about 300 mg/m²/day, about 400 mg/m²/day, about 500 mg/m²/day, about 600 mg/m²/day, about 700 mg/m²/day, about 800 mg/m²/day, about 900 mg/m²/day, about 1000 mg/m²/day, about
25 1500 mg/m²/day or about 2000 mg/m²/day) and specified doses of fludarabine (between 20 mg/m²/day and 900 mg/m²/day, between about 10 mg/m²/day and about 900 mg/m²/day; *e.g.*, about 10 mg/m²/day, about 20 mg/m²/day, about 30 mg/m²/day, about 40 mg/m²/day, about 40 mg/m²/day, about 50 mg/m²/day, about 60 mg/m²/day, about 70 mg/m²/day, about 80 mg/m²/day, about 90 mg/m²/day, about 100 mg/m²/day, about 500 mg/m²/day or about
30 900 mg/m²/day). An exemplary dosing regimen involves treating a patient comprising administering daily to the patient about 300 mg/m²/day of cyclophosphamide in combination or before or after administering about 30 mg/m²/day of fludarabine for three

days prior to administration of a therapeutically effective amount of engineered T cells to the patient.

[0271] In some embodiments, notably in the case when the engineered cells provided herein have been gene edited to eliminate or minimize surface expression of CD52, lymphodepletion further comprises administration of an anti-CD52 antibody, such as alemtuzumab. In some embodiments, the CD52 antibody is administered at a dose of about 1-20 mg/day IV, e.g., about 13 mg/day IV, e.g., about 20 mg/day IV, e.g., about 30 mg/day IV, for 1, 2, 3 or more days. The antibody can be administered in combination with, before, or after administration of other elements of a lymphodepletion regime (e.g., cyclophosphamide and/or fludarabine). Exemplary anti-CD52 antibody sequences are provided in Table 3 as SEQ ID NOs: 67-74.

[0272] In other embodiments, the antigen binding domain, transduced (or otherwise engineered) cells and the chemotherapeutic agent are administered each in an amount effective to treat the disease or condition in the subject.

[0273] In certain embodiments, compositions comprising CAR-expressing immune effector cells disclosed herein may be administered in conjunction with any number of chemotherapeutic agents. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as

denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptapurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanol, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2', 2''-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., paclitaxel (TAXOL™, Bristol-Myers Squibb) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RF S2000; difluoromethylomithine (DMFO); retinoic acid derivatives such as Targretin™ (bexarotene), Panretin™, (alitretinoin); ONTAK™ (denileukin diftitox); esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Combinations of chemotherapeutic agents are also administered where appropriate, including, but not limited to CHOP, i.e., Cyclophosphamide (Cytosan®), Doxorubicin (hydroxydoxorubicin), Vincristine (Oncovin®), and Prednisone.

[0274] In some embodiments, the chemotherapeutic agent is administered at the same time or within one week after the administration of the engineered cell, polypeptide, or nucleic acid. In other embodiments, the chemotherapeutic agent is administered from about

1-7 days, about 1 to about 4 weeks or from about 1 week to about 1 month, about 1 week to about 2 months, about 1 week to about 3 months, about 1 week to about 6 months, about 1 week to about 9 months, or about 1 week to about 12 months after the administration of the engineered cell, polypeptide, or nucleic acid. In other embodiments, the chemotherapeutic agent is administered at least 1 month before administering the cell, polypeptide, or nucleic acid. In some embodiments, the methods further comprise administering two or more chemotherapeutic agents.

[0275] A variety of additional therapeutic agents may be used in conjunction with the compositions described herein. For example, potentially useful additional therapeutic agents include PD-1 inhibitors such as nivolumab (Opdivo®), pembrolizumab (Keytruda®),

[0276] Additional therapeutic agents suitable for use in combination with the disclosure include, but are not limited to, ibrutinib (Imbruvica®), ofatumumab (Arzerra®), rituximab (Rituxan®), bevacizumab (Avastin®), trastuzumab (Herceptin®), trastuzumab emtansine (KADCYLA®), imatinib (Gleevec®), cetuximab (Erbix®), panitumumab (Vectibix®), catumaxomab, ibritumomab, ofatumumab, tositumomab, brentuximab, alemtuzumab, gemtuzumab, erlotinib, gefitinib, vandetanib, afatinib, lapatinib, neratinib, axitinib, masitinib, pazopanib, sunitinib, sorafenib, toceranib, lestaurtinib, axitinib, cediranib, lenvatinib, nintedanib, pazopanib, regorafenib, semaxanib, sorafenib, sunitinib, tivozanib, toceranib, vandetanib, entrectinib, cabozantinib, imatinib, dasatinib, nilotinib, ponatinib, radotinib, bosutinib, lestaurtinib, ruxolitinib, pacritinib, cobimetinib, selumetinib, trametinib, binimetinib, alectinib, ceritinib, crizotinib, aflibercept, adipotide, denileukin diftotox, mTOR inhibitors such as Everolimus and Temsirolimus, hedgehog inhibitors such as sonidegib and vismodegib, CDK inhibitors such as CDK inhibitor (palbociclib).

[0277] In some embodiments, the composition comprising CAR-containing immune cells described herein may be administered with a therapeutic regimen to prevent or reduce cytokine release syndrome (CRS) or neurotoxicity. The therapeutic regimen to prevent cytokine release syndrome (CRS) or neurotoxicity may include lenzilumab, tocilizumab, atrial natriuretic peptide (ANP), anakinra, iNOS inhibitors (e.g., L-NIL or 1400W). In additional embodiments, the composition comprising CAR-containing immune cells can be administered with an anti-inflammatory agent. Anti-inflammatory agents or drugs include, but are not limited to, steroids and glucocorticoids (including betamethasone, budesonide,

dexamethasone, hydrocortisone acetate, hydrocortisone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone), nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, ibuprofen, naproxen, methotrexate, sulfasalazine, leflunomide, anti-TNF medications, cyclophosphamide and mycophenolate.

- 5 Exemplary NSAIDs include ibuprofen, naproxen, naproxen sodium, Cox-2 inhibitors, and sialylates. Exemplary analgesics include acetaminophen, oxycodone, tramadol of propoxyphene hydrochloride. Exemplary glucocorticoids include cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, or prednisone. Exemplary biological response modifiers include molecules directed against cell surface
- 10 markers (e.g., CD4, CD5, etc.), cytokine inhibitors, such as the TNF antagonists, (e.g., etanercept (ENBREL®), adalimumab (HUMIRA®) and infliximab (REMICADE®), chemokine inhibitors and adhesion molecule inhibitors. The biological response modifiers include monoclonal antibodies as well as recombinant forms of molecules. Exemplary
- 15 DMARDs include azathioprine, cyclophosphamide, cyclosporine, methotrexate, penicillamine, leflunomide, sulfasalazine, hydroxychloroquine, Gold (oral (auranofin) and intramuscular) and minocycline. In certain embodiments, the composition described herein can be used for treating an autoimmune disease or disorder.

- [0278] In certain embodiments, the compositions described herein are administered in conjunction with a cytokine. Examples of cytokines are lymphokines, monokines, and
- 20 traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor (HGF); fibroblast
- 25 growth factor (FGF); prolactin; placental lactogen; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors (NGFs) such as NGF-beta; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons
- 30 such as interferon-alpha, beta, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-15, IL-21 a tumor necrosis factor such as TNF-alpha or

TNF-beta; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture, and biologically active equivalents of the native sequence cytokines.

[0279] Kits and Articles of Manufacture

- 5 [0280] The present application provides kits comprising any one of the CD19 CAR containing immune cells described herein, and pharmaceutical compositions of the same. In some embodiments, the CD19 CAR T cells comprise or express a CD70-specific CAR. In an embodiment of a kit the engineered CAR cells (e.g., CAR T cells) are frozen in a suitable medium, such as CryoStor® CS10, CryoStor® CS2 or CryoStor® CS5 (BioLife Solutions).
- 10 [0281] In some exemplary embodiments, a kit of the disclosure comprises allogeneic CD19 CAR-containing T-cells for administering to the subject a lymphodepletion regimen and a CAR-T regimen.

[0282] The present application also provides articles of manufacture comprising any one of the therapeutic compositions or kits described herein. Examples of an article of

- 15 manufacture include bags or vials (e.g., sealed vials).

[0283] Table 3 below shows exemplary sequences.

SEQ ID NO:	Description	Sequence
1	CD8 signal peptide	MALPVTALLPLALLHAARP
2	aCD19 VH	EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWGSETTYNS ALKSRLTI IKDNSKSQVFLKMNSLQTDATAIYYCAKHYYGGSYAMDYWGQTSVTSS
3	aCD19 VL	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSR FSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEIT
4	aCD19 scFv	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSR FSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGSGGGGS EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWGSETTYNS ALKSRLTI IKDNSKSQVFLKMNSLQTDATAIYYCAKHYYGGSYAMDYWGQTSVTSS
5	aCD19 CAR full-length aa sequence underlined indicates signal peptide	MALPVTALLPLALLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNW YQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQG NTLPYTFGGGKLEITGGGSGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVS GVSLPDYGVSWIRQPPRKGLEWLGVWGSETTYNSALKSRLTI IKDNSKSQVFLK MNSLQTDATAIYYCAKHYYGGSYAMDYWGQTSVTSSSTTPAPRPPTPAPTAS QPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQN QLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKMAEAYSEI GMKGERRRGKGDGLYQGLSTATKDTYDALHMQALPPR
6	aCD19 CAR full-length DNA sequence	atggctctgcccgtcaccgcactgctgctcccctggctctgctgctgcacgccgaagaccgatatccagatgaccc agaccaccagcagcctgctgctctctctgggagatagagtactatttctgtaggccagccaggacatctctaagta cctgaactgtaccagcagaaacctgacggcacagtgaactgctgatctaccacacaagccgctgcacagcgcc

		gtccctcttagattcagcgcgagcggtctggaacagattacagcctgacaactcagcaacctggaacaggagacat gccacatacttctgccagcaagggaacacctgccctacacctttggcggaggcaccgaagctggaaatcaccgagc gcgcgcgagcggcgcgaggaagcgaggcgcgagcagaggtgaagctgcaggagagcggtcctggcctg gtggcccttagccagagcctgagcgtgacatgaccgtgtccggtgtccctgctgattacggcggttcttgatcc ggcagcctccaagaaggcgctggaatggctggcggtgactctgggctctgagacaacctactataatagcgtctg aaaagcagactgacctatcaaggacaactctaagagccaagtgttctggaagtgaacagcctccagaccgagc acaccgccatctactactgcgcaagcactactattacggcggtcctacgccatggactactggggacagggcacca gcgtgaccgtgtccagcaccacaacccagcaccaaggccacctacacctgcaccaacctcgctctcagccactg agcctgagaccgaggcctgtaggcctgcagcaggaggagcagtgacacccggggactggactttgctgcgata ctacatctgggcaccactggcaggaacatgtggcgtgctgctgagcctggtcatcacctgtactgcaagagagg caggaagaagctgtgtatatcttcaagcagcccttatgcgccctgtgcagacaacccaggaggaggatggctgct ctgtcggttcccagaggaggaggaggaggatgtgagctgcgctgaagttttccggctgtccgagcaccagcat accagcaggggccagaaccagctgtataacgagctgaatctggccggagagaggagtacgactgctggataaga ggagggggaagagatcccagatgggaggcaagccacggagaaaagaacccaggaggcgctgtacaatgagctg cagaaggataagatggccgaggcctatagcgagatcgcatgaaggagagaggcgccggggcgaaggacacga cggcctgtatcaggcgctgtccaccgctaccaaagacacctatgatgtctgtcacatgcaggctctgccaccaaga
7	aCD19 CAR full-length aa sequence w/o the signal peptide	DIQMTQTSSLSASLGDRVTISCRASQDISKYNWYQQKPDGTVKLLIYHTSRLHS GVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGGGGS GGGSGGGGSEVKLQESGPGLVAPSQLSVTCTVSGVSLPDYGVSWIRQPPRKGLE WLGVIWGSSETTYNSALKSRLTI IKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYG GSYAMDYWGQTSVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR
8	aCD19 CAR full-length DNA sequence w/o the signal peptide	gatatccagatgaccagaccaccagcagcctgtctgtcttctgggagatagagtactatttctgtaggccagcc aggacatctctaagtaactgtaactgttaccagcagaaacctgacggcacagtgaaactcgtgatctaccacacaagc cggctgcacagcggcgctccccttagattcagcgcgagcggcctctggaacagattacagcctgacaatcagcaact ggaacaggaggacatcgccacatacttctgcagcaaggcaacacctgcccctacaccttggcggaggaccaagc tggaatcaccggcgcgcgagcggcgagggaagcggaggcggcgagcagaggtgaagctgcaggag agcggtcctggcctgtgtggccccctagccagagcgtgacatgacagtgctccggcgtgtccctgctgattac ggcgtttcctggatccggcagcctccaagaaggcgctggaatggctggcggtgatctggggctctgagacaacct ctataatagcgtctgaaaagcagactgacctatcaaggacaactctaagagccaagtgttctgaaagtgaaca gcctccagaccgacgacaccgccatctactactgcgccaagcactactattacggcggtcctacgccatggactact ggggacagggcaccagcgtgacctgtccagcaccacaacccagcaccaaggccacctacacctgcaccaacct cgctctcagccactgagcctgagaccgaggcctgtaggcctgcagcaggaggagcagtgacacccggggactg gactttgctgcgatatctacatctgggcaccactggcaggaacatgtggcgtgctgctgagcctggtcatcccc tgtactgcaagagaggcaggaagaagctgctgtatatcttcaagcagcccttatgcgccctgtgcagacaaccagg aggaggatggctgctctgtcggttcccagaggaggaggaggaggatgtgagctgcgctgaagttttccggct gccgagcaccagcataaccagcagggccagaaccagctgtataacgagctgaatctggccggagagaggagtac gacgtgtggataagaggagggaagagatcccagatgggaggcaagccacggagaaaagaacccaggaggg cctgtacaatgagctgcagaaggataagatggccgaggcctatagcgagatcgcatgaaggagagaggcgccg gggcaagggacacgagcgctgtatcaggcgctgtccaccgtactcaaagacacctatgatgtctgcacatgcagg ctctgccaccaaga
9	CD8 hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
10	CD8 transmembr ane	IYIWAPLAGTCGVLLLSLVITLYC
11	CD3z (wt)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
12	4-1BB ICD	KRGRKKLLYIFKQPFMRPVQTTQEEEDGCSCRFEEEEGGCEL

13	CD28 ICD (wt)	RSKRSRLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS
14	P2A	ATNFSLLKQAGDVEENPGP
15	T2A	EGRGSLLTCGDVEENPGP
16	Anti-CD70 VH	QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVTWIRQPPGKALEWLAHIFSN DEKSYSTSLKSRLTISKDTSKTQVVLMTNMDPVDATATYYCARIRDYDISSYYDY WGQGTLSVSVSS
17	Anti-CD70 VL	DIQMTQSPSAMSASVGDRTITCRASQDISNYLAWFQQKPGKVPKRLIYAASSLQS GVPSRFSGSGSGTEFTLTISSLLPEDFATYYCLQLNSFPFTFGGGTKVEIN
18	Anti-CD70 scFv	QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVTWIRQPPGKALEWLAHIFSN DEKSYSTSLKSRLTISKDTSKTQVVLMTNMDPVDATATYYCARIRDYDISSYYDY WGQGTLSVSVSSGGGSGGGGSGGGGSDIQMTQSPSAMSASVGDRTITCRASQDIS NYLAWFQQKPGKVPKRLIYAASSLQSGVPSRFSGSGSGTEFTLTISSLLPEDFATY YCLQLNSFPFTFGGGTKVEIN
19	CD70-binding protein CD70z aa sequence, signal peptide underlined	<u>MALPVTALLLPLALLLHAARPQ</u> VTLKESGPVLVKPTETLTLTCTVSGFSLSNARMG VTWIRQPPGKALEWLAHIFSNDEKSYSTSLKSRLTISKDTSKTQVVLMTNMDPVD TATYYCARIRDYDISSYYDYWGQGTLSVSVSSGGGSGGGGSGGGGSDIQMTQSPS AMSASVGDRTITCRASQDISNYLAWFQQKPGKVPKRLIYAASSLQSGVPSRFSGS GSGTEFTLTISSLLPEDFATYYCLQLNSFPFTFGGGTKVEINTTTPAPRPPTPAPT IASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCR VKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRRGKGHGDLGYQGLSTATKDTYDALHMQALPPR
20	CD70-binding protein CD70z DNA sequence	atggccctgcctgtgaccgccctgctgctgccactcgccctgctgctgcatgccgc tagaccccaggtgacactgaaggaaagtgacactgtgctcgtgaaacctaccgaga cactgacactgacctgtaccgtgtctggcttcagcctgtctaacgcccggatgggc gtgacatggatcagacagcctccaggcaaggccctggaatggctggctcacatctt cagcaatgacgagaagtcctacagcaccagcctgaagagccggctgaccatcagca aggacacaagcaagaccaggttgctgctgacctgacaaacatggaccccgctggac acagccacctaactactgtgccagaatccgggactactatgacatttctagctacta cgattactggggccagggcaccctgggtgtccgtgagcagcgccggaggaggctcag gcggaggaggctccggcgccggaggcagcgacatccagatgacctcctccatct gctatgagcgccagcgtgggtgatagagtgaacatcacctgcagagcctcccagga catcagcaattacctggcctgggtccagcagaagcccggaagtgccaaagagac tgatctacgcccagcagcctgcaaagcgccgtccccagcagattcagcggtct ggcagcggaaccgagttcacccttacaatcagcagcctgctgccagaagatttcgc cacatactactgcctgcagctgaacagctttcctttcaccttcggcgaggcacga aggtggaatcaacaccaccacacctgctcccagacctcctacaccgcacctacc atcgctcccagcccctgtccctgagacctgaggcctgcccggcccgctgctggcgg cgccgtccacaccagaggcctggactgtgctgctgagcctggctacacctgactgcaga gtgaagttcagcagaagcgccgatgcccctgcctaccagcaaggccagaaccagct ttataatgagctgaacctgggacggagggaagagtacgacgtgctggataagcggc ggggcagagatcctgagatgggaggcaagcctagaagaaagaacccccaggagggc ctgtacaacgagctgcaaaaagacaagatggccgaggcctacagcgagatcgcat gaaaggcgagcgccggcgccggaagggccacgacggcctgtaccaaggcctgtcta ccgccaccaaggatacatatgacgcctgcacatgcaggctctgcctcctaga
21	CD70-binding protein CD70z aa sequence w/o the signal peptide	QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVTWIRQPPGKALEWLAHIFSN DEKSYSTSLKSRLTISKDTSKTQVVLMTNMDPVDATATYYCARIRDYDISSYYDY WGQGTLSVSVSSGGGSGGGGSGGGGSDIQMTQSPSAMSASVGDRTITCRASQDIS NYLAWFQQKPGKVPKRLIYAASSLQSGVPSRFSGSGSGTEFTLTISSLLPEDFATY YCLQLNSFPFTFGGGTKVEINTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCRVKFSRSADAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKG ERRRGKGHGDLGYQGLSTATKDTYDALHMQALPPR

22	CD70-binding protein CD70z DNA sequence w/o the signal peptide	cagggtgacactgaaggaaagtggacctgtgctcgtgaaacctaccgagacactgacactgacctgtaccgtgtctggcttcagcctgtctaacgcccggatgggctgacatggatcagacagcctccaggcaaggccctggaatggctggctcacatcttcagcaatgacgagaagtccacagcaccagcctgaagagccggtgaccatcagcaaggacacaagcaagacccagggttgctgctgaccatgacaaacatggaccccgaggacacagccacctactactgtgcccagaatccgggactactatgacatttctagctactacgattactggggccaggggcaccctgggtgtccgtgagcagcggcggaggaggctcaggcggaggaggctccggcggcggaggcagcgcacatccagatgaccagctctccatctgctatgagcgccagcgtgggtgatagagtgaacatcacctgcagagcctcccaggacatcagcaattacctggcctgggtccagcagaagcccggcaagtgccaaagagactgatctacgcccgcagcagcctgcaaagcggcgctcccagcagattcagcggctctggcagcgggaaccgagttcacccttacaatcagcagcctgctgcccagaagatttcgccacatactactgctgcagctgaacagctttcctttcaccttcggcggaggcacgaaggtggaatcaacaccaccacacactgctcccagacctcctacaccgcacctaccatcgctcccagccccctgtccctgagacctgaggcctgcccggcccgtgctggcggcgccgtccacaccagaggcctggactttgctgacatctacatttgggccccctctggccggcacctgcggcgtgctgctgctgagcctggctcatcacctgtactgcagagtgaagttcagcagaagcgccgatgcccctgcctaccagcaaggccagaaccagctttataatgagctgaacctgggacggagggaagagtacgacgtgctggataagcggcggggcagagatcctgagatgggaggcaagcctagaagaaagaacccccaggagggcctgtacaacgagctgcaaaaagacaagatggccgaggcctacagcgagatcggcataaaaggcgagcggcggcgccggaaggccacgacggcctgtaccaaggcctgtctaccgccaccaaggatacatatgacgcctgcacatgcaggctctgcctcctaga
23	(G4S)3	GGGSGGGSGGGSGGGS
24	(G4S)4	GGGSGGGSGGGSGGGSGGGS
25	TPOR transmembrane used in Tail 3 (478-582;H499L, S505N,W515K)	SDPTRVETATETAWISLVTA <u>L</u> LVLGLN <u>NA</u> VLGLLLLR <u>K</u> QFFAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPPKATVSDTCEEVEPSLLEILPKSSERTPLPL
26	IL2R β (339-379,393-433,518-551)	QQDKVPEPASLSSNHSLSCTFTNQGYFFFHLPDALEIEACQDEGVAGAPTGSSPQLPLSGEDDAYCTFPSRDDLLLFSPSGQGEFRALNARLPLNTDAYLSLQELQGQDP THLV
27	Exemplary CCR	<u>M</u> ALPVTALLLPLALLLHAARPSDPTRVETATETAWISLVTA <u>L</u> LVLGLN <u>NA</u> VLGLLLLRKQFFAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPPKATVSDTCEEVEPSLLEILPKSSERTPLPLLEQQDKVPEPASLSSNHSLSCTFTNQGYFFFHLPDALEIEACQDEGVAGAPTGSSPQPLPLSGEDDAYCTFPSRDDLLLFSPSGQGEFRALNARLPLNTDAYLSLQELQGQDP THLV
28	Exemplary CCR DNA sequence	atggccctgcagtgaccgcctgctgctgacctggccctgctgctgcacgcagcaaggccatcagaccctactagagtcgagaccgctaccgagaccgcttgatctctctggtgaccgcctgctgctggtgctgggctgaaccgctgctggcctgctgctgtaggaagcagttccacgacactaccggagactgaggcacgcactgtggccaagcctgcccagctgcacagggtgctgggacagtatctgagggatacagccgctgagcccacctaaggcaaccgtgtccgacatgcgaggaggtggaaccaagtctgctggaaatcctgcctgagcggacaccctgcccctgctgagcagcaggacaagggtcccgcctgctccctgagctccaaccacagcctgacctccttacaacatcagggtactctctttccactgctgacgcccctggagatcaggcctgtcaggatgaggagtggcaggagcacctaccggctcagcccacagccactgcagccactgtctggagaggacgatgcctactgcacattcccagccgggacgatgctgctgtttccctcttggcagggagaggtccgggcccgaagcgaagctgacctgaataccgacgctatctgctctgcaggagctgcaggccaggaccacacacctgtg
29	Exemplary CCR w/o	SDPTRVETATETAWISLVTA <u>L</u> LVLGLN <u>NA</u> VLGLLLLRKQFFAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPPKATVSDTCEEVEPSLLEILPKSSERTPLPLLEQQDKV

	the signal peptide	PEPASLSSNHSLTSCFTNQYFFFHLPDALEIEACQDEGVAGAPTGSSPQLQLPLSGEDDAYCTFPSRDDLLLFSPSQGGEFRALNARLPLNTDAYLSLQELQGQDPHTLV
30	Exemplary CCR w/o the signal peptide DNA sequence	tcagaccctactagagtcgagaccgctaccgagaccgcttgatctctctggtagccctgctgctggctggggcc tgaacgccgtgctgggcctgctgctgctgaggaagcagttccagcacactaccggagactgaggcacgactgtgg ccaagcctgcccacctgcacagggtgctgggacagtatctgaggatacagccgctgagcccactaaggcaac cgtgtccgacacatgcgaggaggtggaaccaagtctgctggaatcctgcaaaatcctctgagcggacacccctgc ccctgctcagcagcaggacaagggtcccagcctgcctccctgagctccaaccacagcctgacctcctgctttacaa atcagggtacttcttttccacctgcctgacgcccctggagatcaggcctgtcaggatgaggagtgaggagcac ctaccggctctagcccacagccactgcagccactgtctggagaggacgatgctactgcacattcccagcgggacg atctgctgctgttttcccttctggacaggagagttccgggcccgaacgcaagactgccactgaataccgacgccta tctgtctcgcaggagctgcagggccaggacccacacacctgggtg
31	EF1a promoter (EFL)	gagtaattcatacaaaaggactcgccccctgccttggggaatcccagggaccgtcgt taaaactcccactaacgtagAACCCAGAGATCGCTGCGTTCGCCGCCCTCACCCGC ccgctctcgtcatcactgaggtggagaagagcatgctgagggctccggtgcccgtc agtgggcagagcgacatcgccacagtcctccgagaagttggggggaggggtcggc aattgaaccggtgcttagagaaggtggcgcggggtaaaactgggaaagtgatgtcgt gtactggctccgctttttcccgagggtgggggagaaacgatatataagtgcagtag tcgcccgtgaacgttctttttcgcaacgggtttgcccgcagaacacaggtaaagtgc gtgtgtggttcccgcgggctggcctctttacgggttatggcccttgctgccttg aattacttccacgcccctggctgcagtgatcttctgatcccgagcttcgggtt ggaagtgggtgggagagttcgaggccttgcgcttaaggagcccccttcgctcgtgc ttgagttgaggcctggcttgggcgctggggcgccgctgcgaatctggtggcacc ttcgcgctgtctcgtgctttcgataagtctctagccattttaaatttttgatga cctgctgcgacgctttttttctggcaagatagtcttgtaaagtgcgggccaagatct gcacactggtatttccgttttttggggcgccggggcgagcggggcccgtgctccc agcgacatgttcggcgaggcggggctgcgagcgccgaccgagaatcggacgg gggtagtctcaagctggccggcctgctctggtgcctggcctcgccgcccgtgat cgccccgccttggcgggcaaggtggcccggctggcaccagttgctgtagcggaaa gatggccgcttcccggccctgctgcaggagctcaaaatggaggacgcggcgctcg ggagagcggcggggtgagtcacccacacaaaggaaaagggccttccgctcctcagc cgtcgcttcatgtgactccacggagtagccggcgccgctcaggcacctcgattagt tctccgagcttttgagtagctcgctcttttaggttgggggaggggttttatgcat ggagtttccccacactgagtggtgggagactgaagttaggacgcttggcacttga tgtaattctccttggaaattgcccctttttgagtttggatcttgggtcattctcaag cctcagacagtggttcaaagttttttcttccatttcagggtgctcgtga
32	EFS promoter	GAGTAATTCATACAAAAGGACTCGCCCCCTGCCTTGGGGAATCCCAGGGACCGTCGT TAAACTCCCCTAACGTAGAACCCAGAGATCGCTGCGTTCGCCGCCCTCACCCGC CCGCTCTCGTCATCACTGAGGTGGAGAAGAGCATGCGTGAGGCTCCGGTGCCCGTC AGTGGGCAGAGCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCCGGC AATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGT GTACTGGCTCCGCCTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAG TCGCCGTGAACGTTCTTTTCGCAACGGGTTTGC GCCCAGAACAC
33	TRAC endogenous promoter	tgctgggccttttccatgcctgcttactctgcagagttatattgctggggtttgaagaagatcctattaaataaa agaataagcagttatttaagtagccctgcatttcagggttccttgagtgaggcaggcctggccgtgaacgttcac tgaaatcatggcctcttgccaagattgatgcttgctgcttcctgagtcacagtcacgagcagctggttcta agatgctatttccgtataaagcatgagaccgtgacttgccagccccacagagcccccttgcctcatcactggcatc tgactccagcctgggttggggcaagagggaaatgagatcatgtcctaaccctgatccttcttccacaga
34	Long PGK promoter Bold/underlined indicates	GGACCTGGGCCTCTTCCAACCTCTGAGAGGTCTCTATTACTAAGTAAGCCTTAAGAAGCAG AATTCCATGAAGGGAGCTAGGAAACCAGGATTTTCCAAAAGGAGGTGGCATTTCATTGAT CCTGGTAGGGCAGCCTCGAATTCACGGGGTTGGGGTTGCGCTTTTCCAAGGCAGCCCTG GGTTTGCGCAGGGACGCGGCTGCTCTGGGCGTGGTTCGGGAAACGACGCGGCGCCGACCC TGGGTCTCGCACATTCTTCACGTCCGTTTCGCAGCGTCACCCGGATCTTCGCCGTACCCCTT GTGGGCCCCCGCGACGCTTCTGCTCCGCCCTAAGTCGGGAAGGTTCTTGCGGTTTCG CGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTACCCTCGCAGACGGACA

	transcription start site	GCGCCAGGGAGCAATGGCAGCGCGCCGACCGCATGGGCTGTGGCCAATAGCGGCTGCTCA GCAGGGCGCGCCGAGAGCAGCGCGCGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGG TAGTGTGGGCCCTGTTCCCTGCCCGCGCGGT GTTCCGCATT CTGCAAGCCTCCGGAGCGCAC GTCGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCCAGCTGTATT
35	PGK SSI-1	TCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCGA TGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGCGCGGGAAGGGG CGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCCTGCCCGCGCGGT GTTCC CGCATT CTGCAAGCCTCCGGAGCGCACGTGCGCAGTCGGCTCCCTCGTTGACCGAATCACCG GACCTCTCTCCCCAGCTGTATT
36	PGK SSI-2	CCCTTGTGGGCCCCCGGCGACGCTTCCTGCTCCGCCCTAAGTCGGGAAGGTTCCCTGCG GTTCCGCGCGGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTACCCTCGCAGAC GGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCATGGGCTGTGGCCAATAGCGGCT GCTCAGCAGGGCGCGCCGAGAGCAGCGCCGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGG GGCGGTAGTGTGGGCCCTGTTCCCTGCCCGCGCGGT GTTCCGCATT CTGCAAGCCTCCGGAG CGCAGTCGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCCAGCTGTATT
37	PGK SSI-3	GGCTGCTCTGGGCGTGGTTCGGGAAACGCAGCGGCGCCGACCTGGGTCTCGCACATTCT TCACGTCCGTTTCGCAGCGTCACCCGGATCTTCGCCGTACCCCTTGTGGGCCCCCGGCGAC GCTTCCTGCTCCGCCCCCTAAGTCGGGAAGGTTCCCTGCGGTTTCGCCGCGTGCCTGACGTGA CAAACGGAAGCCGCACGTCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGG CAGCGCGCCGACCGCATGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAG CAGCGGCCGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTT CTGCCCGCGCGGT GTTCCGCATT CTGCAAGCCTCCGGAGCGCACGTGCGCAGTCGGCTCC TCGTTGACCGAATCACCGACCTCTCTCCCCAGCTGTATT
38	PGK SSI-4	AGGAGGTGGCATTTCATTGATCCTGGTAGGGCAGCCTCGAATTCCACGGGTTGGGGTTG CGCCTTTTCCAAGGCAGCCCTGGGTTTTCGCAGGGACGCGGCTGCTCTGGGCGTGGTTCCG GGAACGCAGCGCGCCGACCTGGGTCTCGCACATTCTTCACGTCCGTTTCGCAGCGTCAC CCGATCTTCGCCGCTACCCCTGTGGGCCCGCGCGACGCTTCCTGCTCCGCCCTAAGT CGGGAAGGTTCCCTGCGGTTTCGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCA CTAGTACCCTCGCAGACGGACAGCGCCAGGAGCAATGGCAGCGCGCCGACCGCATGGG TGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGGCCGGGAAGGGGCGGTG CGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCCTGCCCGCGCGGT GTTCCGCAT TCTGCAAGCCTCCGGAGCGCACGTGCGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCT CTCTCCCCAGCTGTATT
39	UBB300	GCAAGGAAGTTTCCAGAGCTTTCGAGGAAGGTTTCTTCAACTCAAATTCATCCGCTGATA ATTTTCTTATATTTTCTAAAGAAGGAAGAGAAGCGCATAGAGGAGAAGGGAAATAATTTT TTAGGAGCCTTTCTTACGGCTATGAGGAATTTGGGGCTCAGTTGAAAAGCCTAAACTGCCT CTCGGGAGGTTGGGCGCGCGAACTACTTTCAGCGGCGCACGGAGACGGCGTCTACGTGAG GGGTGATAAGTGACGCAACACTCGTTGCATAAATTTGCGCTCCGCCAGCCCGAGCATTTA GGGGCGGTTGGCTTTGTTGGGTGAGCTTGTGTGTCCTGTGGGTGGACGTGGTTGGTGA TTGGCAGGATCCTGGTATCCGCTAAC
40	UBC300	TGGATCCTGCTTACAATAATCGTACCCATTGAAACCAGAGAAATTTCCATGCCTCCCTGTT GGCATCAAGTAGGACCTTTCCATACCTGGCCCTCAGAATGTACAGGAAGGTGGAAGAACAG TCTAGAAGGATGTCGTTTCGCTCAGCCTTTCGTTCCAGCTAAAATAAACTGTGTGGGGTTT CCGCTCTTTTTTCCAATTTAACCTGGACACCCAGCTCCTCTGCAGTGTCTCCCTTGAA AGTTCTCGAGCGTTCCCGAGCTTTAGGGCCACGCCCGCCTGAGATCTGCCGAGTCATTGT CCTTGTCCCGCGGCCCCGGGAGCCCCCGCGACCGGCCTGGGAGGCTCAGGGAGGTTGAAG GGGCTGAGCAAAGGAAGCCCCGTATTACCTCAAATGTGACCCAAAATAAAGACCCGTC CATCTCGCA
41	CypA300	CTGCCCACCCCGTTCCGCACTGACCCTCCCCCGTGCCCGCGTCCCGTACTGCCGCCCCG CCCCGAGTCCCATGCCGCAGCCACCGCGACGGAGCCCGCAGGCGGGAACCTGCCTCCGCGC GTAGCGCGCACGCGCGCCTCATGTGTCGTCCCATCAGCGCGGCTTCCGTCTATAGGCC AGATGCACTGTCACTCTGGCGAAGTCGCAGACCCGATTGGCCGGGACGGAGGCGCGAGACC GGGTTGCGGGCGGGCCGAACGTGGTATAAAAGGGGCGGAGGCCAGGCTCGTGCCGTTTT GCAGACGCCACCGCGAGGAAACCGTGTACTA
42	Exemplary TRAC 5' homology arm	gagtttttagacatcattgaccagagctctgggcagaacctggccattcctgaagcaaggaacagcctgcaagggca ccaaagctgcccttacctgggctggggaagaagggtcttctggaataatgctgttgtaaggcgtttgcacatgcaa agtcagattgttgctccaggccacagcactgttgctcttgaagccatagacctcatgtctagcacagttttgtctgtga

		tatacacatcagaatccttactttgtgacacatttgtttgagaatcaaaatcggtgaataggcagacagacttgcact ggatttagagtctctcagctggttacacggcagggtcggtc
43	Exemplary TRAC 3' homology arm	tagggttctggatatctgtgggacaagaggatcagggttaggacatgatctcatttcctcttggcccaaccaggct ggagtcagatgccagtgatggacaagggcgggctctgtgggctggcaagtacgggtctcatgctttatacggga aatagcatcttagaaccagctgctcgtgatggactgggactcaggacaggcacaagctatcaatcttggccaaga ggccatgatttcagtgaacgttcacggccaggcctggcctgccactcaaggaaacctgaaatgcagggtacttaata atactgcttattcttttatttaatagatcttctcaaaacccagcaatataactctggcagagtaaaggcaggcatgg gaaaaaggcccagca
44	Exemplary CD52 5' homology arm	cttcctcactcagggttgtcttccaagtctcttagttctctattattccacaacacccttgggtgccccaacgcgccta gcacctgtaagtgtctcccagctgtggatacaggttctctcagggcagcccgaagcagtgacagccagccaga agagctggaaggctggcgccaggcccgtgtgtcccagtttctgttggagtcacatctgtgtgtctcaggaga gaaggctgggtctctgggagctcacatctgtatgcctccctccatgccaagcaacttggggagtcctggcagtggt gccaggcgttgctcttacctgtaccataaccagg
45	Exemplary CD52 3' homology arm	gaggctgatggtgagtaggaggaagaggaagcgcttcatttggtaggatcttcgtggctgtcttggtagcagctttt aggtgatctcagggtgtctggaggctcttggtttagctgctttgaaccaggaggcagaggaggcggggtgaggt agggagaggaaggagggttacaagggtggaggagggtgtggcttcttctgcagatctgggggtggataatacca ggctctaggttctaaggccctggctgggggagcaggctagatcgtacaagataggagagaggtcaatccaggagga acgggtgggagtaagtagggcccaaggcctggaatcatgcatcctgcaggctgaatcctggttaatcactgaccagct ctgagaccctgggcca
46	Exemplary CRISPR gRNA for TRAC	UCAGGGUUCUGGAUAUCUGU
47	Exemplary CRISPR gRNA for CD52	CAGAUACAAACUGGACUCUC
48	CD8 signal peptide	ATGGCACTCCCCGTAAGTCTGCTGCTGCCGTTGGCATTGCTCCTGCACGCCGCACGCC CG
49	CD70 aa sequence	MPEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLVVCIQRFAQAQQQLPLESLGWD VAELQLNHTGPQQDPRLYWQGGPALGRSFLHGPDLKQGLRIHRDGIYMVHIQVTL AICSSTTASRHHPTTAVGICSPASRSISLLRLSFHQGCTIASQRLTPLARGDTLC TNLTGTLLPSRNTDETFFGVQWVRP
50	TPOR/MPL R	MPSWALFMVTSCLLLAPQNLAQVSSQDVSLASDSEPLKCFSRTEFEDLTCFWDEEE AAPSGTYQLLYAYPREKPRACPLSSQSMPHFGTRYVCQFPDQEEVRLFFPLHLWVK NVFLNQTRTQRVLFVDSVGLPAPPSIIKAMGGSQPGELQISWEEPAPPEISDFLRYE LRYGPRDPKNSTGPTVIQLIATETCCPALQRPHSASALDQSP CAQPTMPWQDGPQKQTSPSREASALTAEGGSCLISGLQPGNSYWLQLRSEPDGISLG GSWGSWSLPVTVDLPDAVALGLQCFTLDLKNVTCQWQQQDHASSQGFFYHSRARC CPRDRYPIWENCEEEEEKTNPGLQTPQFSRCHFCSRNDIIHILVEVTTAPGTVHSY LGSPFWIHQAVRLPTPNLHWREISSGHLELEWQHPSSWAAQETCYQLRYTGEHQD WKVLEPPLGARGGTLELRPSRYRLQLRARLNGPTYQGPWSSWSDPTRVETATETA WISLVTALHLVLGLSAVLGLLLLLRWQFPAHYRRLRHALWPSLPDLHRVLGQYLRDT AALSPPKATVSDTCEEVEPSLLEILPKSSERTPLPLCSSQAQMDYRRLQPSCLGTM PLSVCPMAESGSCCTTHIANHSYLPLS YWQQP
51	TPOR/MPL R 478-582	SDPTRVETATETAWISLVTALHLVLGLSAVLGLLLLLRWQFPAHYRRLRHALWPSLP DLHRVLGQYLRDTAALSPPKATVSDTCEEVEPSLLEILPKSSERTPLPL
52	PD-1 wt ectodomain (21-170)	PGWFLDSPDRPWNPTTFSPALLVVTEGDNATFTCSFSNTSESFVLNWMSPSNQT DKLAAFPEDRSQPGQDCRFVTLQPNGRDFHMSVVRARRNDSTGYLCGAISLAPKA QIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLV

53	PD-1 high affinity (HA) ectodomain	PGWFLDSPDRPWNPTTFSPALLVVTEGDNATFTCSFSNTSESFHVIWHRESPSGQTDTLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYYCGVISLAPKIQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLV
54	PD-1 WT dominant negative receptor (21-207)	PGWFLDSPDRPWNPTTFSPALLVVTEGDNATFTCSFSNTSESFVLNWMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYYLCGAIAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLVVGVGGLLGSLLVLLVWVLAVICSRAARGTIGARRTGQ
55	PD-1 HA dominant negative receptor	PGWFLDSPDRPWNPTTFSPALLVVTEGDNATFTCSFSNTSESFHVIWHRESPSGQTDTLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYYCGVISLAPKIQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLVVGVGGLLGSLLVLLVWVLAVICSRAARGTIGARRTGQ
56	CD28 transmembrane	FWVLVVVGVLACYSLLVTVAFIIIFWV
57	4-1BB ICD DNA	AAGCGCGGCAGGAAGAAGCTCCTCTACATTTTAAAGCAGCCTTTTATGAGGCCCGTACAGACAACACAGGAGGAAGATGGCTGTAGCTGCAGATTTCCCGAGGAGGAGGAAGGTGGGTGCGAGCTG
58	CD28 ICD DNA	AGATCCAAAAGAAGCCGCCTGCTCCATAGCGATTACATGAATATGACTCCACGCCGCCCTGGCCCCACAAGGAACTACCAGCCTTACGCACCACCTAGAGATTCGCTGCCTATCGGAGC
59	CD3z ICD DNA	AGGGTGAAGTTTTCCAGATCTGCAGATGCACCAGCGTATCAGCAGGGCCAGAACCAACTGTATAACGAGCTCAACCTGGGACGCAGGGAAGAGTATGACGTTTTGGACAAGCGCAGAGGACGGGACCCTGAGATGGGTGGCAAACCAAGACGAAAAACCCCGAGGAGGTCTCTATAATGAGCTGCAGAAGGATAAGATGGCTGAAGCCTATTCTGAAATAGGCATGAAAGGAGAGCGGAGAAGGGGAAAAGGGCACGACGGTTTGTACCAGGGACTCAGCACTGCTACGAAGGATACTTATGACGCTCTCCACATGCAAGCCCTGCCACCTAGG
60	PD-1 transmembrane	VGVVGGLLGSLLVLLVWVLAVI
61	Anti-CD19 Ab CDRH1	DYGVS
62	Anti-CD19 Ab CDRH2	VIWGSETTYYSALKS
63	Anti-CD19 Ab CDRH3	HYYYGGSYAMDY
64	Anti-CD19 Ab CDRL1	RASQDISKYLN
65	Anti-CD19 Ab CDRL2	HTSRLHS
66	Anti-CD19 Ab CDRL3	QQGNTLPYT
67	Anti-CD52 alemtuzumab VH	QVQLQESGPGGLVRPSQTLSTCTVSGFTFTDFYMNWVRQPPGRGLEWIGFIRDKAKGYTTEYNPSVKGRVTMLVDTSKNQFSLRLSSVTAADTAVYYCAREGHTAAPFDYWGQGSLLTVSS
68	alemtuzumab VL	DIQMTQSPSSLSASVGRVTITCKASQNIIDKYLNWYQQKPGKAPKLLIYNTNNLQTVGVPSTRFSGSGSGTDFTFTISSLPEDVATYYCLQHISRPRTEFGQGTKEIKR
69	alemtuzumab CDRH1	DFYMN
70	alemtuzumab CDRH2	FIRDKAKGYTTEYNPSVKG

71	alemtuzuma b CDRH3	EGHTAAPFDY
72	alemtuzuma b CDRL1	KASQNIDKYLN
73	alemtuzuma b CDRL2	NTNNLQT
74	alemtuzuma b CDRL3	LQHISRPT
75	aCD70 Ab CDRH1	NARMGVT
76	aCD70 Ab CDRH2	HIFSNDEKSYSTSLKS
77	aCD70 Ab CDRH3	IRDYYDISSYYDY
78	aCD70 Ab CDRL1	RASQDISNYLA
79	aCD70 Ab CDRL2	AASSLQS
80	aCD70 Ab CDRL3	LQLNSFPFT
81	IL2Rb (333-551)	VTQLLLQQDKVPEPASLSSNHSLSCTFNQGYFFHLPDALEIEACQVYFTYDPYS EEDPDEGVAGAPTGSSPQFLQPLSGEDDAYCTFPSRDDLLLFSPSLLGGPSPSTA PGGSGAGEERMPPSLQERVPRDWDPPQLGPPTPGVPDLVDFQPPPELVREAGEEV PDAGPREGVSPFWSRPPGQGEFRALNARLPLNTDAYLSLQELQGQDPTHLV
82	CD3 zeta variant (dzz)	RVKFSRSADDTQALLRNDQVYQPLRDRDDAQYSHLGGNRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
83	CD3 zeta variant (ezz)	RVKFSRSADERPPVPNPDIYEPPIRKGQRDLYSGLNQRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
84	CD3 zeta variant (gzz)	RVKFSRSADDTQALLRNDQVYQPLRDRDDAQYSHLGGNRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
85	CD3 zeta variant (deg)	RVKFSRSADDTQALLRNDQVYQPLRDRDDAQYSHLGGNRGRDPEMGGKERPPVPNP PDYEPPIRKGQRDLYSGLNQRRGDKQTLLPNDQLYQPLKDREDDQYSHLQGNALPPR
86	CD3 zeta YA	RVKFSRSADAPAYQQGQNQLYAEINLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
87	CD3 zeta YAYAYA	RVKFSRSADAPAYQQGQNQLYAEINLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYAEINLQKDKMAEAYSEIGMKGERRRGKGGHDGLYAGLSTATKDTYDALHMQALPPR
88	CD3 zeta variant (1XX)	RVKFSRSADAPAYQQGQNQLYNELNLRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLFNEINLQKDKMAEAFSEIGMKGERRRGKGGHDGLFQGLSTATKDTFDALHMQALPPR
89	CD3 zeta variant (XX3)	RVKFSRSADAPAYQQGQNQLFNEINLGRREEFDVLDKRRGRDPEMGGKPRRKNPQE GLFNEINLQKDKMAEAFSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
90	CD3 zeta variant (X2X)	RVKFSRSADAPAYQQGQNQLFNEINLGRREEFDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLFQGLSTATKDTFDALHMQALPPR
91	CD3 zeta	RVKFSRSADAPAYKQGQNQLYNELNLRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
92	Exemplary TRAC target sequence	ATCCAGAACCCTGACCCTGCCGTGTACCAG
93	CD28 hinge	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLPFGPSKP

94	CD19 CAR-P2A	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYNSALKSRLTI IKDNSKSQVFLKMNSLQDDTAIYYCAKHYYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGDCSRFPEEEEGGCEL RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPG
95	CD19 CAR P2A without the signal peptide	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYNSALKSRLTI IKDNSKSQVFLKMNSLQDDTAIYYCAKHYYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGDCSRFPEEEEGGCEL RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEENPG
96	CD19 CAR P2A CD70z DNA sequence	ATGGCTCTGCCCCTCACCGCACTGCTGCTGCCCCTGGCTCTGCTGCTGCACGCCGC AAGACCCGATATCCAGATGACCCAGACCAGCAGCCTGTCTGCTTCTCTGGGAG ATAGAGTGACTATTTCTTGTAGGGCCAGCCAGGACATCTCTAAGTACCTGAACTGG TACCAGCAGAAACCTGACGGCACAGTGAACTGCTGATCTACCACACAAGCCGGCT GCACAGCGGCTCCCCTCTAGATTACGCGGCAGCGGCTCTGGAACAGATTACAGCC TGACAATCAGCAACCTGGAACAGGAGGACATCGCCACATACTTCTGCCAGCAAGGC AACACCCCTGCCCTACACCTTTGGCGGAGGCACCAAGCTGGAATCACC GGCGCGG CCGCAGCGCGCGGCGGAGGAAGCGGAGGCGGCGCAGCGAGGTGAAGCTGCAGGAGA GCGGTCTTGGCCTGGTGGCCCCCTAGCCAGAGCCTGAGCGTGACATGCACCGTGTCC GCGGTGTCCCTGCCTGATTACGGCGTTTCTTGGATCCGGCAGCCTCCAAGAAAGGG CCTGGAATGGCTGGGCGTGATCTGGGGCTCTGAGACAACCTACTATAATAGCGCTC TGAAGAGCAGACTGACCATCATCAAGGACAACCTCTAAGAGCCAAGTGTTCCTGAAG ATGAACAGCCTCCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTA TTACGGCGGCTCCTACGCCATGGACTACTGGGGACAGGGCACCAGCGTGACCGTGT CCAGCACCACAACCCAGCACCAAGGCCACCTACACCTGCACCAACCATCGCCTCT CAGCCACTGAGCCTGAGACCCGAGGCCTGTAGGCCTGCAGCAGGAGGAGCAGTGCA CACCCGGGGACTGGACTTTGCCTGCGATATCTACATCTGGGCACCACTGGCAGGAA CATGTGGCGTGCTGCTGCTGAGCCTGGTCATCACCTGTACTGCAAGAGAGGCAGG AAGAAGCTGCTGTATATCTTCAAGCAGCCCTTTATGCGCCCTGTGCAGACAACCCA GGAGGAGGATGGCTGCTCCTGTCCGTTCCAGAGGAGGAGGAGGAGGATGTGAGC TGCGCGTGAAGTTTCCCGGTCTGCCGACGACACAGCATAACCAGCAGGGCCAGAAC CAGCTGTATAACGAGCTGAATCTGGGCCGAGAGAGGAGTACGACGTGCTGGATAA GAGGAGGGGAAGAGATCCCAGAGATGGGAGGCAAGCCACGGAGAAAGAACCCCAAG AGGGCCTGTACAATGAGCTGCAGAAGGATAAGATGGCCGAGGCCTATAGCGAGATC GGCATGAAGGGAGAGAGGCGCCGGGGCAAGGGACACGACGGCCTGTATCAGGGCCT GTCCACCGCTACCAAAGACACCTATGATGCTCTGCACATGCAGGCTCTGCCACCAA GAGGGTCTGGGGCCACCAACTTCAGCTTACTGAAACAGGCCGGCGACGTGGAAGAA AACCTGGCCCTATGGCCCTGCCTGTGACCGCCCTGCTGCTGCCACTCGCCCTGCT GCTGCATGCCGCTAGACCCAGGTGACACTGAAGGAAAGTGGACCTGTGCTCGTGA AACCTACCGAGACACTGACACTGACCTGTACCGTGTCTGGCTTCAGCCTGTCTAAC GCCCGGATGGGCGTGACATGGATCAGACAGCCTCCAGGCAAGGCCCTGGAATGGCT GGCTCACATCTTCAGCAATGACGAGAAGTCCTACAGCACCAGCCTGAAGAGCCGGC TGACCATCAGCAAGGACACAAGCAAGACCCAGGTTGTGCTGACCATGACAAACATG

		GACCCCGTGGACACAGCCACCTACTACTGTGCCAGAATCCGGGACTACTATGACAT TTCTAGCTACTACGATTACTGGGGCCAGGGCACCCTGGTGTCCGTGAGCAGCGGCG GAGGAGGCTCAGGCGGAGGAGGCTCCGGCGGCGGAGGCAGCGACATCCAGATGACC CAGTCTCCATCTGCTATGAGCGCCAGCGTGGGTGATAGAGTGACAATCACCTGCAG AGCCTCCCAGGACATCAGCAATTACCTGGCCTGGTTCCAGCAGAAGCCCCGGCAAAG TGCCAAAGAGACTGATCTACGCCGCCAGCAGCCTGCAAAGCGGCGTCCCCAGCAGA TTCAGCGGCTCTGGCAGCGGAACCGAGTTCACCCTTACAATCAGCAGCCTGCTGCC AGAAGATTTGCCACATACTACTGCCTGCAGCTGAACAGCTTTCCTTTTACCTTCG GCGGAGGCACGAAGGTGGAAATCAACACCACCACACCTGCTCCCAGACCTCCTACA CCCGCACCTACCATCGCCTCCCAGCCCCCTGTCCCTGAGACCTGAGGCGTCCCGGCC CGCTGCTGGCGGCGCCGTCCACACCAGAGGCTGGACTTTGCCCTGCGACATCTACA TTTGGGCCCCCTCTGGCCGGCACCTGCGGCGTGCTGCTGCTGAGCCTGGTCATCACC CTGTACTGCAGAGTGAAGTTCAGCAGAAGCGCCGATGCCCCCTGCCTACCAGCAAGG CCAGAACCAGCTTTATAATGAGCTGAACCTGGGACGGAGGGAAGAGTACGACGTGC TGGATAAGCGCGGGGCGAGAGATCCTGAGATGGGAGGCAAGCCTAGAAGAAAGAAC CCCCAGGAGGGCCTGTACAACGAGCTGCAAAAAGACAAGATGGCCGAGGCCTACAG CGAGATCGGCATGAAAGGCGAGCGGCGGCGCGGCAAGGGCCACGACGGCTGTACC AAGGCCGTGTCTACCGCCACCAAGGATACATATGACGCCCTGCACATGCAGGCTCTG CCTCCTAGATGA
97	CD19 CAR P2A CD70z protein sequence	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNW YQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQG NTLPYTFGGGTKLEITGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVS GVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYNSALKSRLTI IKDNSKSQVFLK MNSLQTDDTAIYYCAKHYYGGSYAMDYWGQTSVTVSSTTTPAPRPPTPAPTAS QPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL RVKFSRSADAPAYQQGQN QLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQAGDVEE NPGPMALPVTALLLPLALLLHAARPQVTLKESGPVLVKPTETLTLTCTVSGFSLN ARMGVTWIRQPPGKALEWLAHIFSNDEKSYSTSLKSRLTISKDTSKTQVVLMTNM DPVDTATYYCARIRDYDIDISSYDYWGQTLVSVSSGGGSGGGGSGGGGSDIQMT QSPSAMSASVGDRVTITCRASQDISNYLAWFQQKPGKVPKRLIYAASSLQSGVPSR FSGSGSGTEFTLTISSLLPEDFATYYCLQLNSFPFTFGGGTKVEINTTTPAPRPPT PAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVIT LYCRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGDGLYQGLSTATKDTYDALHMQAL PPR
98	TRAC endogenous promoter (alternative)	tgctgggcctttttcccatgcctgcctttactctgccagagttatattgctggggt tttgaagaagatcctattaaataaaaagaataagcagattattattaagtagccctgca tttcaggtttccttgagtggcaggccaggcctggccgtgaacgttcactgaaatca tggcctcttgccaagattgatagcttgtgcctgtccctgagtcgccagtcacac gagcagctggtttctaagatgctattttcccgataaagcatgagaccgtgacttgc cagccccacagagccccgccttgtccatcactggcatctggactccagcctgggt tggggcaaagagggaaatgagatcatgtcctaaccctgatcctcttgtccacaga tatccagaacccta
99	CD8 transmembr ane	IYIWAPLAGTCGVLLLSLVIT

EXAMPLES

[0284] Experimental Methods

[0285] To make lentivirus encoding the desired constructs, HEK293T-cells were plated at 0.45 million cells per mL in 2mL of DMEM (Gibco) supplemented with 10% FBS (Hyclone) per well of a 6-well plate the day before transfection. On the day of transfection, the lentivirus was prepared by mixing together lentiviral packaging vectors 1.5 ug psPAX2, 0.5 ug pMD2G, and 0.5 ug of the appropriate transfer CAR vector in 250 uL Opti-MEM (Gibco) per well of the 6-well plate ("DNA mix"). 10 uL Lipofectamine 2000 (Invitrogen) in 250 uL Opti-MEM was incubated at room temperature for 5 minutes and then added to the DNA mix. The mixture was incubated at room temperature for 20 minutes and the total volume of 500 uL was slowly added to the sides of the wells containing HEK293T. 1 day post-transfection, the media from each well of HEK293T-cells in the 6-well plate was replaced with 2mL per well of T-cell transduction media, i.e., X-Vivo-15 supplemented with 10% FBS. 2 days post-transfection, The lentiviral supernatants from HEK293T-cells were harvested and passed through a 0.45 micron filter (EMD Millipore) to remove cell debris, concentrated 25-folds using the Lenti-X Concentrator (Takara Bio) according to manufacturer's instructions and flash-frozen in aliquots. Lentiviral titers were determined by thawing an aliquot of the frozen lentivirus, making 4-fold serial dilutions and performing limiting dilution titration on JurkaT-cells (Clone E6-1; ATCC). On Day 0, purified T-cells were activated in X-Vivo-15 medium (Lonza) supplemented with 100 IU/mL human IL-2 (Miltenyi Biotec), 10% FBS (Hyclone), and human T TransAct (Miltenyi Biotec, Cat# 130-111-160, 1:100 dilution) in a Grex-24 plate (Wilson Wolf, cat# 80192M). On Day2, T-cells were resuspended at 0.5 million cells per mL in T-cell transduction media, transduced with the respective lentiviral stocks at MOI=5 along with 100 IU/mL human IL-2 in a Grex-24 plate. On Day 5 when transduction was complete, cells were harvested and washed to remove residual IL-2. They were then resuspended in T-cell expansion media, i.e., X-Vivo-15 supplemented with 5% human AB serum (Gemini Bio), and each sample was divided equally into 2 parts, with one part receiving 100 IU/mL human IL-2 as per standard protocol, and the other receiving a lower concentration of 25 IU/mL human IL-2. Cells were expanded into larger G-Rex vessels (Wilson Wolf) as needed using T-cell expansion media and the respective concentrations of human IL-2. On Days 5, 9 and 14, the absolute number of T-cells in each sample was counted, and transduction efficiency was determined by detecting the percentage of T-cells that bound a FITC-conjugated v5 tag monoclonal

antibody (Thermo Fisher) using flow cytometry. On Day 14 or 15, the CAR-T-cell products were cryopreserved and thawed as needed for further assays.

[0286] For cells generated with site specific integration with AAV6, two days after T cell activation, TransAct was removed by centrifugation. The cells were then washed in PBS and electroporated using the AMAXA 4D nucleofector electroporation apparatus with 0.5ug of TALEN® mRNA per arm of the nuclease every 1×10^6 cells. Recombinant AAV6 comprising one or more transgenes was added at MOI 10,000-15,000 to the T cells after the gene editing step. After electroporation, cells were incubated at 30°C overnight. Cells were then returned to 37°C and T cell culturing conditions as described above. At day 14 post-activation, TCR α/β depletion was performed using EasySep™ human TCR α/β depletion kit (STEMCELL Technologies) as instructed by the manufacturer protocol. At day 15 or 16 post-activation, T cells were cryopreserved in 90% FBS/10% DMSO using rate-controlled freezing chambers and stored in liquid nitrogen vapor phase.

[0287] AAV-mediated site-specific integration can also be done with a CRISPR endonuclease, e.g., Cas12i endonuclease, or CRISPR reagents. Pan T cell donors were thawed and activated with GMP-grade TransAct and IL-2 for 2 days. Prior to electroporation, Cas12i nuclease and TRAC targeting guide RNA (gRNA) were incubated together at room temperature to form a ribonucleoprotein complex (RNP). T cells were then mixed with the RNP and electroporated. Cells were then immediately incubated with AAV comprising one or more transgenes at a specified MOI overnight, then transferred into IL-2 supplemented complete T cell media in a GREX plate. Cells were then expanded another 12 days before freezing. Flow cytometry analysis was performed 4, 7, and 11 days after electroporation. The transgene cassette was expected to be inserted at the target sequence GACCCTGCC or ATCCAGAACCCTGACCCTGCCGTGTACCAG (SEQ ID NO: 92) within TRAC exon 1. Standard Long-term Killing Assay (LTKA)

[0288] CAR T cells were co-cultured with luciferase-GFP-expressing Raji cells at an effector-to-target ratio of 8:1. Every 2-3 days, half the cells were passaged onto fresh Raji cells. The remaining half of cells was then used to determine target cell killing using Bright-glo reagent (Promega).

[0289] Flow-based serial restimulation assay

[0290] In a 24-well G-rex® (Wilson Wolf, Cat # 80192M), 5×10^5 CAR T cells were co-cultured with 5×10^5 Raji Luciferase-GFP cells in RPMI 1640 + 10% FBS media (Hyclone).

A small aliquot of the co-cultured CAR T and target cells were taken to perform an antibody stain with 123count eBeads™ Counting Beads (Thermo Fisher Scientific, Cat # 01-1234-42) to determine baseline phenotype and cell counts at the start of the assay. Every 3-4 days, 50% of supernatant was removed from the G-rex® and remaining cell suspension was mixed thoroughly, and a small aliquot was taken for phenotyping and cell counting as described above. After cell counts are determined, fresh Raji cells were added back into each well to bring the E:T ratio back to 1:1. Serial restimulation is repeated until CAR T no longer kills the Raji target cells.

[0291] Single-stimulation FACS assay

- 10 [0292] Similar to the flow-based serial stimulation assay, CAR T and Raji cells were co-cultured at a 1:2 or 1:4 E:T ratio in a 24-well G-rex® rather than a 1:1 E:T. Every 3-4 days, 50% of supernatant were removed from the G-rex® and remaining cell suspension were mixed thoroughly, and a small aliquot was taken for phenotyping and cell counting as described above. Instead of adding fresh Raji cells back, only fresh media was added back.
- 15 Timepoints are collected every 3-4 days until Raji cells are either completely cleared by CAR T or until Raji cells outgrow the T cells.

[0293] Primed T mixed lymphocyte reaction (MLR)

- [0294] Human PBMCs (host) were primed against irradiated unedited T cells derived from donors used to make gene edited graft T cells above to promote expansion of
- 20 alloreactive T cell clones. Briefly, graft PBMCs were irradiated at 30 Gy and co-cultured with host PBMCs at a 1:1 ratio in R10 + 20 IU/mL IL-2 + 10 ng/mL IL-7 + 10 ng/mL IL-15 (Miltenyi, Cat # 130-095-765) for 4 days. Media was exchanged to R10 without cytokines and the cells were continued to culture for 3 more days. Afterwards, pan T cells were isolated using MACS negative selection (Miltenyi, human pan T cell isolation kit, Cat
- 25 #130-096-535) per the manufacturer's recommendations. In a 96-well plate, 20,000 gene edited graft T cells were seeded with 20,000 primed host T cells and cultured in R10 + 20 IU/mL IL-2 for 2 days at 37°C, 5% CO₂. Survival of graft T cells was determined by flow cytometry using absolute counts by gating on live TCRαβ-CAR⁺ T cells.

- [0295] Example 1 Generation of CAR T cells expressing an anti-CD19 CAR from a
- 30 lentiviral vector

[0296] Constructs for the expression of second generation anti-CD19 CARs were designed based on the anti-CD19 scFv of SEQ ID NO: 4, CD3z signaling domain and 4-1BB costimulatory domain and cloned in a lentiviral vector (LVV). CAR T cells generated from donor PBMCs either express the CD19 CAR alone, or a chimeric cytokine receptor (CCR) and the CD19 CAR from a bicistronic expression cassette linked via a cleavable P2A peptide (CD19 CAR/CCR). The CCR contains a TPOR transmembrane/JAK binding and activation domain and IL2R intracellular signaling domains exemplified as in SEQ ID NO: 27. Gene expression in all LVV constructs was driven by the elongation factor 1 alpha (EF-1 α) promoter. CAR T cells were generated by transducing activated donor PBMC cells with the LVV. The cytotoxic activities of CAR T cells harboring different constructs generated from three different donors were evaluated by an in vitro long-term killing assay (LTKA) against CD19 positive Raji cells and Daudi cells. As shown in FIGs. 1A-1C, in most cases, co-expressing a CCR improved CD19 CAR T cell cytotoxicity.

[0297] In vivo cytotoxicity of CAR T cells engineered with the selected constructs was evaluated in an orthotopic animal tumor model established by injecting Raji cells into NSG mice. Briefly, luciferase-expressing Raji cells were injected into mice intravenously. CAR T cells were injected 4 days after tumor cell transfer. Tumor burden was measured using an IVIS Spectrum instrument. As shown in FIGs. 2A-2B, CD19 CAR T cells co-expressing a CCR effectively reduced tumor cells in the animal model at a dose level of 3×10^6 CAR T cells.

[0298] Example 2 CAR T cells expressing an anti-CD19 CAR by site-specific integration

[0299] Transduction of LVV constructs into PBMCs results in engineered cells with randomly integrated transgene(s) in the host cell genome. The transgene(s) can be introduced into cells by site-specific integration (SSI) into a predetermined genetic locus to ensure uniformity of the insertion site of the transgene(s) in the genome and limit the number of integration events. Site-specific integration aided by, for example, adeno-associated virus vector (AAV), can also accommodate one or more transgenes that can be larger in size than what can be incorporated into a lentiviral vector, while maintaining high transduction efficiency. The CD19 CAR/CCR construct cloned in an adeno-associated virus vector (AAV) was inserted into the TRAC locus by homologous recombination, and the expression of the CCR and CD19 CAR was driven by a short EF1 α promoter (EFS). The

resulting CAR T cells were evaluated for in vivo cytotoxicity in the Raji orthotopic tumor model in NSG mice, as compared with CAR T cells generated by LVV transduction expressing the same CCR and CD19 CAR (N=8 mice/group, 2.5×10^6 CAR⁺ cells/mouse).

[0300] The results in FIG. 3A show that, unexpectedly, the CAR T cells generated by site-specific integration exhibited significantly reduced tumor control in vivo than the CAR T cells generated by LVV transduction. The results were reproduced in CAR T cells generated using cells from a different donor. Consistent with the poor in vivo cytotoxicity, the CAR T cells generated by site-specific integration failed to expand and failed to persist in the treated mice for the duration of the experiment, in contrast to the CAR T cells generated by LVV transduction (FIG. 3B). Similar in vitro results were also observed in FIGs. 5A-5C, in which the CAR T cells generated by site-specific integration (SSI) driven by the EFS promoter (SSI EFS CD19/CCR) showed reduced cytotoxicity in a standard long-term killing assay, and reduced expansion than the CAR T cells generated by LVV transduction.

[0301] Example 3 Optimization of anti-CD19 CAR construct for site-specific integration

[0302] To improve the in vitro and in vivo anti-tumor activity of our SSI-engineered CAR T cells, we tested a panel of different promoters to drive expression of the CCR and the CAR. All SSI constructs were designed to integrate into the T cell receptor alpha constant (*TRAC*) locus. The surface expression of the CD19 CAR was measured using an anti-idiotypic (anti-ID) antibody (ACROBiosystems) via flow cytometry - to measure the mean fluorescence intensity (MFI) of the CAR - and the anti-tumor activity of the CAR T cells were examined. Several heterologous promoters were tested, including 3 promoters derived from the human phosphoglycerate kinase 1 (*PGK1*) gene, designated PGK-SSI-1, PGK-SSI-2, and PGK-SSI-3.

[0303] The CAR MFI was measured at the end of manufacturing. The CAR construct driven by the human ubiquitin B (*UBB*) promoter (UBB300) had the highest CAR expression at the end of manufacturing (FIG. 4A). The 3 PGK SSI promoters, as well as the human elongation factor 1-alpha 1 short (EFS) promoter and the human cyclophilin A (*CypA300*) promoter resulted in similar CAR expression at the end of manufacturing (FIG. 4A). Next, CAR T cells were analyzed in a flow cytometry-based serial restimulation cytotoxicity assay against a CD19⁺ tumor cell line, Raji, at an effector to target (E:T) ratio

of 1:1. Surprisingly, the PGK SSI promoter constructs led to superior anti-tumor activity compared to all other promoters tested (FIG. 4B).

[0304] To understand the mechanism behind this difference in anti-tumor efficacy, we monitored the CAR expression (MFI) during the serial restimulation assay. We noticed that although the CAR MFI at the end of the process was similar between the EFS and PGK SSI promoters (FIG. 4A), the promoters modulated CAR expression differently after exposure to CD19⁺ tumor cells. All promoters increased CAR expression by day 3 after target exposure (except for the expression constructs controlled under the TRAC endogenous promoter and the UBC300 promoter), with the EFS and UBB promoters achieving the highest level of expression (FIG. 4C). An alternative TRAC endogenous promoter was tested, and similar results were observed (data not shown). By day 17, all promoters down-regulated CAR expression, however the EFS and UBB promoters maintained significantly higher expression throughout the rest of the assay, as compared to the PGK SSI promoters (FIG. 4C). Surprisingly, this sustained high-level CAR expression level after day 17 negatively correlates with CAR T cell expansion (FIG. 4D) and anti-tumor activity (FIG. 4B). In contrast, the PGK SSI promoters, which maintained low-level CAR expression from day 17 on but showed the highest peak expansion (FIG. 4D) and best anti-tumor activity (FIG. 4B) as compared to the other constructs. Without wishing to be bound by specific mechanisms, the results suggest that prolonged high expression of the CAR by the EFS and UBB promoters may have led to exhaustion and dysfunction of the CAR T cells, whereas lower-level expression of the CAR after target exposure by the PGK SSI promoters preserved CAR T cell function and led to better CAR T cells expansion and better anti-tumor activity.

[0305] With these results, we next tested the PGK SSI promoters, e.g., PGK SSI-1, in comparison with the EFS promoter in CAR T cells generated by site-specific integration, and CAR T cells generated by LVV transduction. The results in FIG. 5A show that, when tested in a standard LTKA against wild type Raji cells at an E:T ratio of 4:1, CD19 CAR T cells co-expressing the CCR under the PGK SSI-1 promoter, but not the EFS promoter, performed comparably with that of LVV expressing the same CD19 CAR and CCR. When tested in a single-stimulation FACS based potency assay against wild type Raji cells at an E:T ratio of 1:2, the PGK SSI-1 driven SSI construct outperformed not only the EFS constructs but also the LVV transduced CAR T cells as evidenced by both the number of remaining target cells (FIG. 5B) and the expansion of CAR T⁺ cells (FIG. 5C). All results

shown in FIGs. 5A-C were obtained using CAR T cells generated from the cells of the same donor.

[0306] The results in the in vitro killing assay were confirmed in vivo in the Raji orthotopic tumor NSG mouse model. In the animal tumor model, CD19 CAR T cells driven by the PGK SSI-1 promoter outperformed LVV transduced CAR T cells and the other constructs driven by the Cyp300A and the EFS promoters, at the two different cell doses tested (FIG. 6A-6B).

[0307] Example 4 CD19 CAR T cells co-expressing a CD70-binding protein to reduce rejection and improve persistence

[0308] Allogeneic CAR T cells made from donor cells may be recognized as foreign and rejected by the immune system of the patient who would receive the allogeneic CAR T cells. To reduce rejection by the alloreactive host T cells and to improve persistence and further improve CAR T cell efficacy, a CD70-binding protein was introduced into and expressed in the CD19 CAR T cells. CD70 is a membrane bound protein expressed in immune cells, especially activated T cells, including alloreactive T cells. CAR T cells expressing a CD70 binding protein or a CD70-binding protein, e.g., in this experiment a CD70 specific CAR, could engage CD70 expressed on the surface of alloreactive host T cells and lead to the death of alloreactive host T cells.

[0309] To illustrate, CD19 CAR/CCR T cells generated by site-specific integration into the TRAC locus were tested in an in vitro MLR assay. In the MLR assay, host T cells were first primed for 7 days against unedited allogeneic graft cells before coculturing with genetically edited CAR T cells and target Raji tumor cells. As shown in FIG. 7A, without expressing a CD70-binding protein, CAR T cells were rejected by the alloreactive CD70+ host T cells and failed to expand (FIG. 7D), while alloreactive host T cells expanded (FIG. 7B) and the CD19+ Raji tumor cells continued to grow (FIG. 7C).

[0310] In contrast, when a CD70-binding protein was co-expressed in CD19 CAR/CCR T cells, CAR T cells were able to expand and persist (FIGs. 7E-7H), while alloreactive CD70+ host T cells failed to expand (FIG. 7F) and the CD19+ Raji tumor cells were eliminated (FIG. 7G, comparing squares, without a CD70-binding protein, with circles, with a CD70-binding protein). The CD70-binding protein exemplified in this experiment contains an anti-CD70 scFv, a CD8 hinge/transmembrane domain and a wild type CD3z signaling domain. In this experiment, the construct that expresses the CD70-binding protein

was inserted into the CD52 locus by site-specific integration. The data were pooled from four unique host:graft donor pairs and representative of two independent experiments was shown.

[0311] Example 5 CD19 CAR/CCR T cells co-expressing a CD70-binding protein
5 demonstrated improved cytotoxic activity by preventing antigen escape

[0312] It has been shown that Raji lymphoma cells down-modulate CD19 expression when encountering anti-CD19 effector cells in vitro at low E:T ratios (Schneider, et al. 2017, J. Immunotherapy of Cancer 5:42). As CD70 is expressed on the CD19+ target tumor cells, CD19 and CD70 dual targeting may prevent CD19 antigen escape and enhance anti-
10 tumor activity as compared to CD19 single targeting. To test this hypothesis, Raji cells engineered to express GFP, either CD70 wild type (WT) or CD70 knockout (KO), were first incubated with the TRAC KO CAR T cells or TRAC KO non-transduced (NTD) control cells at a low E:T ratio of 1:3 for 0-4 days in a single antigen stimulation FACS assay (FIG. 8A). Total number of live Raji cells gated on GFP+ and CD20+ were measured on day 0, 1,
15 2 and 4. The data in FIG 8B show that on day 4 Raji cells down-modulated CD19 expression when only CD19 was targeted in both Raji CD70 wild type or Raji CD70 KO cells. The CD19- CD70+ Raji cells were able to escape killing by CD19 CAR/CCR but could not escape killing by CD19 CAR/CCR T cells co-expressing the CD70-binding protein (FIG. 8B). When total live Raji cells were counted in the time course of the
20 experiment, the data in FIG. 8C show that the residual Raji cells on day 4, likely CD19 negative, were eliminated only in the presence of dual targeting CD19 CAR/CCR T cells co-expressing the CD70-binding protein. FIG. 8D examined closely the CD19 negative Raji cells and confirmed the results. The results shown are representative data of two independent experiments.

[0313] The observations were also confirmed in in vivo studies using the orthotopic mouse model. 5×10^6 CD19 CAR T cells were dosed to the NSG mice previously engrafted with Raji cells (N=10 mice per group). Bone marrow was collected and Raji cells were analyzed ex vivo by flow cytometry at terminal end point for the 5×10^6 dose. The data shown in FIGs. 8E-8F confirmed that co-expressing the CD70-binding protein did not
30 negatively impact the cytotoxic activity of CD19 CAR/CCR T cells in vivo, and further, it also demonstrated the added benefit of preventing CD19 antigen escape in vivo.

[0314] Example 6 Analysis of CD70-binding protein -expressing CD19 CAR T cell designs

[0315] We next examined any effects that co-expressing a CD70-binding protein may have on CAR T cell production. As CD70 is expressed on T cells, especially activated T cells, the encounter of CD70 by the CD70-binding protein on the surface of a neighboring cell can cause fratricide (FIG. 9A). T cells expressing the CD70-binding protein, such as T cells singly positive for the CD70-binding protein or CAR T cells doubly positive for both the CD19 CAR and the CD70-binding protein, can be protected from fratricide as a result of the CD70-binding protein binding to, and thus masking, in cis the surface CD70 protein on the same cell, as illustrated in FIG. 9A. T cells that do not express the CD70-binding protein such as CAR T cells singly positive for the CD19 CAR will not be protected from fratricide (FIG. 9A).

[0316] To better understand the masking and fratricide events, we analyzed the CD19CAR/CCR T cells co-expressing the CD70-binding protein along the production process. In this experiment, the cells had the CD70-binding protein integrated into the CD52 locus and the CCR and CD19 CAR bicistronic expression construct integrated into the TRAC locus. As the production process progressed from day 9 to day 14, the CD70-binding protein single positive population, and the CD19 CAR/CCR and CD70-binding protein double positive population enriched while the CD19 CAR/CCR single positive population decreased, likely resulting from fratricide (FIG. 9B).

[0317] Example 7 Optimization and analysis of CD70-binding protein expressing CD19 CAR T cell designs

[0318] We next designed a series of constructs as described in FIG. 10A to enrich the CD19 CAR and CD70-binding protein double positive population and enhance yield. The CAR T cells generated were subjected to phenotypic assays.

[0319] The expression of the CD19 CAR and CD70-binding protein at the end of production process (day 14) using cells of two different donors were analyzed by flow cytometry (FIG. 10B) and the quantified results are shown in FIGs. 10C-10D (symbols represent individual donors). The results of cell phenotype, exhaustion marker analysis and activation marker analysis are shown in FIGs. 10E, 10F, and 10G, respectively. All data were shown for day 14 of the production process, except day 9 for activation marker

analysis. CAR T cell expansion data are shown in FIG. 10H and the CD4:CD8 ratios are shown in FIG. 10I.

[0320] From these experiments, we conclude that CAR T cells expressing CD19 CAR and CD70-binding protein from the constructs of FIG. 10A were successfully produced.

5 The cell populations are shown to be homogenous consisting of mainly double positive cells for both the CD19 CAR and the CD70-binding protein. A higher frequency of CD19 CAR+ cells was achieved using the tandem and dual constructs (FIG. 10D, top panel) as compared to other constructs tested. Among the constructs tested, the PGK SSI-1 CD19/CD70 Dual CAR T cells appear to have the highest amount of double positive cells and consistently
10 good phenotypes compared to other designs.

[0321] The CAR T cells were next examined in an MLR assay against alloreactive T cells (FIG. 11A). The data in FIG. 11B show that on average EFS CD19/CD70 Dual, PGK SSI CD19/CD70 Dual, and the PGK SSI CD70z.CD19/CCR cells have comparable anti-rejection activity and have better graft survival rate than PGK SSI CD19 CAR T cells
15 without expressing a CD70 binding protein. The better graft rate indicates that CD19/CD70 dual CAR T cells were able to deplete CD70+ host T cells.

[0322] The cytotoxic activities of the CAR T cells were tested in a single-stimulation FACS assay. Raji CD70 KO cells were used to allow investigation of the effects of CD19 CAR or CD19 CAR/CCR alone. The data in FIGs. 12A-12B show that PGK SSI
20 CD19/CD70 dual outperformed other formats with a CD70-binding protein in the single stimulation FACS assay against CD70KO Raji cells both in tumor killing and CAR T expansions. The cells were gated at CD19+ CAR T cells and GFP+ cells for Raji. Similar results were observed in the single stimulation FACS assay against CD70 wild type Raji cells (FIGs. 12C-12D). In all of FIGs. 12A-12D, an average of three replicates were shown
25 and the data are representative of two donors.

[0323] Similarly, the data in FIGs. 13A-13B show that PGK SSI CD19/CD70 dual outperformed other designs in the standard long-term killing assay against CD70KO Raji at a E:T ratio of 8:1. The data in FIGs. 14A-14G show that in the mouse tumor model, the PGK SSI CD19/CD70 dual, PGK SSI CD19, and the PGK SSI CD70.CD19/CCR
30 constructs had the best activity and the PGK SSI CD70.CD19/CCR construct outperformed the other designs, at a dose of 5×10^5 CAR T cells (N = 8 NSG mice per group).

[0324] Example 8 Production and analysis of PGK SSI CD19/CD70 dual CAR T cells engineered using CRISPR reagents

[0325] We generated the PGK SSI CD19/CD70 dual CAR T cells engineered using CRISPR gene editing reagents, e.g., the CRISPR endonuclease Cas 12i and gRNA, to knock
5 out TRAC and insert the transgene into the TRAC locus by site-specific integration. The fold expansion of CAR T cells (FIG. 15A) and the number of CAR + cells (FIG. 15B) at the end of process were comparable between the CD19/CD70 dual CAR T cells and CD19 CAR T cells. The fold expansion and number of CAR+ cells at the end of process between CRISPR reagents engineered and TALEN reagents engineered CAR T cells were also
10 comparable (data not shown). The CRISPR reagents engineered CD19/CD70 dual CAR T cells show higher % CAR+ cells and higher peak MFI than CRISPR reagents engineered CD19 CAR T cells (FIGs. 15C-D).

[0326] We next examined the expression level of the CD70 binding protein, or the CD70 CAR, in the CD19/CD70 dual CAR T cells. The data in FIGs. 15E-F show that the
15 expression of the CD70 binding protein, or the CD70 CAR, increased at the end of the production process presented as the % of positive cells (FIG. 15E) and MFI (FIG. 15F), detected by the anti-CD70 scFv anti-id antibody. No meaningful differences were observed between CRISPR reagents engineered CAR T cells and TALEN reagents engineered CAR T cells. The CD4:CD8 ratio decreased during the production process in all groups tested
20 (FIG. 15G) and the end of process ratio was comparable or slightly lower than the ratio shown in FIG. 10I, depending on the donors. The percentage of CD4 or CD8 T cells that is CD25+ and 4-1BB+ was slightly higher in the CD19/CD70 dual CAR T cells than CD19 CAR T cells (FIG. 15H and 15I, respectively), similar to previously observed in FIG. 10G. The percentage of CD4 and CD8 T cells that is Tscm/Tcm was slightly lower in the
25 CD19/CD70 dual CAR T cells than that in CD19 CAR T cells (FIG. 15J and FIG. 15K, respectively).

[0327] We next examined the activities of the CAR T cells in a flow cytometry-based serial restimulation assay against CD19+ human B lymphoblastoid cell line Raji. The data in FIGs. 16A-B show cytotoxic activity against the target cell line (top panel) and fold
30 expansion of CAR T cell during the same timeframe (lower panel) from two donors. In addition, expansion of the dual CAR T cells was not observed in absence of exogenous IL-2 stimulation and target cells (FIG. 16I).

[0328] As the CD19⁺ Raji cell line also expresses CD70, the cell line can be used for confirming that the CRISPR engineered CD19/CD70 dual CAR T cells can effectively target both CD19 and CD70 on the Raji target cells. Target-dependent cytotoxicity was evaluated in short-term killing assays with wild-type (WT) Raji-GFL-Luciferase (RGL), CD19KO RGL, or CD70KO RGL cells so that the effect of the CD19/CD70 dual CAR T cells on each target can be independently investigated. The CD19/CD70 CAR T cells or control cells were co-cultured with target cells at defined effector-to-target (E:T) ratios for 24 hours (FIG. 16C). Target cell killing was determined by luciferase assay. The CAR T cells tested were generated from two different donors. The CD19/CD70 dual CAR T cells efficiently eliminated WT, CD19KO, and CD70KO RGL cells, demonstrating target-dependent cytotoxicity against both CD19 and CD70 (FIG. 16D). As expected, the control CD19 CAR T cells showed cytotoxicity against WT and CD70KO RGL cells but showed no cytotoxicity against CD19KO RGL cells. Dual targeting was also assessed in a mixed tumor model. CD19KO (CD19^{neg}) and CD70KO (CD70^{neg}) RGL cells were mixed at a 1:1 ratio prior to co-culturing with the CAR T cells at an E:T ratio of 1:1. RGL cell growth was monitored over time by flow cytometry (FIG. 16E). When mixed RGL cells were co-cultured with the control CD19 CAR T cells, CD19 positive RGL (i.e., CD70KO RGL) were eliminated (FIG. 16F, bottom right panel), while CD19 negative (CD19KO) RGL cells were able to expand (FIG. 16F bottom left panel). Conversely, the mixed RGL cells were eliminated by the CD19/CD70 CAR T cells through CD19 and CD70 dual targeting and were unable to expand (FIG. 16F, top panel and bottom two panels). When the total RGL cells were measured, only the CD19/CD70 dual CAR T cells, but not the control CD19 CAR T cells, eliminated the target cells, presumably due to the expansion of CD19 negative RGL cells that cannot be eliminated by the control CD19 CAR T cells (FIG. 16F, top panel). The data shown are the combined results from technical replicates from one representative donor.

[0329] The ability of the CD19/CD70 dual CAR T cells in killing target positive cells *in vivo* was tested in a mouse model. 1×10^5 RGL cells were injected intravenously into the NSG mice via tail vein. One day prior to dosing, mice were randomized into groups of ten with similar mean total body bioluminescence values. On Day 4 post tumor cell implantation, cryopreserved CD19/CD70 dual CAR T cells were thawed, washed and resuspended with phenol-red free RPMI 1640 medium. In treatment groups, mice were dosed intravenously with 3.5×10^6 , or 10×10^6 CAR⁺ cells/animal. In control group, mice

were left untreated and served as reference. Tumor bioluminescence, body weight and general animal health were recorded twice weekly. As shown in FIG. 16G, the growth of target cells as shown as tumor volume decreased in a CD19/CD70 dual CAR T dose-dependent manner. The data in FIG. 16H show the expansion of the CD19/CD70 dual CAR T cells in the animals also in a dose-dependent manner.

[0330] Example 9 The CD19/CD70 Dual CAR T cells Effectively Targeted Primary CD19⁺ B Cells and CD70⁺ T Cells

[0331] To test target-dependent cytotoxicity against primary CD19⁺ B cells, short-term killing assays were performed by co-culturing the CD19/CD70 CAR T cells or control cells with PBMCs from a healthy individual or from a systemic lupus erythematosus (SLE) patient. Co-culturing was performed at defined E:T ratios for 24 hours. After 24 hours, killing of CD19⁺ B cells was determined by flow cytometry. CAR T cells tested were generated from two different donors. The CD19⁺ B cells from either the healthy individual or the SLE patient survived and were detected after co-cultured with non-transduced (NTD) control cells (FIG. 17A) but were eliminated when co-cultured with either the control CD19 CAR T cells or the CD19/CD70 CAR T cells (FIGs. 17A & 17B). The data shown are the combined results from technical replicates from one representative donor.

[0332] CD70 is expressed in activated, alloreactive T cells that can reject graft CAR T cells. Mixed lymphocyte reactions (MLRs) were performed to test the anti-allojection function of the CD70 binding protein, or CD70 CAR, expressed in the CD19/CD70 dual CAR T cells as evidence that the dual CAR T cells can effectively target and eliminate CD70⁺ primary T cells. PBMCs from healthy or SLE (systemic lupus erythematosus) patient donors were primed for 7 days against irradiated allogeneic graft T cells. Primed alloreactive T cells were isolated by magnetic separation (Miltenyi) and were then co-cultured at a ratio of 1:1 with CAR T cells derived from the same allogeneic graft T cells used for the priming step. Host alloreactive T cells and graft CAR T cells were assessed over time by flow cytometry. A total of four different host : graft donor pairs were tested, two pairs with healthy host donors and two pairs with SLE host donors. As shown in FIG. 18A, right panels, the NTD or CD19 CAR T cell controls were rapidly rejected by healthy donor host alloreactive T cells, whereas the CD19/CD70 dual CAR T cells resisted allojection and expanded over time. Conversely, CD70⁺ alloreactive host T cells

expanded when co-cultured with NTD control or CD19 CAR T cells but were eliminated when co-cultured with the CD19/CD70 dual CAR T cells, suggesting that the CD70 CAR enhances CAR T cell persistence against host T cell rejection (FIG. 18A, left panel). Similar results were shown using alloreactive T cells from SLE donors (FIG. 18B). The results also demonstrate that the CD19/CD70 dual CAR T cells effectively eliminated CD70+ primary T cells from both healthy and SLE donors. The data are the combined results from technical replicates from a representative host : graft donor pair.

[0333] Example 10 In Vivo Efficacy of the CD19/CD70 Dual CAR T Cells

[0334] Several *in vivo* mouse SLE models were investigated. As illustrated in FIG. 19A, a PBMC humanized mouse model was established by adoptively transferring human PBMC (2×10^7 cells/mouse) into immunodeficient NSG mice (Jackson Laboratory) via intravenous (i.v.) injection. To enhance B cell survival and engraftment, two conditions were tested in addition to the control group, which received PBMC injection only. In one group, mice received human B lymphocyte stimulator (hu BlyS, $10 \mu\text{g}/\text{mouse}$, Peprotech) by intraperitoneal (i.p.) injections four times within one week. In the second group, mice received 1 Gy irradiation one day prior to transplantation. After 10 or 14 days of transplantation, spleens were harvested and analyzed for human immune cells engraftment and phenotype using flow cytometry. As shown in FIG. 19B, successful engraftment of B and T cells was detected in all mice. Among the different conditions tested, irradiation increased the degree of engraftment consistently across different cell types (FIG. 19B) and was chosen for subsequent studies. CD70 expression was detected on both engrafted B and T cells to different levels (FIG. 19C). Data are the combined results from three different donors.

[0335] To monitor the kinetics of B cell reconstitution and differentiation, we engrafted irradiated NSG mice (Jackson Laboratory) with healthy donor PBMCs (5×10^6 , 1×10^7 , 2×10^7 cells/mouse) via i.v. injection (FIG. 20A). Spleens were harvested at different days post injection (day3, 7, and 10) and processed as single-cell suspension. Flow cytometry was used to assess the engraftment and phenotype of human cells. Serum was collected at the terminal end point of the study and human IgG and IgM in mouse serum was measured by ELISA (ThermoFisher). Mice that received different doses of PBMCs showed similar patterns of engraftment. Within 10 days of PBMC adoptive transfer, the engrafted B cells continued to proliferate and undergo differentiation, as indicated by the decrease in naïve

cells and increase in memory phenotype as indicated by the staining of different biomarkers (FIGs. 20B-20D). A significant population of plasmablast and plasma cells were detected as well indicating that the engrafted B cells were activated and underwent further differentiation upon transfer (FIG. 20D). Human antibodies in mouse serum were detected at Day 10 post-adoptive transfer, showing a dose-dependent production of IgG (FIG. 20E) and IgM (FIG. 20F).

[0336] With the development of the PBMC humanized mouse model, *in vivo* efficacy of the CD19/CD70 dual CAR T cells was evaluated in the model according to the protocol shown in FIG. 21A. NSG mice (Jackson Laboratory), aged 7-8 weeks, were pre-conditioned with 1Gy irradiation and injected *i.v.* with 2×10^7 healthy donor PBMCs. After 3 days, mice were randomized to receive either the CD19/CD70 dual CAR T cells at two dose levels (1×10^6 or 4×10^6 CAR T cells per mouse) or no treatment. Spleens and blood were collected on day 4 and 7 post CAR T treatment for flow cytometry. Graft and host populations were distinguished by HLA-A2 cell surface expression. Human IgG and IgM levels in the plasma of control and treated mice were measured by ELISA (ThermoFisher). As shown in FIGs. 21B and 21C, administering the CD19/CD70 dual CAR T cells at both dose levels resulted in CD19⁺ B cell depletion (FIG. 21B and FIG. 21C, top panel) and reduction in antibody production in the mice (FIG. 21C, bottom panel). In addition, the total expansion of host CD3⁺ T cells in the spleen was reduced on Day 7 post CAR T treatment/Day 10 post transfer as compared to the NT control (FIG. 21D) and among which all CD70⁺ T cells were eliminated (FIGs. 21E-21F). In accordance with target killing of CD19⁺ B cells and CD70⁺ T cells, a dose-dependent expansion of CAR T cells was observed in the spleen on Day 7 post treatment/Day 10 post transfer (FIG. 21G) and in the spleen (FIG. 21H, left panel) or blood (FIG. 21H, right panel) of the mice on Day 4 or Day 7 post treatment/Day 7 or Day 10 post transfer.

[0337] Next, *in vivo* efficacy of CD19/CD70 dual CAR T cells was evaluated in a hematopoietic stem cell (HSC) humanized mouse model as illustrated in FIG. 22A. CD34⁺ hu-NSG mice (Jackson Laboratory), aged 16-17 weeks, were treated with the CD19/CD70 dual CAR T cells or non-transduced T cells (6×10^6 cells/mouse) via *i.v.* injection. Changes in the circulating B and T cells in mouse peripheral blood were monitored weekly by flow cytometry. Graft and host populations were distinguished by HLA-A2 cell surface expression. The CD19/CD70 dual CAR T cells eliminated human B cells in the CD34⁺ hu-NSG mice on day 7 post treatment (FIG. 22B) while the CD19/CD70 dual CAR T

expansion was observed (FIG. 22D). Thereafter, B cell depletion persisted until Day 28 post treatment, when host human B cells recovery was detected (FIG. 22B and FIG. 22C, top panel), concomitant with the decline of the CD19/CD70 dual CAR T cells expansion (FIG. 22E). The host human T cells developed in this model were all naïve CD70⁻ T cells (data not shown) and were largely unaffected by the CD19/CD70 dual CAR T treatment (FIG. 22C, bottom panel).

[0338] Example 11 Further Evaluation of the CD19/CD70 Dual CAR T Cells

[0339] Because T cells may be activated upon infection, CD70 CAR activity could suppress host T cell responses against pathogens. For instance, virus-specific T cells may become activated during a productive infection and may upregulate CD70 and susceptible to CD70 CAR targeting. We therefore evaluated the potential for the dual CAR T cells to affect virus-specific T cell (VST) activity. In this experiment, CMV pp65-specific T cells (VST) were co-cultured with the pp65 peptide-pulsed antigen-presenting cells (APCs) and the CD19 CAR T cells, CD19/CD70 dual CAR T cells or non-transduced control cells (NTD) at an 8:1:1 ratio for 48 or 72 hours. APCs alone were also included as a control. See FIG. 23A. The data show that the elimination of the viral peptide-pulsed APCs by VST cells was unaffected by the presence of the CD19/CD70 dual CAR T cells (FIG. 23B), and the VST counts at the end of the experiment were not affected by the presence of the CD19/CD70 dual CAR T cells (FIG. 23C). These results were shown as the average of 3 technical replicates at 48 hrs. Similar results were observed at 72 hours. APCs are defined as HLA-A2⁺CD3^{neg}TCR^{neg}CD20⁺ and VSTs were defined as HLA-A2⁺CD3⁺TCR⁺CD20⁻. The CAR T and NTD cells were gated based on HLA-A2 expression and the results were from a single graft: host donor pair.

[0340] We next investigated whether the cytotoxic activities and expansion of the CD19/CD70 dual CAR T cells can be controlled by an external agent, such as ruxolitinib. Ruxolitinib is a Janus kinase (JAK) inhibitor, modulating the JAK-STAT pathway in immune cells. See, *e.g.* Heine *et al.*, 2013, Blood, 122; 1192-1202; Mantov *et al.*, 2022, Front Pharmacol., 13:896167. In this experiment, the dual CAR T cells were co-cultured with target Raji RGL cells (CD19⁺ CD70KO) at various E:T ratios and incubated in the presence or absence of 10 μ M ruxolitinib for 24 hrs or 96 hrs. The data in FIG. 24A show that ruxolitinib suppressed cytolytic activity of the CD19/CD70 dual CAR T cells in a luciferase-based assay. The data in FIG. 24B show that ruxolitinib reduced proliferation of

the CD19/CD70 dual CAR T cells after 5 days of co-culture at an E:T ratio of 1:2 in a flow-based assay.

[0341] Although the disclosed teachings have been described with reference to various
5 applications, methods, kits, and compositions, it will be appreciated that various changes
and modifications can be made without departing from the teachings herein and the claimed
invention below. The foregoing examples are provided to better illustrate the disclosed
teachings and are not intended to limit the scope of the teachings presented herein. While
the present teachings have been described in terms of these exemplary embodiments, the
10 skilled artisan will readily understand that numerous variations and modifications of these
exemplary embodiments are possible without undue experimentation. All such variations
and modifications are within the scope of the current teachings.

What is claimed is:

1. A polynucleotide comprising a truncated PGK promoter, a first coding sequence and a second coding sequence, wherein the truncated PGK promoter comprises at least 50 nucleotides deletion from the 5' end of a long PGK promoter having the nucleotide sequence of SEQ ID NO: 34, wherein the truncated PGK promoter is operably linked to the first coding sequence and the second coding sequence, and wherein either the first or the second coding sequence encodes an anti-CD19 chimeric antigen receptor (CAR).
2. The polynucleotide of claim 1, wherein the first and second coding sequences are linked by a P2A peptide.
3. The polynucleotide of any one of the preceding claims, wherein the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 4.
4. The polynucleotide of any one of the preceding claims, wherein the anti-CD19 CAR further comprises a CD8 hinge and transmembrane domains.
5. The polynucleotide of any one of the preceding claims, wherein the anti-CD19 CAR further comprises a CD3 signaling domain and/or a 4-1BB costimulatory domain.
6. The polynucleotide of any one of the preceding claims, wherein the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 5 or 7.
7. The polynucleotide of any one of the preceding claims, wherein the coding sequence that encodes the anti-CD19 CAR comprises a nucleotide sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 6 or 8.
8. The polynucleotide of any one of the preceding claims, wherein the truncated PGK promoter comprises at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides, or at least 350 nucleotides deletion from the 5' end of the long PGK promoter having the nucleotide sequence of SEQ ID NO: 34.
9. The polynucleotide of any one of the preceding claims, wherein the truncated PGK promoter comprises a nucleotide sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35, SEQ ID NO: 36, or SEQ ID NO: 37.
10. The polynucleotide of claim 9, wherein the truncated PGK promoter comprises a nucleotide sequence:

- 5 (a) comprising about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, or about 390 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35;
- 10 (b) comprising about 210, about 220, about 230 about 240, about 250, about 260, about 270, about 280, or about 290 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 36;
- 15 (c) comprising about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, or about 190 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 37; or
- (d) comprising about 50, about 60, about 70, about 80, about 90 or about 100 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 38.
- 20 11. The polynucleotide of any one of the preceding claims, wherein the first coding sequence encodes the anti-CD19 CAR and the second coding sequence encodes a CD70-binding protein, or wherein the second coding sequence encodes the anti-CD19 CAR and the first coding sequence encodes a CD70 binding protein.
- 25 12. The polynucleotide of claim 11, wherein the CD70-binding protein comprises an anti-CD70 antibody or an antigen-binding fragment thereof, and a transmembrane domain, and optionally wherein the CD70-binding protein further comprises a hinge domain, and wherein the hinge domain and the transmembrane domain are CD8 hinge domain and CD8 transmembrane domain, or CD28 hinge domain and CD28 transmembrane domain.
- 30 13. The polynucleotide of claim 11 or 12, wherein the CD70-binding protein further comprises a CD3z signaling domain.
14. The polynucleotide of any one of claims 11-13, wherein the CD70-binding protein comprise a CD3z signaling domain and does not comprise a costimulatory domain.

15. The polynucleotide of any one of claims 11-14, wherein the CD70-binding protein comprises the amino acid sequence of SEQ ID: 16 and SEQ ID NO: 17.

16. The polynucleotide of any one of claims 11-15, wherein the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 18.

5 17. The polynucleotide of any one of claims 11-16, wherein the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 19 or 21.

18. The polynucleotide of any one of claims 11-17, wherein the coding sequence that encodes the CD70-binding protein comprises a nucleotide sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to
10 SEQ ID NO: 20 or 22.

19. The polynucleotide of any one of claims 1-10, wherein the first coding sequence encodes a chimeric cytokine receptor (CCR) and the second coding sequence encodes the anti-CD19 CAR, or the first coding sequence encodes the anti-CD19 CAR and the second coding sequence encodes a CCR.

15 20. The polynucleotide of claim 19, wherein the CCR comprises a thrombopoietin receptor/ myeloproliferative leukemia protein receptor (TPOR/MPLR) transmembrane and JAK binding domain and an intracellular recruiting domain.

21. The polynucleotide of claim 20, wherein the TPOR/MPLR transmembrane and JAK binding domain comprises the amino acid sequence of SEQ ID NO: 25 or 51.

20 22. The polynucleotide of claim 20 or 21, wherein the intracellular recruiting domain comprises the amino acid sequence of SEQ ID NO: 26.

23. The polynucleotide of any one of claims 19-22, wherein the CCR comprises the amino acid sequence of SEQ ID NO: 27 or 29.

24. The polynucleotide of any one of claims 19-23, wherein the coding sequence
25 that encodes a CCR comprises the nucleotide sequence having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 28 or 30.

25. The polynucleotide of any one of the preceding claims, further comprising a 5' homology arm and a 3' homology arm, wherein the 5' and 3' homology arms are each at least 100 nucleotides in length.

30 26. The polynucleotide of claim 25, wherein the 5' homology arm and the 3' homology arm each comprises a nucleotide sequence homologous to a nucleotide sequence of a T cell receptor alpha constant region (TRAC) gene.

27. A vector comprising the polynucleotide sequence of any one of claims 1-26.

28. The vector of claim 27, wherein the vector is an adeno-associated virus (AAV) vector or a lentiviral vector.

29. An immune cell comprising the polynucleotide of any one of claims 1-18.

30. The immune cell of claim 29, wherein the polynucleotide is integrated into a human TRAC gene.

31. The immune cell of claim 30, wherein the integration of the polynucleotide into the human TRAC gene is mediated by a CRISPR endonuclease.

32. The immune cell of claim 31, wherein the CRISPR endonuclease is Cas 9 or Cas12.

33. The immune cell of any one of claims 30-32, wherein the CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the TRAC gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration, and optionally wherein the target sequence comprises the nucleic acid sequence of SEQ ID NO: 92.

34. The immune cell of any one of claims 29-33, further comprising an additional polynucleotide that comprises a promoter operably linked to one or more coding sequences, optionally wherein the one or more coding sequence encodes a chimeric cytokine receptor (CCR).

35. The immune cell of claim 34, wherein the additional polynucleotide further comprises a 5' homology arm and a 3' homology arm, wherein the 5' and 3' homology arms each are at least 100 nucleotides in length.

36. The immune cell of claim 35, wherein the 5' and 3' homology arms each comprises a nucleotide sequence homologous to a nucleotide sequence of a CD52 gene.

37. The immune cell of any one of claims 34-36, wherein the additional polynucleotide further comprises an EFS promoter or a truncated PGK promoter operably linked to the one or more coding sequences that encodes the CCR, wherein the truncated PGK promoter comprises at least 50 nucleotides deletion from the 5' end of the long PGK promoter having the nucleotide sequence of SEQ ID NO: 34.

38. The immune cell of claim 37, wherein the additional polynucleotide further comprises a truncated PGK promoter that comprises a nucleotide sequence:

- (a) comprising about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, or about 390 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at

least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35;

5 (b) comprising about 210, about 220, about 230 about 240, about 250, about 260, about 270, about 280, or about 290 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 36;

10 (c) comprising about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, or about 190 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 37; or

15 (d) comprising about 50, about 60, about 70, about 80, about 90 or about 100 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 38.

39. The immune cell of any one of claims 34-38, wherein the additional polynucleotide is integrated into the CD52 gene.

20 40. The immune cell of any one of claims 29-39 expressing the anti-CD19 CAR comprising the amino acid sequence of SEQ ID NO: 5 or 7, and the CD70-binding protein comprising the amino acid sequence of SEQ ID NO: 19 or 21.

41. The immune cell of any one of claims 29-40 further expressing the CCR comprising the amino acid sequence of SEQ ID NO: 27 or 29.

25 42. An immune cell comprising the polynucleotide of any one of claims 19-26.

43. The immune cell of claim 42, wherein the polynucleotide is integrated into a human TRAC gene.

44. The immune cell of claim 43, wherein the integration of the polynucleotide at the human TRAC gene by a CRISPR endonuclease.

30 45. The immune cell of claim 44, wherein the CRISPR endonuclease is Cas 9 or Cas12.

46. The immune cell of any one of claims 43-45, wherein the CRISPR endonuclease cleaves the TRAC gene at a target sequence, and the polynucleotide is integrated into the TRAC gene at the target sequence by AAV-mediated site-specific

integration, optionally wherein the polynucleotide is inserted into the TRAC gene at the target sequence of SEQ ID NO: 92.

47. The immune cell of any one of claims 42-46 further comprising an additional polynucleotide that comprises a promoter operably linked to one or more coding sequences, wherein the one or more coding sequences encodes a CD70-binding protein.

48. The immune cell of 47, wherein the additional polynucleotide further comprises a 5' homology arm and a 3' homology arm, wherein the 5' and 3' homology arms each are at least 100 nucleotides in length.

49. The immune cell of claim 48, wherein the 5' and 3' homology arms each comprises a nucleotide sequence homologous to a nucleotide sequence of a CD52 gene.

50. The immune cell of any one of claims 47-49, wherein the additional polynucleotide further comprises an EFS promoter or a truncated PGK promoter operably linked to the third coding sequence that encodes the CD70-binding protein, wherein the truncated PGK promoter comprises at least 50 nucleotides deletion from the 5' end of the long PGK promoter having the nucleotide sequence of SEQ ID NO: 34.

51. The immune cell of claim 50, wherein the additional polynucleotide further comprises a truncated PGK promoter that comprises a nucleotide sequence:

- (a) comprising about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, or about 390 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 35;
- (b) comprising about 210, about 220, about 230 about 240, about 250, about 260, about 270, about 280, or about 290 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 36;
- (c) comprising about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, or about 190 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 37; or

(d) comprising about 50, about 60, about 70, about 80, about 90 or about 100 nucleotides deletion from the 5' end of SEQ ID NO: 34 and having at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 38.

5 52. The immune cell of any one of claims 42-51 expressing the anti-CD19 CAR comprising the amino acid sequence of SEQ ID NO: 5 or 7, and the CCR comprising the amino acid sequence of SEQ ID NO: 27 or 29.

 53. The immune cell of any one of claims 42-52 further expressing the CD70-binding protein comprising the amino acid sequence of SEQ ID NO: 19 or 21.

10 54. The immune cell of any one of claims 29-53, wherein the immune cell is a T cell, an NK cell, an NK-T cell, a tumor infiltrating lymphocyte (TIL), or a T cell of NK cell derived from an iPSC.

 55. An immune cell comprising a recombinant polynucleotide sequence integrated at a human TRAC gene, wherein the recombinant polynucleotide sequence comprises a 5' region of the human TRAC gene, the polynucleotide of any one of claims 1-18, and a 3' region of the human TRAC gene, wherein the integrated recombinant polynucleotide sequence prevents expression of the human TRAC gene.

 56. The immune cell of claim 55, wherein the integration of the recombinant polynucleotide at the human TRAC gene is mediated a CRISPR endonuclease.

20 57. The immune cell of claim 56, wherein the CRISPR endonuclease is Cas 9 or Cas12.

 58. The immune cell of any one of claims 55-57, wherein the CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the TRAC gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration, and optionally wherein the target sequence comprises the nucleic acid sequence of SEQ ID NO: 92.

 59. The immune cell of any one of claims 55-58 further comprising an additional recombinant polynucleotide sequence integrated at a human CD52 gene, wherein the additional recombinant polynucleotide sequence comprises a 5' region of the human CD52 gene, a coding sequence that encodes a CCR, and a 3' region of the human CD52 gene.

30 60. The immune cell of claim 59, wherein the integration of the additional recombinant polynucleotide at the human CD52 gene is mediated a CRISPR endonuclease.

61. The immune cell of claim 60, wherein the CRISPR endonuclease is Cas 9 or Cas12.

62. The immune cell of claim 55-61, wherein the immune cell expresses the anti-CD19 CAR comprising the amino acid of SEQ ID NO: 5 or 7, and the CD70-binding protein comprising the amino acid of SEQ ID NO: 19 or 21, and optionally further expresses the CCR comprising the amino acid sequence of SEQ ID NO: 27 or 29.

63. An immune cell comprising a recombinant polynucleotide sequence integrated at a human TRAC gene, wherein the recombinant polynucleotide sequence comprises a 5' region of the human TRAC gene, the polynucleotide of any one of claims 19-26, and a 3' region of the human TRAC gene, wherein the integrated recombinant polynucleotide sequence prevents expression of the human TRAC gene.

64. The immune cell of claim 63, wherein the integration of the recombinant polynucleotide at the human TRAC gene is mediated a CRISPR endonuclease.

65. The immune cell of claim 64, wherein the CRISPR endonuclease is Cas 9 or Cas12.

66. The immune cell of any one of claims 63-65, wherein the CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the TRAC gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration, and optionally wherein the target sequence comprises the nucleic acid sequence of SEQ ID NO: 92.

67. The immune cell of any one of claims 63-66 further comprising an additional recombinant polynucleotide sequence integrated at a human CD52 gene, wherein the additional recombinant polynucleotide sequence comprises a 5' region of the human CD52 gene, a coding sequence that encodes a CD70-binding protein, and a 3' region of the human CD52 gene.

68. The immune cell of claim 67, wherein the integration of the additional recombinant polynucleotide at the human CD52 gene is mediated a CRISPR endonuclease.

69. The immune cell of claim 68, wherein the CRISPR endonuclease is Cas 9 or Cas12.

70. The immune cell of any one of claims 63-68 expressing the anti-CD19 CAR comprising the amino acid of SEQ ID NO: 5 or 7, and the CCR comprising the amino acid of SEQ ID NO: 27 or 29, and optionally further expressing the CD70-binding protein comprising the amino acid sequence of SEQ ID NO: 19 or 21.

71. An immune cell comprising a polynucleotide encoding an anti-CD19 CAR and a CD70-binding protein, wherein the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 5 or 6, and the CD70-binding protein comprises the amino acid sequence of SEQ ID NO: 19 or 21.

5 72. The immune cell of claim 71, wherein the polynucleotide is integrated into a human TRAC gene by a CRISPR endonuclease, wherein the CRISPR endonuclease makes a double strand break to create an integration site in a target sequence within the TRAC gene and the polynucleotide is integrated into the integration site by AAV-mediated site-specific integration, and optionally wherein the target sequence comprises the nucleic acid
10 sequence of SEQ ID NO: 92.

73. The immune cell of any one of claims 29-72, wherein the immune cell is from a patient.

74. The immune cell of any one of claims 29-72, wherein the immune cell is from a healthy donor.

15 75. A population of immune cells comprising a plurality of the immune cells of any one of claims 29-74.

76. A pharmaceutical composition comprising the immune cell of any one of claims 29-74, or the population of the immune cell of claim 75, and a pharmaceutically acceptable excipient.

20 77. A method of treating a disease condition in a subject, comprising administering to the subject the immune cell of any one of claims 29-74, the population of immune cells of claim 75, or the pharmaceutical composition of claim 76.

78. The method of claim 77, wherein the immune cell is capable of reducing or depleting B cells, pathogenic B cells, autoreactive B cells, pathogenic T cells, autoreactive
25 T cells, or alloreactive T cells.

79. The method of claim 77 or 78, wherein the disease condition is cancer or an autoimmune disease or disorder.

80. The method of any one of claims 77-79, wherein the disease condition is a non-Hodgkin's lymphoma.

30 81. The method of claim 79 or 80, wherein the disease condition is large B cell lymphoma (LBCL), follicular lymphoma (FL), T cell lymphoma (TCL), B cell acute lymphoblastic leukemia (BALL), T cell acute lymphoblastic leukemia (TALL), primary

central nervous system lymphoma (PCNSL), Mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), peripheral T cell lymphoma (PTCL).

82. The method of claim 79, wherein the autoimmune disease or disorder is lupus, systemic lupus erythematosus (SLE), lupus nephritis, rheumatoid arthritis, systemic sclerosis, scleroderma, multiple sclerosis (MS), relapse remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS), neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis, Sjogren's syndrome, and myositis.

83. The method of any one of claims 77-82, wherein the immune cell is autologous with respect to the subject.

84. The method of any one of claims 77-82, wherein the immune cell is allogeneic with respect to the subject.

85. A method of making an engineered immune cell comprising introducing to the immune cell an engineered endonuclease that cleaves at a target sequence within a human TRAC gene and the polynucleotide of any one of claims 1-26 or the vector of claim 27 or 28, and culturing the immune cell under suitable conditions.

86. The method of claim 85, wherein the polynucleotide is introduced to the immune cell by transfection, electroporation, LVV transduction, or AAV transduction.

87. The method of claim 85 or 86, wherein the engineered endonuclease is a CRISPR endonuclease, wherein the polynucleotide is integrated into the TRAC gene at the target sequence, and optionally wherein the CRISPR endonuclease is Cas12i.

88. An engineered immune cell comprising or expressing an anti-CD19 CAR and an anti-CD70 CAR, wherein the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 5 or 7, and the anti-CD70 CAR comprises the amino acid sequence of SEQ ID NO: 19 or 21.

89. An engineered immune cell comprising a polynucleotide that comprises a nucleic acid sequence encoding an anti-CD19 CAR comprising the amino acid sequence of SEQ ID NO: 5 or 7, and an anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 19 or 21.

90. The engineered immune cell of claim 89 wherein the polynucleotide is integrated into the first exon of a TRAC locus.

91. The engineered immune cell of claim 90, wherein the polynucleotide is integrated into the TRAC locus at an integration site that comprises the nucleic acid sequence of SEQ ID NO: 92.

5 92. The engineered immune cell of any one of claims 89-91, wherein the polynucleotide is operably linked to a truncated PGK promoter.

93. A polynucleotide comprising a nucleic acid sequence that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 97.

94. The polynucleotide of claim 93 comprising the nucleic acid sequence of SEQ ID NO: 96.

10 95. The polynucleotide of claim 93 or 94, wherein the polynucleotide is operably linked to a truncated PGK promoter.