NOVEL CD19 BINDERS, CAR-T CONSTRUCTS COMPRISING THE SAME, AND METHODS OF USING THE SAME

CROSS-REFERENCE

[0001] The present application claims priority from U.S. Provisional Application No. 63/417,220, filed on October 18, 2022, and U.S. Provisional Application No. 63/426,967, filed on November 21, 2022, the contents of which are hereby incorporated by reference in their entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to T cells engineered to express a Chimeric Antigen Receptor (CAR) to treat a disease associated with expression of the Cluster of Differentiation 19 protein (CD19).

BACKGROUND

[0003] Recent developments using chimeric antigen receptor (CAR) modified autologous T cell (CART) therapy, which relies on redirecting T cells to a suitable cell-surface molecule on cancer cells such as B cell malignancies, show promising results in harnessing the power of the immune system to treat B cell malignancies and other cancers. The clinical results of the murine derived CART19 (*i.e.*, "CTL019") have shown promise in establishing complete remissions in patients suffering with chronic lymphocytic leukemia (CLL) as well as in childhood acute lymphoid leukemia (ALL). Despite the clinical success of various CD19 CAR T cell therapies, the therapeutic index of these therapies remains high due to immunogenicity issues, toxicities associated with the infusion of the CAR T cells, and relapse of the tumor.

[0004] Accordingly, there is an urgent need in the art for novel approaches that can solve or mitigate the harmful side effects of CAR T cell therapies and allow for more effective, safe, and efficient adoptive immunotherapy. The present disclosure addresses this need.

SUMMARY OF THE INVENTION

[0004a] According to an aspect, the present invention provides an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR),
wherein the CAR comprises a single chain antibody or a single chain antibody fragment

comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain; and

wherein the anti-CD19 binding domain comprises:

(a) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and

a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6; or

(b) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and

a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0004b] According to an aspect, the present invention provides an isolated nucleic acid molecule comprising:

(a) an scFv comprising an anti-CD19 binding domain, wherein the anti-CD19 binding domain comprises:

(i) LC CDR1 of SEQ ID NO: 1, LC CDR2 of SEQ ID NO: 2, and LC CDR3 of SEQ ID NO: 3, HC CDR1 of SEQ ID NO: 4, HC CDR2 of SEQ ID NO: 5, and HC CDR3 of SEQ ID NO: 6; or

(ii) any LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 disclosed in Table 2;

(b) a transmembrane domain selected from CD28 or CD8 transmembrane domain,

(c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB;

and

(d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0004c] According to an aspect, the present invention provides an isolated nucleic acid molecule comprising:

(a) an scFv comprising an anti-CD19 binding domain, wherein the anti-CD19 binding domain comprises the amino acid sequence of SEQ ID NO: 9, 226, or 201;

(b) a transmembrane domain selected from CD28 or CD8 transmembrane domain,

(c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and

(d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0004d] According to an aspect, the present invention provides an isolated nucleic acid molecule comprising:

(a) an scFv comprising an anti-CD19 binding domain, wherein the anti-CD19 binding domain comprises the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146;

(b) a transmembrane domain comprising the amino acid sequence of selected from the group consisting of SEQ ID NO: 29, 31, and 33;

(c) a costimulatory domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50; and

(d) an intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 52 or SEQ ID NO: 54.

[0004e] According to an aspect, the present invention provides an isolated nucleic acid molecule comprising:

(a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168,or 146;

(b) a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 29;

(c) a costimulatory domain comprising the amino acid sequence of SEQ ID NO:

37; and

(d) an intracellular signaling domain comprising of SEQ ID NO: 52 or 54.

[0004f] According to an aspect, the present invention provides a vector comprising a nucleic acid molecule of the invention.

[0004g] According to an aspect, the present invention provides an isolated polypeptide molecule encoded by the nucleic acid molecule of the invention.

[0004h] According to an aspect, the present invention provides a chimeric antigen receptor (CAR) encoded by the nucleic acid molecule of the invention.

[0004i] According to an aspect, the present invention provides a chimeric antigen receptor (CAR) comprising a single chain antibody or a single chain antibody fragment comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain, and

wherein the anti-CD19 binding domain comprises:

(a) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6; or

(b) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and

a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0004j] According to an aspect, the present invention provides a chimeric antigen receptor (CAR) comprising:

(a) an scFv comprising an anti-CD19 binding domain, wherein the anti-CD19 binding domain comprises:

(i) LC CDR1 of SEQ ID NO: 1, LC CDR2 of SEQ ID NO: 2, and LC CDR3 of SEQ ID NO: 3, HC CDR1 of SEQ ID NO: 4, HC CDR2 of SEQ ID NO: 5, and HC CDR3 of SEQ ID NO: 6; or

(ii) any LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 disclosed in Table 2;

(b) a transmembrane domain selected from CD28 or CD8 transmembrane domain;

(c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and

(d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0004k] According to an aspect, the present invention provides a chimeric antigen receptor (CAR) comprising:

(a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 226, or 201;

(b) a transmembrane domain selected from CD28 or CD8 transmembrane domain;

(c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and

(d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0004l] According to an aspect, the present invention provides a chimeric antigen receptor (CAR) comprising:

(a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146;

(b) a transmembrane domain comprising the amino acid sequence of selected from the group consisting of SEQ ID NO: 29, 31, and 33;

(c) a costimulatory domain comprising an amino acid sequence selected from the

group consisting of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50; and

(d) an intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 52 or SEQ ID NO: 54.

[0004m] According to an aspect, the present invention provides a chimeric antigen receptor (CAR) comprising:

(a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146;

(b) a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 29;

(c) a costimulatory domain comprising the amino acid sequence of SEQ ID NO: 37; and

(d) an intracellular signaling domain comprising of SEQ ID NO: 52 or 54.

[0004n] According to an aspect, the present invention provides a chimeric antigen receptor comprising:

(a) an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 66, 77, 88, 148, 170, 181, 203, 214, 159, 192, 23, and 20; and/or

(b) an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 65, 76, 87, 147, 169, 180, 202, 213, 158, 191, 22, and 19.

[0004o] According to an aspect, the present invention provides a chimeric antigen receptor comprising a sequence selected from the group consisting of SEQ ID NO: 63, 74, 85, 145, 167, 178, 200, 211, 156, 189, 17, 8, 62, 73, 84, 144, 166, 177, 199, 210, 155, 188, 16, and 7.

[0004p] According to an aspect, the present invention provides an anti-CD19 binding domain comprising:

(a) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain

comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6; or

(b) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198; or

(c) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and

a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0005] One example of the present disclosure provides an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), that comprises, consists of, or consists essentially of a single chain antibody or a single chain antibody fragment comprising an anti-

CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain. In some embodiments, the anti-CD19 binding domain comprises: (a) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6; or (b) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198; or (c) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0006] In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 or 199; or an amino acid sequence having at least about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the amino acid sequence of SEQ ID NO: 7 or 199.

[0007] In some embodiments, the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8 or 200, or an amino acid sequence having at least about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the amino acid sequence of SEQ ID NO: 8 or 200.

[0008] In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8. In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 199 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 200. In some embodiments, the CD19 binding domain is a scFv.

[0009] In some embodiments, the anti-CD19 binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9,18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146.

[0010] In some embodiments, the anti-CD19 binding domain comprises: (a) a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216; or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, SEQ ID NO: 24 SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0011] In some embodiments, the anti-CD19 binding domain comprises a light chain variable region or a heavy chain variable region encoded by: (a) a nucleic acid sequence selected from a group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216, or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0012] In some embodiments, the transmembrane domain comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD2, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9.

[0013] In some embodiments, the transmembrane domain comprises an amino acid sequence selected from SEQ ID NO: 29, 31, or 33, or an amino acid sequence or an amino acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 29, 31, or 33.

[0014] In some embodiments, the transmembrane domain comprises a nucleic acid sequence selected from SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 34 or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30, 32, or 34.

[0015] In some embodiments, the transmembrane domain comprises a CD8 transmembrane domain, and/or an amino acid sequence of SEQ ID NO: 29; or an amino acid sequence having about 90% to about 99% identity to SEQ ID NO: 29. In some embodiments, the transmembrane domain comprises a nucleic acid sequence of SEQ ID NO: 30, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30. In some embodiments, the anti-CD19 binding domain is connected to the transmembrane domain by a hinge region.

[0016] In some embodiments, the hinge region: (a) is from a protein selected from the group consisting of an Fc fragment of an antibody, a hinge region of an antibody, a CH2 region of an antibody, a CH3 region of an antibody, an artificial spacer sequence, an IgG hinge, a CD8 hinge, and any combination thereof; or (b) comprises the amino acid sequence of SEQ ID NO: 27 or SEQ ID NO: 35, or a sequence having about 90% to about 99% identity to SEQ ID NO: 27 or 35.

[0017] In some embodiments, the hinge region comprises a CD8 hinge region and/or the amino acid sequence of SEQ ID NO: 27, or a sequence having about 90%, about 91%, about

92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 27.

[0018] In some embodiments, the hinge region comprises a nucleic acid sequence selected from SEQ ID NO: 28, or SEQ ID NO: 36 or a sequence having about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 28 or 36.

[0019] In some embodiments, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of a TNFR superfamily member, OX40 (CD134), CD2, CD5, CD7, CD27, CD28, CD30, CD40, PD-1, CD8, ICAM-1, lymphocyte function-associated antigen-1 (LFA-1), CD11a, CD18, ICOS (CD278), LIGHT, NKG2C, B7-H3, a ligand that specifically binds to CD83, DAP10, DAP12, Lck, Fas and 4-1BB (CD137).

[0020] In some embodiments, the costimulatory domain comprises an amino acid sequence selected from SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, or SEQ ID NO: 50, or a sequence having about 90% to about 99% identity to SEQ ID NO: 37, 39, 41, 43, 46, 48, or 50.

[0021] In some embodiments, the costimulatory domain comprises a nucleic acid sequence selected from SEQ ID NO: 38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO: 45, SEQ ID NO:47, or SEQ ID NO:49, or a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 38, 40, 42, 44, 45, 47, or 49.

[0022] In some embodiments, the intracellular signaling domain comprises a signaling domain of a protein selected from the group consisting of CD3 zeta, FcyRIII, FcsRI, a cytoplasmic tail of an Fc receptor, an immunoreceptor tyrosine-based activation motif (ITAM) bearing cytoplasmic receptor, TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

[0023] In some embodiments, the intracellular signaling domain comprises the intracellular signaling domain of CD3 zeta, the amino acid sequence of SEQ ID NO: 52 or 54, or a sequence having about 90% to about 99% identity to SEQ ID NO: 52 or 54.

[0024] In some embodiments, the intracellular signaling domain comprises the nucleic acid sequence of SEQ ID NO: 53 or 55, or a sequence having about 90% to about 99% identity to

SEQ ID NO: 53 or 55. In some embodiments, the CAR comprises a functional 4-1BB costimulatory domain and a functional CD3 zeta intracellular signaling domain. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52, or SEQ ID NO:54 or a sequence having about 90% to about 99% identity to an amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52 or SEQ ID NO:54.

[0025] In some embodiments, the intracellular signaling domain comprises the sequence of SEQ ID NO: 37 and the sequence of SEQ ID NO: 52 or SEQ ID NO: 54, or a sequence having about 90% to about 99% identity to SEQ ID NO: 37, SEQ ID NO: 52 or SEQ ID NO: 54. In that embodiment, the sequences are expressed in the same frame and as a single polypeptide chain.

[0026] In some embodiments of the isolated nucleic acid molecule disclosed herein, (a) the nucleic acid sequence comprises a sequence of SEQ ID NO: 38, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 38, and/or (b) a sequence of SEQ ID NO: 53 or SEQ ID NO: 55, or a sequence having about 90% to about 99% identity to SEQ ID NO: 53 or 55.

[0027] In some embodiments of the isolated nucleic acid molecule disclosed herein, the CAR further comprises a leader sequence. In some embodiments, the leader sequence comprises the amino acid of SEQ ID NO: 25.

[0028] Another example of the present disclosure provides an isolated nucleic acid molecule comprising: (a) an scFv comprising an anti-CD19 binding domain, where the anti-CD19 binding domain comprises: (i) LC CDR1 of SEQ ID NO: 1, LC CDR2 of SEQ ID NO: 2, and LC CDR3, HC CDR1 of SEQ ID NO: 4, HC CDR2 of SEQ ID NO: 5, and HC CDR3 of SEQ ID NO: 6; or (ii) LC CDR1 of SEQ ID NO: 193, LC CDR2 of SEQ ID NO: 194, LC CDR3 of SEQ ID NO: 195; HC CDR1 of SEQ ID NO: 196, HC CDR2 of SEQ ID NO: 197, and HC CDR3 of SEQ ID NO: 198; or(iii) any LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 disclosed in Table 2; (b) a transmembrane domain selected from CD28 or CD8 transmembrane domain; (c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2,

CD28, ICOS, and 4-1BB; and (d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0029] Another example of the present disclosure provides an isolated nucleic acid molecule comprising: (a) an scFv comprising an anti-CD19 binding domain, wherein the anti-CD19 binding domain comprises the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; (b) a transmembrane domain selected from CD28 or CD8 transmembrane domain; (c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and (d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0030] Another example of the present disclosure provides an isolated nucleic acid molecule comprising: (a) an scFv comprising an anti-CD19 binding domain, wherein the anti-CD19 binding domain comprises the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; (b) a transmembrane domain comprising the amino acid sequence of selected from the group consisting of SEQ ID NO: 29, 31, and 33; (c) a costimulatory domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50; and (d) an intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 52 or SEQ ID NO: 54.

[0031] Another example of the present disclosure provides an isolated nucleic acid molecule comprising: (a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168,or 146; (b) a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 29; (c) a costimulatory domain comprising the amino acid sequence of SEQ ID NO: 37; and (d) an intracellular signaling domain comprising of SEQ ID NO: 52 or 54.

[0032] In some embodiments, the isolated nucleic acid comprises: (a) an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 66, 77, 88, 148, 170, 181, 203, 214, 159, 192, 23, and 20; and/or (b) an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 65, 76, 87, 147, 169, 180, 202, 213, 158, 191, 22, and 19.

[0033] Another example of the present disclosure provides an isolated polypeptide molecule encoded by the nucleic acid molecule disclosed herein.

[0034] In some embodiments, the isolated polypeptide comprises a sequence selected from the group consisting of SEQ ID NO: 63, 74, 85, 145, 167, 178, 200, 211, 156, 189, 17, 8, 62, 73, 84, 144, 166, 177, 199, 210, 155, 188, 16, and 7.

[0035] Another example of the present disclosure provides a chimeric antigen receptor (CAR) comprising a single chain antibody or a single chain antibody fragment comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain where the anti-CD19 binding domain comprises: (a) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6; or (b) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198; or (c) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0036] In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 or 199; or an amino acid sequence having at least about 90% to about 99% identity to SEQ ID NO: 7 or 199. In some embodiments, the heavy chain variable

region comprises the amino acid sequence of SEQ ID NO: 8 or 200, or an amino acid sequence having at least about 90% to about 99% identity to SEQ ID NO: 8 or 200. In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8. In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 199 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 200.

[0037] In some embodiments, the CD19 binding domain is a scFv. In some embodiments, the anti-CD19 binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146.

[0038] In some embodiments, the anti-CD19 binding domain comprises: (a) a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216; or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0039] In some embodiments, the anti-CD19 binding domain comprises a light chain variable region or a heavy chain variable region encoded by: (a) a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216, or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID

NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0040] In some embodiments, the transmembrane domain comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD2, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD 154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9.

[0041] In some embodiments, the transmembrane domain comprises an amino acid sequence selected from SEQ ID NO: 29, 31, or 33, or an amino acid sequence about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 29, 31, or 33.

[0042] In some embodiments, the transmembrane domain comprises a nucleic acid sequence selected from SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 34 or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30, 32, or 34.

[0043] In some embodiments, the transmembrane domain comprises a CD8 transmembrane domain, and/or an amino acid sequence of SEQ ID NO: 29; or an amino acid sequence having about 90% to about 99% identity to SEQ ID NO: 29. In some embodiments, the transmembrane domain comprises a nucleic acid sequence of SEQ ID NO: 30, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30.

[0044] In some embodiments, the anti-CD19 binding domain is connected to the transmembrane domain by a hinge region. In some embodiments of the CAR disclosed herein, the hinge region: (a) is from a protein selected from the group consisting of an Fc fragment of an antibody, a hinge region of an antibody, a CH2 region of an antibody, a CH3 region of an antibody, an artificial spacer sequence, an IgG hinge region, a CD8 hinge, and any combination thereof; or (b) comprises the amino acid sequence of SEQ ID NO: 27 or SEQ ID NO: 35, or a sequence having about 90%, about 91%, about 92%, about 93%, about

94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 27 or 35.

[0045] In some embodiments, the hinge region comprises a CD8 hinge region and/or the amino acid sequence of SEQ ID NO: 27, or a sequence having about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 27. In some embodiments, the hinge region comprises a nucleic acid sequence selected from SEQ ID NO: 28, or SEQ ID NO: 36 or a sequence having about 95%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 28 or 36.

[0046] In some embodiments of the CAR disclosed herein, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of a TNFR superfamily member, OX40 (CD134), CD2, CD5, CD7, CD27, CD28, CD30, CD40, PD-1, CD8, ICAM-1, lymphocyte function-associated antigen-1 (LFA-1), CD11a, CD18, ICOS (CD278), LIGHT, NKG2C, B7-H3, a ligand that specifically binds to CD83, DAP10, DAP12, Lck, Fas and 4-1BB (CD137).

[0047] In some embodiments, the costimulatory domain comprises an amino acid sequence selected from SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, or SEQ ID NO: 50, or a sequence having about 90% to about 99% identity to SEQ ID NO: 37, 39, 41, 43, 46, 48, or 50.

[0048] In some embodiments, the costimulatory domain comprises a nucleic acid sequence selected from SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 47, or SEQ ID NO: 49, or a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 38, 40, 42, 44, 45, 47, or 49.

[0049] In some embodiments, the intracellular signaling domain comprises a signaling domain of a protein selected from the group consisting of CD3 zeta, FcyRIII, FcsRI, a cytoplasmic tail of an Fc receptor, an immunoreceptor tyrosine-based activation motif (ITAM) bearing cytoplasmic receptor, TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

[0050] In some emnodiments, the intracellular signaling domain comprises a CD3 zeta intracellular domain, the amino acid sequence of SEQ ID NO: 52 or 54, or a sequence having about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 52 or 54.

[0051] In some embodiments, the intracellular signaling domain comprises the nucleic acid sequence of SEQ ID NO: 53 or 55, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 53 or 55. In some embodiments, the CAR comprises a functional 4-1BB costimulatory domain and a functional CD3 zeta intracellular signaling domain. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52, or SEQ ID NO: 54 or a sequence having about 90, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to an amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52 or SEQ ID NO: 54.

[0052] In some embodiments, the intracellular signaling domain comprises the sequence of SEQ ID NO: 37 and the sequence of SEQ ID NO: 52 or SEQ ID NO:54, or a sequence having about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to an amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52, or SEQ ID NO:54. In that embodiment, the sequences are expressed in the same frame and as a single polypeptide chain.

[0053] In some embodiments, the nucleic acid sequence comprises a sequence of SEQ ID NO: 38, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 38, and/or a sequence of SEQ ID NO: 53 or SEQ ID NO:55, or a sequence having about 90%, , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 53 or 55. In some embodiments, the CAR further comprises a leader sequence. In some embodiments, the leader sequence comprises SEQ ID NO: 25.

[0054] One example of the present disclosure provides a chimeric antigen receptor (CAR) comprising: (a) an scFv comprising an anti-CD19 binding domain, where the anti-CD19 binding domain comprises: (i) LC CDR1 of SEQ ID NO: 1, LC CDR2 of SEQ ID NO: 2, and

LC CDR3, HC CDR1 of SEQ ID NO: 4, HC CDR2 of SEQ ID NO: 5, and HC CDR3 of SEQ ID NO: 6; or (ii) LC CDR1 of SEQ ID NO: 193, LC CDR2 of SEQ ID NO: 194, LC CDR3 of SEQ ID NO: 195; HC CDR1 of SEQ ID NO: 196, HC CDR2 of SEQ ID NO: 197, and HC CDR3 of SEQ ID NO: 198; any LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 disclosed in Table 2; (b) a transmembrane domain selected from CD28 or CD8 transmembrane domain; (c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and (d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0055] One example of the present disclosure provides a chimeric antigen receptor (CAR) comprising: (a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; (b) a transmembrane domain selected from CD28 or CD8 transmembrane domain; (c) a costimulatory domain comprising an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and (d) an intracellular signaling domain comprising of CD3-zeta or FcR gamma.

[0056] One example of the present disclosure a chimeric antigen receptor (CAR) comprising: (a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; (b) a transmembrane domain comprising the amino acid sequence of selected from the group consisting of SEQ ID NO: 29, 31, and 33; (c) a costimulatory domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50; and (d) an intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 52 or SEQ ID NO: 54.

[0057] Another example of the present disclosure provides a chimeric antigen receptor (CAR) comprising: (a) an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; (b) a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 29; (c) a costimulatory domain comprising the amino acid sequence of SEQ ID NO: 37; and (d) an intracellular signaling domain comprising of SEQ ID NO: 52 or 54.

[0058] Another example of the present disclosure provides a chimeric antigen receptor comprising: (a) an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 66, 77, 88, 148, 170, 181, 203, 214, 159, 192, 23, and 20; and/or (b) an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 65, 76, 87, 147, 169, 180, 202, 213, 158, 191, 22, and 19.

[0059] Another example of the present disclosure provides a chimeric antigen receptor comprising a sequence selected from the group consisting of SEQ ID NO: 63, 74, 85, 145, 167, 178, 200, 211, 156, 189, 17, 8, 62, 73, 84, 144, 166, 177, 199, 210, 155, 188, 16, and 7.

[0060] Another example of the present disclosure provides an anti-CD19 binding domain comprising: (a) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6; or (b) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198; or (c) a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0061] In some embodiments, the anti-CD19 binding domain is a scFv comprising: (a) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 7 or 199, or a

sequence having about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity y to SEQ ID NO: 7 or 199; and/or (b) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 8, or 200, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 8 or 200.

[0062] Another example of the present disclosure provides a vector comprising a nucleic acid molecule disclosed herein. In some embodiments, the vector is selected from the group consisting of a DNA, a RNA, a plasmid, a lentivirus vector, an adenoviral vector, or a retroviral vector. In some embodiments, the vector further comprises a promoter, a rev response element (RRE), a poly(A) tail, a 3' UTR, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE); and/or a cPPT sequence.

[0063] In some embodiments of the vector disclosed herein, the promoter: (a) is a constitutive promoter; (b) is selected from the group consisting of an EF-1alpha promoter, a PGK-1 promoter, a truncated PGK-1 promoter, an UBC promoter, a CMV promoter, a CAGG promoter, and an SV40 promoter; (c) is an EF-1 promoter; or (d) comprises the sequence of SEQ ID NO: 101.

[0064] In some embodiments, the WPRE comprises the sequence of SEQ ID NO: 100. In some embodiments, the vector is a lentiviral vector. In some embodiments, the vector is an in vitro transcribed vector.

[0065] In some embodiments, the vector comprises the isolated nucleic acid molecule disclosed herein operably linked via a linker peptide to a nucleic acid sequence encoding a switch receptor and/or a dominant negative receptor. In that embodiment, the linker peptide: (a) is selected from F2A, E2A, P2A, T2A, or Furin-(G4S)2-T2A (F-GS2-T2A); (b) comprises the amino acid sequence of SEQ ID NO: 92, SEQ ID NO:94, SEQ ID NO:96, or SEQ ID NO: 99; or (c) comprises the nucleic acid sequence of SEQ ID NO: 93, 95, 97, or 98.

[0066] Another example of the present disclosure provides a modified cell comprising: (a) the isolated nucleic acid molecule disclosed herein; (b) the isolated polypeptide disclosed herein; (c) the CAR disclosed herein; (d) the anti-CD19 binding domain disclosed herein; or (b) the vector disclosed herein. In some embodiments, the modified cell is a modified immune cell, a modified natural killer (NK) cell, a modified natural killer T (NKT) cell, or a

modified T cell. In some embodiments, the modified cell is a modified T cell or a modified human T cell. In some embodiments, the modified T cell is a CD8+ T cell. In some embodiments, the modified cell is an autologous cell, heterologous cell, or an allogeneic cell.

[0067] In some embodiments, the modified cell disclosed herein further comprises:(a) a switch receptor comprising a first polypeptide that comprises at least a portion of an inhibitory molecule selected from the group consisting of PD1, TGFβR, TIM-2 and BTLA, conjugated to a second polypeptide that comprises a positive signal from an intracellular signaling domain selected from the group consisting of OX40, CD27, CD28, IL-12R, ICOS, and 4-1BB; (b) a dominant negative receptor comprising a truncated variant of a receptor selected from the group consisting of PD1, TGFβR, TIM-2 and BTLA; and/or (c) a polypeptide that enhances an immune cell function, or a functional derivative thereof selected from the group consisting of a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, Interleukin -7 (IL-7), Interleukin-7 receptor (IL-7R), Interleukin-15 (IL-15), Interleukin-15 receptor (IL-15R), Interleukin-21 (IL-21), Interleukin-18 (IL-18), Interleukin-18 receptor (IL-18R), CCL21, CCL19, and a combination thereof.

[0068] Another example of the present disclosure provides a composition comprising a modified cell or a population of modified cells disclosed herein.

[0069] Another example of the present disclosure provides a method of making a modified cell comprising transfecting a cell with: (a) the isolated nucleic acid molecule disclosed herein; (b) a nucleic acid encoding the CAR disclosed herein; (c) a nucleic acid encoding the anti-CD19 binding domain disclosed herein; or (d) a vector disclosed herein.

[0070] Another example of the present disclosure provides a method of generating a population of RNA-engineered cells comprising transfecting a cell with an in vitro transcribed RNA or synthetic RNA, where the RNA comprises: (a) the isolated nucleic acid molecule disclosed herein; (b) a nucleic acid encoding the CAR disclosed herein; or (c) a nucleic acid encoding the anti-CD19 binding domain disclosed herein.

[0071] Another example of the present disclosure provides a method of providing an anti-tumor immunity in a mammal comprising administering to the mammal an effective amount of: (a) a composition comprising a modified cell expressing a CAR disclosed herein; (b) the modified cell disclosed herein; or (c) the composition disclosed herein.

[0072] Another example of the present disclosure provides a method of treating a mammal having a disease associated with expression of CD19 comprising administering to the mammal an effective amount of: (a) a composition comprising a modified cell expressing a CAR disclosed herein; (b) the modified cell disclosed herein; or (c) the composition disclosed herein.

[0073] In some embodiments, the modified cell is an autologous modified T cell. In some embodiments, the modified cell is an allogeneic modified T cell. In some embodiments, the mammal is a human.

[0074] In some embodiments, the disease associated with CD19 expression is selected from: (a) a proliferative disease, a malignancy, a precancerous condition, or a non-cancer related indication associated with expression of CD19; or (b) a cancer, an atypical and/or a non-classical cancer, a myelodysplasia, a myelodysplastic syndrome, or a preleukemia.

[0075] In some embodiments, the disease is a hematologic cancer selected from the group consisting of: (a) an acute leukemia, a chronic leukemia, a hematologic condition, and combinations thereof; or (b) B-cell acute lymphoid leukemia ("BALL"), T-cell acute lymphoid leukemia ("TALL"), acute lymphoid leukemia (ALL), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, mantle cell lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin's lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, ineffective production (or dysplasia) of myeloid blood cells, and combinations thereof.

[0076] In some embodiments of the method of treatment disclosed herein, the modified cells or the composition are administered in combination with: (a) an agent that increases the efficacy of a cell expressing a CAR molecule; (b) an agent that ameliorates one or more side effects associated with administration of a cell expressing a CAR molecule; or (c) an agent that treats the disease associated with CD19.

BRIEF DESCRIPTION OF THE DRAWINGS

[0077] FIG. 1 shows a schematic outlining the identification of unique CD19-specific antibody clones from phage display libraries followed by biotinylated baculovirus binding, SIGLEC binding, and/or NALM6 tumor cells selection.

[0078] FIGs. 2A-2E show an alignment of the nucleic acid sequences of the novel CD19 binders of the present disclosure.

[0079] FIG. 2F shows a percent identity matrix illustrating the similarity of the novel binders at the nucleic acid level.

[0080] FIGs. 3A-B show bar graphs quantifying the surface expression of and tonic signaling induced by CD19 CARs comprising CD19 binders 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 and 53 (**FIG. 3A**) or optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP (**FIG. 3B**). The CD19 CARs were transduced in Jurkat-NFAT-GFP reporter cells. Tonic signaling was observed in over 60% of transduced cells. Notably, most expressed CD19 CARs induced some tonic signaling, except for original clone 42, which was highly expressed (74.6%), but induced negligible tonic signaling (1.50%). *See also* Table 4 and Table 11.

[0081] FIGs. 4A-B show line graphs illustrating the expansion or growth curve (**FIG. 4A**) and the mean cell size (*i.e.*, contraction) (**FIG. 4B**) of T cells from doner ND607 transduced with a CAR comprising an antigen binding domain of any of clones 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 and 53 when compared to untransduced cells on Day 0 (D0), Day 5 (D5), Day 7 (D7), Day 9 (D9), and Day 11 (D11) after transduction.

[0082] FIGs. 5A-B show bar graphs quantifying cytokine production (IL-2, TNF-α, IFN-γ) in ND607 CD19 CAR T cells expressing CAR comprising original CD19 binders 42, 43, 44, 45, 46, and 52 (**FIG. 5A**) and ND518 CD19 CAR T cells expressing CAR comprising optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP, and 52OP following a 4 hr-stimulation with Nalm6 nine days after transduction. *See also* **Table 6**, **Table 14, and Table 24**.

[0083] FIGs. **6A-D** show graphs illustrating that the expansion profiles of CAR T cells expressing CARs comprising CD19 binders 42, 43, 44, 45, 46, 50, 51, and 52 (**FIGs. 6A-B**),

and the mean CAR T cell size (**FIGs. 6C-D**) were substantially similar over time. The CD19 CARs were transduced in ND539 and ND572 donor T cells. CD19 CARs comprising binder 42 (clone 42) showed a slightly higher expansion and contracted (e.g., rested down) earlier.

[0084] FIGs. 7A-C show bar graphs quantifying CD19 binder CAR T cells manufacturing expansion over time; and demonstrating that the CD19 binder CAR T cells showed similar T cell doubling in manufacturing expansion, with CD19 CAR T cells expressing a CAR comprising the 42 clone showing the greatest expansion (**FIG. 7A**). In addition, the CD19 binder CAR T cells showed similar percent reduction in $CD4^+$ T cells population over time (**FIGs. 7B-C**). The CD19 CAR binders were tested in two different donor T cells, ND539 (**FIG. 7B**) and ND572 (**FIG. 7C**).

[0085] FIGs. 8A-D shows graphs illustrating changes in the percentage of CD19 CAR comprising CD19 binders 42, 43, 44, 46, 50, 51, and 52 staining on T cells from two donors (**FIGs. 8 A-B**) and the percentage of CD19 CAR expressed in the $CD4^+$ T cell population during expansions (**FIGs. 8C-D**). These figures demonstrate that the percentage of $CD4^+$ T cells expressing a novel CD19 CAR binder was the highest on Day 6 but stabilized at later time point.

[0086] FIG. 9 shows a schematic illustrating the timing of T cell isolation, transduction, and expansion of ND539 donor T cells for evaluating the CD19 CARs at the nucleic acid (e.g., RNA) and protein levels. In particular, ND539 donor CAR T cells expressing CARs comprising CD19 binders 42, 43, 44, 45, 46, and 52 were evaluated at the nucleic acid level (RNA) by RT PCR using primers and probe sets for WPRE sequence and at the protein levels using western blot and probed with an anti-CD3 zeta antibody.

[0087] FIGs. 10A-B show bar graphs illustrating relative fold changes in total RNA levels for each tested CAR comprising the disclosed CD19 binders over time. The RNA was analyzed by Real Time PCR at Days 6, 9, and 12. The individual “day wise” analysis (F**IG. 10A**) and the overall comparison, normalized to a positive control (**FIG. 10B**) showed that the CD19 CARs were expressed at relatively similar RNA levels by Real time PCR. The total RNA level of CARs comprising the CD19 binder 42 was consistently a fold higher than CARs comprising CD19 binders 43, 44, 45, 46, and 52.

[0088] FIGs. 11A-B shows western blot results demonstrating the total protein levels of CARs comprising original (**FIG. 11A**) and optimized (**FIG. 11B**) CD19 binders 42, 43, 44, 45, 46, and 52 expressed in T cells from donor ND539 (**FIG. 11A**) and ND518 (**FIG. 11B**). **Table 9** shows raw data of the corresponding CAR surface expression on ND539 CD19 CAR T cells (**FIG. 11A**) analyzed by FACS. Surface protein and total protein expression levels of the CD19 CARs comprising the novel CD19 binders did not correlate with the RNA levels shown in **FIGs. 10A-B**. Expression levels and sized profiles were assessed on day 6 post-transduction. Comparison of total protein profiles between original vs optimized CD19 binders suggested that CAR optimization appeared to have enhanced the expression of the isoforms or species of the CD19 CAR protein with the higher molecular weight (e.g., the larger band is dominant) in **FIG.11B** for some of the CD19 binders.

[0089] FIGs. 12A-B show bar graphs demonstrating that the anti-FMC63 antibody is not an anti-idiotypic antibody for the novel CD19 binders (**FIG. 12A**) and the anti-FMC63 antibody did not block the binding of the CD19 CARs to a recombinant CD19-GFP molecule (**FIG. 12B**). *See also* **Tables 9-10**. The binding of anti-FMC63 antibody or CD19-GFP on ND539 CD19 CAR T cells comprising CD19 binder 42, 43, 44, 45, 46, or 52 on Day 9 after transduction is shown.

[0090] FIG. 13 shows a graph illustrating the activation kinetics of CARs comprising optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP transduced in Jurkat NFAT-GFP reporter cell line following a co-culture with Nalm6 cells. The percent transduction was selected at 8-17% for activation of single integration event. **T**he activation of the optimized CD19 CARs began at about 2-3 hours and maximized at about 10 hrs of co-culture with Nalm6 cells. The activation kinetics of CARs comprising the optimized CD19 binder 42 (42 op) was the fastest and showed the highest induction levels of NFAT. CARs comprising CD19 binders 45OP and 52OP had similar and middle NFAT induction kinetics. CARs comprising CD19 binders 44OP and 46OP had low NFAT induction kinetics. CARs comprising CD19 binders 43OP and 51OP had minimal NFAT induction. *See also* **Table 12**.

[0091] FIGs. 14A-B show bar graphs showing the quantification of the percentage of $CD4^+$ and $CD8^+$ T cells that expressed relevant CD19 CARs on Day 11 during expansion shown in **FIG. 15**. Specifically, the percentages of $CD4^+$ and $CD8^+$ T cells were similar in all

optimized CD19 CAR-binders tested regardless of the donor. T cells were from two donors, ND518 (**FIG. 14A**) and ND528 (**FIG. 14B**) were used. *See also* **Table 13**.

[0092] FIGs. 15A-D show the expansion profiles of CARs comprising optimized CD19 binders transduced in ND518 and ND528 donor T cells. ND518 donor T cells transduced with CARs comprising CD19 binder 42OP, 51OP, or 52OP showed the fastest and highest expansion doublings (**FIG. 15A**). ND518 donor T cells transduced with CARs comprising CD19 binder 42OP, 51OP, or 52OP showed the fastest and highest expansion doublings (**FIG. 15C**). In all cases, the expansion sizes picked at about 7 days and decreased from thereon with the similar kinetics (**FIG. 15B and D**).

[0093] FIGs. 16A-B show schematics illustrating the timeline (**FIG. 16A**) of the activation stress test and gating strategy (**FIG. 16B**) used to evaluate the cytotoxic effectiveness (*e.g.*, killing) of the CD19 CAR T cells. Re-stimulation stress test of ND528 CAR T cells expressing optimized CD19 binder T cells was performed using optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP. The killing target was Nalm6 cells. At the end of each stimulation process, CD19 CAR T cells were stained and the number of live cells were determined by flow; new co-cultures were established and CAR T cells were evaluated using flow cytometry for T cell phenotypes (cytotoxicity). Thawed ND528 cells were used for serial re-stimulation studies and for evaluating cell killing properties.

[0094] FIGs. 17A-D show the killing profiles of optimized ND528 CAR T cells expressing CARs comprising optimized CD19 binders 42OP (**FIG. 17A**), 44OP (**FIG. 17B**), 45OP (**FIG. 17C**), and 52OP (**FIG. 17D**) targeted against Nalm6 cells at 3:1, 1:1, 1:3 and 1:10 CAR^+:Nalm6 wt ratios. All tested CD19 binders effectively killed Nalm6 cells within 45 minutes. *See also* **Table 15**, **Table 16**, **Table 17**, **Table 18**, **Table 19**, and **Table 20.**

[0095] FIGs. 18A-B show growth curves or expansion (**FIG. 18A**) and cell size (contraction) graphs (**FIG. 18B**) of ND608 donor CD19 CAR T cells expressing CARs comprising either original CD19 binders 42, 44, 45, and 52 or optimized CD19 binders opt 42 (also referred to as 42OP), Opt 44, Opt 45, and Opt 52. Each pair of original and optimized CD19 binders showed similar growth and size profiles.

[0096] FIGs. 19A-B show graphs quantifying the surface expression of CD19 CAR comprising original and optimized CD19 binders 42, 44, 45, and 52 on ND608 CAR T cells

(**FIG. 19A**); and their tumor growth suppression in the Jeko NSG mouse model (**FIG. 19B**). Tumor growth was suppressed at the greatest level by CD19 binder 42 original CAR T cells. CD19 binder 42 optimized CAR T cells, CD19 binder 52 original CAR T cells, and CD19 binder 52 optimized CAR T cells were also effective at suppressing tumor growth.

[0097] FIG. 20 shows the location of the non-overlapping epitopes of the CD19 42 original (42 og) scFv on sequences of the extracellular domain of CD19 when compared to epitopes of three known anti-CD19 antibodies, the FMC63 antibody, the 4G7 antibody, and the 3B10 antibody.

[0098] FIGs. 21A-B show graphs demonstrating that CD19 42og scFv selectively bound to CD19 when tested on a membrane proteome array (MPA)comprising over 5,220 human membrane proteins. **FIG. 21A** shows the results of the MPA screen highlighting binding to CD19 and FCGR protein (FCGR1A), which served as a positive control. **FIG. 12B-C** show validation of the titration results demonstrating that 42og scFv-Fc bound strongly to Protein A (positive control) and CD19 with MFI signals that were respectively 750-fold and 400-fold higher than the negative control (empty vector). **FIG. 21C** shows that the isotype control did not bind to CD19, or any other targets tested, but bound strongly to Protein A and FCGR1A, with MFI signals that were respectively 190-fold and 45-fold higher than the negative control.

DETAILED DESCRIPTION

I. OVERVIEW

[0099] The present disclosure provides novel anti-CD19 chimeric antigen receptors with low affinity and fast off-rate when compared to CD19 CARs known in the prior art or the clinically approved FMC63-based CARs. FMC63 is an IgG2a mouse monoclonal antibody specific for CD19, which is a target for the immunotherapy of B lineage leukemias and lymphomas.

[0100] Chimeric antigen receptor-modified T cells (CAR T cells) directed against CD19 have shown promise as a novel therapy for hematological malignancies. Remarkable antitumor responses have been achieved from anti-CD19 CAR-T therapies against B-cell acute lymphoblastic leukemia (B-ALL) and other refractory B-cell malignancies. Complete remission (CR) has been achieved in as many as 70–90% of cases of relapsed/refractory acute

lymphoblastic leukemia (R/R B-ALL). In light of these outstanding experimental results, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved several CD19-directed CAR T cell products, including tisagenlecleucel (KYMRIAH®, Novartis), axicabtagene ciloleucel (YESCARTA®, Kite Pharma-Gilead), and lisocabtagene maraleucel (BREYANZI®, Juno Therapeutics-Celgene-BMS) for treating large B-cell lymphoma. In addition, brexucabtagene autoleucel (TECARTUS®, Kite Pharma-Gilead) was approved for treating relapsed/refractory mantle cell lymphoma.

[0101] Despite the range of validated CAR T cell products, the success of these approved CAR T cell products has been limited. This is because about 40–50% of patients responding to CD19 CAR T cell therapy relapse within 1 year, and nearly half of these relapses included CD19-positive leukemic cells. Recent evidence suggests that resistance to CD19 chimeric antigen receptor (CAR)-modified T cell therapy may be due to the presence of CD19 isoforms that lose binding to the single-chain variable fragment (scFv) in current use. Additional resistance mechanisms that limit current CAR T cell therapies include T-cell exhaustion, immunosuppression, antigen loss, cytokine-release syndrome (CRS), immune effector cell–associated neurotoxicity syndrome, and/or neurotoxicity.

A. Identification of novel CD19 binders

[0102] To resolve these issues, the present disclosure relates to improved CD19 binders using an immunization-independent antibody generation methods based on a large yeast display human antibodies libraries. *See e.g.*, AvantGen Inc., avantgen.com/therapeutic-antibodies.

[0103] The novel CD19 binders (e.g., antibody, antibody fragment, or scFv) were specifically screened to have desired characteristics. In particular, the novel CD19 binders were screened to have low affinity and fast off-rate. While low affinity binding can be determined by either on-rate (K_{on}) or an off rate (K_{off}), the anti-CD19 binders (e.g., scFv) disclosed herein were selected for a fast off rate. This fast off-rate allows the CD19 CAR to rapidly dissociate from CD19, thereby resulting in a shorter CAR T cell-tumor interaction. This shorter interaction time can then reduce cytokine release and thereby reducing toxicity. In one example, the CD19 binders disclosed herein have a K_D value of about 1nM to about 50nM. In another example, the CD19 binders disclosed herein have a K_{off} value of about 1.0 $x10^{-3}$ s^{-1} to about 5.0 $x10^{-3}$ s^{-1}.

[0104] In addition, the short interaction time can reduce T-cell exhaustion, which may enhance CAR T-cell persistence. As such, the novel binders were identified by specifically screening a human antibody library for CD19-specific antibodies or antibody fragments for low binding affinity (e.g., a K_D of 1nM to about 50nM) and a fast off-rate (e.g., K_{off} about 1.0 $x10^{-3}$ s^{-1} to about 5.0 $x10^{-3}$ s^{-1}). **FIG. 1** shows a schematic outlining the general steps used to identify the 12 unique CD19 binders from phage display libraries and yeast display screening to selection by biotinylated baculovirus binding, SIGLEC binding, and/or NALM6 tumor cells binding.

[0105] This screen yielded about 13 novel binders shown in **Table 3** and **FIGs 2A-2E**. The nucleic acid sequences of the novel CD19 binders disclosed herein are about 58% to about 97% identical to each other as shown in **FIG. 2F**.

[0106] T cells expressing CD19 CARs comprising the novel binders of the present disclosure can exhibit higher efficacy, enhanced *in vivo* persistence, and low toxicity when compared to T cells expressing the FMC63-based CARs. However, T cells expressing the low affinity CD19 CAR of the present disclosure can kill target cells as well as T cells expressing a high affinity CD19 CAR. Furthermore, T cells expressing the low affinity CD19 CARs of the present disclosure can show similar cytokine production (e.g., interferon γ or IL-2 production) and proliferation as T cells expressing a high affinity CD19 CAR (e.g., FMC63-based CAR).

B. Characterization of the novel CD19 binders

[0107] Selection of the top of the 12 novel CD19 binder candidates was ultimately based on the following functional characteristics in view of known CD19 binders: (1) low tonic signal; (2) strong activation rate; (3) healthy expansion profiles; (4) robust stable surface expression; and (5) cytokine production. Based on these criteria, CD19 binders 42 (P1) and 52 (P11 and P13) appeared to be exemplary candidates.

[0108] Preliminary analyses showed that the 12 novel CD19 binders produced similar transcriptional profiles. As described herein, these 12 novel CD19 binders exhibited unusual and unique functional characteristics, signaling, pharmacology, and tumor suppression properties. The new properties described herein will addressed current CD19 CAR issues,

such as *e.g.*, T-cell exhaustion, immunosuppression, antigen loss, cytokine-release syndrome (CRS), immune effector cell–associated neurotoxicity syndrome, and/or neurotoxicity.

[0109] As described herein, CD19 binder 42 exhibited desired and unique CD19 binder characteristics. For example, CART cells expressing CARs comprising the CD19 binder 42 were able to maintain higher level of CAR RNA transcripts. Using real time PCR, the relative transcript levels for all CD19 binders were found to be comparable (**FIGs. 10A-B**). The CD19 binder 42 had twice the transcript levels of all tested binders, yet its total protein levels were similar to other CD19 binders. The mechanism of this high transcript level is not known. It was speculated that these cells had either a higher transcription rate or their RNA transcripts were more stable (less degradation).

[0110] The translated products of the CD19 binders (*e.g.*, total protein levels) were evaluated by Westerns. The Western blots results captured two protein bands with similar sizes but various expression levels (**FIGs. 11A-B**). For example, CAR T cells comprising a CD19 binder 52 CAR only expressed the larger molecular weight band. While two isoforms of the CD19 binder 42 were identified, and the smaller isoform was highly expressed. Two CAR isoforms were also detected in CAR T cells expressing a control CAR. So far, no correlation between the ratio of protein band sizes and cytokine production or tumor clearance was found.

[0111] Most CD19 binders were well expressed in Jurkat NFAT cells or human primary T cells (**Table 4)**. However, the majority of expressed CD19 CARs induced tonic signaling in these cells (**Table 4** and **FIG. 3A-B)**. CD19 binder 42 was an exception because expression of the CD19 binder 42 (original) CAR produced no tonic signaling. CD19 binder 52 produced some tonic signaling. Consistent with the expression pattern, the novel CD19 binders were also able to induce cytokine production when expressed in primary human T cells (**FIG. 5A-B**; and **Tables 6, 14**, and **24**).

[0112] The scFvs of the CD19 binders 42, 43, 44, 45, 46, 50, 51, and 52 were also optimized based on codon usage and GC content. Codon optimization was performed to determine if a more stable robust expression could be obtained. Codon optimization reduced tonic signaling induced by original CD19 binder 52 (**FIG. 3B**).

[0113] Furthermore, CAR T cells expressing CARs comprising original or optimized CD19 binders 42 and 52 effectively controlled tumor growth in Jeko NSG mouse model (**FIG. 19B**). Tumor growth was suppressed at the greatest level by CD19 binder 42 original CAR T cells. CD19 binder 42opt CAR T cells, CD19 binder 52 original CAR T cells, and CD19 binder 52op CAR T cells also suppressed tumor growth.

C. Epitope mapping of the novel CD19 binders

[0114] An initial evaluation of epitope binding region of CD19 binders was also conducted as shown in **FIG. 12 and Tables 9-10.** Specifically, binding assays were performed to determine if the novel CD19 binders bound to the anti-FMC63 antibody and if they shared the same binding site (e.g., epitope) or if they bound to the same region. These data showed that the anti-FMC63 antibody was not an idiotype antibody for the novel CD19 binders. For example, the anti-FMC63 antibody did not bind to any cells expressing a CAR comprising a the novel CD19 binder described herein. In addition, the anti-FMC63 antibody did not block the interaction between any of the novel CD19 binders tested and a recombinant CD19 protein.

[0115] In addition, a high-throughput shotgun mutagenesis analysis was performed to map the epitope of the novel CD19 binders on the extracellular domain of the full-length CD19 protein (SEQ ID NO: 217). The high-throughput shotgun mutagenesis analysis of the CD19 42original (42og) showed that CD19 42og bound to a distinct epitope on the extracellular domain of CD19 (FIG. 20, Table 28, and Table 29). CD19 42og scFv bound to a completely region of the extracellular domain of CD19 that did not overlap with regions bound by well characterized CD19 antibodies, such as FMC63, 4G7, or 3B10.

[0116] Klesmith et al. (Biochemistry 58:4869-4881 (2019)) characterized the conformational epitopes of FMC63, 4G7, and 3B10 (e.g., anti-CD19 clinical antibodies) using high-throughput screening strategies to comprehensively map the binding sequences of these antibodies to the extracellular domain of CD19 variant CD19.1. These extensive analyses of conformational epitope maps of FMC63, 4G7 and 3B10 showed that all three antibodies have partially overlapping epitopes near the published epitope of antibody B43 co-crystallized with CD19. As shown in FIG. 20, two main regions were identified. The first region comprises amino acid sequence WAKDRPEIWEGEP (SEQ ID NO: 219) located at positions

159-171 of the full-length CD19 protein (SEQ ID NO:217). The second region comprises the amino acid sequence of PKGPKSLLSLE (SEQ ID NO: 220) and was located at positions 219-229 of SEQ ID NO: 217.

[0117] In contrast, CD19 42og scFv bound primarily to amino acid sequence of QPGPPSEKAWQP (SEQ ID NO: 221) located at positions 98-109 of SEQ ID NO: 217. CD19 42og scFv also interacted with another region comprising the amino acid sequence VPPDSVSRGPL (SEQ ID NO: 222) located at positions 202-212 of SEQ ID NO: 217 (Full-length CD19). Accordingly, CD19 42og does not bind to the same epitope as FMC63, 4G7, 3B10, or B43 (e.g., anti-CD19 clinical antibodies).

[0118] These results further demonstrate the unique functional characteristics of the novel CD19 binders described herein, in particular CD19 42og. The novel binders disclosed herein have uncovered new clinically relevant CD19 epitopes that do not overlap with epitopes from at least three well-characterized clinically relevant antibodies, namely, the FMC63, 4G7, and 3B10 (**Table 29**).

[0119] Lastly, the specificity and selectivity of the novel CD19 disclosed herein was assessed using a high-throughput membrane proteome array (Integral Molecular). These experiments demonstrated that CD19 42og selectively bound to CD19 when assessed for cross-reactivity against an array of 5,220 human membrane proteins, which represented over 94% of the human membrane proteome (**FIGs. 21A-C**). The assays described in Example 13 did not identify binding to a non-CD19 protein. These experiments were well controlled as shown in the Examples below (**FIGs. 21B-C**).

Table 3: P1-P12 Novel CD19 binders

Name	**Description**	**Other names**	**scFv SEQ ID NO.**		**V_H SEQ ID NO.**		**V_L SEQ ID NO.**	
			Amino acid	Nucleic acid	Amino acid	Nucleic acid	Amino acid	Nucleic acid
P1	CD19 42og	42 or A2	9	21	8	20	7	19
P2	CD19 A4	43	18	24	17	23	16	22
P3	CD19 E4	44	64	102	63	66	62	65
P4	CD19 E7	45	75	103	74	77	73	76

Table 3: P1-P12 Novel CD19 binders								
Name	**Description**	**Other names**	**scFv SEQ ID NO.**		**V_H SEQ ID NO.**		**V_L SEQ ID NO.**	
			Amino acid	Nucleic acid	Amino acid	Nucleic acid	Amino acid	Nucleic acid
P5	CD19 7	46	86	104	85	88	84	87
P6	CD19 10	47	146	118	145	148	144	147
P7	CD19 11	48	157	119	156	159	155	158
P8	CD19 14	49	168	114	167	170	166	169
P9	CD19 15	50	179	115	178	181	177	180
P10	CD19 16	51	190	120	189	192	188	191
P11	CD19 18	52	201	116	200	203	199	202
P12	CD19 23	53	212	117	21		214	210
P13	CD19 52 Opt or OP	Optimized 52	201	216	200		188	
P14	CD19 42 Opt or OP	Optimized 42	226	225				

[0120] Accordingly, one example of the present disclosure provides isolated nucleic acid molecules encoding a chimeric antigen receptor (CAR) comprising a CD19 binder disclosed herein. In some embodiments, the CAR comprises an anti-CD19 binding domain selected from P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, or P13. In some embodiments, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0121] In some embodiments, a novel anti-CD19 binding domain disclosed herein (e.g., CD19 binders 42, 43, 44, 45, 46, or 52) binds to a different epitope of human CD19 than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from known CD19 antibodies (e.g., FMC63, 4G7, 3B10, or B43). In some embodiments, a novel

anti-CD19 binding domain disclosed herein (e.g., CD19 binders 42, 43, 44, 45, 46, or 52) binds to the same epitope of human CD19 as the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from t known CD19 antibodies (e.g., FMC63, 4G7, 3B10, or B43).

[0122] In some embodiments, a novel anti-CD19 binding domain disclosed in Table 3 binds to a different epitope of human CD19 than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from known CD19 antibodies (e.g., FMC63, 4G7, 3B10, or B43). In some embodiments, a novel anti-CD19 binding domain disclosed in Table 3 binds to the same epitope of human CD19 as the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from known CD19 antibodies (e.g., FMC63, 4G7, 3B10, or B43).

[0123] In some embodiment, a novel anti-CD19 binding domain disclosed in Table 3 binds to a CD19 polypeptide comprising the amino acid sequence of SEQ ID NOs: 219, 220, 221, 222, 223, and/or 224. In some embodiments, a novel anti-CD19 binding domain disclosed herein does not bind a CD19 polypeptide comprising the amino acid sequence of SEQ ID NO: 219 and/or SEQ ID NO: 220. In some embodiments, a novel anti-CD19 binding domain disclosed herein binds to a residue located at positions 90-120, 95-115, or 95-110 of SEQ ID NO: 217. In some embodiments, an epitope of a novel anti-CD19 binding domain disclosed herein comprises a sequence of amino acids selected from the amino acids 41-120, 180-215, 90-120, 95-110, 98-106, 200-215, 200-208, 200-210, 205-210, 200-226, 200-230, or 200-240 of SEQ ID NO: 217 or any combination thereof. In some embodiments, an epitope of a novel anti-CD19 binding domain disclosed herein comprises a sequence of amino acids selected from the amino acids 41-120, 180-215, 90-120, 95-110, or 98-106 and a sequence of amino acids selected from the amino acids 200-215, 200-208, 200-210, 205-210, 200-226, 200-230, or 200-240 of SEQ ID NO: 217.

[0124] In some embodiments, an epitope of a novel anti-CD19 binding domain disclosed herein comprises a residue selected from Q98, E104, K105, A106, or V207 or any combination thereof. In one embodiment, an epitope of a novel anti-CD19 binding domain disclosed herein comprises a residue selected from Q98, E104, K105, or A106. In one embodiment, an epitope of a novel anti-CD19 binding domain disclosed herein comprises

residues Q98, E104, K105, A106, and V207. In one embodiment, an epitope of a novel anti-CD19 binding domain disclosed herein comprises a residue selected from Q98, or K105. In one embodiment, an epitope of a novel anti-CD19 binding domain disclosed herein comprises residues Q98, E104, K105, and A106. In one embodiment, an epitope of a novel anti-CD19 binding domain disclosed herein comprises residues Q98 and K105.

[0125] Another example of the present disclosure provides an isolated polypeptide molecule encoded by the nucleic acid molecule disclosed in Table 3 or Table 1.

II. CHIMERIC ANTIGEN RECEPTORS (CARS)

[0126] One example of the present disclosure provides compositions of matter and methods of use for the treatment of a disease such as cancer using anti-CD19 chimeric antigen receptors (CAR). In particular, the present disclosure provides a number of chimeric antigen receptors (CAR) comprising an antibody or antibody fragment engineered for enhanced binding to a CD19 protein. In some embodiments, the CAR comprises an amino acid sequence of any one of SEQ ID NO: 63, SEQ ID NO: 74, SEQ ID NO: 85, SEQ ID NO: 145, SEQ ID NO: 167, SEQ ID NO: 178, SEQ ID NO: 200, SEQ ID NO: 211, SEQ ID NO: 156, SEQ ID NO: 189, SEQ ID NO: 17, SEQ ID NO: 8, SEQ ID NO: 62, SEQ ID NO: 73, SEQ ID NO: 84, SEQ ID NO: 144, SEQ ID NO: 166, SEQ ID NO: 177, SEQ ID NO: 199, SEQ ID NO: 210, SEQ ID NO: 155, SEQ ID NO: 188, SEQ ID NO: 16, and SEQ ID NO: 7. In some embodiments, the CAR comprises a polypeptide encoded by the nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216; or a nucleic sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 21, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0127] In some embodiments, the CARs of the present disclosure, comprising an anti-CD19 antigen binding domain described herein, have a low affinity and a fast Off-rate when

compared to CARs comprising anti-CD19 antigen binding domain known in the art.

[0128] Accordingly, the present disclosure provides a cell (e.g., T cell) engineered to express a CAR, wherein the CAR T cell ("CART") exhibits an antitumor property. The cell is transformed with the CAR and the CAR is expressed on the cell surface. The cell (e.g., T cell) is transduced with a viral vector encoding a CAR. The viral vector is a retroviral vector. In some embodiments, the viral vector is a lentiviral vector. The cell may stably express the CAR. The cell (e.g., T cell) may be transfected with a nucleic acid (e.g., mRNA, cDNA, DNA, encoding a CAR). In some embodiments, the cell may transiently express the CAR.

[0129] In some embodiments, the anti-CD19 protein binding portion of the CAR is an scFv antibody fragment. Such antibody fragments may be functional in that they retain the equivalent binding affinity. For example, they bind the same antigen with comparable efficacy as the IgG antibody from which they were derived. Such antibody fragments may be functional in that they provide a biological response that can include, but is not limited to, activation of an immune response, inhibition of signal-transduction origination from its target antigen, inhibition of kinase activity, and the like, as will be understood by a skilled artisan. In some embodiments, the anti-CD19 antigen binding domain of the CAR is a scFv antibody fragment that is human derived.

[0130] The novel CD19 antigen binding domains were engineered to have low affinity and a fast off-rate. The CD19 antigen binding domains were identified based on binding to CD19 on HEK 293 cells followed by binding to NALM6 expressing or lacking CD19 expression. In some embodiments, the novel anti-CD19 antigen binding domain described herein may have a binding affinity for the human CD19 (hCD19) antigen. For example, the anti-CD19 antigen binding domain described herein may have an association rate constant or K_{on} rate (antibody (Ab) + antigen (Ag) $^{ko}\rightarrow$ Ab-Ag) of at least about 2 x 10^5 $M\text{-}^1s^1$, at least about 5 x 10^5 $M^{-1}s^{-1}$, at least about 10^6 $M^{-1}s^{-1}$, at least about 5 x 10^6 $M^{-1}s^{-1}$, at least about 10^7 $M^{-1}s^{-1}$at least about 5 x 10^7 $M^{-1}s^{-1}$, or at least about 10^8 $M^{-1}s^{-1}$.

A. Chimeric antigen receptor

[0131] The present disclosure provides engineered immune effector cells (for example, T cells or NK cells) comprising one or more CARs that direct the immune effector cells to cancer. In some embodiments, the CAR comprises an antigen-binding domain, a

transmembrane domain, a co-stimulatory domain, and an intracellular domain. The CAR may comprise any antigen binding domain, any hinge, any transmembrane domain, any costimulatory domain, and any intracellular signaling domain described herein.

[0132] The antigen binding domain may be operably linked to another domain of the CAR, such as the transmembrane domain or the intracellular domain, both described herein, for expression in any immune cell described herein. In one embodiment, a first nucleic acid sequence encoding the antigen binding domain is operably linked to a second nucleic acid encoding a transmembrane domain, and further operably linked to a third a nucleic acid sequence encoding an intracellular domain.

[0133] The antigen binding domains described herein can be combined with any of the transmembrane domains described herein, any of the intracellular domains or cytoplasmic domains described herein, or any of the other domains described herein that may be included in a CAR of the present invention. A subject CAR of the present invention may also include a spacer domain as described herein. In some embodiments, each of the antigen binding domain, transmembrane domain, and intracellular domain is separated by a linker.

[0134] One example of the present disclosure provides a chimeric antigen receptor (CAR) comprising a single chain antibody or a single chain antibody fragment comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain. The anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6.

[0135] Alternatively, the anti-CD19 binding domain can comprise a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and

a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198.

[0136] In another embodiment, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

Table 2: P1-P12 CDR Amino Acid sequences

Name	SEQ ID NO	Description	Sequences
P1 42 or A2	1	42og CDR-L1	RASQTISNYLN
	2	42og CDR-L2	AASSLQS
	3	42og CDR-L3	QQSYSTPPT
	4	42og CDR-H1	AASGFTFSNYAIS
	5	42og CDR-H2	VSVITASGVDTYYADSV
	6	42og CDR-H3	GGTPYFITTYDYYGFDV
P2 43	10	A4 CDR-L1	RASQSVSSNYLA
	11	A4 CDR-L2	GASSRAT
	12	A4 CDR-L3	QQYESSPSWT
	13	A4 CDR-H1	KASGGTFSNYYIS
	14	A4 CDR-H2	MGGIIPLFGTTNYAQ
	15	A4 CDR-H3	GTWYAGDI
P3 44	56	E4 CDR-L1	RASQSISSYLN
	57	E4 CDR-L2	GASSLQS
	58	E4 CDR-L3	QQSYRTPVT
	59	E4 CDR-H1	KASGGTFSNYAIN
	60	E4 CDR-H2	MGRIVPLLGIANYAQ
	61	E4 CDR-H3	EHIAYRPTSAGYYYYMDI
P4 45	67	E7 CDR-L1	RASQDITRYLN
	68	E7 CDR-L2	AASSLQS
	69	E7 CDR-L3	QQSYSYPPT
	70	E7 CDR-H1	AASGFTFRDYGMH
	71	E7 CDR-H2	VAVISYEGSNEYYADSV

Table 2: P1-P12 CDR Amino Acid sequences			
Name	**SEQ ID NO**	**Description**	**Sequences**
	72	E7 CDR-H3	DRGFAGWYDYAFDP
P5 46	78	7 CDR-L1	RASQSISKYLN
	79	7 CDR-L2	DASSLQS
	80	7 CDR-L3	QQSYTIPLT
	81	7 CDR-H1	KASGGTFSSYAFS
	82	7 CDR-H2	MGGIVPLFGAVEYAQ
	83	7 CDR-H3	EKGFYRYFDH
P6 47	89	10 CDR-L1	RASQTISRYLN
	90	10 CDR-L2	AASSLQS
	91	10 CDR-L3	QQSYRPPLT
	141	10 CDR-H1	AASGFTFSSYAMS
	142	10 CDR-H2	VSTISAGGHGTYYADSV
	143	10 CDR-H3	GAGYFDY
P7 48	149	11 CDR-L1	RASQSISSYLN
	150	11 CDR-L2	AASSLQS
	151	11 CDR-L3	QQTGAVPYTF
	152	11 CDR-H1	AASGFTFRDYAMS
	153	11 CDR-H2	VSAISESGIDTYYADSV
	154	11 CDR-H3	VAGYDSDSSTYYDYMDV
P8 49	160	14 CDR-L1	RASQSISNYLN
	161	14 CDR-L2	AASSLQS
	162	14 CDR-L3	QQAYSAPIT
	163	14 CDR-H1	AASGFTFGDYAMS
	164	14 CDR-H2	VSAISRGGHGTYYADSV
	165	14 CDR-H3	LVGYGLDY
P9 50	171	15 CDR-L1	RASQPIRPYLN
	172	15 CDR-L2	DASSLQS
	173	15 CDR-L3	QQSYSAPYT
	174	15 CDR-H1	AASGFTFSSYAMS
	175	15 CDR-H2	VSVISGGGANTYYADSVK
	176	15 CDR-H3	DWRYFDH
P10 51	182	16 CDR-L1	TGSSSNIGAGYDVH
	183	16 CDR-L2	GNNNRPS
	184	16 CDR-L3	QSYDVSLGVWV
	185	16 CDR-H1	TVSGGSISSPSYYWG
	186	16 CDR-H2	IGSIYYTGATYYNPSL
	187	16 CDR-H3	YGPAGVGFDY
P11	193	18 CDR-L1	TGSSSNIGAGYDVH

Table 2: P1-P12 CDR Amino Acid sequences			
Name	**SEQ ID NO**	**Description**	**Sequences**
52	194	18 CDR-L2	GTKNRPS
	195	18 CDR-L3	QSYDVRLKGWV
	196	18 CDR-H1	TVSGGSITSSSYYWG
	197	18 CDR-H2	IGSIYYTGTTYYNPSL
	198	18 CDR-H3	YVGLSGGFDY
P12 53	204	23 CDR-L1	RASQSIYSYLN
	205	23 CDR-L2	DASSLQS
	206	23 CDR-L3	QQSYTAPPT
	207	23 CDR-H1	AASGFTFSNYAMS
	208	23 CDR-H2	VSAISESGHGTYYADSV
	209	23 CDR-H3	LDWAGFDV

[0137] In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 or 199; or an amino acid sequence having at least about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 7 or 199. In some embodiments, the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8 or 200, or an amino acid sequence having at least about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 8 or 200.

[0138] In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8. In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 199 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 200. In some embodiments, the CD19 binding domain is a scFv.

[0139] In some embodiments, the anti-CD19 binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146.

[0140] In some embodiments, the anti-CD19 binding domain comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID

NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216; or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0141] In some embodiments, the anti-CD19 binding domain comprises a light chain variable region or a heavy chain variable region encoded by (a) a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216, or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0142] In some embodiments, a novel anti-CD19 binding domain disclosed herein (e.g., CD19 binders 42, 43, 44, 45, 46, or 52) binds to a different epitope of human CD19 than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody.

[0143] In some embodiments, a novel anti-CD19 binding domain disclosed herein (e.g., CD19 binders 42, 43, 44, 45, 46, or 52) binds to the same epitope of human CD19 than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody.

[0144] In some embodiments, a novel anti-CD19 binding domain disclosed in Table 2 or Table 3 binds to a different epitope of human CD19 than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody.

[0145] In some embodiments, a novel anti-CD19 binding domain disclosed in Table 2 or Table 3 binds to the same epitope of human CD19 than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody.

[0146] In some embodiments, the anti-CD19 binding domain comprises a light chain variable region or a heavy chain variable region encoded by (a) a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216, or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216; and binds to a different epitope on the human CD19 protein than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody.

[0147] In some embodiments, the anti-CD19 binding domain comprises a light chain variable region or a heavy chain variable region encoded by (a) a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216, or (b) a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216; and binds to the same epitope on the human CD19 protein than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody.

[0148] In some embodiments, the novel anti-CD19 binding domain described herein competes for binding to human CD19 with an antigen binding domain comprising a sequence from a known CD19 scFv binder (e.g., FMC63 binder), e.g., in a competition assay.

[0149] In some embodiments, the competition assay can be an SPR-based assay. Briefly, the antigen, e.g., human CD19, can be immobilized on a surface. Through a microflow system, a reference antibody (e.g., FMC63) is injected over the antigen layer. Upon binding of the reference antibody to the antigen, an increase in signal, typically expressed in response units (RU) is detected, e.g., reference signal. After a desired time, a novel CD19 binder described herein is injected over the antigen layer. If the test antibody binds to a different region or epitope of the antigen, then an additional increase in signal is detected, e.g., a 5% or more, 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35%, or more, 40% or more, 45% or more, 50% or more, 55% of more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, or 95% or more increase in signal, e.g., RU, as compared to the highest signal detected upon binding of the reference antibody, e.g., the reference signal.

[0150] If the test antibody binds to the same region or epitope of the antigen, then little or no increase in signal, e.g., RU, will be detected, e.g., less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% increase in signal, e.g., RU, as compared to the highest signal detected upon binding of the reference antibody, e.g., the reference signal.

[0151] When using this SPR-based competition assay, an antibody is said to compete with the reference antibody when less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% increase in signal, e.g., RU, is detected when compared to the reference signal detected upon binding of the reference antibody to the antigen. An antibody is said to not compete, or compete poorly, with a reference antibody when a 5% or more, 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35%, or more, 40% or more, 45% or more, 50% or more, 55% of more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, or 95% or more increase in signal, e.g., RU, is detected when compared to the reference signal detected upon binding of the reference antibody to the antigen.

[0152] Identification of the epitope bound by the novel CD19 antigen binding domains described herein can be determined by various methods known in the art. For example, crystal structures can be generated containing the antigen binding domain bound to, or in complex with, the antigen. In another example, assays, e.g., a protection assay, can be performed to identify the regions of the antigen contribute to the epitope, or to identify the epitope. An exemplary protection assay, a hydrogen/deuterium exchange (HDX) mass spectrometry assay can be used.

1. Antigen binding domain

[0153] The antigen binding domain of a CAR is an extracellular region of the CAR for binding to a specific target antigen including proteins, carbohydrates, and glycolipids. In some embodiments, the CAR comprises affinity to a target antigen (e.g. a tumor associated antigen) on a target cell (e.g., a cancer cell). The target antigen may include any type of protein, or epitope thereof, associated with the target cell. For example, the CAR may comprise affinity to a target antigen on a target cell that indicates a particular status of the target cell.

[0154] As described herein, a CAR of the present disclosure having affinity for a specific target antigen on a target cell may comprise a target-specific binding domain. In some embodiments, the target-specific binding domain is a murine target-specific binding domain, e.g., the target-specific binding domain is of murine origin. In some embodiments, the target-specific binding domain is a human target-specific binding domain, e.g., the target-specific binding domain is of human origin.

[0155] The antigen binding domain can include any domain that binds to the antigen and may include, but is not limited to, a monoclonal antibody, a polyclonal antibody, a synthetic antibody, a human antibody, a humanized antibody, a non-human antibody, and any fragment thereof. Thus, in one embodiment, the antigen binding domain portion comprises a mammalian antibody or a fragment thereof. In some embodiments, the antigen binding domain comprises a full-length antibody. In some embodiments, the antigen binding domain comprises an antigen binding fragment (Fab), e.g., Fab, Fab', F(ab')2, a monospecific Fab2, a bispecific Fab2, a trispecific Fab2, a single-chain variable fragment (scFv), dAb, tandem scFv, VhH, V-NAR, camelid, diabody, minibody, triabody, or tetrabody. In some

embodiments, the antigen-binding domain is selected from the group consisting of (a) a full-length antibody or antigen-binding fragment thereof, (b) a Fab, (c) a single-chain variable fragment (scFv), and (d) a single-domain antibody.

[0156] In some embodiments, a CAR of the present disclosure may have affinity for one or more target antigens on one or more target cells. In some embodiments, a CAR may have affinity for one or more target antigens on a single target cell. In such embodiments, the CAR is a bispecific CAR, or a multispecific CAR. In some embodiments, the CAR comprises one or more target-specific binding domains that confer affinity for one or more target antigens. In some embodiments, the CAR comprises one or more target-specific binding domains that confer affinity for the same target antigen. For example, a CAR comprising one or more target-specific binding domains having affinity for the same target antigen could bind distinct epitopes of the target antigen. When a plurality of target-specific binding domains is present in a CAR, the binding domains may be arranged in tandem and may be separated by linker peptides. For example, in a CAR comprising two target-specific binding domains, the binding domains are connected to each other covalently on a single polypeptide chain, through a polypeptide linker, an Fc hinge region, or a membrane hinge region.

[0157] In some instances, the antigen binding domain may be derived from the same species in which the CAR will ultimately be used. For example, for use in humans, the antigen binding domain of the CAR may comprise a human antibody as described elsewhere herein, or a fragment thereof.

[0158] Accordingly, a CAR encoded by a lentiviral vector or retroviral vector of the present disclosure may target one of the following cancer associated antigens (tumor antigens): CD19; CD20; CD22 (Siglec 2); CD37; CD 123; CD22; CD30; CD 171; CS-1 (also referred to as CD2 subset 1, CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule- 1 (CLL-1 or CLECL1); CD33; CD133; epidermal growth factor receptor (EGFR); epidermal growth factor receptor variant III (EGFRvIII); human epidermal growth factor receptor (HER1); ganglioside G2 (GD2); ganglioside GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(l - 4)bDGlcp(l-l)Cer); TNF receptor family member B cell maturation (BCMA); Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)); prostate-specific membrane antigen (PSMA); Receptor tyrosine kinase-like orphan receptor 1 (ROR1); Fms- Like Tyrosine Kinase 3 (FLT3); Tumor-associated glycoprotein 72 (TAG72); CD38; CD44v6; Carcinoembryonic antigen (CEA);

Epithelial cell adhesion molecule (EPCAM); B7H3 (CD276); KIT (CD117); Interleukin-13 receptor subunit alpha-2 (IL- 13Ra2 or CD213A2); Mesothelin; Interleukin 11 receptor alpha (IL- l lRa); prostate stem cell antigen (PSCA); Protease Serine 21 (Testisin or PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta (PDGFR- beta); Stage- specific embryonic antigen-4 (SSEA-4); Folate receptor alpha; Receptor tyro sine-protein kinase ERBB2 (Her2/neu); Mucin 1, cell surface associated (MUC 1); GalNAca1-O-Ser/Thr (Tn) MUC 1 (TnMUC1); neural cell adhesion molecule (NCAM); Prostase; prostatic acid phosphatase (PAP); elongation factor 2 mutated (ELF2M); Ephrin B2; fibroblast activation protein alpha (FAP); insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX); Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2); glycoprotein 100 (gp100); oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl); tyrosinase; ephrin type-A receptor 2 (EphA2); Fucosyl GM1; sialyl Lewis adhesion molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGalp(l-4)bDGlcp(l-l)Cer); transglutaminase 5 (TGS5); high molecular weight-melanoma-associated antigen (HMWMAA); o-acetyl-GD2 ganglioside (OAcGD2); Folate receptor beta; tumor endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R); claudin 6 (CLDN6); thyroid stimulating hormone receptor (TSHR); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); CD97; CD179a; anaplastic lymphoma kinase (ALK); Polysialic acid; placenta- specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); mammary gland differentiation antigen (NY-BR-1); uroplakin 2 (UPK2); tyrosine-protein kinase Met (c-Met); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex, locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); Cancer/testis antigen 1 (NY-ESO-1); Cancer/testis antigen 2 (LAGE-la); Melanoma- associated antigen 1 (MAGE-A1); ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML); sperm protein 17 (SPA17); X Antigen Family, Member 1A (XAGEl); angiopoietin-binding cell surface receptor 2 (Tie 2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen

1; tumor protein p53 (p53); p53 mutant; prostein; surviving; telomerase; prostate carcinoma tumor antigen- 1 (PCTA-1 or Galectin 8), melanoma antigen recognized by T cells 1 (MelanA or MARTI); Rat sarcoma (Ras) mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N-Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B l; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Tyrosinase-related protein 2 (TRP-2); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger Protein)- Like (BORIS or Brother of the Regulator of Imprinted Sites), Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES l); lymphocyte- specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Receptor for Advanced Glycation Endproducts (RAGE-1); renal ubiquitous 1 (RU1); renal ubiquitous 2 (RU2); legumain; human papilloma virus E6 (HPV E6); human papilloma virus E7 (HPV E7); intestinal carboxyl esterase; heat shock protein 70-2 mutated (mut hsp70-2); CD79a; CD79b; CD72; Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR or CD89); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module- containing mucin-like hormone receptor-like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-2 (GPC2); Glypican-3 (GPC3); NKG2D; KRAS; GDNF family receptor alpha-4 (GFRa4); IL13Ra2; Fc receptor-like 5 (FCRL5); and immunoglobulin lambda-like polypeptide 1 (IGLL1).

[0159] In some embodiments, the CAR targets CD19, CD20, CD22, BCMA, CD37, Mesothelin, PSMA, PSCA, Tn-MUC1, EGFR, EGFRvIII, c-Met, HER1, HER2, CD33, CD133, GD2, GPC2, GPC3, NKG2D, KRAS, or WT1. In some embodiments, the antigen-binding domain specifically binds a target antigen selected from the group consisting of CD4, CD19, CD20, CD22, BCMA, CD123, CD133, EGFR, EGFRvIII, mesothelin, Her2, PSMA, CEA, GD2, IL-13Ra2, glypican-3, GPC2, TnMuc1, CIAX, LI-CAM, CA 125, CTAG1B, Mucin 1, and Folate receptor-alpha. In some embodiments, the CAR targets CD19.

[0160] Accordingly, one example of the present invention provides an anti-CD19 binding domain comprising a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6. Alternatively, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198. In another embodiment, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0161] In some embodiments, the anti-CD19 binding domain is a scFv comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO: 7 or 199, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 7 or 199; and/or a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 8, or 200, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 7 or 200.

[0162] One example of the present disclosure provides an anti-CD19 binding domain (e.g., scFv)comprising a light chain variable domain or a heavy variable domain encoded by the

nucleic acid sequence selected from SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216. In some embodiments, the nucleic acid sequence of the light chain variable domain or the heavy variable domain of the anti-CD19 binding domain (e.g., scFv) is encoded by a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, SEQ ID NO: 24 SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0163] The anti-CD19 antigen binding domain of the present disclosure may have a k_{off} rate ((Ab- Ag)ko→ antibody (Ab) + antigen (Ag)) of less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, or less than about $10^{-1}s^{-1}$. In an another embodiment, an antibody of the invention has a k_{off} of less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, less than about 5 x $10^{-1}s^{-1}$, less than about $10^{-1}s^{-1}$, or less than about $10^{-1}s^{-1}$.

[0164] The anti-CD19 antigen binding domain of the present disclosure may have an affinity constant or K_a (k_{on}/k_{off}) of at least about 10^2 M^{-1}, at least about 5 x 10^2 M^{-1}, at least about 10^3 M^{-1}, at least about 5 x 10^3 M^{-1}, at least about 10^4 M^{-1}, at least about 5 x 10^4 M^{-1}, at least about 10^5 M^{-1}, at least about 5 x 10^5 M^{-1}, at least about 10^6 M^{-1}, at least about 5 x 10^6 M^{-1}, at least about 10^7 M^{-1}, at least about 5 x 10^7 M^{-1}, at least about 10^8 M^{-1}, at least about 5 x 10^8 M^{-1}, at least about 10^9 M^{-1}, at least about 5 x 10^9 M^{-1}, at least about 10^{10} M^{-1}, at least about 5 x 10^{10} M^{-1}, at about least 10^{11} M^{-1}, at least about 5 x 10^{11} M^{-1}, at least about 10^{12} M^{-1}, at least about 5 x 10^{12} M^{-1}, at least about 10^{13} M^{-1}, at least about 5 x 10^{13} M^{-1}, at least about 10^{14} M^{-1}, at least about 5 x 10^{14} M^{-1}, at least about 10^{15} M^{-1}, or at least about 5 x 10^{15} M^{-1}.

[0165] The anti-CD19 antigen binding domain of the present disclosure may have a dissociation constant or K_D (k_{off}/k_{on}) of less than about 5 x 10^{-2} M, less than about 10^{-2} M, less than about 5 x 10^{-3} M, less than about 10^{-3} M, less than 5 x 10^{-4} M, less than about 10^{-4} M, less than about 5 x 10^{-5} M, less than about 10^{-5} M, less than 5 x 10^{-6} M, less than about 10^{-}

^{6} M, less than about 5 x 10^{-7} M, less than about 10^{-7} M, less than about 5 x 10^{-8} M, less than about 10^{-8} M, less than about 5 x 10^{-9} M, less than about 10^{-9} M, less than about 5 x 10^{-10} M, less than about 10^{-10} M, less than about 5 x 10^{-11} M, less than about 10^{-11} M, less than about 5 x 10^{-12} M, less than about 10^{-12} M, less than about 5 x 10^{-13}M, less than about 10^{-13} M, less than about 5 x 10^{-14} M, less than about 10^{-14} M, less than about 5 x 10^{-15} M, or less than about 10^{-15} M.

[0166] When used with the method described herein, the anti-CD19 antigen binding domain of the present disclosure may specifically bind to human CD19 with a dissociation constant (K_d) of less than about 3000 nM, less than about 2500 nM, less than about 2000 nM, less than about 1500 nM, less than about 1000 nM, less than about 750 nM, less than about 500 nM, less than about 250 nM, less than about 200 nM, less than about 150 nM, less than about 100 nM, or less than about 75 nM as assessed using a method described herein or known to one of skill in the art (e.g., a BIAcore assay, ELISA) (Biacore International AB, Uppsala, Sweden).

[0167] In some embodiments, the anti-CD19 antigen binding domain of the present disclosure may specifically bind to a human CD19 antigen with a dissociation constant (K_d) of between about 25 to about 3400 nM, about 25 to about 3000 nM, about 25 to about 2500 nM, about 25 to about 2000 nM, about 25 to about 1500 nM, about 25 to about 1000 nM, about 25 to about 750 nM, about 25 to about 500 nM, about 25 to about 250 nM, about 25 to about 100 nM, about 25 to about 75nM, about 25 to about 50 nM as assessed using a method described herein or known to one of skill in the art (e.g., a BIAcore assay, ELISA). In another embodiment, the anti-CD19 antigen binding domain may specifically bind to hCD19 with a dissociation constant (K_d) of at least about 500 nM, at least about 100 nM, at least about 75 nM or at least about 50 nM as assessed using a method described herein or known to one of skill in the art (e.g., a BIAcore assay, ELISA).

2. Transmembrane Domain

[0168] A CAR encoded of the present disclosure can be designed to comprise a transmembrane domain that connects the antigen binding domain of the CAR to the intracellular domain. The transmembrane domain of a subject CAR is a region that is capable of spanning the plasma membrane of a cell (*e.g.*, an immune cell or precursor thereof). The transmembrane domain is for insertion into a cell membrane, *e.g.*, a eukaryotic cell

membrane. In some embodiments, the transmembrane domain is interposed between the antigen-binding domain and the intracellular domain of a CAR.

[0169] In one embodiment, the transmembrane domain is naturally associated with one or more of the domains in the CAR. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0170] In some embodiments, 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, *e.g.*, a Type I transmembrane protein. Where the source is synthetic, the transmembrane domain may be any artificial sequence that facilitates insertion of the CAR into a cell membrane, *e.g.*, an artificial hydrophobic sequence. In some embodiments, the transmembrane domain of particular use in this invention includes, without limitation, a transmembrane domain derived from (the alpha, beta or zeta chain of the T-cell receptor, CD28, CD2, CD3 epsilon, CD45, CD4, CD5, CD7, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and a killer immunoglobulin-like receptor (KIR).

[0171] In some embodiments, the transmembrane domain comprises at least a transmembrane region of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD2, CD3 epsilon, CD45, CD4, CD5, CD7, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and a killer immunoglobulin-like receptor (KIR).

[0172] In some embodiments, the transmembrane domain may be synthetic. In some embodiments, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. In certain exemplary embodiments, a triplet of

phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.

[0173] The transmembrane domains described herein can be combined with any of the antigen binding domains described herein, any of the costimulatory signaling domains described herein, any of the intracellular signaling domains described herein, or any of the other domains described herein that may be included in a subject CAR.

[0174] In one embodiment, the transmembrane domain comprises a CD8α transmembrane domain. In some embodiments, the transmembrane domain comprises a CD8α transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 29. In some embodiments, the transmembrane domain comprises the nucleotide sequence set forth in SEQ ID NO: 30.

[0175] In some embodiments, the transmembrane domain comprises a CD28 transmembrane domain. In some embodiments, the CAR comprises a CD28 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 31. In some embodiments, the CD28 transmembrane domain comprises the nucleotide sequence set forth in SEQ ID NO: 32.

[0176] In some embodiments, the transmembrane domain comprises a CD28 transmembrane domain. In some embodiments, the CAR comprises a ICOS transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 33. In some embodiments, the ICOS transmembrane domain comprises the nucleotide sequence set forth in SEQ ID NO: 34.

[0177] Tolerable variations of the transmembrane and/or hinge domain will be known to those of skill in the art, while maintaining its intended function. In some embodiments, the transmembrane domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, 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%, at least about 99% sequence identity to any of the amino acid sequences set forth in SEQ ID NOs: 29, 31, and/or 33. In some embodiments the transmembrane domain is encoded by a nucleic acid sequence comprising the nucleotide sequence that has at least about 80%, at least about 81%,

at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, 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%, at least about 99% sequence identity to any of the nucleotide sequences set forth in SEQ ID NOs: 30, 32, and/or 34. The transmembrane domain may be combined with any hinge domain and/or may comprise one or more transmembrane domains described herein.

[0178] In some embodiments, the CAR comprises: any transmembrane domain selected from the group consisting of the transmembrane domain of alpha, beta or zeta chain of the T-cell receptor, CD28, CD2, CD3 epsilon, CD45, CD4, CD5, CD7, CD8, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and a killer immunoglobulin-like receptor (KIR); any costimulatory signaling domains, and any intracellular domains or cytoplasmic domains described herein, or any of the other domains described herein that may be included in the CAR, and optionally a hinge domain.

[0179] In some embodiments, the CAR further comprises a spacer domain between the extracellular domain and the transmembrane domain of the CAR, or between the intracellular domain and the transmembrane domain of the CAR. In some embodiments, the spacer domain may be a short oligo- or polypeptide linker, *e.g.*, between about 2 and about 10 amino acids in length. For example, glycine-serine doublet provides a particularly suitable linker between the transmembrane domain and the intracellular signaling domain of the subject CAR. Accordingly, the CAR of the present disclosure may comprise any of the transmembrane domains, hinge domains, or spacer domains described herein.

3. Hinge domain

[0180] In some embodiments, a CAR of the present disclosure further comprises a hinge region. The hinge region of the CAR is a hydrophilic region which is located between the antigen binding domain and the transmembrane domain. In some embodiments, the hinge domain facilitates proper protein folding for the CAR. In some embodiments, the hinge domain is an optional component for the CAR. In some embodiments, the hinge domain

comprises a domain selected from Fc fragments of antibodies, hinge regions of antibodies, CH2 regions of antibodies, CH3 regions of antibodies, artificial hinge sequences or combinations thereof. In some embodiments, the hinge domain is selected from but not limited to, a CD8a hinge, artificial hinges made of polypeptides that may be as small as, three glycines (Gly). In some embodiments, the hinge region is a hinge region polypeptide derived from a receptor. In some embodiments, the hinge region is a CD8-derived hinge region). In one embodiment, the hinge domain comprises an amino acid sequence derived from human CD8, or a variant thereof. In some embodiments, a subject CAR comprises a CD8α hinge domain and a CD8α transmembrane domain. In some embodiment, the CD8α hinge domain comprises the amino acid sequence set forth in SEQ ID NO: 27 or 35. In some embodiments, the CD8α hinge domain comprises the nucleotide sequence set forth in SEQ ID NO: 28 or 36.

[0181] In some embodiments the hinge domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, 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%, at least about 99% sequence identity to any of the amino acid sequences set forth in SEQ ID NO: 27 or 35.

[0182] In some embodiments the hinge domain is encoded by a nucleic acid sequence comprising the nucleotide sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, 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%, at least about 99% sequence identity to any of the nucleotide sequences set forth in SEQ ID NO: 28 or 36.

[0183] In some embodiments, the hinge domain connects the antigen-binding domain to the transmembrane domain, which, is linked to the intracellular domain. In exemplary embodiments, the hinge region is capable of supporting the antigen binding domain to recognize and bind to the target antigen on the target cells. In some embodiments, the hinge region is a flexible domain, thus allowing the antigen binding domain to have a structure to optimally recognize the specific structure and density of the target antigens on a cell such as

tumor cell. The flexibility of the hinge region permits the hinge region to adopt many different conformations.

[0184] In some embodiments, the hinge domain has a length selected from about 4 to about 50, from about 4 to about 10, from about 10 to about 15, from about 15 to about 20, from about 20 to about 25, from about 25 to about 30, from about 30 to about 40, or from about 40 to about 50 amino acids. Suitable hinge regions can be readily selected and can be of any of a number of suitable lengths, such as from about 1 amino acid (e.g., Glycine (Gly) to about 20 amino acids, from about 2 to about 15, from about 3 to about 12 amino acids, including about 4 to about 10, about 5 to about 9, about 6 to about 8, or about 7 to about 8 amino acids, and can be about 1, about 2, about 3, about 4, about 5, about 6, or about 7 amino acids.

[0185] In some embodiments, the amino acid is a glycine (Gly). Glycine and glycine-serine polymers can be used; both Gly and Ser are relatively unstructured, and therefore can serve as a neutral tether between components. Glycine polymers can be used; glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains. In some embodiment, the hinge regions comprises glycine polymers (G)n, glycine-serine polymers. In some embodiments, the hinge region comprises glycine-serine polymers selected from the group consisting of (GS)n, (GSGGS)n and (GGGS)n, where n is an integer of at least one). In some embodiments, the hinge domain comprises an amino acid sequence of including, but not limited to, GGSG (SEQ ID NO: 121), GGSGG (SEQ ID NO: 122), GSGSG (SEQ ID NO: 123), GSGGG (SEQ ID NO: 124), GGGSG (SEQ ID NO: 125), GSSSG (SEQ ID NO: 126). In some embodiment, the hinge region comprises glycine-alanine polymers, alanine-serine polymers, or other flexible linkers known in the art.

[0186] In some embodiments, the hinge region is an immunoglobulin heavy chain hinge region. Immunoglobulin hinge region amino acid sequences are known in the art. In some embodiments, an immunoglobulin hinge domain comprises an amino acid sequence selected from the group consisting of DKTHT (SEQ ID NO: 130); CPPC (SEQ ID NO: 131); CPEPKSCDTPPPCPR (SEQ ID NO: 132) (see, e.g., Glaser et al., J. Biol. Chem. (2005) 280:41494-41503); ELKTPLGDTTHT (SEQ ID NO: 133); KSCDKTHTCP (SEQ ID NO: 134); KCCVDCP (SEQ ID NO:135); KYGPPCP (SEQ ID NO: 136); EPKSCDKTHTCPPCP (SEQ ID NO: 137) (human IgG1 hinge); ERKCCVECPPCP (SEQ ID NO: 138) (human

IgG2 hinge); ELKTPLGDTTHTCPRCP (SEQ ID NO: 139) (human IgG3 hinge); SPNMVPHAHHAQ (SEQ ID NO: 49) (human IgG4 hinge); and the like.

[0187] In some embodiments, the hinge region is an immunoglobulin heavy chain hinge region. In some embodiments, the hinge is selected from CH1 and CH3 domains of IgGs (such as human IgG4). In some embodiments, the hinge domain comprises an amino acid sequence of a human IgG1, IgG2, IgG3, or IgG4 hinge domain. In some embodiments, the hinge region can include one or more amino acid substitutions and/or insertions and/or deletions compared to a wild-type (naturally-occurring) hinge region. In some embodiment, histidine at position 229 (His229) of human IgG1 hinge is substituted with tyrosine (Tyr). In some embodiments, the hinge domain comprises the amino acid sequence EPKSCDKTYTCPPCP (SEQ ID NO: 137).

4. Intracellular Domain

[0188] A CAR encoded of the present disclosure also comprises an intracellular domain. The intracellular domain or otherwise the cytoplasmic domain of the CAR is responsible for activation of the cell in which the CAR is expressed. The term "intracellular domain" is thus meant to include any portion of the intracellular domain sufficient to transduce the activation signal. In one embodiment, the intracellular domain includes a domain responsible for an effector function. The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. In one embodiment, the intracellular domain of the CAR includes a domain responsible for signal activation and/or transduction. The intracellular domain may transmit signal activation via protein-protein interactions, biochemical changes or other response to alter the cell's metabolism, shape, gene expression, or other cellular response to activation of the chimeric intracellular signaling molecule.

[0189] Examples of an intracellular domain for use in the invention include, but are not limited to, the cytoplasmic portion of a T cell receptor (TCR), and any co-stimulatory molecule, or any molecule that acts in concert with the TCR to initiate signal transduction in the T cell, following antigen receptor engagement, as well as any derivative or variant of these elements and any synthetic sequence that has the same functional capability.

[0190] In certain embodiments, the intracellular domain comprises an intracellular signaling

domain. Examples of the intracellular domain include a fragment or domain from one or more molecules or receptors including, but are not limited to, TCR, CD3 zeta, CD3 gamma, CD3 delta, CD3 epsilon, CD86, common FcR gamma, FcR beta (Fc Epsilon Rib), CD79a, CD79b, Fc gamma R11a, DAP10, DAP12, T cell receptor (TCR), CD2, CD8, CD27, CD28, 4-1BB (CD137), OX9, OX40, CD30, CD40, PD-1, ICOS, a KIR family protein, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, CD5, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), CD127, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD1Id, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD lib, ITGAX, CD11c, ITGBl, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD 162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, syk family tyrosine kinases (Syk, ZAP 70, etc.), src family tyrosine kinases (Lck, Fyn, Lyn, etc.), other co-stimulatory molecules described herein, any derivative, variant, or fragment thereof, any synthetic sequence of a co-stimulatory molecule that has the same functional capability, and any combination thereof.

[0191] In some embodiments, the intracellular signaling domain comprises an intracellular domain selected from the group consisting of cytoplasmic signaling domains of a human CD2, CD3 zeta chain (CD3ζ), FcγRIII, FcsRI, a cytoplasmic tail of an Fc receptor, an immunoreceptor tyrosine-based activation motif (ITAM) bearing cytoplasmic receptor, TCR zeta, FcR gamma, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d, or a variant thereof. In some embodiments, the intracellular signaling domain comprises CD3 zeta intracellular signaling domain.

[0192] Additional examples of intracellular domains include, without limitation, intracellular signaling domains of several types of various other immune signaling receptors, including, but not limited to, first, second, and third generation T cell signaling proteins including CD3, B7 family costimulatory, and Tumor Necrosis Factor Receptor (TNFR) superfamily

receptors. Additionally, intracellular signaling domains may include signaling domains used by NK and NKT cells such as signaling domains of NKp30 (B7-H6), and DAP 12, NKG2D, NKp44, NKp46, DAP10, and CD3z.

[0193] Intracellular signaling domains suitable for use in the CAR of the present invention include any desired signaling domain that transduces a signal in response to the activation of the CAR (i.e., activated by antigen and dimerizing agent). In some embodiments, a distinct and detectable signal e.g. comprises increased production of one or more cytokines by the cell; change in transcription of a target gene; change in activity of a protein; change in cell behavior (e.g., cell death); cellular proliferation; cellular differentiation; cell survival; and/or modulation of cellular signaling responses. e.g. In some embodiments, the intracellular signaling domain includes DAP10/CD28 type signaling chains. In some embodiments, the intracellular signaling domain is not covalently attached to the membrane bound CAR, but is instead diffused in the cytoplasm.

[0194] Intracellular signaling domains suitable for use in the CAR of the present invention include immunoreceptor tyrosine-based activation motif (ITAM)-containing intracellular signaling polypeptides. In some embodiments, the intracellular signaling domain includes at least one at least two, at least three, at least four, at least five, or at least six ITAM motifs as described below. In some embodiments, an ITAM motif is repeated twice in an intracellular signaling domain, where the first and second instances of the ITAM motif are separated from one another by 6 to 8 amino acids. In one embodiment, the intracellular signaling domain of a subject CAR comprises 3 ITAM motifs. In some embodiments, intracellular signaling domains includes the signaling domains of human immunoglobulin receptors that contain immunoreceptor tyrosine based activation motifs (ITAMs) such as, but not limited to, Fc gamma RI, Fc gamma RIIA, Fc gamma RIIC, Fc gamma RIIIA, FcRL5.

[0195] A suitable intracellular signaling domain can be an ITAM motif-containing portion that is derived from a polypeptide that contains an ITAM motif. For example, a suitable intracellular signaling domain can be an ITAM motif-containing domain from any ITAM motif-containing protein. Thus, a suitable intracellular signaling domain need not contain the entire sequence of the entire protein from which it is derived. Examples of suitable ITAM motif-containing polypeptides include, but are not limited to: DAP12, FCER1G (Fc epsilon

receptor I gamma chain), CD3D (CD3 delta), CD3E (CD3 epsilon), CD3G (CD3 gamma), CD3Z (CD3 zeta), and CD79A (antigen receptor complex-associated protein alpha chain).

[0196] In one embodiment, the intracellular signaling domain is derived from DAP12 (also known as TYROBP; TYRO protein tyrosine kinase binding protein; KARAP; PLOSL; DNAX-activation protein 12; KAR-associated protein; TYRO protein tyrosine kinase-binding protein; killer activating receptor associated protein; killer-activating receptor-associated protein; etc.). In one embodiment, the intracellular signaling domain is derived from FCER1G (also known as FCRG; Fc epsilon receptor I gamma chain; Fc receptor gamma-chain; fc-epsilon RI-gamma; fcR gamma; fceR1 gamma; high affinity immunoglobulin epsilon receptor subunit gamma; immunoglobulin E receptor, high affinity, gamma chain; etc.). In one embodiment, the intracellular signaling domain is derived from T-cell surface glycoprotein CD3 delta chain (also known as CD3D; CD3-DELTA; T3D; CD3 antigen, delta subunit; CD3 delta; CD3d antigen, delta polypeptide (TiT3 complex); OKT3, delta chain; T-cell receptor T3 delta chain; T-cell surface glycoprotein CD3 delta chain; etc.). In one embodiment, the intracellular signaling domain is derived from T-cell surface glycoprotein CD3 epsilon chain (also known as CD3e, T-cell surface antigen T3/Leu-4 epsilon chain, T-cell surface glycoprotein CD3 epsilon chain, AI504783, CD3, CD3epsilon, T3e, etc.). In one embodiment, the intracellular signaling domain is derived from T-cell surface glycoprotein CD3 gamma chain (also known as CD3G, T-cell receptor T3 gamma chain, CD3-GAMMA, T3G, gamma polypeptide (TiT3 complex), etc.). In one embodiment, the intracellular signaling domain is derived from T-cell surface glycoprotein CD3 zeta chain (also known as CD3Z, T-cell receptor T3 zeta chain, CD247, CD3-zeta, CD3H, CD3Q, T3Z, TCRZ, etc.). In one embodiment, the intracellular signaling domain is derived from CD79A (also known as B-cell antigen receptor complex-associated protein alpha chain; CD79a antigen (immunoglobulin-associated alpha); MB-1 membrane glycoprotein; Ig-alpha; membrane-bound immunoglobulin-associated protein; surface IgM-associated protein; etc.). In one embodiment, an intracellular signaling domain suitable for use in the CAR of the present disclosure includes a DAP10/CD28 type signaling chain. In one embodiment, an intracellular signaling domain suitable for use in a subject CAR of the present disclosure includes a ZAP70 polypeptide. In some embodiments, the intracellular signaling domain includes a cytoplasmic signaling domain of TCR zeta, FcR gamma, FcR beta, CD3 gamma,

CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, or CD66d. In one embodiment, the intracellular signaling domain in the CAR includes a cytoplasmic signaling domain of human CD3 zeta.

[0197] While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The intracellular signaling domain includes any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

[0198] The intracellular signaling domains described herein can be combined with any of the costimulatory signaling domains described herein, any of the antigen binding domains described herein, any of the transmembrane domains described herein, or any of the other domains described herein that may be included in the CAR. In some embodiment, the intracellular domain of the CAR comprises dual signaling domains. The dual signaling domains may include a fragment or domain from any of the molecules described herein. In some embodiments, the intracellular domain comprises 4-1BBcostimulatory domain and CD3 zeta signaling domain; CD28 costimulatory domain and CD3 zeta signaling domain; CD2 costimulatory domain and CD3 zeta signaling domain. In some embodiments, the intracellular domain of the CAR includes any portion of a co-stimulatory molecule, such as at least one signaling domain from CD3, CD27, CD28, ICOS, 4-1BB, PD-1, T cell receptor (TCR), any derivative or variant thereof, any synthetic sequence thereof that has the same functional capability, and any combination thereof.

[0199] Further, variant intracellular signaling domains suitable for use in a subject CAR are known in the art. The YMFM motif is found in ICOS and is a SH2 binding motif that recruits both p85 and p50alpha subunits of PI3K, resulting in enhanced AKT signaling. In one embodiment, a CD28 intracellular domain variant may be generated to comprise a YMFM motif.

[0200] In one embodiment, the intracellular domain of a subject CAR comprises a CD3 zeta intracellular signaling domain comprising the amino acid sequence set forth in SEQ ID NO:

52 or SEQ ID NO: 54, which may be encoded by a nucleic acid sequence comprising the nucleotide sequence set forth in SEQ ID NO: 53 or SEQ ID NO: 55, respectively.

[0201] Tolerable variations of the intracellular domain will be known to those of skill in the art, while maintaining specific activity. In some embodiments, the intracellular domain comprises an amino acid sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to any of the amino acid sequences set forth in SEQ ID NO: 52 or 54. In some embodiments, the intracellular domain is encoded by a nucleic acid sequence comprising a nucleotide sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to any of the nucleotide sequences set forth in SEQ ID NO: 53 or 55.

5. Costimulatory Domain

[0202] In some embodiments, the intracellular domain comprises a costimulatory signaling domain and an intracellular signaling. In certain embodiments, the intracellular domain comprises a costimulatory signaling domain. In one embodiment, the intracellular domain of the CAR comprises a costimulatory signaling domain selected from the group consisting of a portion of a signaling domain from proteins in the TNFR superfamily, CD27, CD28, 4-1BB (CD137), OX40 (CD134), PD-1, CD7, LIGHT, CD83L, DAP10, DAP12, CD27, CD2, CD5, ICAM-1, LFA-1, Lck, TNFR-I, TNFR-II, Fas, CD30, CD40, ICOS (CD278), NKG2C, B7-H3 (CD276), and an intracellular domain derived from a killer immunoglobulin-like receptor (KIR, any derivative or variant thereof, any synthetic sequence thereof that has the same functional capability, and any combination thereof.

[0203] In some embodiments, the costimulatory domain comprises one or more of a costimulatory domain of a protein selected from the group consisting of proteins in the TNFR superfamily, CD28, 4-1BB (CD137), OX40 (CD134), PD-1, CD7, LIGHT, CD83L, DAP10, DAP12, CD27, CD2, CD5, ICAM-1, LFA-1, Lck, TNFR-I, TNFR-II, Fas, CD30, CD40, ICOS (CD278), NKG2C, B7-H3 (CD276), and an intracellular domain derived from a killer

immunoglobulin-like receptor (KIR), or a variant thereof. In some embodiments, the costimulatory domain comprises one or more of a costimulatory domain of a protein selected from the group consisting of proteins in the CD28, 4-1BB (CD137), OX40 (CD134), CD27, CD2, or a combination thereof. In some embodiments, the costimulatory signaling domain comprises 4-1BB costimulatory domain. In some embodiments, the costimulatory signaling domain comprises CD2 costimulatory domain. In some embodiments, the costimulatory signaling domain comprises CD28 costimulatory domain.

[0204] In some embodiments, the costimulatory domain comprises an amino acid sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to any of the amino acid sequences set forth in SEQ ID NO: 37, 39, 41, 43, 46, 48, or 50. In some embodiments, the intracellular domain is encoded by a nucleic acid sequence comprising a nucleotide sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to any of the nucleotide sequences set forth in SEQ ID NO: 38, 40, 42, 44, 45, 47, 49, or 51.

[0205] In one embodiment, the intracellular domain of a subject CAR comprises an ICOS costimulatory domain and a CD3 zeta intracellular signaling domain. In one embodiment, the intracellular domain of a subject CAR comprises a CD28 costimulatory domain and a CD3 zeta intracellular signaling domain. In one embodiment, the intracellular domain of a subject CAR comprises a CD28 YMFM variant costimulatory domain and a CD3 zeta intracellular signaling domain. In one embodiment, the intracellular domain of a subject CAR comprises a CD27 costimulatory domain and a CD3 zeta intracellular signaling domain. In one embodiment, the intracellular domain of a subject CAR comprises a OX40 costimulatory domain and a CD3 zeta intracellular signaling domain. In one exemplary embodiment, the intracellular domain of a subject CAR comprises a 4-1BB costimulatory domain and a CD3 zeta intracellular signaling domain. In one exemplary embodiment, the intracellular domain of a subject CAR comprises a CD2 costimulatory domain and a CD3 zeta intracellular signaling domain.

B. Additional Antigen-binding polypeptides

[0206] In some embodiments, the modified T cell expresses an antigen-binding polypeptide, a cell surface receptor ligand, or a polypeptide that binds to a tumor antigen. In some instances, the antigen-binding domain comprises an antibody that recognizes a cell surface protein or a receptor expressed on a tumor cell. In some instances, the antigen-binding domain comprises an antibody that recognizes a tumor antigen. In some instances, the antigen-binding domain comprises a full length antibody or an antigen-binding fragment thereof, a Fab, a F(ab)2, a monospecific Fab2, a bispecific Fab2, a trispecific Fab2, a single-chain variable fragment (scFv), a diabody, a triabody, a minibody, a V-NAR, or a VhH.

C. Cell surface receptor ligands

[0207] In some embodiments, a lentiviral vector or retroviral vector of the present disclosure further comprises a nucleic acid encoding a cell surface receptor ligand. In some instances, the ligand binds to a cell surface receptor expressed on a tumor cell. In some cases, the ligand comprises a wild-type protein or a variant thereof that binds to the cell surface receptor. In some instances, the ligand comprises a full-length protein or a functional fragment thereof that binds to the cell surface receptor. In some cases, the functional fragment comprises about 90%, about 80%, about 70%, about 60%, about 50%, or about 40% in length as compared to the full length version of the protein but retains binding to the cell surface receptor. In some cases, the ligand is a *de novo* engineered protein that binds to the cell surface receptor. Exemplary ligands include, but are not limited to, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or Wnt3A.

D. Tumor Antigens

[0208] In some embodiments, a lentiviral vector or retroviral vector of the present disclosure further comprises a nucleic acid encoding a polypeptide that binds to a tumor antigen. In some embodiments, the tumor antigen is associated with a hematologic malignancy. Exemplary tumor antigens include, but are not limited to, CD19, CD20, CD22, CD33/IL3Ra, ROR1, mesothelin, c-Met, PSMA, PSCA, Folate receptor alpha, Folate receptor beta, EGFRvIII, GPC2, Tn-MUC1, GDNF family receptor alpha-4 (GFRa4), fibroblast activation protein (FAP), and IL13Ra2. In some instances, the tumor antigen comprises CD19, CD20, CD22, BCMA, CD37, Mesothelin, PSMA, PSCA, Tn-MUC1, EGFR, EGFRvIII, c-Met,

HER1, HER2, CD33, CD133, GD2, GPC2, GPC3, NKG2D, KRAS, or WT1. In some instances, the polypeptide is a ligand of the tumor antigen, e.g., a full-length protein that binds to the tumor antigen, a functional fragment thereof, or a *de novo* engineered ligand that binds to the tumor antigen. In some instances, the polypeptide is an antibody that binds to the tumor antigen.

E. Engineered T cell Receptors

[0209] In some embodiments, the antigen binding domain of a CAR described herein can be grafted to one or more constant domains of a T cell receptor ("TCR") chain (e.g., a TCR alpha or TCR beta chain), to create a chimeric TCR. Chimeric TCRs can signal through the TCR complex upon antigen binding. For example, an scFv as disclosed herein, can be grafted to the constant domain, or at least a portion of the extracellular constant domain, the transmembrane domain of a TCR chain. As another example, an antibody fragment, for example a VL domain as described herein, can be grafted to the constant domain of a TCR alpha chain. Such chimeric TCRs may be produced, for example, by methods known in the art (For example, Willemsen RA et al, Gene Therapy 2000; 7: 1369-1377; Zhang T et al, Cancer Gene Ther 2004; 11: 487-496; Aggen et al, Gene Ther. 2012 Apr;19(4):365-74).

F. Switch Receptors and Dominant Negative Receptors

[0210] In one example, a lentiviral vector or retroviral vector of the present disclosure further comprises a nucleic acid encoding a dominant negative receptor, a switch receptor, or a combination thereof. In some embodiments, the lentiviral vector or retroviral vector described herein comprises a chimeric antigen receptor (CAR), and/or a dominant negative receptor. In some embodiments, the lentiviral vector or retroviral vector comprises a CAR, and/or a switch receptor. In some embodiments, the lentiviral vector or retroviral vector described herein comprises an engineered TCR, and a switch receptor. In some embodiments, the lentiviral vector or retroviral vector described herein comprises an engineered TCR, and a dominant negative receptor. In some embodiments, the lentiviral vector or retroviral vector described herein comprises a KIR, and a switch receptor. In some embodiments, the lentiviral vector or retroviral vector described herein further comprises a KIR, and a dominant negative receptor.

1. Switch Receptors

[0211] The present disclosure provides quick and efficient manufacturing processes for engineering modified immune cells comprising a CAR, or an exogenous TCR and/or a switch receptor. In some embodiments, the CAR, the TCR and/or the switch receptor are encoded by one or more nucleic acids. In some embodiments, the lentiviral vector or retroviral vector disclosed herein comprises one or more nucleic acid sequence encoding the CAR, the TCR and/or the switch receptor. In some embodiments, the nucleic acid sequence encoding the CAR is operably linked to a nucleic acid sequence encoding the switch receptor. In some embodiments, the switch receptor can enhances the efficiency of the CAR or the CAR expressing cell.

[0212] Tumor cells generate an immunosuppressive microenvironment that serves to protect them from immune recognition and elimination. This immunosuppressive microenvironment can limit the effectiveness of immunosuppressive therapies such as CAR-T or TCR-T cell therapy. For example, the secreted cytokine Transforming Growth Factor β (TGF β) directly inhibits the function of cytotoxic T cells and additionally induces regulatory T cell formation to further suppress immune responses. T cell immunosuppression due to TGFβ in the context of prostate cancers has been previously demonstrated. To reduce the immunosuppressive effects of TGF on the immune cells can be modified to express an engineered TGFβR comprising the extracellular ligand-binding domain of the TGFβR fused to the intracellular signaling domain of, for example, Interleukin-12 receptor (IL12R; TGFβR-IL12R). Therefore, a modified immune cell comprising a switch receptor may bind a negative signal transduction molecule in the microenvironment of the modified immune cell, and convert the negative signal transduction signal of an inhibitory molecule may have on the modified immune cell into a positive signal that stimulate the modified immune cell. A switch receptor of the present disclosure may be designed to reduce the effects of a negative signal transduction molecule, or to convert the negative signal into a positive signal, by virtue of comprising an intracellular domain associated with the positive signal.

[0213] As used herein, the term "switch receptor" refers to a molecule designed to reduce the effect of a negative signal transduction molecule on a modified immune cell of the present invention. The switch receptor comprises: a first domain that is derived from a first polypeptide that is associated with a negative signal (a signal transduction that suppresses or inhibits a cell or T cell activation); and a second domain that is derived from a second

polypeptide that is associated with a positive signal (a signal transduction signal that stimulate a cell or a T cell). In some embodiments, the protein associated with the negative signal is selected from the group consisting of CTLA4, PD-1, TGFβRII, BTLA, VSIG3, VSIG8, and TIM-3. In some embodiments, the protein associated with the positive signal is selected from the group consisting of CD28, 4-1BB, IL12Rβ1, IL12Rβ2, CD2, ICOS, and CD27.

[0214] In one embodiment, the first domain comprises at least a portion of the extracellular domain of the first polypeptide that is associated with a negative signal, and the second domain comprises at least a portion of the intracellular domain of the second polypeptide that is associated with a positive signal. As such, a switch receptor comprises an extracellular domain associated with a negative signal fused to an intracellular domain associated with a positive signal. In some embodiments, the switch receptor comprises an extracellular domain of a signaling protein associated with a negative signal, a transmembrane domain, and an intracellular domain of a signaling protein associated with a positive signal. In some embodiments, the transmembrane domain of the switch receptor is selected from the transmembrane of the protein associated with a negative signal or the transmembrane domain of the protein associated with the negative signal. In some embodiments, the transmembrane domain of the switch receptor is selected from a transmembrane domain of a protein selected from the group consisting of CTLA4, PD-1, VSIG3, VSIG8, TGFβRII, BTLA, TIM-3, CD28, 4-1BB, IL12Rβ1, IL12Rβ2, CD2, ICOS, and CD27.

[0215] In some embodiments, the switch receptor is selected from the group consisting of PD-1-CD28, PD-1A132L-CD28, PD-1-CD27, PD-1A132L-CD27, PD-1-4-1BB, PD-1A132L-4-1BB, PD-1-ICOS, PD-1A132L-ICOS, PD-1-IL12Rβ1, PD-1A132L-IL12Rβ1, PD-1-IL12Rβ2, PD-1A132L-IL12Rβ2, VSIG3-CD28, VSIG8-CD28, VSIG3-CD27, VSIG8-CD27, VSIG3-4-1BB, VSIG8-4-1BB, VSIG3-ICOS, VSIG8-ICOS, VSIG3-IL12Rβ1, VSIG8-IL12Rβ1, VSIG3-IL12Rβ2, VSIG8-IL12Rβ2, TGFβRII-CD27, TGFβRII-CD28, TGFβRII-4-1BB, TGFβRII-ICOS, TGFβRII-IL12Rβ1, and TGFβRII-IL12Rβ2.

2. Dominant Negative Receptors

[0216] The present disclosure provides a quick and efficient manufacturing process for engineering modified immune cells comprising a CAR, or an exogenous TCR and a dominant

negative receptor. In some embodiments, the CAR, the TCR and/or the switch receptor are encoded by one or more nucleic acid, In some embodiments, the lentiviral vector or retroviral vector disclosed herein comprises one or more nucleic acid sequence encoding the CAR, the TCR and/or the dominant negative receptor. In some embodiments, the nucleic acid sequence encoding the CAR is operably linked to a nucleic acid sequence encoding the dominant negative receptor. In some embodiments, the dominant negative receptor enhances the efficiency of the CAR or the CAR expressing cell.

[0217] As used herein, the term “dominant negative receptor” refers to a molecule designed to reduce the effect of a negative signal transduction molecule (*e.g.*, the effect of a negative signal transduction molecule on a modified immune cell of the present invention). A dominant negative receptor is a truncated variant of a wild-type protein associated with a negative signal. In some embodiments, the protein associated with a negative signal he protein associated with the negative signal is selected from the group consisting of CTLA4, PD-1, BTLA, TGFβRII, VSIG3, VSIG8, and TIM-3.

[0218] A dominant negative receptor of the present invention may bind a negative signal transduction molecule (*e.g.*, CTLA4, PD-1, BTLA, TGFβRII, VSIG3, VSIG8, and TIM-3) by virtue of an extracellular domain associated with the negative signal, may reduce the effect of the negative signal transduction molecule. For example, a modified immune cell comprising a dominant negative receptor may bind a negative signal transduction molecule in the microenvironment of the modified immune cell, but this binding will not transduce this signal inside the cell to modify the activity of the modified T cell. Rather, the binding sequesters the negative signal transduction molecule and prevents its binding to endogenous receptor/ligand, thereby reducing the effect of the negative signal transduction molecule may have on the modified immune cell. As such, to reduce the immunosuppressive effects of certain molecule, immune cells can be modified to express a dominant negative receptor that is a dominant negative receptor.

[0219] In some embodiments, the dominant negative receptor comprises a truncated variant of a wild-type protein associated with a negative signal. In some embodiments, the dominant negative receptor comprises a variant of a wild-type protein associated with a negative signal comprising an extracellular domain, a transmembrane domain, and substantially lacking an intracellular signaling domain. In some embodiments, the dominant negative receptor

comprises an extracellular domain of a signaling protein associated with a negative signal, and a transmembrane domain. In some embodiments, the dominant negative receptor is PD-1, CTLA4, BTLA, TGFβRII, VSIG3, VSIG8, or TIM-3 dominant negative receptor. In some embodiments, the dominant negative receptor is PD-1, or TGFβRII dominant negative receptor. Tolerable variations of the dominant negative receptor will be known to those of skill in the art, while maintaining its intended biological activity (*e.g.*, blocking a negative signal and/or sequestering a molecule having a negative signal when expressed in a cell).

G. Chemokine and Cytokine as Immune Enhancing Factors for Improved Fitness

[0220] The present disclosure provides quick and efficient manufacturing processes for engineering modified immune cells comprising a CAR, or an exogenous TCR and/or an immune enhancing factor that improves the fitness of the engineered immune cells. In some embodiments, the immune enhancing factor or a functional derivative thereof is a polypeptide that enhances the immune cell function.

[0221] In some embodiments, a polypeptide that enhances the immune cell function, or a functional derivative thereof is selected from a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, Interleukin -7 (IL-7), Interleukin-7 receptor (IL-7R), Interleukin-15 (IL-15), Interleukin-15 receptor (IL-15R), Interleukin-21 (IL-21), Interleukin-18 (IL-18), Interleukin-18 receptor (IL-18R),CCL21, CCL19, or a combination thereof. In some embodiments, a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, IL-18R, C-C Motif Chemokine Ligand 21 (CCL21), or C-C Motif Chemokine Ligand 19 (CCL19) is an immune function-enhancing factor that improves the fitness of the claimed modified immune cell. Without wishing to be bound by theory, the addition of a nucleic acid encoding a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, IL-18R, CCL21, or CCL19 to the modified immune cell of the present disclosure enhances the immunity-inducing effect and antitumor activity of the modified immune cell.

1. T cell infiltration

[0222] Without wishing to be bound by theory, interleukins and chemokines, may promote increase T cell priming and/or T cell infiltration in a solid tumor. For instance, in microsatellite stable colorectal cancers (CRCs) with low T cell infiltration, IL-15 promotes T

cell priming. In some embodiments, the combination of a CAR and chemokine/interleukine receptor complex promotes T cell priming. Furthermore, IL-15 may induce NK cell infiltration. In some embodiments, response to an IL-15/IL-15RA complex can result in NK cell infiltration. In certain embodiments, the modified immune cell described herein further comprises an IL-15/IL-15Ra complex. In some embodiments, the IL-15/IL-15Ra complex is chosen from NIZ985 (Novartis), ATL-803 (Altor) or CYP0150 (Cytune). In some embodiments, the IL-15/IL-15RA complex is NIZ985. In some embodiments, IL-15 stimulates Natural Killer cells to eliminate (e.g., kill) pancreatic cancer cells. In some embodiments, therapeutic response to a modified immune cell described herein further comprising IL-15/IL15Ra is associated with Natural Killer cell infiltration in an animal model of colorectal cancer. In some embodiments, the IL-15/IL-15Ra complex comprises human IL-15 complexed with a soluble form of human IL-15Ra. The complex may comprise IL-15 covalently or noncovalently bound to a soluble form of IL-15Ra. In a particular embodiment, the human IL-15 is noncovalently bonded to a soluble form of IL-15Ra.

[0223] The ineffectiveness of CAR T cell therapy against solid tumors is partially caused by the limited recruitment and accumulation of immune cells and CAR T cells in solid tumors. One approach to solve this problem is to engineer CAR T cells that mimic the function of T-zone fibroblastic reticular cells (FRC). The lymph node is responsible for detecting pathogens and immunogens. The T-zone contains three types of cells: (1) innate immunity cells such as dendritic cells, monocytes, macrophages, and granulocytes; (2) adaptive immunity cells, such as CD4 and CD8 lymphocytes, and (3) stromal cells (FRCs). These cells cooperate to mount an effective immune response against a pathogen by facilitating the activation, differentiation and maturation of CD4 T cells. FRCs are particularly important because they form a network that allows dendritic cells and T cells to travel throughout the lymph node, and attracts B cells. In particular, FRCs provide a network for: (i) the recruitment of naive T cells, B cells and dendritic cells to the lymph node by releasing two chemokines (CCL21 and CCL19); (ii) T cell survival by secreting IL-7, which is a survival factor particularly for naive T cells; and (iii) trafficking of CD4 T cells toward the germinal center (GC; a different part of the lymph node). Accordingly, a CAR armored with exogenous CCL21, or CCL19 and IL-7, will enhance the recruitment of T cells, B cells and dendritic cells to solid tumors. In some embodiments, the modified immune cells engineered by the method disclosed herein

comprises a lentiviral vector or retroviral vector comprising a nucleic acid encoding an immune function-enhancing factor, and a CAR. In that embodiment, the nucleic acid encoding the immune function-enhancing factor is a nucleic acid encoding interleukin-7 and a nucleic acid encoding CCL19 or CCL21.

[0224] In some embodiments, the nucleic acid of the immune function-enhancing factor (i.e. chemokine, the chemokine receptor, the cytokine, the cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, CCL21, or CCL19) is fused to a CAR. In some embodiments, the chemokine, the chemokine receptor, the cytokine, the cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, CCL21, or CCL19 is fused to a CAR via a self-cleaving peptide, such as a P2A, a T2A, an E2A, or an F2A.

2. T cell priming (IL-18)

[0225] The present disclosure provides quick and efficient manufacturing processes for engineering modified immune cells comprising a CAR, or an exogenous TCR and/or polypeptide which enhances T cell priming (i.e., T cell priming polypeptide). In some embodiments, the polypeptide that enhances T cell priming (ETP) is selected from the group consisting of a costimulatory molecule, a soluble cytokine, a polypeptide involved in antigen presentation, a polypeptide involved in trafficking and/or migration, or a polypeptide involved in dendritic cell targeting, or a functional fragment or variant thereof. In an embodiment, the T cell priming costimulatory molecule is selected from the group consisting of CD70, CD83, CD80, CD86, CD40, CD154, CD137L (4-1BBL), CD252 (OX40L), CD275 (ICOS-L), CD54 (ICAM-1), CD49a, CD43, CD48, CD112 (PVRL2), CD150 (SLAM), CD155 (PVR), CD265 (RANK), CD270 (HVEM), TL1A, CD127, IL-4R, GITR-L, CD160, CD258, TIM-4, CD153 (CD30L), CD200R (OX2R), CD44, ligands thereof, and functional fragments and variants thereof. In an embodiment, the soluble cytokine is selected from the group consisting of: IL-2, IL-12, IL-6, IL-7, IL-15, IL-18, IL-21, GM-CSF, IL-18, IL-21, IL-27, and functional fragments and variants thereof. In an embodiment, the polypeptide involved in antigen presentation is selected from the group consisting of CD64, MHC I, MHC II, and functional fragments and variants thereof. In an embodiment, the polypeptide involved in trafficking and/or migration is selected from the group consisting of CD183, CCR2, CCR6, CD50, CD197, CD58, CD62L, and functional fragments and variants thereof. In an embodiment, the polypeptide involved in DC targeting is selected from the group

consisting of TLR ligands, anti-DEC-205 antibody, an anti-DC-SIGN antibody, and functional fragments and variants thereof.

[0226] In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 2 (IL-2) (e.g., GenBank Acc. No. AAB46833.1), or a nucleic acid sequence of IL-2 (e.g., GenBank Acc. No. S82692.1). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 12 (IL-12) (e.g., GenBank Acc. No. AAD16432.1), or a nucleic acid sequence of IL-12 (e.g., GenBank Acc. No. AF101062.1). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 6 (IL-6) (e.g., GenBank Acc. No. AAD13886.1 or NP_000591.1), or a nucleic acid sequence of IL-6 (e.g., GenBank Acc. No. S56892.1 or NM_000600.3). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 7 (IL-7) (e.g., GenBank Acc. No. AAH47698.1 or NP_000871.1), or a nucleic acid sequence of IL-7 (e.g., GenBank Acc. No. BC047698.1 or NM_000880.3). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 15 (IL-15) (e.g., GenBank Acc. No. AAU21241.1), or a nucleic acid sequence of IL- 15 (e.g., GenBank Acc. No. AY720442.1). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 18 (IL- 18) (e.g., GenBank Acc. No. AAK95950.1), or a nucleic acid sequence of IL- 18 (e.g., GenBank Acc. No. AY044641.1). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of interleukin 21 (IL-21) (e.g., GenBank Acc. No. AAG29348.1), or a nucleic acid sequence of IL-21 (e.g., GenBank Acc. No. AF254069.1). In some embodiments, the T cell priming polypeptide comprises an amino acid sequence of GM-CSF (e.g., GenBank Acc. No. AAA52578.1), or a nucleic acid sequence of GM-CSF (e.g., GenBank Acc. No. Ml 1220.1). In some embodiments, the T cell priming polypeptide is an IL-18.

[0227] In some embodiments, the expression of the CAR or CARs does not substantially affect the level of expression of the T cell priming polypeptide in the armored CAR T cell. In some embodiments, the CAR comprises an antigen binding domain that binds the antigen, and the expression of the T cell priming polypeptide does not substantially affect the level of expression or cell-killing function of the CAR or CARs in the armored CAR T cell.

[0228] In some embodiments, the lentiviral vector or retroviral vector disclosed herein comprises and delivers more than one T cell priming polypeptides. In an embodiment, the

lentiviral vector or retroviral vector comprises 2, 3, 4, 5, 6 or more nucleic acids encoding one or more T cell priming polypeptides; and further comprises a nucleic acid sequence encoding a CAR. In some embodiments, the co-delivery of one or more T cell priming polypeptides does not affect (e.g., substantially decrease or substantially inhibit), the expression or activity of the co-expressed CAR in the armored CAR T cell or armored CAR-expressing immune cell. In some embodiments, the CAR does not affect (e.g., substantially decrease or substantially inhibit), the expression or activity of the co-expressed T cell priming polypeptide.

III. NUCLEIC ACIDS AND EXPRESSION VECTORS

A. Nucleic acid encoding a CAR

[0229] The present disclosure provides nucleic acid molecules encoding one or more CAR constructs described herein. The nucleic acid molecule can be a messenger RNA transcript. The nucleic acid molecule can also be a DNA construct.

[0230] In one example, the present disclosure provides an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), which may comprise a single chain antibody or a single chain antibody fragment comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain. In some embodiments, the anti-CD19 binding domain is encoded by a nucleic acid sequence selected from SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216. In some embodiments, the anti-CD19 binding domain is encoded by a nucleic acid isolated nucleic acid molecule having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, SEQ ID NO:24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 21, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 21. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 24. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO:

102. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 103. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 104. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 114. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 115. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 116. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 117. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 118. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 119. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 120. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 216. In some embodiments, the anti-CD19 binding domain comprise a nucleotide sequence of SEQ ID NO: 225.

[0231] In some embodiments, the CAR comprises an anti-CD19 binding domain comprising a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6.

[0232] In some embodiments, the CAR comprises an anti-CD19 binding domain comprising a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198. In another embodiment, the anti-CD19 binding domain comprises a light chain variable domain

comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0233] The light chain variable region may comprise the amino acid sequence of SEQ ID NO: 7 or 199; or an amino acid sequence having at least about 90% to about 99% identity to the amino acid sequence of SEQ ID NO: 7 or 199. Alternatively, the heavy chain variable region may comprise the amino acid sequence of SEQ ID NO: 8 or 200, or an amino acid sequence having at least about 90% to about 99% identity to the amino acid sequence of SEQ ID NO: 8 or 200.

[0234] In some embodiments, the anit-CD19 binding domain comprises the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8.

[0235] In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 199 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 200. In some embodiments, the CD19 binding domain may be a scFv.

[0236] In some embodiments, the anti-CD19 binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146.

[0237] In some embodiments, the anti-CD19 binding domain comprises a light chain variable region or a heavy chain variable region encoded by a nucleic acid sequence selected from a group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216. The anti-CD19 binding

domain may comprise a light chain variable region or a heavy chain variable region encoded by a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0238] In some embodiments of the isolated nucleic acid molecule described herein, the transmembrane domain of the CAR may comprise a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD2, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9. In some embodiments, the transmembrane domain comprises an amino acid sequence selected from SEQ ID NO: 29, 31, or 33, or an amino acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 29, 31, or 33. In some embodiments, the transmembrane domain comprises a nucleic acid sequence selected from SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 34, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30, 32, or 34. In some embodiments, the transmembrane domain comprises a CD8 transmembrane domain, and/or an amino acid sequence of SEQ ID NO: 29; or an amino acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 29. In some embodiments, the transmembrane domain comprises a nucleic acid sequence of SEQ ID NO: 30, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30.

[0239] In some embodiments of the isolated nucleic acid molecule described herein, the CAR further comprises a hinge domain as described herein. In some embodiments, the anti-CD19 binding domain is connected to the transmembrane domain by a hinge region. In some embodiments, the hinge region may be from a protein selected from the group consisting of an Fc fragment of an antibody, a hinge region of an antibody, a CH2 region of an antibody, a

CH3 region of an antibody, an artificial spacer sequence, an IgG hinge, a CD8 hinge, and any combination thereof.

[0240] In some embodiments of the isolated nucleic acid molecule described herein, the CAR comprises a costimulatory domain, which may be a functional signaling domain of a protein selected from the group consisting of a TNFR superfamily member, OX40 (CD134), CD2, CD5, CD7, CD27, CD28, CD30, CD40, PD-1, CD8, ICAM-1, lymphocyte function-associated antigen-1 (LFA-1), CD11a, CD18, ICOS (CD278), LIGHT, NKG2C, B7-H3, a ligand that specifically binds to CD83, DAP10, DAP12, Lck, Fas and 4-1BB (CD137). In some embodiments, the costimulatory domain comprises an amino acid sequence selected from SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, or SEQ ID NO: 50, or a sequence having about 90% to about 99% identity to SEQ ID NO: 37, 39, 41, 43, 46, 48, or 50. In some embodiments, the costimulatory domain comprises a nucleic acid sequence selected from SEQ ID NO: 38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO: 45, SEQ ID NO:47, or SEQ ID NO:49, or a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 38, 40, 42, 44, 45, 47, or 49.

[0241] In some embodiments of the isolated nucleic acid molecule described herein, the CAR may comprise an intracellular signaling domain. The signaling domain may be from a protein selected from the group consisting of CD3 zeta, FcyRIII, FcsRI, a cytoplasmic tail of an Fc receptor, an immunoreceptor tyrosine-based activation motif (ITAM) bearing cytoplasmic receptor, TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. In that embodiments, the intracellular signaling domain comprises the intracellular signaling domain of CD3 zeta, the amino acid sequence of SEQ ID NO: 52 or 54, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 52 or 54. Alternatively, the intracellular signaling domain comprises the nucleic acid sequence of SEQ ID NO: 53 or 55, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 53 or 55.

[0242] In some embodiments, the CAR comprises a functional 4-1BB costimulatory domain

and a functional CD3 zeta intracellular signaling domain. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52, or SEQ ID NO:54 or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to an amino acid sequence of SEQ ID NO: 37, SEQ ID NO: 52 or SEQ ID NO:54.

[0243] The intracellular signaling domain may comprise the sequence of SEQ ID NO: 37 and the sequence of SEQ ID NO: 52 or SEQ ID NO: 54, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identityto SEQ ID NO: 37, SEQ ID NO: 52 or SEQ ID NO: 54. These sequences may be expressed in the same frame and as a single polypeptide chain. In some embodiments, the nucleic acid sequence comprises a sequence of SEQ ID NO: 38, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 38. In some embodiments, the nucleic acid sequence comprises a sequence of SEQ ID NO: 53 or SEQ ID NO: 55, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 53 or 55.

[0244] In some embodiments of the isolated nucleic acid molecule described herein, the CAR further comprises a leader sequence. The leader sequence may comprises the amino acid of SEQ ID NO: 25.

[0245] One example of the present disclosure provides an isolated nucleic acid molecule comprising an scFv comprising an anti-CD19 binding domain described herein. One example of the present disclosure provides an isolated nucleic acid molecule comprising a CAR comprising an anti-CD19 binding domain described herein, a transmembrane domain, a costimulatory domain and an intracellular domain. In some embodiments, the anti-CD19 binding domain may comprise LC CDR1 of SEQ ID NO: 1, LC CDR2 of SEQ ID NO: 2, and LC CDR3, HC CDR1 of SEQ ID NO: 4, HC CDR2 of SEQ ID NO: 5, and HC CDR3 of SEQ ID NO: 6; or LC CDR1 of SEQ ID NO: 193, LC CDR2 of SEQ ID NO: 194, LC CDR3 of SEQ ID NO: 195; HC CDR1 of SEQ ID NO: 196, HC CDR2 of SEQ ID NO: 197, and HC CDR3 of SEQ ID NO: 198; or any LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 disclosed in Table 2, the transmembrane domain is selected from CD28 or CD8 transmembrane domain, the costimulatory domain comprises an intracellular signaling

domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and the intracellular signaling domain comprises CD3-zeta or FcR gamma.

[0246] In another embodiment, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0247] One example of the present disclosure provides an isolated nucleic acid molecule comprising a CAR comprising an anti-CD19 binding domain, a transmembrane domain, an costimulatory domain, and an intracellular domain. The anti-CD19 binding domain comprises the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146. The transmembrane domain is selected from CD28 or CD8 transmembrane domain, the costimulatory domain may comprise an intracellular signaling domain of a protein selected from the group consisting of OX40, CD27, CD2, CD28, ICOS, and 4-1BB; and an intracellular signaling domain comprising CD3-zeta or FcR gamma.

[0248] One example of the present disclosure provides an isolated nucleic acid molecule comprising an scFv comprising an anti-CD19 binding domain. One example of the present disclosure provides an isolated nucleic acid molecule comprising a CAR comprising an anti-CD19 binding domain (e.g., scFv), a transmembrane domain, a costimulatory domain and an intracellular domain. The anti-CD19 binding domain comprises the amino acid sequence of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; the transmembrane domain comprising the amino acid sequence of selected from the group consisting of SEQ ID NO: 29, 31, and 33; the costimulatory domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50; and the intracellular signaling domain comprising the amino acid sequence of SEQ ID NO: 52 or SEQ ID NO: 54.

[0249] One example of the present disclosure provides an isolated nucleic acid molecule comprising an anti-CD19 binding domain comprising the amino acid sequence of SEQ ID

NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146; a transmembrane domain comprising the amino acid sequence of SEQ ID NO: 29; a costimulatory domain comprising the amino acid sequence of SEQ ID NO: 37; and
an intracellular signaling domain comprising of SEQ ID NO: 52 or 54.

[0250] One example of the present disclosure provides an isolated nucleic acid comprising an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 66, 77, 88, 148, 170, 181, 203, 214, 159, 192, 23, and 20; and/or an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 65, 76, 87, 147, 169, 180, 202, 213, 158, 191, 22, and 19.

[0251] One example of the present disclosure provides an isolated nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216.

[0252] One example of the present disclosure provides an isolated polypeptide molecule encoded by the nucleic acid molecule described herein. The isolated polypeptide may comprise a sequence selected from the group consisting of SEQ ID NO: 63, 74, 85, 145, 167, 178, 200, 211, 156, 189, 17, 8, 62, 73, 84, 144, 166, 177, 199, 210, 155, 188, 16, and 7.

B. Expression vectors

[0253] One example of the present disclosure provides a vector (e.g., expression vector) comprising the isolated nucleic acid described. The vector may be selected from the group consisting of a DNA, a RNA, a plasmid, a lentivirus vector, an adenoviral vector, or a retroviral vector.

[0254] The lentiviral vector may be based on a virus selected from the group consisting of a retrovirus, an alpha retrovirus, a beta retrovirus, a gamma retrovirus, a delta retrovirus, and an epsilon retrovirus. For example, the lentiviral vector may be based on a Human immunodeficiency virus (HIV), an Equine infectious anemia virus (EIAV), a visna-maedi virus (VMV) virus, a caprine arthritis-encephalitis virus (CAEV), a feline immunodeficiency virus (FIV), a bovine immune deficiency virus (BIV), a VISNA virus, and a simian immunodeficiency virus (SIV). In some embodiments, the lentiviral vector may be

pseudotyped with an envelope glycoprotein (Env) from a virus selected from the group consisting of a murine leukemia virus (MLV), a vesicular stomatitis virus (VSV) Indiana strain, VSV New Jersey strain, Cocal virus, Chandipura virus, Piry virus, spring viremia of carp virus (SVCV), Sigma virus, infectious hematopoietic necrosis virus (IHNV), Mokola virus, rabies virus CVS virus, Isfahan virus, Alagoas virus, Calchaqui virus, Jurona vrus, La Joya virus, Maraba virus, Feline Endogenous Retrovirus (RD114) Envelope Protein, Perinet virus, Yug Bugdanovac virus, a prototypic foamy virus (PFV), and gibbon ape leukemia virus (GaLV). In some embodiments, the lentiviral vector may be pseudotyped with an envelope glycoprotein (Env) selected from the group consisting of vesicular stomatitis virus (VSV) Indiana strain, VSV New Jersey strain, and Cocal virus.

[0255] In some embodiments of the lentiviral vector described herein, the viral envelope protein (Env) comprises a VSV-G glycoprotein selected from the group consisting of VSV-G of the Indiana strain, VSV-G of the New Jersey strain, the Cocal virus envelope protein, the Isfahan virus envelope protein, Chandipura virus envelope protein, Pyri virus envelope protein, a murine leukemia virus (MLV) envelope glycoprotein, a SVCV virus envelope protein, and a variant thereof. The lentiviral vector may also comprise a nucleotide sequence encoding a heterologous VSV-G envelope protein.

[0256] The heterologous VSV G envelope protein may be codon-optimized for human expression. Alternatively, the heterologous VSV G envelope protein may be a VSV G protein variant. In some embodiments, the lentiviral vector comprises a nucleotide sequence encoding the VSV-G envelope protein, or a VSV G protein variant.

[0257] In some embodiments of the lentiviral vector described herein, the heterologous envelope protein may be under the control of a transcriptional regulatory element. The transcriptional regulatory element maybe a promoter selected from an eukaryotic promoter or a constitutive promoter.

[0258] The lentiviral vector described herein can further comprise a transcriptional regulatory element and the transcriptional regulatory element may be upstream of the heterologous envelope glycoprotein (i.e. in the 5’ direction of the nucleotide sequence encoding the heterologous envelope glycoprotein). For example, the transcriptional regulatory element may control the expression (i.e. transcription and, accordingly, but optionally, translation) of

the nucleic acid encoding the heterologous envelope glycoprotein. In some embodiments, the transcriptional regulatory element is constitutively active or is a constitutive promoter. In exemplary embodiments, the constitutively active transcriptional regulatory element or the constitutive promoter may be a cytomegalovirus (CMV) promoter, such as the CMV major immediate early promoter (CMV IE1), a murine stem cell virus promoter, Elongation Factor-1 alpha promoter (EF-1 alpha), a viral simian virus 40 (SV40) (e.g., early or late), a Moloney murine leukemia virus (MoMLV), an ubiquitin C promoter, a phosphoglycerokinase (PGK) promoter, a Rous sarcoma virus (RSV), or herpes simplex virus (HSV) (thymidine kinase) promoter.

[0259] In other embodiments, the activity of the transcriptional regulatory element may be inducible or the promoter may be an inducible promoter. In some embodiments, the transcriptional regulatory element may be a eukaryotic promoter, such as phosphoglycerate kinase promoter. Other transcriptional regulatory elements, including prokaryotic and eukaryotic, constitutive and inducible promoters, and origins of replication are known in the art.

[0260] In some embodiments, the lentiviral vector described herein can be structured and arranged so that the expression of the proteins, enzymes, and viral elements necessary for producing retroviral particles (i.e. cis- acting and trans-acting genes) are under the control of a transcriptional regulatory element. In a preferred embodiment, the lentiviral vector can further comprise a transcriptional regulatory element and the transcriptional regulatory element is upstream (i.e. in the 5’ direction) of the proteins, enzymes, and viral elements necessary for producing retroviral particles (i.e. cis-acting and trans- acting genes) and, optionally, the transcriptional regulatory element controls the expression (i.e. transcription or translation) of the nucleic acid encoding proteins, enzymes, and viral elements necessary for producing retroviral particles (i.e. cis-acting and trans-acting genes). In some embodiments, the transcriptional regulatory element may be constitutively active or may be a constitutive promoter.

[0261] In some embodiments, the lentiviral vectors described herein and nucleic acids encoding the heterologous envelope protein may be amplified or produced prior to the introduction into producer cells and, accordingly, prior to the production of viral particles. In some embodiment, the lentiviral vectors and nucleic acids encoding the other proteins,

enzymes, and elements necessary for retroviral particle production may be amplified or produced prior to the introduction into producer cells, and, accordingly, the production of the retroviral proteins.

[0262] In some embodiments, the lentiviral vectors and nucleic acids encoding the heterologous envelope protein may be structured and arranged such that a transcriptional control element drives the transcription, and therefore translation, of the heterologous envelope protein in a producer cell to facilitate the production the lentiviral particles. In some embodiments, the lentiviral vectors and nucleic acids encoding the proteins, enzymes, viral elements (i.e. cis- and trans-acting genes, including rev and gag/pol) necessary for the production of the retroviral particles may be structured and arranged so that a transcriptional control element may drive the transcription, and therefore translation, of the proteins, enzymes, viral elements (i.e. cis- and trans-acting genes, including rev and gag/pol) in a producer cell so that the producer cell produces the retroviral particles.

[0263] In some embodiments, the vector comprises the isolated nucleic acid molecule comprising a CAR described herein operably linked via a linker peptide to a nucleic acid sequence encoding a switch receptor, a dominant negative receptor, or a polypeptide that can enhance an immune cell function, or a functional derivative.

[0264] In some embodiments, the linker peptide is selected from F2A, E2A, P2A, T2A, or Furin-(G4S)2-T2A (F-GS2-T2A). Alternatively, the linker may comprise the amino acid sequence of SEQ ID NO: 92, SEQ ID NO:94, SEQ ID NO:96, or SEQ ID NO: 99. The linker may comprise the nucleic acid sequence of SEQ ID NO: 93, 95, 97, or 98.

C. Methods of introducing nucleic acids into a cell

[0265] Methods of introducing nucleic acids into a cell include physical, biological, chemical methods, and combination thereof. Expression vectors including a nucleic acid of the present disclosure can be introduced into a host cell by any means known to persons skilled in the art. The expression vectors may include viral sequences for transfection, if desired. Alternatively, the expression vectors may be introduced by fusion, electroporation, biolistics (*e.g.*, gene gun), transfection, lipofection (*e.g.*, cationic liposome), polymer encapsulation, or the like. The host cell (*e.g.*, immune cell or $CD4^+$ and $CD8^+$ cell) may be grown and expanded in culture before introduction of the expression vectors, followed by the appropriate treatment

for introduction and integration of the vectors. The host cells (*e.g.*, immune cells) may then be expanded and may be screened by virtue of a marker present in the vectors. Methods for producing cells including vectors and/or exogenous nucleic acids are well-known in the art.

[0266] In some embodiments, the host cell (e.g., immune cell, $CD4^+$ and $CD8^+$ cell) or population of host cells (e.g., population of immune cells, or $CD4^+$ and $CD8^+$ cells) can be modified using any method known in the art, such as activation, expansion, induction of apoptosis, genetic manipulation, induction of antigen-specificity. In some embodiments, the host cell (e.g., immune cell, $CD4^+$ and $CD8^+$ cell) or population of host cells (e.g., population of immune cells, or $CD4^+$ and $CD8^+$ cells) can be modified by the addition of cytokines, cross-linking specific receptors, addition of antigens, introduction of nucleic acid molecules (DNA, RNA, and/or modified versions thereof), protein agents, addition of drugs or small molecules, or any combination thereof. In some embodiments, the introduction of exogenous nucleic acid molecules comprises viral transfection (transduction), non-viral transfection, electroporation, lipofection, cationic liposome mediated transfection using lipofection, polymer encapsulation, peptide mediated transfection, or biolistic particle delivery systems such as "gene guns".

[0267] Regardless of the method used to introduce isolated nucleic acids molecule described herein into a host cell or otherwise expose a cell to the CD19 CAR of the present invention, to confirm the presence of the nucleic acids in the host cell, a variety of assays may be performed. Such assays include, for example, molecular biological assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; biochemical assays, such as detecting the presence or absence of a particular peptide (*e.g.*, immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

[0268] Moreover, the nucleic acids may be introduced by any means, such as transducing the expanded host cells (*e.g.*, immune cells), transfecting the expanded host cells (*e.g.*, immune cells), and electroporating the expanded host cells (*e.g.*, immune cells). One isolated nucleic acid molecule may be introduced by one method and another nucleic acid may be introduced into the host cell (*e.g.*, immune cells) by a different method.

1. Biological methods

[0269] Biological methods for introducing a polynucleotide of interest into a host cell (*e.g.* immune cell) include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors (viral transfection), have become the most widely used method for inserting genes into mammalian (*e.g.*, human cells). Viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like.

[0270] In some embodiments, a nucleic acid encoding a subject CAR, a subject engineered TCR, a subject KIR, a subject antigen-binding polypeptide, a subject cell surface receptor ligand, a subject tumor antigen, a subject switch receptor, a subject dominant negative receptor, and/or a subject polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration) can be introduced into a cell with an expression vector (viral transfection). Expression vectors (e.g., lentiviral vector or retroviral vector) comprising a nucleic acid encoding a subject CAR, a subject engineered TCR, a subject KIR, a subject antigen-binding polypeptide, a subject cell surface receptor ligand, a subject tumor antigen, a subject switch receptor, a subject dominant negative receptor, and/or a subject polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration) are provided herein. Suitable expression vectors include lentivirus vectors, gamma retrovirus vectors, foamy virus vectors, adeno associated virus (AAV) vectors, adenovirus vectors, engineered hybrid viruses, naked DNA, including but not limited to transposon mediated vectors, such as Sleeping Beauty, Piggyback, and Integrases such as Phi31. Some other suitable expression vectors include herpes simplex virus (HSV) and retrovirus expression vectors.

[0271] In some embodiments, the nucleic acids, encoding a subject CAR (e.g., a CD-19 CAR), a subject engineered TCR, a subject KIR, a subject antigen-binding polypeptide, a subject cell surface receptor ligand, a subject tumor antigen, a subject switch receptor, a subject dominant negative receptor, and/or a subject polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration), are introduced into the immune cell by viral transduction. In some embodiments, the viral vector is selected from the group consisting of a retroviral vector, sendai viral vectors, adenoviral vectors, adeno-associated virus vectors, and lentiviral vectors. Various markers that may be used are known in the art, and may include hprt, neomycin resistance, thymidine kinase, hygromycin resistance, etc.

[0272] The modified immune cell, $CD4^+$ and $CD8^+$ cell or population of immune cells, or $CD4^+$ and $CD8^+$ cells of the present disclosure (*e.g.*, comprising a nucleic acid encoding a

subject CAR, a subject engineered TCR, a subject KIR, a subject antigen-binding polypeptide, a subject cell surface receptor ligand, a subject tumor antigen, a subject switch receptor, subject dominant negative receptor, and/or a subject polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration)) may be produced by stably transfecting host cells (*e.g.* immune cells) with an expression vector including a nucleic acid of the present disclosure.

[0273] Transfected cells (i.e. immune cells) expressing a nucleic acid encoding a CAR, a KIR, a TCR, a KIR, an antigen-binding polypeptide, a cell surface receptor ligand, a tumor antigen, a subject switch receptor, a subject dominant negative receptor, and/or a subject polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration) of the present disclosure may be expanded *ex vivo*. In some embodiments, transfected cells (i.e. immune cells) expressing a nucleic acid encoding a CAR, a KIR, a TCR, a KIR, an antigen-binding polypeptide, a cell surface receptor ligand, a tumor antigen, a subject switch receptor, subject dominant negative receptor, and/or a subject polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration) of the present disclosure are not expanded *ex vivo*.

[0274] Additional methods for generating a modified cell of the present disclosure include, without limitation, chemical transformation methods (*e.g.*, using calcium phosphate, dendrimers, liposomes and/or cationic polymers), non-chemical transformation methods (*e.g.*, electroporation, optical transformation, gene electrotransfer and/or hydrodynamic delivery) and/or particle-based methods (*e.g.*, impalefection, using a gene gun and/or magnetofection).

2. Physical methods

[0275] Physical methods for introducing a polynucleotide (RNA, or DNA) or an expression vector into a host cell (e.g., an immune cell) include lipofection, particle bombardment, microinjection, electroporation, and the like. The expression vector or polynucleotide can be introduced into target cells using commercially available methods which include electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, MA) or the Gene Pulser II (BioRad, Denver, CO), Multiporator (Eppendorf, Hamburg Germany).

IV. CAR T CELLS

[0276] One example of the present disclosure provides a modified cell, a modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell engineered by the methods described herein. The modified cell is a modified immune cell, a modified natural killer (NK) cell, a modified natural killer T (NKT) cell, or a modified T cell. The modified cell is a modified T cell or a modified human T cell. The modified T cell can be a $CD8^+$ T cell. The modified cell contemplated herein can be an autologous cell, heterologous cell, or an allogeneic cell.

[0277] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a chimeric antigen receptor (CAR) comprising a single chain antibody or a single chain antibody fragment comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain

[0278] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises the isolated nucleic acid molecule described herein. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, 24 SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0279] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises an isolated polypeptide encoded by the nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216.

[0280] In some embodiments, the modified cell (e.g., modified immune cell, or a modified

$CD4^+$ and $CD8^+$ cell) comprises a CAR comprising a single chain antibody or a single chain antibody fragment comprising an anti-CD19 binding domain, a transmembrane domain, a costimulatory, and an intracellular signaling domain. In that embodiment, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 1, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 2, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 3; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 4, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 5, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 6. In another embodiment, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1) of SEQ ID NO: 193, a light chain complementary determining region 2 (LC CDR2) of SEQ ID NO: 194, and a light chain complementary determining region 3 (LC CDR3) of SEQ ID NO: 195; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1) of SEQ ID NO: 196, a heavy chain complementary determining region 2 (HC CDR2) of SEQ ID NO: 197, and a heavy chain complementary determining region 3 (HC CDR3) of SEQ ID NO: 198. In another embodiment, the anti-CD19 binding domain comprises a light chain variable domain comprising a light chain complementary determining region 1 (LC CDR1), a light chain complementary determining region 2 (LC CDR2), and a light chain complementary determining region 3 (LC CDR3) disclosed in Table 2; and a heavy chain variable domain comprising a heavy chain complementary determining region 1 (HC CDR1), a heavy chain complementary determining region 2 (HC CDR2), and a heavy chain complementary determining region 3 (HC CDR3) disclosed in Table 2.

[0281] In some embodiments, the anti-CD19 binding domain comprises the light chain variable region comprising the amino acid sequence of SEQ ID NO: 7 or 199; or an amino acid sequence having at least about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 7 or 199; and/or the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8 or 200, an amino acid sequence having at least about 90%, about 91%, about 92%,

about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 8 or 200.

[0282] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CAR comprising the light chain variable region comprises the amino acid sequence of SEQ ID NO: 7 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 8. Alternatively, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 199 and the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 200. In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CAR comprising a CD-19 scFv. The anti-CD19 binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, and 146, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 9, 18, 64, 75, 86, 190, 157, 212, 201, 226, 179, 168, or 146.

[0283] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CAR comprising the anti-CD19 binding domain encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, and SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216. In some embodiments, the anti-CD19 binding domain is encoded by a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, or 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0284] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CAR comprising the anti-CD19 binding domain comprising a light chain variable region or a heavy chain variable region encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, and SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116,

SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216. Alternatively, the anti-CD19 binding domain comprising a light chain variable region or a heavy chain variable region encoded by a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 19-24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 21, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216.

[0285] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CAR comprising the anti-CD19 binding domain comprising a light chain variable region or a heavy chain variable region encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216 and transmembrane domain comprising a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD2, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134 (OX-40), CD137 (4-1BB), CD 154 (CD40L), CD278 (ICOS), CD357 (GITR), Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9. In that embodiment, the transmembrane domain may comprise an amino acid sequence selected from SEQ ID NO: 29, 31, or 33, or an amino acid sequence about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 29, 31, or 33. Alternatively, the transmembrane domain may comprise a nucleic acid sequence selected from SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 34 or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30, 32, or 34. The transmembrane domain may comprise a CD8 transmembrane domain, and/or an amino acid sequence of SEQ ID NO: 29; or an amino acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 29. The

transmembrane domain may comprise a nucleic acid sequence of SEQ ID NO: 30, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 30.

[0286] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CAR comprising the anti-CD19 binding domain comprising a light chain variable region or a heavy chain variable region encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, and SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216; and the anti-CD19 binding domain may be connected to the transmembrane domain by a hinge region. The hinge region may be from a protein selected from the group consisting of an Fc fragment of an antibody, a hinge region of an antibody, a CH2 region of an antibody, a CH3 region of an antibody, an artificial spacer sequence, an IgG hinge region, a CD8 hinge, and any combination thereof. The hinge may comprises the amino acid sequence of SEQ ID NO: 27 or SEQ ID NO: 35, or a sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 27 or 35. The hinge region may comprise a CD8 hinge region and/or the amino acid sequence of SEQ ID NO: 27, or a sequence having about 90% , about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 27. In that embodiment, the CAR further comprises a functional signaling domain (e.g., the costimulatory domain) of a protein selected from the group consisting of a TNFR superfamily member, OX40 (CD134), CD2, CD5, CD7, CD27, CD28, CD30, CD40, PD-1, CD8, ICAM-1, lymphocyte function-associated antigen-1 (LFA-1), CD11a, CD18, ICOS (CD278), LIGHT, NKG2C, B7-H3, a ligand that specifically binds to CD83, DAP10, DAP12, Lck, Fas and 4-1BB (CD137).

[0287] In some embodiments, the modified cell (e.g., modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell) comprises a CD194BBz CAR, a CD19CD2z, a CD19CD2z CAR, a CD19CD27z CAR, a CD19Ox40z CAR, a CD1928z YMFM, a CD19ICOSz, a CD19ICOS-1z.

[0288] One example of the present disclosure provides a modified cell, a modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell comprising a chimeric antigen receptor (CAR) comprising an anti-CD19 binding domain, a switch receptor, a dominant negative receptor, and/or a polypeptide that enhances an immune cell function. In some embodiments, the switch receptor comprises a first polypeptide that comprises at least a portion of an inhibitory molecule selected from the group consisting of PD1, TGFβR, TIM-2 and BTLA, conjugated to a second polypeptide that comprises a positive signal from an intracellular signaling domain selected from the group consisting of OX40, CD27, CD28, IL-12R, ICOS, and 4-1BB. In some embodiments, the switch receptor is selected from the group consisting of PD-1-CD28, PD-1A132L-CD28, PD-1-CD27, PD-1A132L-CD27, PD-1-4-1BB, PD-1A132L-4-1BB, PD-1-ICOS, PD-1A132L-ICOS, PD-1-IL12Rβ1, PD-1A132L-IL12Rβ1, PD-1-IL12Rβ2, PD-1A132L-IL12Rβ2, VSIG3-CD28, VSIG8-CD28, VSIG3-CD27, VSIG8-CD27, VSIG3-4-1BB, VSIG8-4-1BB, VSIG3-ICOS, VSIG8-ICOS, VSIG3-IL12Rβ1, VSIG8-IL12Rβ1, VSIG3-IL12Rβ2, VSIG8-IL12Rβ2, TGFβRII-CD27, TGFβRII-CD28, TGFβRII-4-1BB, TGFβRII-ICOS, TGFβRII-IL12Rβ1, and TGFβRII-IL12Rβ2.

[0289] In some embodiments, the dominant negative receptor comprises a truncated variant of a receptor selected from the group consisting of PD1, TGFβR, TIM-2 and BTLA. In some embodiments, the dominant negative receptor is PD-1, CTLA4, BTLA, TGFβRII, VSIG3, VSIG8, or TIM-3 dominant negative receptor.

[0290] In some embodiments, a polypeptide that enhances the immune cell function, or a functional derivative thereof is selected from a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, Interleukin -7 (IL-7), Interleukin-7 receptor (IL-7R), Interleukin-15 (IL-15), Interleukin-15 receptor (IL-15R), Interleukin-21 (IL-21), Interleukin-18 (IL-18), Interleukin-18 receptor (IL-18R),CCL21, CCL19, or a combination thereof. In some embodiments, a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, IL-18R, C-C Motif Chemokine Ligand 21 (CCL21), or C-C Motif Chemokine Ligand 19 (CCL19) is an immune function-enhancing factor that improves the fitness of the claimed modified immune cell. Without wishing to be bound by theory, the addition of a nucleic acid encoding a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, IL-18R, CCL21, or CCL19 to the modified immune cell of the present disclosure enhances the immunity-inducing effect

and antitumor activity of the modified immune cell.

[0291] In some embodiments, the polypeptide that enhances an immune cell function, or a functional derivative thereof selected from the group consisting of a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, Interleukin -7 (IL-7), Interleukin-7 receptor (IL-7R), Interleukin-15 (IL-15), Interleukin-15 receptor (IL-15R), Interleukin-21 (IL-21), Interleukin-18 (IL-18), Interleukin-18 receptor (IL-18R), CCL21, CCL19, and a combination thereof.

[0292] In some embodiments, the modified cell, the modified immune cell, or the modified $CD4^+$ and $CD8^+$ cell comprises a CAR (e.g., CD19 CAR), an engineered TCR (e.g., a CD19 TCR), a KIR (a CD19 KIR), an antigen-binding polypeptide, a cell surface receptor ligand, a tumor antigen, a switch receptor, a dominant negative receptor, and/or a polypeptide that enhances immune function (e.g., T cell priming or T cell infiltration).

[0293] In some embodiments, a polypeptide that enhances immune function is selected from the group consisting of a chemokine, a chemokine receptor, a cytokine, a cytokine receptor, IL-7, IL-7R, IL-15, IL-15R, IL-21, IL-18, IL-18R, CCL21, CCL19, or a combination thereof.

[0294] Another example of the present disclosure provides a population of modified cells, a population of modified immune cells, or a population of modified $CD4^+$ and $CD8^+$ cells comprising a lentiviral vector described herein. In some embodiments, the modified $CD4^+$ and $CD8^+$ cell engineered described herein is for use in the production of a protein of interest (e.g., a CD19 CAR).

[0295] In some embodiments of the modified $CD4^+$ and $CD8^+$ cell engineered described herein, the protein of interest may be selected from the group consisting of an industrial protein, or a therapeutic protein. In some embodiments, the protein of interest may be selected from the group consisting of enzymes, regulatory proteins, receptors, peptides, peptide hormones, cytokines, membrane or transport proteins, vaccine antigens, antigen-binding proteins, immune stimulatory proteins, allergens, full-length antibodies or antibody fragments or derivatives; single chain antibodies, (scFv), Fab fragments, Fv fragments, single domain antibodies (VH or VL fragment), domain antibodies, camelid single domain antibodies (VHH), nanobodies and a combination thereof.

[0296] One example of the present disclosure provides a method of making a modified cell comprising transfecting a cell with the isolated nucleic acid molecule described herein. In

some embodiments, the isolated nucleic acid molecule encoded a CAR described herein. In some embodiments, the isolated nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, and SEQ ID NO: 216.

[0297] In some embodiments, the isolated nucleic acid molecule comprises a nucleic acid sequence having about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 225, or SEQ ID NO: 216.

[0298] One example of the present disclosure provides a method of making a modified cell comprising transfecting a cell with a nucleic acid encoding the anti-CD19 binding domain described herein; or a vector comprising the isolated nucleic acid described herein.

V. COMPOSITIONS

[0299] One example of the present disclosure provides a composition comprising a modified cell, modified lymphocyte, a modified immune cell, or a modified $CD4^+$ and $CD8^+$ cell produced by the methods described herein. Another example of the present disclosure provides a composition comprising a population of modified lymphocytes, a population of modified cells, a population of modified immune cells, or a population of modified $CD4^+$ and $CD8^+$ cells generated by the methods described herein. Another example of the present disclosure provides a composition comprising a lentiviral vector described herein. In some embodiments, the composition further comprises one or more pharmaceutically or physiologically acceptable carriers, diluents, or excipients.

[0300] In some embodiments, the compositions described herein are used in medicaments for use in treating a disease or a described herein (e.g., cancer, any malignancy, autoimmune diseases involving cells or tissues which express a tumor antigen as described herein). In some embodiments, the compositions described herein is used in methods for treating, treating a disease or a described herein (e.g., cancer, any malignancy, autoimmune diseases

involving cells or tissues which express a tumor antigen as described herein). In some embodiments, provided herein are pharmaceutical compositions comprising a CAR-expressing cell, for example, a plurality of CAR-expressing cells, made by a manufacturing process described herein (for example, the cytokine process, or the activation process described herein).

VI. METHOD OF TREATMENT

[0301] In one example, the present disclosure provides a method for adoptive cell transfer therapy comprising administering to a subject in need thereof a modified immune cell engineered by the methods described herein. In some embodiments, disclosed herein is a method of treating a disease or a condition in a subject, which comprises administering to the subject a population of modified T cells described herein, *e.g.*, a population of modified unstimulated T cells or a population of modified stimulated T cells described herein. In some embodiments, the invention includes a method of treating a disease or condition in a subject comprising administering to a subject in need thereof a composition comprising the modified immune cells described herein.

[0302] One example of the present disclosure provides a method of treating a disease or condition in a subject, the method comprising administering to the subject in need thereof a therapeutically effective amount of the modified cell, the modified immune cell, or the modified $CD4^+$ and $CD8^+$ cell, thereby treating the disease or condition in the subject. The method of treating a disease or condition in a subject may also comprise administering to the subject in need thereof a therapeutically effective amount of the population of modified cells, the population of modified immune cells, or the population of modified $CD4^+$ and $CD8^+$ cells made by the methods described herein. The method of treating a disease or condition in a subject may also comprise administering to the subject in need thereof a therapeutically effective amount of the composition described herein.

[0303] In some embodiments, the modified immune cell, or the modified $CD4^+$ and $CD8^+$ cell. In some embodiments, the modified immune cell, or the modified $CD4^+$ and $CD8^+$ cell is allogeneic to the subject. In some embodiments, the modified immune cell, or the modified $CD4^+$ and $CD8^+$ cell is a xenogeneic to the subject. In some embodiments, the subject is a human.

A. Diseases and conditions

[0304] One example of the present disclosure provides a method of providing an anti-tumor immunity in a mammal comprising administering to the mammal an effective amount of a composition, or a modified cell described herein. In some embodiments, the composition comprises a modified cell expressing a CAR as described herein. The composition may also comprise a modified cell or a population of modified cells.

[0305] Another example of the present disclosure provides a method of treating a mammal having a disease associated with expression of CD19 comprising administering to the mammal an effective amount of a composition or a modified cell described herein. In some embodiments, the composition comprises a modified cell expressing a CAR described herein.

[0306] The modified cell may be an autologous modified T cell or an allogeneic modified T cell. In some embodiments, the mammal is a human.

[0307] In some embodiments, the disease associated with CD19 expression is selected from a proliferative disease, a malignancy, a precancerous condition, or a non-cancer related indication associated with expression of CD19. In some embodiments, the disease associated with CD19 expression is a cancer, an atypical and/or a non-classical cancer, a myelodysplasia, a myelodysplastic syndrome, or a preleukemia.

[0308] In some embodiments, the disease is a hematologic cancer selected from the group consisting of an acute leukemia, a chronic leukemia, a hematologic condition, and combinations thereof. The disease may also be a B-cell acute lymphoid leukemia ("BALL"), T-cell acute lymphoid leukemia ("TALL"), acute lymphoid leukemia (ALL), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, mantle cell lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin's lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, ineffective production (or dysplasia) of myeloid blood cells, and combinations thereof.

[0309] In some embodiments, the modified cells or the composition are administered in combination with an agent that increases the efficacy of a cell expressing a CAR molecule. In some embodiments, the modified cells or the composition are administered in combination with an agent that ameliorates one or more side effects associated with administration of a cell expressing a CAR molecule. In some embodiments, the modified cells or the composition are administered in combination with an agent that treats the disease associated with CD19.

[0310] One example of the present disclosure provides an adoptive cell transfer therapy method for a disease or condition. In some embodiments, the disease or condition may be selected from the group consisting of cancer, an autoimmune disease, Lupus, a neurodegenerative disease or condition, Alzheimers disease, multiple sclerosis, an infectious disease, a fibrotic condition, liver fibrosis, lung fibrosis, post-ischemic fibrosis, a genetic disorder, sickle cell anemia, hemophilia, and/or beta-thalassemia. In some embodiments, the disease or condition selected from a cancer, any malignancy, autoimmune diseases involving cells or tissues which express a tumor antigen as described herein.

B. Combination therapy

[0311] In some embodiments, the method of treating a disease further comprises administering to the subject an additional therapeutic agent or an additional therapy. In some cases, an additional therapeutic agent disclosed herein comprises a chemotherapeutic agent, an immunotherapeutic agent, a targeted therapy, radiation therapy, or a combination thereof. Illustrative additional therapeutic agents include, but are not limited to, alkylating agents such as altretamine, busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, lomustine, melphalan, oxalaplatin, temozolomide, or thiotepa; antimetabolites such as 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, hydroxyurea, methotrexate, or pemetrexed; anthracyclines such as daunorubicin, doxorubicin, epirubicin, or idarubicin; topoisomerase I inhibitors such as topotecan or irinotecan (CPT-11); topoisomerase II inhibitors such as etoposide (VP- 16), teniposide, or mitoxantrone; mitotic inhibitors such as docetaxel, estramustine, ixabepilone, paclitaxel, vinblastine, vincristine, or vinorelbine; or corticosteroids such as prednisone, methylprednisolone, or dexamethasone.In some cases, the additional therapeutic agent comprises a first-line therapy. As used herein, “first-line therapy”

comprises a primary treatment for a subject with a cancer. In some instances, the cancer is a primary cancer. In other instances, the cancer is a metastatic or recurrent cancer. In some cases, the first-line therapy comprises chemotherapy. In other cases, the first-line treatment comprises radiation therapy. A skilled artisan would readily understand that different first-line treatments may be applicable to different type of cancers. In some cases, the additional therapeutic agent comprises an immune checkpoint inhibitor. In some instances, the immune checkpoint inhibitor comprises an inhibitors such as an antibody or fragments (*e.g.*, a monoclonal antibody, a human, humanized, or chimeric antibody) thereof, RNAi molecules, or small molecules to PD-1, PD-L1, CTLA4, PD-L2, LAG3, B7-H3, KIR, CD137, PS, TFM3, CD52, CD30, CD20, CD33, CD27, OX40, GITR, ICOS, BTLA (CD272), CD160, 2B4, LAIR1, TIGHT, LIGHT, DR3, CD226, CD2, or SLAM.Exemplary checkpoint inhibitors include pembrolizumab, nivolumab, tremelimumab, or ipilimumab. In some embodiments, the additional therapy comprises radiation therapy.

[0312] In some embodiments, the additional therapy comprises surgery.

VII. KITS

[0313] One example of the present disclosure provides a kit comprising a population of modified immune cells or a population of modified $CD4^+$ and $CD8^+$ cells, or a population of engineered by the methods described herein. Another example of the present disclosure provides a kit comprising a lentiviral vector comprising a CAR described herein.

VIII. DEFINITIONS

[0314] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

[0315] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described herein. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0316] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of tissue culture, immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art.

[0317] As used herein, the singular forms “a”, “an,” and “the” include plural referents unless the context clearly indicates otherwise. For example, the term “a cell” includes a plurality of cells, including mixtures thereof, and means one cell or more than one cell

[0318] As used herein, the term “About” refers to a value includes the standard deviation of error for the device or method being employed to determine the value. The term “about” when used before a numerical designation, *e.g.*, temperature, time, amount, and concentration, including range, indicates approximations which may vary by (+) or (–) (±) 20%, 15%, 10%, 5%, 3%, 2%, or 1 %. Preferably ±5%, more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

[0319] As used herein, the term “Activation” refers to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. Activation can also be associated with induced cytokine production, and detectable effector functions. The term “activated T cells” refers to, among other things, T cells that are undergoing cell division.

[0320] As used herein, the term “Affinity” means a measure of the binding strength between antibody and a simple hapten or antigen determinant. Without being bound to theory, affinity depends on the closeness of stereochemical fit between antibody combining sites and antigen determinants, on the size of the area of contact between them, and on the distribution of charged and hydrophobic groups. Affinity also includes the term "avidity," which refers to the strength of the antigen-antibody bond after formation of reversible complexes. Methods for calculating the affinity of an antibody for an antigen are known in the art, including use of binding experiments to calculate affinity. In the case of an antibody (Ab) binding to an antigen (Ag), the affinity constant is used (expressed as inverted dissociation constant).

[0321] Ab + Ag = AbAg Ka = i[AbAg][Ab] [Ag] = 1 K_a

[0322] The chemical equilibrium of antibody binding is also the ratio of the on-rate ($k_{forward}$) and off-rate (k_{back}) constants. Two antibodies can have the same affinity, but one may have

both a high on- and off-rate constant, while the other may have both a low on- and off-rate constant.

[0323] Antibody activity in functional assays (e.g., cell lysis assay) may also be reflective of antibody affinity. In some embodiments, the antigen recognizing receptor has low affinity. Low affinity includes micromolar and nanomolar affinities. A low affinity may comprise 10^{-3}, 10^{-4}, 10^{-5}, 5×10^{-5}, 5x 10^{-6}, 10^{-6}, 5×10^{-7}, 10^{-7}, 5×10^{-8}, 10^{-8}, 5×10^{-9}, or 10^{-9} M. Antibody and affinities can be phenotypically characterized and compared using functional assay (e.g., cell lysis assay). A wide variety of methods for determining binding affinity are known in the art. An exemplary method for determining binding affinity employs surface plasmon resonance. Surface plasmon resonance is an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.).

[0324] As used herein, the term “Allogeneic” refers to any material derived from a different animal of the same species as the individual to whom the material is introduced. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical. In some embodiments, allogeneic material from individuals of the same species may be sufficiently unlike genetically to interact antigenically.

[0325] As used herein, the term “Analogue”, in relation to polypeptides or polynucleotides includes any mimetic, that is, a chemical compound that possesses at least one of the endogenous functions of the polypeptides or polynucleotides which it mimics. Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required activity or ability. Amino acid substitutions may include the use of non-naturally occurring analogues.

[0326] Proteins used in the present disclosure may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues as long as the endogenous function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively

charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include asparagine, glutamine, serine, threonine and tyrosine. Conservative substitutions may be made.

[0327] As used herein, the term “Antibody” refers to an immunoglobulin molecule, which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)2, as well as single chain antibodies (scFv) and humanized antibodies. In some embodiments, antibody refers to such assemblies (*e.g.*, intact antibody molecules, immunoadhesins, or variants thereof) which have significant known specific immunoreactive activity to an antigen of interest (*e.g.* a tumor associated antigen). Antibodies and immunoglobulins comprise light and heavy chains, with or without an interchain covalent linkage between them. Basic immunoglobulin structures in vertebrate systems are relatively well understood.

[0328] The term “Antibody fragment” refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

[0329] In some embodiments the term antibody fragment refers to at least one portion of an intact antibody, or recombinant variants thereof, and refers to the antigen binding domain, e.g., an antigenic determining variable region of an intact antibody, that is sufficient to confer recognition and specific binding of the antibody fragment to a target, such as an antigen. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, and Fv fragments, scFv antibody fragments, linear antibodies, single domain antibodies such as sdAb (either VL or VH), camelid VHH domains, and multi- specific antibodies formed from antibody fragments. The term "scFv" refers to a fusion protein comprising at least one antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked via a short flexible polypeptide linker, and capable of being expressed as a single chain polypeptide, and wherein the scFv retains the specificity

of the intact antibody from which it is derived. Unless specified, as used herein an scFv may have the VL and VH variable regions in either order, e.g., with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise VL-linker-VH or may comprise VH-linker-VL.

[0330] The portion of the CAR composition of the invention comprising an antibody or antibody fragment thereof may exist in a variety of forms where the antigen binding domain is expressed as part of a contiguous polypeptide chain including, for example, a single domain antibody fragment (sdAb), a single chain antibody (scFv), human derived, and a humanized antibody (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426). In one example, the antigen binding domain of a CAR composition of the invention comprises an antibody fragment. In a further example, the CAR comprises an antibody fragment that comprises a scFv.

[0331] As used herein, the term "Antibody heavy chain," as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

[0332] As used herein, an "Antibody light chain," refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations. α and β light chains refer to the two major antibody light chain isotypes. The antigen binding domain of (*e.g.*, a chimeric antigen receptor) includes antibody variants. As used herein, the term "antibody variant" includes synthetic and engineered forms of antibodies which are altered such that they are not naturally occurring, *e.g.*, antibodies that comprise at least two heavy chain portions but not two complete heavy chains (such as, domain deleted antibodies or minibodies); multi-specific forms of antibodies (*e.g.*, bi-specific, tri-specific, etc.) altered to bind to two or more different antigens or to different epitopes on a single antigen); heavy chain molecules joined to scFv molecules and the like. In addition, the term "antibody variant" includes multivalent forms of antibodies (*e.g.*, trivalent, tetravalent, etc., antibodies that bind to three, four or more copies of the same antigen.

[0333] As used herein, the term “Antigen” or “Ag” is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequence or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an “antigen” as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full-length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to elicit a desired immune response. Moreover, the skilled artisan will understand that an antigen need not be encoded by a “gene” at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

[0334] As used herein, the term "Antigen presenting cell" or "APC" refers to an immune system cell such as an accessory cell (e.g., a B-cell, a dendritic cell, and the like) that displays a foreign antigen complexed with major histocompatibility complexes (MHC's) on its surface. T-cells may recognize these complexes using their T-cell receptors (TCRs). APCs process antigens and present them to T-cells.

[0335] As used herein, the term “Anti-tumor effect” refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. In some embodiments, an “anti-tumor effect” can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies of the invention in prevention of the occurrence of tumor in the first place.

[0336] As used herein, the term “Autoimmune disease” as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases

include but are not limited to, Addision's disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, cancer, Crohn's disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barr syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

[0337] As used herein, the term “Autologous” is meant to refer to any material derived from the same individual into whom the material may later be re-introduced.

[0338] As used herein, the term “Cancer” refers to a disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. As used herein, the term “cancer” refers to a disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, metastatic castrate-resistant prostate cancer, melanoma, synovial sarcoma, advanced TnMuc1 positive solid tumors, neuroblastoma, neuroendocrine tumors, and the like. In certain embodiments, CD19-positive tumor, the cancer is medullary thyroid carcinoma. In certain embodiments, the cancer is prostate cancer. In certain embodiments, the cancer is mesothelioma or a mesothelin expressing cancer. In some embodiments, the cancer is metastatic castrate-resistant prostate cancer. The terms "cancer" and "tumor" are used interchangeably herein, and both terms encompass solid and liquid tumors, diffuse or circulating tumors. In some embodiments, the cancer or tumor includes premalignant, as well as malignant cancers and tumors.

[0339] As used herein, the term “Cancer associated antigen” or “Tumor antigen” interchangeably refers to a molecule (typically a protein, carbohydrate or lipid) that is expressed on the surface of a cancer cell, either entirely or as a fragment (*e.g.*, MHC/peptide), and which is useful for the preferential targeting of a pharmacological agent to the cancer

cell. In some embodiments, a tumor antigen is a marker expressed by both normal cells and cancer cells (*e.g.*, a lineage marker such as CD19 on B cells). In some embodiments, a tumor antigen is a cell surface molecule that is overexpressed in a cancer cell in comparison to a normal cell, for instance, 1-fold over expression, 2-fold overexpression, 3 -fold overexpression or more in comparison to a normal cell. In some embodiments, a tumor antigen is a cell surface molecule that is inappropriately synthesized in the cancer cell, for instance, a molecule that contains deletions, additions or mutations in comparison to the molecule expressed on a normal cell. In some embodiments, a tumor antigen will be expressed exclusively on the cell surface of a cancer cell, entirely or as a fragment (*e.g.*, MHC/peptide), and not synthesized or expressed on the surface of a normal cell. In some embodiments, the CARs of the present invention includes CARs comprising an antigen binding domain (*e.g.*, antibody or antibody fragment) that binds to a MHC presented peptide. Normally, peptides derived from endogenous proteins fill the pockets of Major histocompatibility complex (MHC) class I molecules, and are recognized by T cell receptors (TCRs) on $CD8^+$ T lymphocytes. The MHC class I complexes are constitutively expressed by all nucleated cells. In cancer, virus-specific and/or tumor-specific peptide/MHC complexes represent a unique class of cell surface targets for immunotherapy. TCR-like antibodies targeting peptides derived from viral or tumor antigens in the context of human leukocyte antigen (HLA)-A1 or HLA-A2 have been described. For example, TCR-like antibody can be identified from screening a library, such as a human scFv phage displayed library.

[0340] As used herein, the term “Cancer-supporting antigen” or “tumor-supporting antigen” interchangeably refers to a molecule (typically a protein, carbohydrate or lipid) that is expressed on the surface of a cell that is, itself, not cancerous, but supports the cancer cells by promoting their growth or survival (*e.g.*, resistance to immune cells). Exemplary cells of this type include stromal cells and myeloid-derived suppressor cells (MDSCs). The tumor-supporting antigen itself need not play a role in supporting the tumor cells so long as the antigen is present on a cell that supports cancer cells.

[0341] As used herein, a “Cell-surface marker” refers to any molecule that is expressed on the surface of a cell. Cell-surface expression usually requires that a molecule possesses a transmembrane domain. Many naturally occurring cell-surface markers are termed “CD” or “cluster of differentiation” molecules. Cell-surface markers often provide antigenic

determinants to which antibodies can bind.

[0342] As used herein, the term "Chimeric Antigen Receptor" or alternatively a "CAR" refers to a recombinant polypeptide construct comprising at least an extracellular antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain (also referred to herein as "an intracellular signaling domain") comprising a functional signaling domain derived from a stimulatory molecule as defined below.

[0343] In some embodiments, CAR refers to an artificial T cell receptor that is engineered to be expressed on an immune effector cell or precursor cell thereof and specifically bind an antigen. CARs may be used in adoptive cell therapy with adoptive cell transfer. In some embodiments, adoptive cell transfer (or therapy) comprises removal of T cells from a patient, and modifying the T cells to express the receptors specific to a particular antigen. In some embodiments, the CAR has specificity to a selected target, for example, CD19, ROR1, mesothelin, c-Met, PSMA, PSCA, Folate receptor alpha, Folate receptor beta, EGFR, EGFRvIII, GPC2, GPC2, Mucin 1(MUC1), Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)), TnMUC1, GDNF family receptor alpha-4 (GFRa4), fibroblast activation protein (FAP), or Interleukin-13 receptor subunit alpha-2 (IL-13Ra2 or CD213A2).

[0344] In some embodiments, the stimulatory molecule is the zeta chain associated with the T cell receptor complex. I In some embodiments, the cytoplasmic signaling domain further comprises one or more functional signaling domains derived from at least one costimulatory molecule as defined below. In some embodiments, the costimulatory molecule is chosen from 4-1BB (i.e., CD137), CD27 and/or CD28. In some embodiments, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a stimulatory molecule. In one example, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a co- stimulatory molecule and a functional signaling domain derived from a stimulatory molecule.

[0345] As used herein, the term "Signaling domain" refers to the functional portion of a protein which acts by transmitting information within the cell to regulate cellular activity via

defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers.

[0346] As used herein, the term "CD19" refers to the Cluster of Differentiation 19 protein, which is an antigenic determinant detectable on leukemia precursor cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequence of human CD 19 can be found as UniProt/Swiss-Prot Accession No. P15391 and the nucleotide sequence encoding of the human CD19 can be found at Accession No. NM_001178098. CD19 is expressed on most B lineage cancers, including, e.g., acute lymphoblastic leukemia, chronic lymphocyte leukaemia and non-Hodgkin's lymphoma. Other cells with express CD 19 are provided below in the definition of "disease associated with expression of CD19." It is also an early marker of B cell progenitors. See, e.g., Nicholson et al. Mol. Immun. 34 (16-17): 1157-1165 (1997). In some embodiments, the antigen-binding portion of the CART recognizes and binds an antigen within the extracellular domain of the CD19 protein. In some embodiments, the CD19 protein is expressed on a cancer cell.

[0347] As used herein, the term “Conservative sequence modifications” is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of an antibody can be replaced with other amino acid

residues from the same side chain family and the altered antibody can be tested for the ability to bind antigens using the functional assays described herein.

[0348] As used herein, the term "Co-stimulatory ligand," includes a molecule on an antigen presenting cell (*e.g.*, an aAPC, dendritic cell, B cell, and the like) that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A co-stimulatory ligand can include, but is not limited to, CD2, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD- L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, HVEM, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co- stimulatory molecule present on a T cell, such as, but not limited to, CD27, CD28, 4- 1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA- 1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

[0349] As used herein, a "Co-stimulatory molecule" refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules are cell surface molecules other than antigen receptors or their ligands that are contribute to an efficient immune response. Costimulatory molecules include, but are not limited to an MHC class I molecule, BTLA, a Toll ligand receptor, CD28, 4-1BB (CD137), OX40 (CD134), PD-1, CD7, LIGHT, CD83L, DAP10, DAP12, CD27, CD2, CD5, ICAM-1, LFA-1, Lck, TNFR-I, TNFR-II, Fas, CD30, CD40, ICOS (CD278), NKG2C, B7-H3 (CD276), and an intracellular domain derived from a killer immunoglobulin-like receptor (KIR). In some embodiments, a co-stimulatory molecule includes OX40, CD27, CD2, CD28, ICOS (CD278), and 4-1BB (CD137). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R 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, NKG2C, TNFR2, TRANCE RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83.

[0350] As used herein, the term "Co-stimulatory signal" refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation and/or upregulation or downregulation of key molecules. A costimulatory intracellular signaling domain can be the intracellular portion of a costimulatory molecule. A costimulatory molecule can be represented in the following protein families: TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), and activating NK cell receptors. Examples of such molecules include CD27, CD28, 4-lBB (CD137), OX40, GITR, CD30, CD40, ICOS, BAFFR, HVEM, ICAM-1, lymphocyte function-associated antigen-1 (LFA-1), CD2, CDS, CD7, CD287, LIGHT, NKG2C, NKG2D, SLAMF7, NKp80, NKp30, NKp44, NKp46, CD160, B7-H3, and a ligand that specifically binds with CD83, and the like.

[0351] As used herein, the term "Derived from" refers to a relationship between a first and a second molecule. It generally refers to structural similarity between the first molecule and a second molecule and does not connotate or include a process or source limitation on a first molecule that is derived from a second molecule. For example, in the case of an intracellular signaling domain that is derived from a CD3zeta molecule, the intracellular signaling domain retains sufficient CD3zeta structure such that is has the required function, namely, the ability to generate a signal under the appropriate conditions. It does not connotate or include a limitation to a particular process of producing the intracellular signaling domain, for example, it does not mean that, to provide the intracellular signaling domain, one must start with a CD3zeta sequence and delete unwanted sequence, or impose mutations, to arrive at the intracellular signaling domain.

[0352] As used herein, the term "Disease" refers to a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the

animal’s health continues to deteriorate. In contrast, the term “disorder” in an animal refers to a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

[0353] As used herein, “Disease associated with expression of a tumor antigen” includes, but is not limited to, a disease associated with expression of a tumor antigen or condition associated with cells which express a tumor antigen including, but not limited to proliferative diseases such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells, which express a tumor antigen. In some embodiments, a cancer associated with expression of a tumor antigen is a hematological cancer. In some embodiments, a cancer associated with expression of a tumor antigen is a solid cancer. Further diseases associated with expression of a tumor antigen include, but not limited to, atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of a tumor antigen. Non-cancer related indications associated with expression of a tumor antigen include, but are not limited to, autoimmune disease, (*e.g.*, lupus), inflammatory disorders (allergy and asthma) and transplantation. In some embodiments, the tumor antigen-expressing cells express, or at any time expressed, mRNA encoding the tumor antigen. In some embodiments, the tumor antigen-expressing cells produce the tumor antigen protein (*e.g.*, wild-type or mutant), and the tumor antigen protein may be present at normal levels or reduced levels. In some embodiment, the tumor antigen-expressing cells produced detectable levels of a tumor antigen protein at one point, and subsequently produced substantially no detectable tumor antigen protein.

[0354] As used herein, the term "Disease associated with expression of CD19" includes, but is not limited to, a disease associated with expression of CD19 or condition associated with cells which express CD19 including, proliferative diseases such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplasia syndrome or a preleukemia; or a noncancer related indication associated with cells which express CD19. In some embodiments, a cancer associated with expression of CD 19 is a hematolical cancer. In one example, the hematolical cancer is a leukemia or a lymphoma. In one example, a cancer

associated with expression of CD19 includes cancers and malignancies including, but not limited to, e.g., one or more acute leukemias including but not limited to, e.g., B-cell acute Lymphoid Leukemia ("BALL"), T-cell acute Lymphoid Leukemia ("TALL"), acute lymphoid leukemia (ALL); one or more chronic leukemias including but not limited to, e.g., chronic myelogenous leukemia (CML), Chronic Lymphoid Leukemia (CLL). Additional cancers or hematologic conditions associated with expression of CD 19 comprise, but are not limited to, e.g., B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, Follicular lymphoma, Hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, mantle cell lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplasia syndrome, non-Hodgkin's lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, and "preleukemia" which are a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells, and the like. Further diseases associated with expression of CD19 expression include, but not limited to, e.g., atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of CD19. Non-cancer related indications associated with expression of CD19 include, but are not limited to, e.g., autoimmune disease, (e.g., lupus), inflammatory disorders (allergy and asthma) and transplantation.

[0355] As used herein, the term “Downregulation” refers to the decrease or elimination of gene expression of one or more genes.

[0356] As used herein, the term “Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide (such as a gene, a cDNA, or an mRNA), to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*e.g.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product

of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or a RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

[0357] As used herein, the terms "Effective amount" and "Therapeutically effective amount" are used interchangeably herein, refer to an amount of a compound, formulation, material, pharmaceutical agent, or composition, as described herein effective to achieve a desired physiological, therapeutic, or prophylactic outcome in a subject in need thereof. Such results may include, but are not limited to an amount that when administered to a mammal, causes a detectable level of immune response compared to the immune response detected in the absence of the composition of the invention. The immune response can be readily assessed by a plethora of art-recognized methods. The skilled artisan would understand that the amount of the composition administered herein varies and can be readily determined based on a number of factors such as the disease or condition being treated, the age and health and physical condition of the mammal being treated, the severity of the disease, the particular compound being administered, and the like. The effective amount may vary among subjects depending on the health and physical condition of the subject to be treated, the taxonomic group of the subjects to be treated, the formulation of the composition, assessment of the subject's medical condition, and other relevant factors.

[0358] As used herein, the term "Endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

[0359] As used herein, the term "Expand" as used herein refers to increasing in number, as in an increase in the number of immune cells (*e.g.* T cells). In some embodiments, the immune cells (*e.g.* T cells) that are expanded ex vivo increase in number relative to the number originally present in the culture. In another embodiment, the immune cells (*e.g.* T cells) that are expanded ex vivo increase in number relative to other cell types in the culture.

[0360] As used herein, the term "Expression" refers to the transcription and/or translation of a particular nucleotide sequence driven by a promoter.

[0361] As used herein, the term “Exogenous” refers to any material introduced from or produced outside an organism, cell, tissue or system.

[0362] As used herein, the term “Expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., Sendai viruses, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

[0363] As used herein, the term “Extended packaging signal” or “Extended packaging sequence” refers to the use of sequences around the psi sequence with further extension into the gag gene. The inclusion of these additional packaging sequences may increase the efficiency of insertion of vector RNA into viral particles. As an example, for the Murine Leukemia Virus (MoMLV) the minimum core packaging signal is encoded by the sequence (counting from the 5' LTR cap site) from approximately nucleotide 144, up through the Pst I site (nucleotide 567). The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the gag/pol gene (nucleotide 621), and beyond nucleotide 1040. These sequences include about a third of the gag gene sequence.

[0364] As used herein, the term “ex vivo,” refers to cells that have been removed from a living organism, (*e.g.*, a human) and propagated outside the organism (*e.g.*, in a culture dish, test tube, or bioreactor).

[0365] As used herein, “Fab” refers to a fragment of an antibody structure that binds to an antigen but is monovalent and does not have a Fc portion, for example, an antibody digested by the enzyme papain yields two Fab fragments and an Fc fragment (e.g., a heavy (H) chain constant region; Fc region that does not bind to an antigen).

[0366] As used herein, the term “Flexible polypeptide linker” or “linker” as used in the context of a scFv refers to a peptide linker that consists of amino acids such as glycine and/or serine residues used alone or in combination, to link variable heavy and variable light chain regions together. In one embodiment, the flexible polypeptide linker is a Gly/Ser linker and

comprises the amino acid sequence (Gly-Gly-Gly-Ser)n, where n is a positive integer equal to or greater than 1. For example, n=l, n=2, n=3. n=4, n=5 and n=6, n=7, n=8, n=9 and n=10. Exemplary linkers are shown in Table 1.

[0367] As used herein, a “Fragment” is also a variant and the term typically refers to a selected region of a polypeptide or polynucleotide that is of interest either functionally or, for example, in an assay. “Fragment” thus refers to an amino acid or nucleic acid sequence that is a portion of a full-length polypeptide or polynucleotide.

[0368] As used herein, “Functional variant” refers to a polypeptide that has a substantially identical amino acid sequence to a reference amino acid sequence, or is encoded by a substantially identical nucleotide sequence, and is capable of having one or more activities of the reference amino acid sequence.

[0369] s used herein, the term "Host cell" includes cells transfected, infected, or transduced in vivo, ex vivo, or in vitro with a recombinant vector or a polynucleotide of the invention. Host cells may include packaging cells, producer cells, and cells infected with viral vectors. In some embodiments, host cells infected with the lentiviral vector of the disclosure are administered to a subject in need of therapy. In some embodiments, the term "target cell" is used interchangeably with host cell and refers to transfected, infected, or transduced cells of a desired cell type. In preferred embodiments, the target cell is a T cell.

[0370] As used herein, the term “Homologous” refers to the subunit sequence identity between two polymeric molecules (*e.g.*, between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules), or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, then they are homologous at that position. For example, if a position in each of two DNA molecules is occupied by adenine, then the two DNA molecules are homologous. The homology between two sequences is a direct function of the number of matching or homologous positions. For example, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (*e.g.*, 9 of 10), are matched or homologous, the two sequences are 90% homologous.

[0371] As used herein, the term “Homologue” means an entity having a certain homology with the wild type amino acid sequence and the wild type nucleotide sequence. The term “homology” can be equated with “identity”. In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 50%, 55%, 65%, 75%, 85% or 90% identical, preferably at least 95% or 97% or 99% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

[0372] A homologous sequence is taken to include a nucleotide sequence which may be at least 50%, 55%, 65%, 75%, 85% or 90% identical, preferably at least 95% or 97% or 99% identical to the subject sequence. Although homology can also be considered in terms of similarity, in the context of the present disclosure it is preferred to express homology in terms of sequence identity. Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate percentage homology or identity between two or more sequences.

[0373] Percentage homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an “ungapped” alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues. Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion in the nucleotide sequence may cause the following codons to be put out of alignment, thus potentially resulting in a large reduction in percent homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalizing unduly the overall homology score. This is achieved by inserting “gaps” in the sequence alignment to try to maximize local homology.

[0374] However, these more complex methods assign “Gap penalties” to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence

alignment with as few gaps as possible, reflecting higher relatedness between the two compared sequences, will achieve a higher score than one with many gaps. “Affine gap costs” are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is −12 for a gap and −4 for each extension.

[0375] Calculation of maximum percentage homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux et al. (1984) Nucleic Acids Research 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching. However, for some applications, it is preferred to use the GCG Bestfit program. Another tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences.

[0376] Although the final percentage homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix—the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62. Once the software has produced an optimal alignment, it is possible to calculate percentage homology, preferably percentage sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0377] As used herein, the term “Hybrid vector” refers to a vector, LTR or other nucleic acid containing both retroviral sequences (e.g., lentiviral), and non-retroviral sequences (e.g., lentiviral viral sequences). In one embodiment, a hybrid vector refers to a vector or transfer plasmid comprising retroviral (e.g., lentiviral) sequences for reverse transcription, replication, integration and/or packaging.

[0378] Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5′ and 3′ flanking regions corresponding to the naturally-occurring sequence either side of the insertion site may be made. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

[0379] As used herein, the term “Identity” refers to the subunit sequence identity between two polymeric molecules particularly between two amino acid molecules, such as, between two polypeptide molecules. When two amino acid sequences have the same residues at the same positions, then they are identical at that position. For example, if a position in each of two polypeptide molecules is occupied by an Arginine, then the two polypeptides are identical. The identity or extent to which two amino acid sequences have the same residues at the same positions in an alignment is often expressed as a percentage. The identity between two amino acid sequences is a direct function of the number of matching or identical positions. For example, if half (*e.g.*, five positions in a polymer ten amino acids in length) of the positions in two sequences are identical, the two sequences are 50% identical; if 90% of the positions (*e.g.*, 9 of 10), are matched or identical, the two amino acids sequences are 90% identical.

[0380] As used herein, the term “Immunoglobulin” or “Ig,” defines a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of proteins are IgA, IgG, IgM, IgD, and IgE. IgA is the primary antibody that is present in body secretions, such as saliva, tears, breast milk, gastrointestinal secretions and mucus secretions of the

respiratory and genitourinary tracts. IgG is the most common circulating antibody. IgM is the main immunoglobulin produced in the primary immune response in most subjects. It is the most efficient immunoglobulin in agglutination, complement fixation, and other antibody responses, and is important in defense against bacteria and viruses. IgD is the immunoglobulin that has no known antibody function, but may serve as an antigen receptor. IgE is the immunoglobulin that mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to allergen.

[0381] As used herein, the term “Immune response” as used herein is defined as a cellular response to an antigen that occurs when lymphocytes identify antigenic molecules as foreign and induce the formation of antibodies and/or activate lymphocytes to remove the antigen.

[0382] As used herein, the term “Immune effector cell,” refers to a cell that is involved in an immune response, *e.g.*, in the promotion of an immune effector response. Examples of immune effector cells include T cells (*e.g.*, alpha/eta T cells and gamma/delta T cells), B cells, natural killer (NK) cells, natural killer T (NKT) cells, mast cells, and myeloic-derived phagocytes.

[0383] As used herein, the term “Immune effector function or immune effector response,” refers to a function or response that enhances or promotes an immune attack of a target cell. In some embodiment, an immune effector function or response refers to a property of a T or NK cell that promotes the killing or the inhibition of growth or proliferation, of a target cell. In the case of a T cell, primary stimulation and co-stimulation are examples of immune effector function or response.

[0384] As used herein, the term “Inhibitory molecule” refers to a molecule, which when activated, causes or contributes to an inhibition of cell survival, activation, proliferation and/or function; and the gene encoding said molecule and its associated regulatory elements (*e.g.*, promoters). In some embodiments, an inhibitory molecule is a molecule expressed on an immune effector cell (*e.g.*, on a T cell). Non-limiting examples of inhibitory molecules are PD-1, PD-L1, PD-L2, CTLA4, TIM3, LAG3, CEACAM (*e.g.*, CEACAM-1, CEACAM-3 and/or CEACAM-5), VISTA, TGFβIIR, VSIG3, VSIG 8, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7- H4 (VTCN1), HVEM (TNFRSF14 or CD107), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGF beta. It will be

understood that the term inhibitory molecule refers to the gene (and its associated regulatory elements) encoding an inhibitory molecule protein when it is used in connection with a target sequence or gRNA molecule. In some embodiments, gene encoding the inhibitory molecule is BTLA, PD-1, TIM-3, VSIG3, VSIG8, CTLA4, or TGFβIIR. In some embodiments, the gene encoding the inhibitory molecule is VSIG3. In some embodiments, the gene encoding the inhibitory molecule is PD-1. In some embodiments, the gene encoding the inhibitory molecule is TGFβIIR.

[0385] As used herein, the term "Induced pluripotent stem cell" or "iPS cell" refers to a pluripotent stem cell that is generated from adult cells, such immune cells (i.e. T cells). The expression of reprogramming factors, such as Klf4, Oct3/4 and Sox2, in adult cells convert the cells into pluripotent cells capable of propagation and differentiation into multiple cell types.

[0386] As used herein, the term "Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

[0387] As used herein, "*In vitro* transcribed RNA" refers to RNA that has been synthesized in vitro. In some embodiments the RNA is mRNA. Generally, the *in vitro* transcribed RNA is generated from an in vitro transcription vector. The in vitro transcription vector comprises a template that is used to generate the in vitro transcribed RNA.

[0388] As used herein, the term "Knockout" refers to the ablation of gene expression of one or more genes.

[0389] The term "K_D" refers to the equilibrium dissociation constant between an antibody and its antigen. In particular, $K_D$is the equilibrium dissociation constant, a ratio of k_{off}/k_{on}, between the antibody and its antigen. K_D and affinity are inversely related. The K_D value relates to the concentration of antibody (the amount of antibody needed for a particular experiment) and so the lower the K_D value (lower concentration) and thus the higher the affinity of the antibody. Most antibodies have K_D values in the low micromolar (10^{-6}) to

nanomolar (10^{-7} to 10^{-9}) range. High affinity antibodies generally considered to be in the low nanomolar range (10^{-9}) with very high affinity antibodies being in the picomolar (10^{-12}) range.

[0390] The term "K_{on}", or "association reaction," is the "on-rate," which is a constant a constant used to characterize how quickly an antibody binds to its target.

[0391] The term "K_{off}", or "disassociation reaction," is the "off-rate," which is a constant used to characterize how quickly an antibody dissociates from its target. The ratio of experimentally measured off- and on- rates (K_{off}/ K_{on}) is used to calculate the K_D value.

[0392] As used herein, the term "Lentiviral vector" refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, including LTRs that are primarily derived from a lentivirus. In some embodiments, the terms "Lentiviral vector," and "Lentiviral expression vector" may be used to refer to lentiviral transfer plasmids and/or infectious lentiviral particles. Where reference is made herein to elements such as cloning sites, promoters, regulatory elements, heterologous nucleic acids, etc. In some embodiments, the sequences of these elements are present in RNA form in the lentiviral particles of the invention and are present in DNA form in the DNA plasmids of the invention.

[0393] As used herein, the term "Lentivirus" refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer in vivo.

[0394] The lentivirus family differs from retroviruses in that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis et al. (1992); Lewis and Emerman (1994)). In contrast, retroviruses, such as MLV, are unable to infect non-dividing or slowly dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

[0395] A lentiviral or lentivirus vector, as used herein, is a vector which comprises at least one component part derivable from a lentivirus. Preferably, that component part is involved in the biological mechanisms by which the vector infects cells, expresses genes or is replicated. The lentiviral vector may be a "non-primate" vector, i.e., derived from a virus

which does not primarily infect primates, especially humans. The non-primate lentivirus may be any member of the family of lentiviridae, which does not naturally infect a primate and may include a feline immunodeficiency virus (FIV), a bovine immunodeficiency virus (BIV), a caprine arthritis encephalitis virus (CAEV), a Maedi visna virus (MVV) or an equine infectious anemia virus (EIAV).

[0396] As used herein, the term "Modified" means a changed state or structure of a molecule or cell of the invention. Molecules may be modified in many ways, including chemically, structurally, and functionally. Cells may be modified through the introduction of nucleic acids.

[0397] As used herein, the term "Modulating," means mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

[0398] In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

[0399] As used herein, a "Naive T cell" refers to a T cell that is antigen-inexperienced. In some embodiments, an antigen-inexperienced T cell has encountered its cognate antigen in the thymus but not in the periphery. In some embodiments, naive T cells are precursors of memory cells. In some embodiments, naive T cells express both CD45RA and CCR7, but do not express CD45RO. In some embodiments, naive T cells may be characterized by expression of CD62L, CD27, CCR7, CD45RA, CD28, and CD127, and the absence of CD95 or CD45RO isoform. In some embodiments, naive T cells express CD62L, IL-7 receptor-a, IL-6 receptor, and CD132, but do not express CD25, CD44, CD69, or CD45RO. In some embodiments, naive T cells express CD45RA, CCR7, and CD62L and do not express CD95 or IL-2 receptor β. In some embodiments, surface expression levels of markers are assessed using flow cytometry.

[0400] Unless otherwise specified, a "Nucleotide sequence encoding an amino acid

sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

[0401] As used herein, the term "Operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

[0402] As used herein, the term "Overexpressed" tumor antigen or "overexpression" of a tumor antigen is intended to indicate an abnormal level of expression of a tumor antigen in a cell from a disease area like a solid tumor within a specific tissue or organ of the patient relative to the level of expression in a normal cell from that tissue or organ. Patients having solid tumors or a hematological malignancy characterized by overexpression of the tumor antigen can be determined by standard assays known in the art.

[0403] As used herein, the term "Parenteral" administration of an immunogenic composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

[0404] As used herein, the terms "Peptide," "Polypeptide," and "Protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically

active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

[0405] As used herein, a “poly(A)” is a series of adenosines attached by polyadenylation to the mRNA. In some embodiments of a construct for transient expression, the poly(A) is between 50 and 5000. In some embodiments the poly (A) is greater than 64. In some embodiments the poly(A)is greater than 100. In some embodiments the poly(A) is greater than 300. In some embodiments the poly(A) is greater than 400. poly(A) sequences can be modified chemically or enzymatically to modulate mRNA functionality such as localization, stability or efficiency of translation.

[0406] As used herein, “Polyadenylation” refers to the covalent linkage of a polyadenylyl moiety, or its modified variant, to a messenger RNA molecule. In eukaryotic organisms, most messenger RNA (mRNA) molecules are polyadenylated at the 3' end. The 3' poly(A) tail is a long sequence of adenine nucleotides (often several hundred) added to the pre-mRNA through the action of an enzyme, polyadenylate polymerase. In higher eukaryotes, the poly(A) tail is added onto transcripts that contain a specific sequence, the polyadenylation signal. The poly(A) tail and the protein bound to it aid in protecting mRNA from degradation by exonucleases. Polyadenylation is also important for transcription termination, export of the mRNA from the nucleus, and translation. Polyadenylation occurs in the nucleus immediately after transcription of DNA into RNA, but additionally can also occur later in the cytoplasm. After transcription has been terminated, the mRNA chain is cleaved through the action of an endonuclease complex associated with RNA polymerase. The cleavage site is usually characterized by the presence of the base sequence AAUAAA near the cleavage site. After the mRNA has been cleaved, adenosine residues are added to the free 3' end at the cleavage site.

[0407] As used herein, the term “Transient” refers to expression of a non-integrated transgene for a period of hours, days or weeks, wherein the period of time of expression is less than the period of time for expression of the gene if integrated into the genome or contained within a stable plasmid replicon in the host cell.

[0408] As used herein, the term “Polynucleotide” as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric “nucleotides.” The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means.

[0409] As used herein, the term “Promoter” is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

[0410] As used herein, the term “Promoter/regulatory sequence” means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

[0411] As used herein, the term “Constitutive promoter” is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

[0412] As used herein, the term “Inducible promoter” is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

[0413] As used herein, the term “Tissue-specific promoter” is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene

product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

[0414] As used herein, the term "Pseudotype" or "Pseudotyping" refers to a virus whose viral envelope proteins have been substituted with those of another virus possessing preferable characteristics. For example, HIV can be pseudotyped with vesicular stomatitis virus G-protein (VSV-G) envelope proteins, which allows HIV to infect a wider range of cells because HIV envelope proteins (encoded by the env gene) normally target the virus to $CD4^+$ presenting cells. In a preferred embodiment of the invention, lentiviral envelope proteins are pseudotyped with VSV-G. In one embodiment, the invention provides packaging cells, which produce recombinant retrovirus, e.g., lentivirus, pseudotyped with the VSV-G envelope glycoprotein.

[0415] As used herein, the term "Recombinant antibody" refers to an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage or yeast expression system. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using recombinant DNA or amino acid sequence technology which is available and well known in the art.

[0416] As used herein, the term “Recombinant viral vector” (RRV) refers to a vector with sufficient viral genetic information to allow packaging of an RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. The RRV carries non-viral coding sequences which are to be delivered by the vector to the target cell. A RRV is incapable of independent replication to produce infectious viral particles within the final target cell. Usually the RRV lacks a functional gag-pol and/or env gene and/or other genes essential for replication. The vector of the present invention may be configured as a split-intron vector. Preferably the RRV vector of the present disclosure has a minimal viral genome.

[0417] As used herein, the term “Retroviral vector” refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, that are primarily

derived from a retrovirus.

[0418] In some embodiments, an additional safety enhancement is provided by replacing the U3 region of the 5' LTR with a heterologous promoter to drive transcription of the viral genome during production of viral particles. The heterologous promoters may be selected from the group consisting of viral simian virus 40 (SV40) (e.g., early or late), cytomegalovirus (CMV) (e.g., immediate early), Moloney murine leukemia virus (MoMLV), Rous sarcoma virus (RSV), and herpes simplex virus (HSV) (thymidine kinase) promoters. Typical promoters are able to drive high levels of transcription in a Tat-independent manner. This replacement reduces the possibility of recombination to generate replication-competent virus because there is no complete U3 sequence in the virus production system. In some embodiments, the heterologous promoter has additional advantages in controlling the manner in which the viral genome is transcribed. For example, the heterologous promoter can be inducible, such that transcription of all or part of the viral genome will occur only when the induction factors are present. Induction factors include, but are not limited to, one or more chemical compounds or the physiological conditions such as temperature or pH, in which the host cells are cultured.

[0419] As used herein, the term "Sendai virus" refers to a genus of the Paramyxoviridae family. Sendai viruses are negative, single stranded RNA viruses that do not integrate into the host genome or alter the genetic information of the host cell. Sendai viruses have an exceptionally broad host range and are not pathogenic to humans. Used as a recombinant viral vector, Sendai viruses are capable of transient but strong gene expression.

[0420] As used herein, the term "Signal transduction pathway" refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. The phrase "cell surface receptor" includes molecules and complexes of molecules capable of receiving a signal and transmitting signal across the plasma membrane of a cell.

[0421] As used herein, the term "Single chain antibodies" refer to antibodies formed by recombinant DNA techniques in which immunoglobulin heavy and light chain fragments are linked to the Fv region via an engineered span of amino acids. Various methods of generating single chain antibodies are known.

[0422] As used herein, the term “Single-chain variable fragment” or “scFv” is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of an immunoglobulin (e.g., mouse or human) covalently linked to form a VH::VL heterodimer. The heavy (VH) and light chains (VL) are either joined directly or joined by a peptide-encoding linker or spacer, which connects the N-terminus of the VH with the C-terminus of the VL, or the C-terminus of the VH with the N-terminus of the VL. The terms “linker” and “spacer” are used interchangeably herein. In some embodiments, the antigen binding domain (e.g., Tn-MUC1 binding domain, PSMA binding domain, or mesothelin binding domain) comprises an scFv having the configuration from N-terminus to C-terminus, VH – linker – VL. In some embodiments, the antigen binding domain (e.g., a Tn-MUC1 binding domain, a PSMA binding domain, or a mesothelin binding domain) comprises an scFv having the configuration from N-terminus to C-terminus, VL – linker – VH. Those of skill in the art would be able to select the appropriate configuration for use in the present invention.

[0423] The linker is typically rich in glycine for flexibility, as well as serine or threonine for solubility. The linker can link the heavy chain variable region and the light chain variable region of the extracellular antigen-binding domain. Various linker sequences are known in the art, including, without limitation, glycine serine (GS) linkers such as (GS)n, (GSGGS)n, (GGGS)n, and (GGGGS)n, where n represents an integer of at least 1. Exemplary linker sequences can comprise amino acid sequences including, without limitation, GGSG (SEQ ID NO: 121), GGSGG (SEQ ID NO:122), GSGSG (SEQ ID NO: 123), GSGGG (SEQ ID NO: 124), GGGSG (SEQ ID NO: 125), GSSSG (SEQ ID NO: 126), GGGGS (SEQ ID NO: 127), or GGGGSGGGGSGGGGS (SEQ ID NO: 128), and the like. Those of skill in the art would be able to select the appropriate linker sequence for use in the present invention. In one embodiment, an antigen binding domain (e.g., a CD19 binding domain) of the present disclosure comprises a heavy chain variable region (VH) and a light chain variable region (VL). In some embodiments, the VH and VL is separated by the linker sequence having the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO:128). In some embodiments, the linker nucleic acid sequence comprises the nucleotide sequence GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTGGCGGCGGATCT (SEQ ID NO: 129).

[0424] Despite removal of the constant regions and the introduction of a linker, scFv proteins retain the specificity of the original immunoglobulin. Single chain Fv polypeptide antibodies can be expressed from a nucleic acid comprising VH- and VL-encoding sequences. Antagonistic scFvs having inhibitory activity have been described.

[0425] As used herein, the term "Specificity" refers to the ability to specifically bind (*e.g.*, immunoreact with) a given target antigen (*e.g.*, a human target antigen). A chimeric antigen receptor may be monospecific and contain one or more binding sites, which specifically bind a target or a chimeric antigen receptor may be multi-specific and contain two or more binding sites which specifically bind the same or different targets. In certain embodiments, a chimeric antigen receptor is specific for two different (*e.g.*, non-overlapping) portions of the same target. In certain embodiments, a chimeric antigen receptor is specific for more than one target.

[0426] As used herein, the term "Spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular domain or, the intracellular domain in the polypeptide chain. A spacer domain may comprise up to about 300 amino acids, *e.g.*, about 10 to about 100 amino acids, or about 25 to about 50 amino acids.

[0427] As used herein, the term "Specifically binds," with respect to an antibody, means an antibody or binding fragment thereof (*e.g.*, scFv) which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "Specific binding" or "Specifically binding," can be used in reference to the interaction of an antibody, a protein, a chimeric antigen receptor, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (*e.g.*, an antigenic determinant or epitope) on the chemical species; for example, a chimeric antigen receptor recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A," the presence of a

molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled “A” and the antibody, will reduce the amount of labeled A bound to the antibody.

[0428] As used herein, the term “Stimulation,” means a primary response induced by binding of a stimulatory molecule (*e.g.*, a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex. Stimulation can mediate altered expression of certain molecules, such as downregulation of TGF-beta, and/or reorganization of cytoskeletal structures, clonal expansion, and differentiation into distinct subsets.

[0429] As used herein, the term “Stimulatory molecule” means a molecule on a T cell that specifically binds with a cognate stimulatory ligand present on an antigen presenting cell. Stimulatory molecule may be expressed by a T cell that provides the primary cytoplasmic signaling sequence(s) that regulate primary activation of the TCR complex in a stimulatory way for at least some example of the T cell signaling pathway. For example, the primary signal is initiated by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, and which leads to mediation of a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A primary cytoplasmic signaling sequence (also referred to as a "primary signaling domain") that acts in a stimulatory manner may contain a signaling motif which is known as immunoreceptor tyrosine- based activation motif or IT AM. Examples of an IT AM containing primary cytoplasmic signaling sequence that is of particular use in the invention includes, but is not limited to, those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta , CD3 epsilon, CD5, CD22, CD79a, CD79b, CD278 (also known as "ICOS") and CD66d. In a specific CAR of the invention, the intracellular signaling domain in any one or more CARS of the invention comprises an intracellular signaling sequence, e.g., a primary signaling sequence of CD3-zeta. In a specific CAR of the invention, the primary signaling sequence of CD3-zeta is the sequence provided as SEQ ID NO: 52, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like. In a specific CAR of the invention, the primary signaling sequence of CD3-zeta is the sequence as provided in SEQ ID NO: 54, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like.

[0430] As used herein, the term “Stimulatory ligand” means a ligand that when present on an antigen presenting cell (*e.g.*, an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a “stimulatory molecule”) on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands are well-known in the art and encompass, inter alia, an MHC Class I molecule loaded with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

[0431] As used herein, the terms “Subject” refers to a vertebrate. A vertebrate can be a mammal, such as a non-primate (*e.g.*, cows, pigs, horses, cats, dogs, rats, etc.) or a primate (*e.g.*, monkey and human). Mammals can include, without limitation, humans, non-human primates, wild animals, feral animals, farm animals, sport animals, and pets. In some embodiments, “Subject” and “Patient” are used interchangeably. Any living organism in which an immune response can be elicited may be a subject or patient. In certain exemplary embodiments, a subject is a human.

[0432] As used herein, the term “Substantially identical”, in the context of a nucleotide sequence, refers to a first nucleic acid sequence that contains a sufficient or minimum number of nucleotides that are identical to aligned nucleotides in a second nucleic acid sequence such that the first and second nucleotide sequences encode a polypeptide having common functional activity, or encode a common structural polypeptide domain or a common functional polypeptide activity, for example, nucleotide sequences having at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to a reference sequence, for example, a sequence provided herein.

[0433] In some embodiments, the context of an amino acid sequence, the term “Substantially identical” refers to a first amino acid sequence that contains a sufficient or minimum number of amino acid residues that are i) identical to, or ii) conservative substitutions of aligned amino acid residues in a second amino acid sequence such that the first and second amino acid sequences can have a common structural domain and/or common functional activity, for example, amino acid sequences that contain a common structural domain having at least about 85%, 90%. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to a reference sequence, for example, a sequence provided herein.

[0434] As used herein, the term “Substantially purified” cell is a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some embodiments, the cells are cultured in vitro. In other embodiments, the cells are not cultured in vitro.

[0435] As used herein, “Substantially Complementary” refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are complementary, or one or more bases are non- complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of a gene that is normally transcribed in cells (the open reading frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a gene that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR are generated by synthetic methods that are well known in the art. “Forward primers” are primers that contain a region of nucleotides that are substantially complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. “Upstream” is used herein to refer to a location 5, to the DNA sequence to be amplified relative to the coding strand. “Reverse primers” are primers that contain a region of nucleotides that are substantially complementary to a double-stranded DNA template that are downstream of the DNA sequence that is to be amplified. “Downstream” is used herein to refer to a location 3' to the DNA sequence to be amplified relative to the coding strand.

[0436] As used herein, the term “Target site” or “Target sequence” refers to a genomic nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule may specifically bind under conditions sufficient for binding to occur.

[0437] As used herein, the term “Targeting domain” used in connection with a gRNA, refers to a portion of the gRNA molecule that recognizes, or is complementary to, a target sequence. For example, a target sequence within the nucleic acid of a cell (*e.g.*, within a gene).

[0438] As used herein, the term “Target sequence” refers to a sequence of nucleic acids complimentary, for example fully complementary, to a gRNA targeting domain. In some embodiments, the target sequence is disposed on genomic DNA. In some embodiment the target sequence is adjacent to (either on the same strand or on the complementary strand of DNA) a protospacer adjacent motif (PAM) sequence recognized by a protein having nuclease or other effector activity, *e.g.*, a PAM sequence recognized by Cas9. In some embodiments, the target sequence is a target sequence of an allogeneic T cell target. In some embodiments, the target sequence is a target sequence of an inhibitory molecule. In some embodiments, the target sequence is a target sequence of a downstream effector of an inhibitory molecule.

[0439] As used herein, the term “T cell receptor” or “TCR” refers to a complex of membrane proteins that participate in the activation of T cells in response to the presentation of antigen. The TCR is responsible for recognizing antigens bound to major histocompatibility complex molecules. TCR is composed of a heterodimer of an alpha (a) and beta (β) chain, coupled to three dimeric modules CD3δ/CD3ε, CD3γ /CD3ε, and CD3ζ/CD3ζ. In some cells the TCR consists of gamma and delta (γ/δ) chains (CD3γ /CD3ε). In some embodiments, TCRs may exist in alpha/beta and gamma/delta forms, which are structurally similar but have distinct anatomical locations and functions. Each chain is composed of two extracellular domains, a variable and constant domain. In some embodiments, the TCR may be modified on any cell comprising a TCR, including, for example, a helper T cell, a cytotoxic T cell, a memory T cell, regulatory T cell, natural killer T cell, and gamma delta T cell.

[0440] The term “Therapeutic” as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

[0441] As used herein, the term “Therapy” refers to any protocol, method and/or agent (*e.g.*, a CAR-T) that can be used in the prevention, management, treatment and/or amelioration of a disease or a symptom related thereto. In some embodiments, the terms “therapies” and “therapy” refer to a biological therapy (*e.g.*, adoptive cell therapy), supportive therapy (*e.g.*, lymphodepleting therapy), and/or other therapies useful in the prevention, management, treatment and/or amelioration of a disease or a symptom related thereto, known to one of skill in the art such as medical personnel.

[0442] As used herein, the term "Tumor antigen" or "Hyperproliferative disorder antigen" or "Antigen associated with a hyperproliferative disorder" refers to antigens that are common to specific hyperproliferative disorders. In certain examples, the hyperproliferative disorder antigens of the present invention are derived from, cancers including but not limited to primary or metastatic melanoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, non-Hodgkin's lymphoma, non-Hodgkins lymphoma, leukemias, uterine cancer, cervical cancer, bladder cancer, kidney cancer and adenocarcinomas such as breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, and the like.

[0443] As used herein, the term "Transfected" or "transformed" or "transduced" refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny. [00126] The term "specifically binds," refers to an antibody, or a ligand, which recognizes and binds with a cognate binding partner (e.g., a stimulatory and/or costimulatory molecule present on a T cell) protein present in a sample, but which antibody or ligand does not substantially recognize or bind other molecules in the sample.

[0444] As used herein the term “Transduction” refers to the delivery of a gene(s) or other polynucleotide sequence using a retroviral or lentiviral vector by means of viral infection rather than by transfection. In some embodiments, the lentiviral vectors of the present disclosure are transduced into a cell through infection and provirus integration. In some embodiments, a target cell, e.g., a T cell, is "transduced" if it comprises a gene or other polynucleotide sequence delivered to the cell by infection using a viral or retroviral vector. In particular embodiments, a transduced cell comprises one or more genes or other polynucleotide sequences delivered by a retroviral or lentiviral vector in its cellular genome.

[0445] As used herein, the terms “Treat,” “Treatment” and “Treating” refer to the reduction or amelioration of the progression, severity, frequency and/or duration of a disease or a symptom related thereto, resulting from the administration of one or more therapies (including, but not limited to, a CAR-T therapy directed to the treatment of solid tumors). The term “treating,” as used herein, can also refer to altering the disease course of the subject being treated. Therapeutic effects of treatment include, without limitation, preventing occurrence or recurrence of disease, alleviation of symptom(s), diminishment of direct or

indirect pathological consequences of the disease, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

[0446] As used herein, the term a “Variant of any given sequence” is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues) has been modified in such a manner that the polypeptide or polynucleotide in question retains at least one of its endogenous functions. A variant sequence can be obtained by addition, deletion, substitution, modification, replacement and/or variation of at least one residue present in the naturally-occurring protein. In some embodiments, a “Variant” refers to a polypeptide that has a substantially identical amino acid sequence to a reference amino acid sequence, or is encoded by a substantially identical nucleotide sequence. In some embodiments, the variant is a functional variant. As used herein, the term “Functional variant” refers to a polypeptide that has a substantially identical amino acid sequence to a reference amino acid sequence, or is encoded by a substantially identical nucleotide sequence, and is capable of having one or more activities of the reference amino acid sequence.

[0447] As used herein, the term “Vector” refers to a nucleic acid molecule capable transferring or transporting another nucleic acid molecule. The transferred nucleic acid is generally linked to, e.g., inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication in a cell, or may include sequences sufficient to allow integration into host cell DNA. In some embodiments, a vector is a composition of matter that comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non- viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, viral vectors, Sendai viral vectors, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, plasmids (e.g., DNA plasmids or RNA plasmids), transposons, cosmids, bacterial artificial chromosomes, and variant viral vectors.

[0448] As used herein, the term “Xenogeneic” refers to a graft derived from an animal of a different species.”

[0449] An "intracellular signaling domain," as the term is used herein, refers to an intracellular portion of a molecule. The intracellular signaling domain generates a signal that promotes an immune effector function of the CAR containing cell, e.g., a CART cell. Examples of immune effector function, e.g., in a CART cell, include cytolytic activity and helper activity, including the secretion of cytokines. In an embodiment, the intracellular signaling domain can comprise a primary intracellular signaling domain. Exemplary primary intracellular signaling domains include those derived from the molecules responsible for primary stimulation, or antigen dependent simulation. In an embodiment, the intracellular signaling domain can comprise a costimulatory intracellular domain. Exemplary costimulatory intracellular signaling domains include those derived from molecules responsible for costimulatory signals, or antigen independent stimulation. For example, in the case of a CART, a primary intracellular signaling domain can comprise a cytoplasmic sequence of a T cell receptor, and a costimulatory intracellular signaling domain can comprise cytoplasmic sequence from co-receptor or costimulatory molecule.

[0450] A primary intracellular signaling domain can comprise a signaling motif which is known as an immunoreceptor tyrosine-based activation motif or IT AM. Examples of ΓΤAM containing primary cytoplasmic signaling sequences include, but are not limited to, those derived from CD3 zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d DAP10 and DAP12.

[0451] As used herein, a 5' cap (also termed an RNA cap, an RNA 7-methylguanosine cap or an RNA m G cap) is a modified guanine nucleotide that has been added to the "front" or 5' end of a eukaryotic messenger RNA shortly after the start of transcription. The 5' cap consists of a terminal group which is linked to the first transcribed nucleotide. Its presence is critical for recognition by the ribosome and protection from RNases. Cap addition is coupled to transcription, and occurs co-transcriptionally, such that each influences the other. Shortly after the start of transcription, the 5' end of the mRNA being synthesized is bound by a cap-synthesizing complex associated with RNA polymerase. This enzymatic complex catalyzes the chemical reactions that are required for mRNA capping. Synthesis proceeds as a multi-step biochemical reaction. The capping moiety can be modified to modulate functionality of mRNA such as its stability or efficiency of translation.

[0452] Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. As another example, a range such as 95-99% identity, includes something with 95%, 96%, 97%, 98% or 99% identity, and includes subranges such as 96-99%, 96-98%, 96-97%, 97-99%, 97-98% and 98-99% identity. This applies regardless of the breadth of the range.

EXAMPLES

[0453] The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

[0454] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples specifically point out various examples of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Materials and methods

[0455] *Primary cells and Cells lines.* Purified $CD4^+$ and $CD8^+$ T cells obtained from de-identified healthy human donors by Human Immunology Core at the University of Pennsylvania. Leukemic cell line (Nalm6.CBG.GFP), CRISPR knocked out leukemic cell line (Nalm6-CD19KO, CD19-Ve Nalm6.CBG.GFP), Jeko-1(CBG-GFP) and were established available from ATCC authenticated cell lines. These leukemic cell lines were maintained in culture with RPMI-1640 (Life Technologies) supplemented with 10% FBS

(Seradigm), 50 UI/ml penicillin/streptomycin (Life Technologies), 1% of 2mM GlutaMAX™ (Life Technologies) and 1% of 25mM of HEPES (Life Technologies). Fresh primary cells ($CD4^+$ and $CD8^+$ T cells) from HIC were used for all studies. Jurkat NFAT-GFP reporter cell line was obtained from Saar Gill.

[0456] *Cell culture*. Human T cells were purified by negative selection by using RosetteSep™ Human $CD3^+$ T cells Enrichment Cocktails (Stem-Cell Technologies) according to the manufacturer protocol. T cells were cultured at 1 x 10^6 cells per ml in either complete RPMI: RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum (Seradigm), 1% Penicillin-Streptomycin (Pen Strep) (Life Technologies), 2 mM GlutaMAX™ (Life Technologies), and 25 mM HEPES buffer (Life Technologies) or in CTS™ OpTmizer™ T-Cell Expansion SFM (Gibco) added with T-cell expansion supplement provided with Media, 1% Penicillin-Streptomycin, 2 mM GlutaMAX™ and 25 mM HEPES buffer. T cells were stimulated either with anti-CD3/CD28 Dynabeads™ (Life Technologies) at 1:3 (cell/bead) ratio or with irradiated K562.OKT3.64.86 at 2:1 (T cells/K562) ratio with 100-300 IU/mL of recombinant human interleukin-2 (Proleukin® from Clinigen). 20 hrs after stimulation, medium was reduced by and replaced with 200 µl of the appropriate lentivirus supernatant. Alternatively, 24 hrs after stimulation, titered virus was added at a 3:1 ratio (infectious particles: T cell). On day 3 of T cell activation, volume was doubled with fresh media. Expansions were de-beaded on day 5, counted every other day adjusting cell counts to 0.5×10^6 cells per ml with fresh media. When irradiated K562 were used for T cells activation, after day 2 of T cells activation, the medium was quadruplet and medium was changed every second day up to resting stage of T cells (day 10-11).

[0457] *Flow cytometry.* BD LSRFortessa™ instruments using BD FACSDiva™ Software v.8.0.1 (BD Biosciences) was used to acquire samples for different assays. Data were analyzed using FlowJo™ v.10 software. Anti-human antibodies were purchased from BD and BioLegend. CD19 binder CAR expression was evaluated on transduced T cells using FITC-Labeled human CD19 protein (ACROBiosystem, Cat-CD9-HF251).

[0458] *Intracellular cytokine assay and protein binding to binders.* Functionality of CART cells were evaluated following co-cultures of 2×10^5 CAR or NTD T cells with 4×10^5 Nalm6.CBG.GFP, Nalm6-CD19KO, or Jeko-1. One hour after start of co-culture, 1X Brefeldin A and Monensin Solution (BioLegend). After 6hrs co-culture at 37°C, intracellular

cytokine production was measured by flow cytometry staining with anti-human antibodies specific for IFN-γ, TNF-α and IL-2.

[0459] In another assay, killing of target cells (GFP) was assayed by intracellular staining with active caspase3 (564096, BD). In another assays, to see the binding specificity of CD19 protein towards all CD19 binders, FITC-labeled human CD19 protein was incubated with anti-CD19Ab (clone-FMC63) for 15min at 1:1 and 1:3 ratio. All original binders were stained with this cocktail to determine if detection of CAR CD19 binders was blocked.

[0460] *Lentivirus production and transfection*. Lentiviral packaging mix containing Rev, Gag/Pol and Cocal-G glycoprotein along with the appropriate pTRPE transfer vector were transfected into HEK293T cells using Lipofectamine™ 2000 (Life Technologies). At 24 hrs and 48 hrs after transfection, the HEK293T cell supernatant was collected, filtered through a 0.45-μm syringe-driven filter and then concentrated the lentivirus by ultracentrifugation at 25,000 r.p.m. for 2.5hrs at 4 °C. The supernatant was discarded and the lentivirus pellet was resuspended in 1000μl of complete RPMI and stored at −80 °C.

[0461] *RNA Electroporation of truncated CD19 antigen (CD19Ag) in K562 cell line*. K562-wt cells were transfected by electroporation with varying amounts of truncated CD19 antigen (CD19Ag) RNA (20ug, 5ug or 0.5ug) for 500us at 300V using BTX. After electroporation, cells were incubated overnight in 37ºC incubator. Next day, K562 expressing different level of human CD19 (huCD19) were stained for expression and co-cultured with CD19 Binder's CART cells at 1:2 ratio to measure stimulated intracellular cytokine staining.

[0462] *Western blot*. CART cells were lysed in a 70μl of RIPA lysis buffer (1X protease and phosphatase inhibitor cocktail (Thermo Scientific™ Halt™ Protease and Phosphatase Inhibitor Cocktail), incubated at 4°C for 30 min and centrifugation at 12000 x g (at 4°C) for 30 min. The supernatants were collected and protein concentration determined with BCA protein assay kit (Thermofisher). 30-60μg of protein was mixed with appropriate amount of reducing agent (10x) and LDS sample buffer (4x) and heated the samples at 95°C for 5 min. 30ul of samples was loaded in 4-12% PAGE gel at 100V for 2hrs. The gel was transferred onto Immnobilon-P membranes for overnight at 25V. The membranes were blocked with 5% skim milk at room temperature for 30 min, probed with anti-CD247 or anti-CD3z, (BD Pharmingen™) and incubated at 4°C for overnight. Subsequently, the membranes were

incubated with diluted secondary antibodies at room temperature for 1 h. Detection of transferred proteins visualized by the SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit (Thermofisher) according to the manufacturer's protocols.

[0463] *CART CD19binders cell in vitro stress test.* 2E6 CAR CD19 binder positive T cells were co-cultured with 8E6 IRF720$^+$ Nalm6-wt or IRF720$^+$ Nalm6-CD19 knockout (KO) cells for a 1:4 CAR$^+$:Target ratio. After 3 - 4 days, 0.5ml of the co-cultures were collected and stained to determine T cell number and phenotype by flow cytometry. CD19 binder T cell phenotypes were evaluated for viability using Live/dead violet fixable viability kit (Life Technologies) following the manufacturer's protocol and then stained with the following anti-human antibodies: BV605-CD45, PE-CD4, BV510-CD8, BV650-CD45RO, PerCP-CCR7, BV711-PD1, BV785-CD69, PE-Cy7-ICOS, and FITC-CD19 protein. CountBright™ Absolute Counting Beads (Invitrogen) were used as an internal standard to calculate absolute cell counts in cell suspensions. After calculations, CD19 binder T cells were seeded with fresh IRF720$^+$ Nalm6-wt cells at a ratio 1:4 (CD45$^+$:Nalm6-wt). This process was repeated every 3-4 days for 25 days, total 6 rounds. Flow cytometric data was acquired on an LSRII Fortessa™ Cytometer (BD Bioscience) and analyzed with FlowJo™ v10 software (FlowJo, LLC).

[0464] *Mouse Experiments*. NSG mice (NOD/scid/IL2rg) were purchased from Jackson Laboratory and bred in the animal facility at the University of Pennsylvania. 8–12 weeks old, male or female mice, were used in this study. For the Jeko-1 lymphoma mouse model, each mouse was tail vein injected with 1E6 Jeko1-CBG-GFP cells and seven days later, with 1E5 human CAR CD19binder$^+$ T cells. Mice were health monitored twice per week with tumor BLI and weight measured weekly. Mice were bled for T cells engraftment. Endpoint euthanization for study were disease progression (BLI>1E13 P/S), 20% weight loss and lethargic activity or hunched posture.

[0465] *TruCount™ assay.* The TruCount™ assay was performed to determine absolute numbers of huCD45$^+$ cells circulating in mouse whole blood. Anti-human mAbs mix of CD45-BV605, CD4-BV711, CD8-V500, PE-conjugated huCD19 protein were added to TruCount™ tubes followed by 50ul of anticoagulated whole blood. Samples were vortexed gently and incubated for 15 min in the dark at room temperature. Then 450µL of BD FACS™ Lysing Solution was added to each tube, vortexed and incubated for 15 min in the dark at

room temperature. Data was acquired within 1–3 hrs of staining on a LSRII Fortessa™ flow cytometer (BD Bioscience).

Example 2: Generation of CD19 binders

[0466] This example describes the identification of the novel CD19 binders disclosed herein.

[0467] Affinity tuning of CAR binding domains can reduce targeting of cells expressing lower levels of the targeted antigen. An affinity tuning platform to generate low affinity variants of CD19 binders was generated by comprehensively mutating heavy and light CDR3 regions in combination with high-throughput screening using a large yeast display human antibodies libraries and antibody characterization assays. *See e.g.,* AvantGen Inc., avantgen.com/therapeutic-antibodies. Multiple single amino acid substitution variants were generated, with the goal of only maintaining the CD19 scFv binding specificity and key CAR properties, including high CAR T cell expansion and lack of tonic signaling, while also having a low affinity and a fast off-rate.

[0468] Identification of antibodies with reduced affinity represents a relatively uncommon objective in antibody discovery and poses unique challenges when developing appropriate screening approaches. However, the comprehensive mutagenesis approach taken enabled the identification of 12 binders showing various levels of reduced binding. **Table 3**, and **FIGs 2A-E**.

Example 3 Identification of Unique CD19-binders

[0469] As shown in **FIG. 1,** six general steps were taken to identify the unique CD19-specific antibody clones used to generate the novel CD19 binders described herein. Specifically, human antibody library for CD19-specific antibody clones were screened and induced on phage display in approximately 100 million yeast cells (Step 1). In step 2, enrichment for clones that bound biotinylated CD19-Fc using a streptavidin microbead column (MACS technology) was performed twice (2x). FACS screen of 6 phage display libraries for enrichment for binding to CD19-Fc was performed. Multiple rounds of FACS enrichment with fluor-labeled CD19 to enrich for CD19-specific clones were conducted. From these screens, a panel of up to thirty (30) single-chain variable fragments (scFv) clones CD19-specific unique clones were identified. The sequences of these CD19 scFv clones were selected based on their specific binding to CD19 expressed on recombinant HEK 293F cells

transfected with a human GFP-tagged CD19 (huCD19-GFP) plasmid.

[0470] These up to 30 scFv clones were further screened based on their ability to bind baculovirus or SIGLEC. Clones that bound biotinylated baculovirus or SIGLEC 7, 8 and 9 were removed from consideration. From this selection, clones A4 (clone 43 or 43), E4 (clone 44 or 44), and E7 (clone 45 or 45) were identified (Step 3).

[0471] To ensure that the up to (30) clones could recognize native CD19 expressed on tumor cells, the clones (scFv) were also screened against NALM6 tumor cell line positive (+) CD19 expression (NALM6 cell line $^{+}$ CD19). CD19 knockout Nalm6 cell line (NALM6 cell line $^{-}$ CD1; or CD19KO Nalm6 cell line) were used as a negative control for the selection. CD19KO Nalm6 cell lines used for the screen were engineered CRISPR knocked-out (KO) Nalm6-CD19KO cells. Clones that bound $CD19^{+}$ NALM6 tumor cells, but not $CD19^{-}$ NALM6 tumor cells (CD19KO) were selected (Step 4). From the $CD19^{+}$ NALM6 tumor cells screen, the 12 CD19-specific antibody clones were identified. The clones, disclosed in Table 3 are called: A2 (clone 42; or 42); A4 (clone 43; or 43); E4 (clone 44 or 44); E7 (clone 45 or 45); 7 (clone 46 or 46); 10 (clone 47 or 47); 11 (clone 48 or 48); 14 (clone 49 or 49); 15 (clone 50 or 50); 16 (clone 51 or 51); 18 (clone 52 or 52); 23 (clone 53 or 53). **FIG. 1** and **Table 3**. The nucleic acid sequences of these 12 novel CD19 binders are shown in the sequence alignment disclosed in **FIGs. 2A-2E**, and **Table 1**. The sequence similarities between these clones are shown in **FIG. 2F**.

[0472] Thus, the original up to 30 scFvs were reduced to twelve (12) novel CD19 scFvs that specifically bound to wild type NALM6-wt cells but not engineered CRISPR Nalm6-CD19KO cells. These twelve CD19 binders were designated 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 and 53 (**FIG. 1; Table 3**).

[0473] The 12 novel CD19 binders were used to engineer novel anti-CD19 chimeric antigen receptors (CD19) for further analysis. These selected scFvs were engineered to be functioning chimeric antigen receptors (CARs) designed to use structural components of the CD8 leader, hinge and transmembrane domain TM for membrane expression and signaling domains of 4-1BB and CD3zeta to direct activation responses. The novel anti-CD19 CARs comprised an antigen binding domain comprising an anti-CD19 antibody fragment or scFv selected from the group consisting of 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 and 53 or

optimized variant thereof; a CD8 hinge domain; a CD8 transmembrane domain; the intracellular domain of a 4-1BB costimulatory molecule; and a CD3 zeta intracellular domain (anti-CD19 scFv-BBz CAR constructs).

[0474] Each of these CARs was cloned into a lentiviral expression plasmid pTRPE (pTRPE anti-CD19 scFv-BBz CAR constructs; **FIG. 1**). Each of the twelve CAR constructs was packaged and transduced in both Jurkat NFAT reporter cells and primary human T cells; and characterized as described herein.

Example 4: Functional Characterization of the 12 CD19 binders.

[0475] *Evaluation I: Expression and cytokine response in Jurkat cells and primary human T cells (ND607 donor)*

[0476] CD19 CARs comprising the novel binders were initially transduced into Jurkat NFAT-GFP reporter cell line to evaluate CAR expression, detection, and their ability to activate NFAT transcription pathway and express GFP. These cells were transduced at high levels, for example greater than 70%, which allowed for greater sensitivity of ligand-independent activation of the NFAT reporter, known as tonic signaling. **Table 4** shows the raw data for CAR expression and tonic signaling.

Table 4: CD19 CAR Expression and Tonic Signaling - Jurkat NFAT-GFP reporter cell

Novel CD19 binders-Clone #	**CD19 CAR Surface expression (% mean fluorescence intensity (mfi))**	**Tonic Signaling (mfi)**	**Remarks**
42	74.600	1.500	No tonic signaling Medium surface detection
43	83.300	6.450	Some tonic signaling Low surface detection
44	78.400	5.370	Some tonic signaling Med surface detection
45	86.800	3.820	Some tonic signaling Low surface detection
46	75.100	10.800	Tonic signaling Medium surface detection
47	0.170	13.500*	*Tonic signaling No surface detection
48	0.000	18.100*	*Tonic signaling No surface detection

Table 4: CD19 CAR Expression and Tonic Signaling - Jurkat NFAT-GFP reporter cell			
Novel CD19 binders- Clone #	**CD19 CAR Surface expression (% mean fluorescence intensity (mfi))**	**Tonic Signaling (mfi)**	**Remarks**
49	0.000	5.240*	Some tonic signaling No surface detection
50	76.700	2.72	Low tonic signaling Medium surface detection
51	73.900	8.510	Some tonic signaling Medium surface detection
52	73.400	5.170	Some tonic signaling Medium surface detection
53	0.0120	2.840*	*Low tonic signaling No surface detection

[0477] A CAR comprising a clone 43 antigen binding domain was weakly expressed (low mfi surface detection) on the surface of the transduced cells and the clone 43 CAR appeared to induce some tonic signaling. A CAR comprising a clone 44 antigen binding domain was expressed on the surface of the transduced cells (medium mfi surface detection) and the clone 44 CAR appeared to induce some tonic signaling. A CAR comprising a clone 45 antigen binding domain was weakly expressed on the surface of the cells (low mfi surface detection) and the clone 45 CAR appeared to induce some tonic signaling. A CAR comprising a clone 46 antigen binding domain expressed on the surface of the transduced cells (medium mfi surface detection) and the clone 46 CAR appeared to induce high tonic signaling. While a CAR comprising a clone 47 antigen binding domain was not detectable on the surface of the transduced cells, the clone 47 CAR appeared to induce tonic signaling in the transduced cells. Like clone 47, a CAR comprising clone 48 was also not detectable on the surface of transduced cells, yet the clone 48 CAR also appeared to induce tonic signaling. A CAR comprising a clone 49 antigen binding domain was also not detected on the surface of transduced cells, but the clone 49 CAR appeared to induce some tonic signaling. A CAR comprising a clone 50 antigen binding domain was expressed on the surface of the transduced cells (medium mfi surface detection) and the clone 50 CAR appeared to induce low or weak tonic signaling. A CAR comprising a clone 51 antigen binding domain was expressed on the surface of the transduced cells (medium mfi surface detection) and the clone 51 CAR

appeared to induce some tonic signaling. A CAR comprising a clone 52 antigen binding domain was expressed on the surface of the transduced cells (medium mfi surface detection) and the clone 52 CAR appeared to induce some tonic signaling. A CAR comprising a clone 53 antigen binding domain was not detected on the surface of the transduced cells (medium mfi surface detection), but the clone 53 CAR appeared to induce low or weak tonic signaling.

[0478] FIG. 3A shows the quantification of the tonic signaling induced by CARs comprising an antigen binding domain of any of clone 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 and 53 relative to CAR expression. Detectable surface expression of the CD19 CAR (CAR CD19binders) varied in levels among the 12 tested binders, with CARs 43 and 45 having the highest expression. The expression of a CAR comprising a clone 42 antigen binding domain was highly detected on the surface of transduced Jurkat-NFAT-GFP reporter cells (mean fluorescence intensity (mfi) of 74.6%), but the clone 42 CAR did not appear to induce tonic signaling (e.g., 1.5% mfi) in the transduced cells. **Table 4**.

[0479] Relative to a control CD19 CAR comprising a known binder (*e.g.*, FMC63 binder), a CAR comprising a clone 42 antigen binding domain (CAR42, CAR CD19 42, or CAR CD19binder 24, CD19 CAR 42) produced a high amount of GFP expression, and no tonic signaling. Interestingly, CAR expression was not readily detectable in CAR T cells transduced with CARs comprising CD19 binders 47, 48, 49, and 53 (**FIG. 3A**), but these CAR T cells did show varying levels of tonic signaling **Table 4**.

[0480] Together, these data demonstrated that CARs comprising the novel CD19 binders disclosed herein can activate the NFAT pathway without stimulation with CD19 ligand (tonic signaling or ligand independent activation). CD19-42 CARs showed the lowest level of CAR-induced tonic signaling. Other CARs appeared to induce tonic signaling even though the surface expression of these CARs was not readily detected on the CAR T cell surface. Generally, most CARs tested expressed on the cell surface and activated the NFAT pathway.

[0481] For the first characterization in human primary T cells, the twelve CAR CD19 binders were transduced into ND607 donor T cells and their expansion was monitored to assess their proliferation or expansion (e.g., in cell numbers) **(FIG. 4A)** and changes in blast size or contraction **(FIG. 4B)**. The expansion of primary CAR T cells was substantially similar among all 12 novel binders. A similar trend was observed for the contraction of the CAR T

cells at day 11 post-transduction. For example, the mean cell size for CD19 binder 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 50 was respectively, 200 μm^3, 236 μm^3, 329 μm^3, 324 μm^3, 286 μm^3, 298 μm^3, 309 μm^3, 243 μm^3, 261 μm^3, 269 μm^3, 332 μm^3, 292 μm^3, or 261 μm^3.

[0482] **Table 5** shows the expression profiles of CD19 CARs comprising the novel CD19 binders described herein on transduced $CD4^+$ T cells and $CD8^+$ T cells from the ND607 donor on day 7 (D7) after transduction.

Table 5: Expression Profiles of ND607 CD19 CAR T Cells on Day 7

Novel CD19 binders-Clone #	Gated on CD4+ T cells	Gated on CD8+ T cells
Untransduced (NTD)	0.22	0.15
42	70.60	63.70
43	39.50	30.50
44	38.70	39.20
45	69.50	63.70
46	18.10	15.30
47	11.60	9.17
48	0.76	0.80
49	0.85	1.49
50	50.90	39.70
51	27.70	16.20
52	53.20	54.60
53	1.35	1.77

[0483] Further analyses of percent of detectable CAR transduced T cells and the MFI of their expression on both the $CD4^+$ and $CD8^+$ T cells at different times in expansion (*e.g.*, D7 vs D11; data not shown) showed that CD19 CARs 44, 45, and 52 appeared to be the most stable in detectable transduction over time, but these CD19 CARs appeared to lose their MFI towards the end of expansion. Part of this loss was attributed to normal T cell size reduction. Similar results were observed with a control CD19 binder. While the CD19 control CAR and CD19 CARs comprising binders 44, 45, and 52 appeared to be the most stable in detectable transduction over time, all tested CAR T cells seemed to lose their MFI towards the end of expansion. Part of this loss could be a result of normal T cell size reduction.

[0484] *Cytokine production*

[0485] The ability of CARs comprising the novel CD19 binders to produce cytokines after a 4 hr co-culture with Nalm6 cells was also evaluated by intracellular detection using flow

cytometry. The cytokines tested were IL-2, TNFα, and/or IFNγ. **Table 6** shows that only CAR T cells expressing CD19 CARs comprising CD19 binder 42, 43, 44, 45, 46, or 52 induced significant and quantifiable levels of cytokine. CAR T cells expressing a positive CD19 CAR control also produced cytokines.

Table 6: Cytokine Response of ND607 CD19 CAR T cells upon Nalm6 stimulation for 4 hrs on Day 9

Novel CD19 binders	**Unstimulated Cells**			**Nalm6 stimulation**			**PMA/Ionomycin stimulation**		
	IL2	**TNFα**	**IL2 and TNFα**	**IL2**	**TNFα**	**IL2 and TNFα**	**IL2**	**TNFα**	**IL2 and TNFα**
NTD*	0.80	0.080	0.91	0.35	0.075	0.75	14.1	2.88	73.2
42	0.870	0.320	1.060	4.020	2.240	5.930	11.100	3.360	80.100
43	0.600	0.310	0.330	4.420	5.170	6.170	3.920	6.740	83.000
44	1.750	0.510	0.900	4.290	6.000	7.760	3.720	6.890	84.000
45	1.480	0.330	0.950	2.970	3.750	13.400	8.210	3.100	82.700
46	1.230	0.530	0.940	2.390	3.680	7.440	7.430	4.340	84.800
47	0.930	0.038	0.620	0.350	0.100	0.430	5.200	5.310	84.400
48	0.540	0.024	1.110	0.390	0.250	0.920	9.780	2.150	83.100
49	0.460	0.097	0.790	0.480	0.300	0.480	10.100	2.610	83.800
50	0.350	0.078	0.570	0.350	0.330	0.720	8.620	4.540	82.500
51	0.520	0.520	0.660	0.750	1.150	0.770	4.890	8.780	80.200
52	0.640	0.360	0.550	5.210	5.840	15.700	4.970	4.350	86.700
53	0.780	0.048	1.220	0.410	0.140	0.860	13.300	2.430	80.400

NTD = untransduced

[0486] As expected, CAR T cells did not produce cytokines in the absence of stimulation (*e.g.*, before co-culture), all CAR T cells tested had equal potential to produce cytokines as shown with PMA/ionomycin stimulation. **FIG. 5A** shows the quantification of the relative production of IL-2 and TNFγ either alone or in combination following Nalm6 co-culture for 4 hrs.

[0487] The activation states of the CD19 CAR T cells were determined on day 11 (D11) post-transduction by assessing the expression of HLA-DR and 4-1BB. **Table 7** shows the raw results of this analysis.

Table 7: Expression of HLA-DR and 4-1BB on ND607 CD19 CAR T cells on Day 11				
Novel CD19 binders-Clone #	**HLA-DR expression**		**4-1BB expression**	
Gated on	**CD4+ T cells**	**CD8+ T cells**	**CD4+ T cells**	**CD8+ T cells**
Untransduced (NTD)	6.21	2.11	0.43	0.23
42	21.40	14.10	0.70	0.57
43	50.50	55.40	4.34	7.21
44	41.10	46.10	4.25	5.80
45	37.00	43.90	4.17	5.27
46	33.00	39.90	4.23	6.37
47	49.10	47.90	4.34	5.07
48	17.30	12.70	3.06	2.34
49	27.40	24.40	1.95	1.89
50	30.60	26.70	2.65	1.72
51	45.40	56.60	2.42	3.92
52	35.90	39.30	3.99	6.23
53	30.30	23.60	2.88	2.05

[0488] All tested ND307 CD19 CAR T cells showed HLA-DR expression for both $CD4^+$ and $CD8^+$T cells. These results indicated that the ND307 CD19 CAR T cells were still in a more activated state than untransduced (NTD T) cells. All tested ND307 CD19 CAR T cells also showed 4-1BB expression in both $CD4^+$ and $CD8^+$ T cells. 4-1BB (CD137) was used as an early activation surface marker and its expression or lack thereof indicated the resting state of the ND307 CD19 CAR T cells when compared to control CD19 CART cells. CAR T cells expressing CARs comprising a CD19 binder 42 had the lowest 4-1BB expression levels (0.70 and 0.57 mfi).

[0489] **Table 7** shows that on Day 7, CD19 CAR T cells were still in a more activated state when compared to untransduced (NTD) T cells. However, the observed level of activity may not have been enough to produce cytokines in some CAR T cells. The CD19 CAR T cells were also tested to assess their activation level using an early activation surface marker 4-1BB (CD137). The early activation surface marker 4-1BB (CD137) was detected at Day 11 following transduction, which was an indication of their resting state.

[0490] Together, the expression of the CD19 CARs, HLA-DR and 4-1BB, and cytokine profiles analyses confirmed that CD19 CAR T cells expressing CARs comprising CD19 binders 42, 43, 44, 45, 46 and 52 produced cytokines in a CAR-dependent manner. CAR T

cells expressing CARs comprising the CD19 binders 50 and 51 had a good detectable CAR surface expression, but a weaker CAR-induced cytokine production. As shown below, CARs comprising CD19 binders 42, 43, 44, 45, 46 50, 51, and 52 were further analyzed using additional primary T cells derived from two different donors.

[0491] CD19 CAR T cells expressing CD19 CARs comprising CD19 binders 47, 48, 49 and 53 did not have detectable CAR expression on Jurkat and primary human T cells (**FIG. 3A and Table 4**) and they did not result in significant cytokine production (**Table 4**). Nonetheless, additional data demonstrated that these novel binders were functional despite the absence of any detectable expression. For example, CAR T cells expressing CARs comprising CD19 binders 47, 48, 49 and 53 showed induced NFAT activity (**Table 4**) and maintained higher levels of HLA-DR and CD137 over untransduced cells (**Table 7**).

[0492] *Evaluation II: Expansion and Cytokine production in two primary human T cells (ND572 and ND539 donors)*

[0493] A second round of evaluation of the CD19 binders was performed using two additional human primary T cells (e.g., normal) from two different donors (ND572 and ND539. In particular, CAR comprising CD19 binders 42, 43, 44, 45, 46, 50, 51, and 52 were evaluated, ND572, and ND539 donors T cells.

[0494] The expansion profiles of CAR T cells expressing CD19 CARs comprising CD19 binders 42, 43, 44, 45, 46, 50, 51, and 52 were assessed in ND539 and ND572 donor T cells as shown in **FIG. 6**. In both donor T cells, the T cells expansions were very similar among all the CD19 binders tested. The CAR T cells expressing a CAR comprising the CD19 binder 42 showed a slightly higher expansion based on the expansion doubling number (**FIGs. 6A and B**). In addition, CD19 binder 42 CAR T cells appeared to possibly rest down (contract) earlier based on the mean cell size analysis (**FIGs. 6C and D**).

[0495] At the end of the expansion period (e.g., on day 13 and day 14), the number of T cell doublings during manufacturing for T cells from donor ND539 and ND572 was also evaluated. As shown in **FIG. 7A**, CAR T cells comprising a CD19 binder 42 showed the greatest expansion when compared to CD19 CAR T cells expressing a CD19 CAR comprising CD19 binders 43, 44, 45, 46, 50, 51, and 52. The expansion of the latter CAR T cells was highly variable between binders.

[0496] The CD19 CAR T cells were also assessed to determine the percent change of $CD4^+$ T cells population. As shown in **FIGs. 7B and C**, the percent change of $CD4^+$ T cells showed a similar decline among all the CAR T cells tested regardless of donors. However, CAR T cells expressing a CAR comprising a CD19 binder 46 (CAR46) consistently showed a lower percent change in the $CD4^+$ T cells population when compared to CAR T cells expressing CARs comprising CD19 binders 42, 43, 44, 45, 50, 51, and 52. The percent of $CD4^+$ T cells population expressing the CARs was the highest on Day 6 of the expansion, but it stabilized out in later timepoints **(FIGs. 8A-D)**. The observed higher level on day 6 may be due to lingering pseudo-transduction on day 6. As seen in ND608 donor and **Table 8**, the detectable MFI of CAR^+ CD19 binders decreased over time on both the ND572 $CD4^+$ and $CD8^+$ T cells.

Table 8: Changes in Cell Surface Expression of CD19 CARs on ND572 CAR T cells during Expansion

Novel CD19 binders-Clone #	**CD4+ T cells**		**CD8+ T cells**	
	Day 6	**Day 12**	**Day 6**	**Day 12**
42	75.6	64.5	69.7	35.7
43	51.7	35.1	41.2	17.4
44	53.8	41.1	44.8	29.5
45	65.9	48.9	46.1	17.0
46	52.8	54.1	44.8	31.7
50	53.0	50.6	38.3	33.5
51	64.9	58.4	55.0	24.5
52	63.2	54.1	55.7	32.2

[0497] As with ND572, the CAR surface expression in donor ND539, as seen by the contour plots also showed a drop in MFI over time. To further understand this reduction in expression, codon optimized versions of the novel CD19 scFvs were generated to potentially enhance their stability as described below. CARs expressed in T cells from donor ND539 were also tested to evaluate their cytokine response and HLA-DR expression on Day 12. Flow cytometric analyses of these CAR T cells demonstrated that donor ND539 T cells expressing a control CD19 CAR or a CAR comprising the CD19 binders 42, 44, 45 and 52 produced cytokine when co-cultured with Nalm6. ND539 CAR T cells expressing a CAR comprising CD19 binders 43 and 46 did not produce cytokine when stimulated. This response was unexpected because ND608 CAR T cell expressing CARs comprising CD19 binders 43 and 46 did produce cytokine.

[0498] CARs comprising CD19 binders 42, 43, 44, 45, 46 and 52 were evaluated further in the ND539 donor for CAR expression, RNA transcript levels, total protein produced and cytokine response in Nalm6 co-cultures. CD19 binders 50 and 51 were removed from this analysis because of their lack of cytokine response in ND608 **(Table 6)**. CARs comprising CD19 binders 50 and 51 showed a cytokine response that was similar to untransduced cells. These binders were set aside until the cytokine unresponsiveness could be resolved.

Example 5: Characterization of Novel CD19 binders RNA levels, Protein levels and epitope binding regions.

[0499] *Detection of total RNA*

[0500] CARs comprising the novel CD19 binders 42, 43, 44, 45, 46, and 52 were characterized to determine the relative levels of CD19 binder RNA transcripts, the total protein produced, and uniformity of the protein species in ND539 CAR T cells. The overall experimental scheme for generating the CD19 ND539 CAR T cells from the timing of cell isolation and characterization to RT PCR and western blot is shown in **FIG. 9**. Total RNA from each CAR population was isolated and subjected to real-time PCR analysis using WPRE and CD3ε primer/sets. The WPRE primers were used to detect the CAR , while the anti- CD3ε primers were used to detect total T cells. The relative number of CAR specific transcripts and total T cell loading control were also determined. **FIG. 10A** shows the relative expression level of RNA transcripts for each CAR comprising a novel CD19 binder in the donor T cells normalized to a control CAR (e.g., FMC63 CAR) levels. The relative amounts of the RNA transcripts were substantially similar for all CD19 binders tested over time. F**IGs. 10A and B**. However, CAR comprising the CD19 binder 42 consistently had twice the amount RNA transcripts detected in the control CD19 CAR.

[0501] In addition, the relative overall RNA transcript amounts decreased with time (**FIG. 10B,** compare day 6 to Day 12). This decrease was probably a result of normal contraction in the blasting transcriptional activity of T cells resting down. Indeed, the results showed a unique characteristic of CARs comprising the CD19 binder 42. CAR T cells expressing CARs comprising the CD19 binder 42 were able to maintain higher level of CAR RNA transcripts. The mechanism of this high transcript level is not known, but it was speculated that these cells had either a higher transcription rate or their RNA transcripts were more

stable (less degradation). The RNA transcript levels of CD19 binders 43, 44, 45, 46, and 52 were relatively similar between binders, while the RNA transcripts of CD19 binder 42 was 2 fold higher than even the positive control. As described herein, CD19 binders 42 exhibited the desired and unique CD19 binder characteristics that was screened for.

[0502] *Protein Expression*

[0503] The total protein levels of each transduced CAR were also evaluated on day 6 following transduction. This analysis showed a wider variation in both surface protein expression and total protein amounts (**FIG. 11A**). For example, the CD19 CAR surface expression on ND539 CAR T cells, based on flow cytometry (mfi) was 0.18% in untransduced T cells compared to 47.6% for control CD19 CAR; 57.4% for CD19-42 CAR; 16.6% for CD19-43 CAR; 25.4% for CD19-44 CAR; 48.5% for CD19-45 CAR; 13.3 % for CD19-46 CAR; and 44.9% for CD19-52 CAR. A western blot analysis of CAR T cells expressing these binders detected two isoforms of the expressed CARs as illustrated by two bands around the expected protein size of about 50KDa. The CAR isoforms were detected using an anti-CD3ζ antibody (**FIG. 11A**) TCR-CD3z was used as an internal control and showed a uniform protein expression in all tested samples. The amount of each isoforms varies depending on the CD19 binder. The CD19-52 CAR T cells only expressed the highest molecular weight isoform. The highest molecular weight isoform was also dominant in CD19-43, CD19-44, and CD19-45 CAR T cells. In contrast, the lowest molecular weight isoform was dominant in CD19-42 and CD19-46 CAR T cells.

[0504] , The protein expression levels of CARs comprising the CD19 binder 46 (CAR46) did not directly correlate with the RNA transcript levels shown in **FIGs. 10A-B**. This is because the western blot showed that CD19-46 CAR was highly expressed on Day 6 post transduction, yet these cells showed similar RNA levels of the transgene, which was similar to the RNA levels of CD19 binders 43, 44, 45, and 52. In fact, CD19-binder 42 showed a 2 fold increase in RNA levels compared to other binders, but this increase did not correlate with the western blot protein expression. Further analyses of the stability of CD19 binder surface expression showed that the MFI appeared to decrease over time. These observations were consistent with observations made using donor ND572 CAR T cells.

[0505] *CAR CD19 binders were detected with a recombinant CD19 protein.*

[0506] To further detect the expression of CARs comprising the novel CD19 binders on CAR T cells. CARs comprising CD19 binder 42, 43, 44, 45, 46, or 52 were expressed on ND539 T cells. At day 9 (D9) post-transduction, frozen ND539 CAR T cells were incubated with a fluorescently labeled recombinant CD19 protein. The CD19 CAR T cells were then analyzed by flow cytometry to determine the binding of the recombinant molecules to the CD19 CAR T cells. ND539 CAR T cells expressing a CAR comprising a FMC63 binder were used as control. Flow was gated for CD19 binder CAR. As shown in **Table 9** (CD19-FITC Protein only), the recombinant CD19 protein effectively bound to the CD19 CAR T cells.

[0507] FIG. 12B shows the quantification of the mfi detected on the ND539 CAR T cells. The recombinant CD19 protein weakly recognized CD19 CAR comprising binder 43 (mfi = 1.17 compared to 0.85 (untransduced)), and binder 46 (mfi = 3.57) compared to 0.85 (untransduced)). CD19-GFP bound CD19 binder 52 (mfi = 31.1) as well as the positive CD19 control (mfi = 31.1). CD19-GFP binding to CD19 binders 42 (mfi = 16.3), 44 (mfi = 23.2), and 45 (mfi = 23.1) was intermediate. These results confirmed the novel CD19 binders' ability to detect their target antigen-CD19.

Table 9: CD19 binders Epitope analysis on ND539 Frozen Cells on Day 9

Incubated with	**Anti-FMC63**	**CD19-FITC Protein**	**CD19-FITC Protein addition after anti-FMC63Ab PE**	
	Gated for Anti-FMC63 Ab Expression	Gated for CD19 -CAR expression	Gated for Anti-FMC63 Ab Expression	Gated for CD19 CAR Expression
Untransduced (NTD)	0.47	0.85	0.49	0.86
CAR-FMC63	38.60	31.10	46.80	26.60
42	0.18	16.30	1.37	12.10
43	0.19	1.17	1.28	1.37
44	0.52	23.20	1.15	26.20
45	0.43	23.10	1.19	17.70
46	0.39	3.57	0.83	0.83
52	2.66	30.40	0.90	20.10

[0508] *CAR CD19 binders were not detected by an anti-FMC63 idiotype.*

[0509] To determine the relationship between the novel binders and a well characterized CD19 binder (CD19-FMC63), the effect of the anti-FMC63 idiotype antibody on the binders was determined. FMC63 is a common scFv used in research and clinic. The anti-FMC63 antibody is a very good anti-idiotype probe for the control CD19 CAR (e.g., FMC63 CAR). For these experiments, the anti-FMC63 antibody was incubated with ND539 CD19 CAR T

cells expressing CARs comprising CD19 binder 42, 43, 44, 45, 46, or 52 at day 9 (D9) post-transduction. The binding of the anti-FMC63 antibody to the CAR was assessed by flow cytometry and gated for anti-FMC63.

[0510] As shown in **Table 9** (Anti-FMC63 only), the anti-FMC63 antibody did not detect any CAR T cells comprising a novel CD19 binder 42, 43, 44, 45, 46, or 52. These results are illustrated in **FIG. 12A**. The anti-FMC63 antibody effectively bound to the control FMC63 CAR T cells (mfi = 38.6%) when compared to untransduced cells (background, mfi = 0.47%). The anti-FMC63 antibody weakly bound to CD19-52 binder (mfi = 2.66). The anti-FMC63 antibody did not bind CAR T cells expressing the novel CD19 binders 42, 43, 44, 45, and 46. The binding for novel CD19 binders 42, 43, 44, 45, and 46 were respectively, 0.18, 0.19, 0.52, 0.43, and 0.39 (background). These results were in sharp contrast to the binding of the recombinant CD19 protein to the same CAR T cells, which recognized many (42, 44, 45, and 30.4), but not all these binders (43 and 46).

[0511] *The CD19 binders 42, 43, 44, 45, 46, and 52 may not bind to the same region as the anti-FMC63 on the CD19 protein.*

[0512] To determine whether the anti-FMC63 antibody and the CD19 binders could bind the same region on the CD19 protein, competitive assays were performed. In the first set of experiments, the CD19 CAR T cells were first incubated with 1ul of anti-FMC63 antibody PE for 20 minutes. After the incubation period, 2ul of the CD19-FITC protein was added to the CAR T cells, which were further incubated for another 20 minutes. The CAR T cells were washed and assessed by Flow cytometry. The CAR T cells were gated for anti-FMC63 antibody (idiotype antibody binding) and the CD19 CAR binders (CD19-GFP detection).

[0513] As shown in **Table 9** (CD19-FITC Protein addition after anti-FMC63Ab PE), pre-incubation with the anti-FMC63 antibody weakly reduced the binding of the recombinant CD19 protein to the binders (**FIG. 12B**). This weak effect was also observed in the positive control CD19 binder (26.6 vs 31.1). CD19 binder 52 showed the greatest binding reduction (20.1 vs 30.4). The changes for CD19 binders 42, 44, 45, and 46 were respectively, 12.1 vs 16.3; 26.2 vs 23.2; 17.7 vs 23.1; 0.83 vs 3.57. The sequential co-incubation weakly enhanced the binding of the CD19-GFP protein to cells expressing CD19 binder 44 (**FIG. 12B**). No changes were detected for the CD19 binder 43(**FIG. 12B**). The sequential co-incubation did

not change the interaction of the anti-FMC63 antibody with the CD19 CAR T cells (**FIG. 12A**).

[0514] In the second set of assays, CAR T cells were co-incubated with the CD19-FTC protein and the anti-CD19 PE (FMC63 clones) at 1:1 or 1:3 ratio for 15 minutes. The CAR T cells were then stained and incubated for 20 minutes before imaging by flow cytometry. At 1:1 ratio, 2 ul CD19-FITC protein and 2ul Anti CD19 (FMC63 Clone)-PE were added to the CAR T cells. At 1:3 ratio, 2 ul CD19-FITC protein and 6ul anti CD19 (FMC63 Clone)-PE were added to the CAR T cells. The CAR T cells were gated for anti-CD19 PE (anti-FMC63 antibody; idiotype antibody binding) and the CD19 binder CAR binder FITC (CD19-GFP detection).

[0515] As shown in **Table 10**, the co-incubation blocked the binding of the anti-FMC63 idiopatic antibody to the positive control CD19 FMC63 CAR T cells at 1:1 and 1: 3 ratio (**FIG. 12A**). For the positive control, the mfi was 0.80 at 1:1 ratio and 1.63 at 1:3 ratio when compared to untransduced cells (2.25 and 2.95). The mfi from CAR T cells expressing binders 42, 43, 44, 46, and 52 were respectively 0.045, 0.041, 1.73, 0.12, and 3.29. The co-incubation increased the binding of the anti-FMC63 idiopatic antibody to CD19 CAR-46 T cells (antiCD19 PE mfi =15.9 (1:1 ratio) and 18.1 (1:3 ratio)) when compared to the binding to the control CD19 CAR-FMC63 T cells (antiCD19 PE mfi =0.8 and 1.63) (**FIG. 12A**).

[0516] At 1:1 ratio, the recombinant CD19-GFP protein was still able to bind CAR T cells expressing CD19 binders 42, 43, 44, 45, 46, and 52. The mfi of these cells at 1:1 ratio were respectively 6.23, 0.23, 15.9, 9.64, 0.43, and 14.5. At 1:3 ratio, the mfi of these cells were respectively 1.08, 0.23, 3.01, 4.76, 0.19, and 5.56. The perceived reduction in binding when compared to sequential co-incubation or no co-incubation may have been caused by the interaction of the recombinant CD19-GFP molecules to the anti-FMC63 antibody, independent of the CAR T cells.

Table 10: Anti-FMC63 blocked CD19 protein-CD19 CAR interaction on ND539 Frozen Cells- Day 9 post transduction

	1:1 ratio		**1:3 ratio**	
Novel CD19 binders-Clone #	Gated for Anti-CD19 PE Expression	Gated for CD19 -CAR - FITC expression	Gated for Anti-CD19 PE Expression	CD1 -CAR - FITC expression
Untransduced (NTD)	2.25	0.21	2.95	0.21

Table 10: Anti-FMC63 blocked CD19 protein-CD19 CAR interaction on ND539 Frozen Cells- Day 9 post transduction				
	1:1 ratio		**1:3 ratio**	
Novel CD19 binders-Clone #	Gated for Anti-CD19 PE Expression	Gated for CD19 -CAR - FITC expression	Gated for Anti-CD19 PE Expression	CD1 -CAR - FITC expression
CAR-FMC63	0.800	14.000	1.630	2.290
42	0.045	6.270	0.480	1.080
43	0.041	0.230	0.970	0.230
44	1.730	15.900	1.260	3.010
45	15.900	9.640	18.100	4.760
46	0.120	0.430	0.780	0.190
52	3.290	14.500	2.350	5.560

[0517] The co-incubation experiments of the anti-FMC63 and the CD19 protein showed a similar pattern of binding reduction in all tested groups (**FIG. 12B**).

[0518] Generally, the anti-FMC63 did not block the recombinant CD19-GFP protein's ability to detect the novel CD19 binders (**FIG. 12A**). Additional competitive assays are needed to determine whether the novel CD19 binders bind to the same epitope as the FMC63 antibody. It is most likely that that at least one novel CD19 binder binds to one or more novel epitopes on the CD19 proteins. Additional epitope mapping using SPR based assays (SPR sensograms) comparing the novel CD19 binders to known CD19 binder (e.g, FMC63 binder) would likely show that at least one novel CD19 protein described herein binds to a different epitope on the human CD19 protein than the epitope of human CD19 targeted by the antigen binding domain comprising a scFv from the FMC63 antibody or any other known CD19 binder.

[0519] Additional assays may include, a protection assay, such as e.g., a hydrogen/deuterium exchange (HDX) mass spectrometry assay.

Example 6: Codon optimized CD19binders 42, 43, 44, 45, 46, 51, and 52

[0520] Based on the results shown in Examples 1-5, seven out of twelve novel CD19 binders were selected for further evaluation based on their expression level, including CD19 binders 42, 43, 44, 45, 46, 51, and 52. These seven novel scFvs were codon optimized with the goal of enhancing their binding affinity and stabilizing the CAR expression. The codon optimized CD19 binders were then evaluated on two different donors.

[0521] Codon optimized CD19 binders 42, 43, 44, 45, 46, 51, 52 were generated and CARs comprising antigen binding domain comprising each of the optimized CD19 binders were engineered. These optimized CD19 binders were evaluated for tonic signaling, their expansion profiles, the stability of surface detection (e.g., CAR surface mfi and stability), functional performance in cell killing, persistence of performance in long-term co-cultures and ability to produce cytokines. As described herein, the codon optimized versions of the CD19 binders stabilized and/or enhanced the CD19 CARs performance.

[0522] *NFAT Activation Kinetics*

[0523] The optimized CARs were transduced at high levels on the Jurkat NFAT-GFP reporter cells to enhance the observation of tonic signaling. Most of these optimized CD19 CARs were well expressed on the Jurkat NFAT-GFP reporter cells (**Table 11**). The mfi for CD19 CAR 42OP, 43OP, 44OP, 45OP, 45OP, 46OP, 51OP, and 52OP were respectively, 61.4, 60.2, 47.4, 44.2, 36.1, 71.5, and 70.9. Analysis of tonic signaling was conducted based on the percentage of CAR T cells that were GPF and CAR positive (GFP^+ CAR^+).

Table 11: Surface expression and Tonic Signaling in optimized CD19 CAR T cells

Novel CD19 binders-Clone #	CD19 CAR Surface expression(mfi)	Tonic Signaling (mfi)
42OP	61.40	7.20
43OP	60.20	7.05
44OP	47.40	32.00
45OP	44.20	35.90
46OP	36.10	33.90
51OP	71.50	8.06
52OP	70.90	7.98

[0524] As shown in **Table 11** and illustrated in **FIG. 3B**, tonic signaling was low in CAR T cells expressing the 42OP binder (mfi =7.20), 43OP binder (mfi =7.05), 51OP binder (mfi =8.06), and 52OP binder (mfi =7.98). However, CAR T cells expressing the 44OP binder (mfi =32.0), 45OP binder (mfi =35.9), and 46 OP binder (mfi =33.9) showed enhanced tonic signaling.

[0525] Thus, expression of these optimized CD19 CARs induced tonic signaling and NFAT activation without ligand activation. CD19 binders 42OP, 43OP, 51OP, 52OP had relatively low levels of tonic signaling. This low signaling correlated with the level of tonic signaling

observed in a control CD19 CAR (e.g., a CAR comprising a scFv from the anti-FMC63 antibody).

[0526] To determine whether optimization enhanced the activation kinetics of the optimized CD19 CARs, CD19 CARs comprising optimized CD19 binder 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP were transduced in Jurkat NFAT-GFP reporter cells. Transduced CAR T cells were then co-culture with wildtype Nalm6 cells, expressing a CD19 antigen. All transduced T cells had a similar MOI range. T cells with low CAR transduction were used to monitor the kinetics of NFAT activation. The percent transduction was selected at 8-17% for activation of single integration event by flow cytometry analysis. As shown in **Table 12**, the transduction efficiency of 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP CD19 CARs, based on anti-CD19 staining (PE-A), was respectively 15.7%, 17.1%, 10.9%, 16.9%, 7.28%, 7.79%, and 8.23%.

Table 12: CD19 CAR Transduction efficiency for Activation Kinetics Assay

	CD19 CAR Surface expression(Count)
42OP	15.70
43OP	17.10
44OP	10.90
45OP	16.90
46OP	7.28
51OP	7.79
52OP	8.23

[0527] After co-culturing the CAR^+ T cells with Nalm6-wt cells, the induction of NFAT (e.g., GFP) was measured as intensity over time using the IncuCyte® live-cell analysis system (**FIG. 13**). The NFAT induction, which correlated to the activation of the optimized CD19 CARs was observed for about 24 hours. The activation of the optimized CD19 CARs began at about 2-3 hours and maximized at about 10 hrs of co-culture with Nalm6 cells (**FIG. 13**). Specifically, the activation kinetics of CARs comprising the optimized CD19 binders 42 (42 op) was the fastest and showed the highest induction levels of NFAT. At about 14 hrs, the integrated green intensity of CD19 42OP was about $4x10^4$ to about $5x10^4$ when compared to about $2x10^4$ for CD19 52OP and CD19 45OP and less than $1x10^4$ for CD19 44OP, CD19 46OP, 51OP, and CD19 43OP. At the end of the assay, the integrated green intensity of

CD19 42OP was about $3x10^4$ to about $4x10^4$ when compared to about $1x10^4$ for CD19 52OP and CD19 45OP and less than $1x10^4$ for CD19 44OP, CD19 46OP, 51OP, and CD19 43OP.

[0528] CARs comprising CD19 binders 45OP and 52OP had similar and intermediate NFAT induction kinetics (**FIG. 13**). CARs comprising CD19 binders 44op and 46op had low NFAT induction kinetics. CARs comprising CD19 binders 43OP and 51OP had minimal NFAT induction. These results suggested that the 42OP, 45OP, and 52OP generated a good kinetic NFAT response following activation by Nalm6 cells. This response was the same or higher than NFAT kinetic response observed with existing CD19 CARs.

[0529] *Expansion profile of optimized CD19 binders*

[0530] CAR comprising the codon optimized CD19 binders were transduced into two normal donors ND518 and ND528 and their doublings (expansion) and sizes (contraction) were evaluated over the expansion period which lasted about 15 days. **FIGs. 15A and C** show that the doubling in manufacturing was higher for optimized CD19 binders 42OP and 52OP for both donors. The observed population doubling was substantially similar to and/or better than a positive control CD19 CAR. **FIGs. 15B and D** show that the contraction of cell sizes was substantially similar in all groups tested. In particular, the contraction of cell sizes correlated with the population doubling data in that CAR T cells expressing a CAR with the CD19 binder 42OP and 52OP rested down (contracted) first. The surface expression profile of the CARs were monitored by flow cytometry on day 7 and day 11 (**FIG. 14)**.

Table 13: Expression profiles (percent) for optimized CAR-CD19 binders in ND518 and ND528 expansion shown in FIG. 14

	ND518		**ND528**	
	Day 7(D7)	**Day 11(D11)**	**Day 7(D7)**	**Day 11(D11)**
42OP	61.30	N/A	54.30	N/A
43OP	24.90	N/A	13.10	N/A
44OP	50.30	N/A	42.10	N/A
45OP	72.40	N/A	60.70	N/A
46OP	18.50	N/A	19.90	N/A
51OP	13.50	N/A	8.74	N/A
52OP	59.50	N/A	58.60	N/A

[0531] On Day 7, the expression of most CARs in ND518 or ND528 remained relatively stable in transduction percentages. For example, in ND518, the MFI for CARs comprising optimized CD19 binders 42, 44, 45 and 52 was respectively 60.1%, 50.3%, 72.4%, and

59.5%. CARs comprising optimized CD19 binders 43, 46 and 51 had low detectable levels on CAR T cell surface, which were respectively, 24.9%, 18.5%, and 13.5%. Substantially similar expression profiles were observed in ND528 T cells. Specifically, the MFI for CARs comprising optimized CD19 binders 42, 44, 45 and 52 was respectively 54.3%, 42.1%, 60.7% and 58.6%. CARs comprising optimized CD19 binders 43, 46 and 51 had low detectable levels on CAR T cell surface which were respectively, 13.1%, 19.9%, and 8.74%. The surface expression profiles remained relatively stable at day 7 and day 11.

[0532] The total CAR protein levels of optimized CD19 CARs expressed in ND518 CAR T cells were evaluated using western blot (**FIG. 11B**). Optimized CD19 binders 52, 51, and 43 were expressed as a single isoform. Only the higher molecular weight species was expressed. Two CD19 CAR isoforms were detected in CAR T cells expressing optimized CD19 binders 42, 44, 45, and 46. However, the highest molecular weight isoform appeared to be dominantly expressed in these cells when compared to cells expressing their original counterparts (**FIG. 11A**).

[0533] To determine whether codon optimization affected the CAR expression, the optimized CD19 CAR expression levels were compared to the original CD19 CAR expression (**FIG. 11B**). The two CD19 CAR isoforms were observed by western blot in both groups. However, the expression levels of the two CAR band sizes showed a shift in the expression of the isoform with the highest molecular weight in CAR T cells expressing the optimized CD19 CARs (**FIG. 11B** vs **11A**). The upper band (higher molecular weight isoform) was the most dominant band across all tested optimized CD19 binder CARs. A CAR comprising the CD19 binder 52 (52OP) only showed a single upper band in both original and optimized versions.

[0534] The percentages of $CD4^+$ and $CD8^+$ T cells in ND518 and ND528 that expressed the CD19 CARs were similar across all tested CD19 binders (**FIGs. 24A-B**). However, $CD8^+$ T cells showed a reduced expression of CAR comprising the CD19 binder 45OP (CAR45OP). Indeed, the percent T cell population expressing the CAR45OP shifted with less $CD8^+$ when compared to $CD4^+$ (**FIGs. 24A-B**).

[0535] *Cytokine production*

[0536] The ability of CARs comprising the optimized CD19 binders to produce cytokines after a 4 hr co-culture with Nalm6 cells was also evaluated by intracellular detection using

flow cytometry of IL-2, TNFα, and/or IFNγ staining. The CD19 binders were transduced in ND518 donor T cells, which were then stimulated with Nalm6 cells, PMA/ionomycin or left unstimulated.

Table 14: Cytokine response from Optimized CD19 ND518 CAR T Cells						
	Nalm6 Stimulation					
	IL-2	**TNF-α**	**IL-2 & TNF-α**	**IFN-γ**	**TNF-α**	**IFN-γ & TNF-α**
42OP	1.270	6.980	5.990	8.130	8.73	3.960
43OP	2.010	9.960	6.590	5.240	11.900	3.920
44OP	2.790	14.100	10.300	6.670	17.800	5.660
45OP	4.500	13.800	23.100	7.010	25.600	10.400
46OP	1.600	9.010	5.810	3.470	10.400	3.850
51OP	0.100	1.740	0.390	1.760	1.450	0.460
52OP	1.890	11.300	8.400	6.300	13.900	5.080
	No stimulation (Unstimulated)					
	IL-2	**TNF-α**	**IL-2 & TNF-α**	**IFN-γ**	**TNF-α**	**IFN-γ & TNF-α**
42OP	0.400	0.160	0.380	1.090	0.160	0.310
43OP	0.150	0.810	0.300	0.770	0.680	0.160
44OP	0.300	1.500	0.720	1.130	1.410	0.290
45OP	0.490	0.830	0.840	1.440	0.960	0.300
46OP	0.094	0.990	0.210	0.770	0.890	0.110
51OP	0.068	0.260	0.140	0.770	0.210	0.130
52OP	0.160	0.110	0.370	2.130	0.180	0.280
	PMA/Ionomycin Stimulated					
	IL-2	**TNF-α**	**IL-2 & TNF-α**	**IFN-γ**	**TNF-α**	**IFN-γ & TNF-α**
42OP	7.150	12.900	42.700	5.470	34.200	20.900
43OP	8.940	19.400	53.800	6.860	43.700	28.700
44OP	13.700	16.500	52.700	8.430	43.800	24.500
45OP	10.600	14.100	63.300	6.020	48.700	27.700
46OP	8.600	21.100	51.100	6.120	43.000	28.600
51OP	10.900	21.000	44.700	10.300	38.100	26.800
52OP	9.280	16.500	47.800	7.940	38.900	25.000

[0537] Table 14 shows raw cytokine production data of CAR T cells expressing a CAR comprising an optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP and 52OP. These data show that these optimized CAR T cells produced significant levels of IL-2, TNFα, and IFNγ when stimulated with Nalm6 cells. *See also* **Table 6** and **Table 24**. Optimized CD19 CAR T cells cytokine production is also illustrated in **FIG. 5B**. Cytokine production in CAR T cells expressing the CD19 binder 51OP was not significantly higher than background. This lack of cytokine production was consistent with the cytokine production of CAR T cells expressing the original (un-optimized,) CD19 binder 51 (**FIG. 5A; Table 6**). **Table 14** shows that all optimized CD19 CAR T cells tested produced IL-2, TNFα, and IFNγ upon PMA/ionomycin stimulation.

[0538] *Conclusion*

[0539] Generally, no absolute correlation was observed between the detectable surface expression of a CAR comprising a novel CD19 binder and the total protein expression level and/or cytokine production. Low detectable surface expressed CAR CD19 binders produced similar levels of protein as well expressed CD19 binders. This was the case for CAR T cells expressing CD19 binders 46 and 51. In addition, some lowly expressed CD19 CAR (*e.g.*, those with detectable surface expressed CD19 CAR or those with the lowest levels of total proteins) produced levels of cytokines that were comparable to well-expressed CD19 binders. For example, CD19 binder 46 produced cytokines and CD19 binder 51 did not produced cytokine.

[0540] These results indicated that low detectable surface CAR CD19 binder expression could produce similar levels of total protein as higher detectable binders, e.g., CD19 CAR 46 and 51 (**Table 13** and **FIGs. 11A-B**). Conversely, good detection of a CAR surface expression did not guarantee its ability to produce cytokines as shown with e.g., CD19 binders 46 and 51 (**Tables 13-14**). In addition, low detectable surface expression of CAR CD19 binder 43 produced the lowest levels of protein when compared to other novel CD19 binders. Yet, CAR T cells expressing a CAR comprising CD19 binder 43 produced comparable levels of cytokines (**Tables 13-14, FIGs. 11A-B**). Together, these results suggested that the CD19 binders showed unique functional profiles when compared to known CD19 binders. In particular, CAR comprising optimized CD19 binders 42 and 52 showed functional profiles that were similar to each other. In addition, CD19 binders 42 and 52 appeared to be more efficient at killing tumor cells than a control CD19 binder (*e.g.*, a CD19 binder comprising FMC63 scFv).

Example 7: Effective CD19 CAR T cell killing and persistence

[0541] An effective CAR T cell is one that can kill and persist. To determine which of the novel CD19 binders would endow CAR T cells with these two properties (e.g., the best candidates matching this criterion) an activation stress test was performed. Thawed ND528 cells transduced with the optimized CD19 CARs were used for serial re-stimulation studies and for evaluating cell killing properties.

[0542] Re-stimulation stress test of ND528 CAR T cells expressing optimized CD19 binders

was performed using optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP. The killing target cells were Nalm6 cells. The low expression of CD19 binders 43OP, 46OP, and 51OP made testing difficult. At the end of each stimulation process, CD19 CAR T cells were stained and the numbers of live cells were determined by flow. New co-cultures were established and CAR T cells were evaluated using flow cytometry for T cell phenotypes (cytotoxicity).

[0543] In the activation stress test, CAR T cells expressing a novel CD19 binder (*e.g.*, 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52OP) were co-cultured with wild type (wt) Nalm6 at a 4:1 ratio of Targets:CAR T cells on day 0 (D0). The cultures were evaluated every 3 to 4 days by flow cytometry and selected based on CD45 expression ($CD45^+$) (**FIG. 16A**). These co-cultures were re-stimulated (*i.e.*, re-established co-cultures) at 4:1 for 6 rounds. Specifically, the cells were re-stimulated on Day 4, (Round 1, D4), Day 7 (Round 2, D7), Day 11 (Round 3, D11), Day 14, (D14, R4), Day 18 (Round 5, R5), and Day 21 (Round 6, R6).

[0544] The scheme for screening activated cells is shown in **FIG. 16B**. The cells were serially gated as shown in **FIG. 16B** to identify the correct population of CD19 CAR T cells. Initially, CAR T cells were gated for live cells and selected. Then, the live cells were gated for Nalm6 and Nalm6 negative ($Nalm6^-$) cells were selected. These $Nalm6^-$cells were then gated for human CD45 (huCD45), and $huCD45^+$ cells were selected. Lastly, $huCD45^+$ cells were gated for CD8 (CD137, CCR7, and PD1) and CD4 (ICOS, CD45RO, and CD69).

[0545] *Titrated cell killing*

[0546] To determine the ability of each of the engineered CART cells to kill target cells (*e.g.*, Nalm6) from the start, the thawed ND528 CAR T cells used for restimulation stress test, were evaluated for real time cell killing of one cycle at 3:1, 1:1, 1:3 and 1:10 CAR^+:Nalm6 wt ratios.

[0547] The titrated cell killing results shown in **FIG. 17** indicated that all CD19 CAR T cells tested killed target cells at relatively the same level. The initial count per image was about 1,000 for CD19-42OP CAR T cells. At 45 minutes, the count was less than 50 in 1:1 and 3: 1 CAR^+:Nalm6 wt ratios. However, in the 1:10 CAR^+:Nalm6 wt ratio, the count was over 2500; and at 1:3 CAR^+:Nalm6 wt ratio, the count was about 1500.

[0548] In the CD19-45OP CAR T cells, the count per image was about 900. At 45 minutes, the count was less than 50 in 1:1 and 3: 1 CAR^+:Nalm6 wt ratios. However, in the 1:10 CAR^+:Nalm6 wt ratio, the count was over 2500; and at 1:3 CAR^+:Nalm6 wt ratio, the count was about 500. In the CD19-44OP CAR T cells, the count per image was about 900. At 45 minutes, the count was about 0 in 3: 1 CAR^+:Nalm6 wt ratio. In 1:1 CAR^+:Nalm6 wt ratio, the count was less than 50. However, in the 1:10 CAR^+:Nalm6 wt ratio, the count was over 1100; and at 1:3 CAR^+:Nalm6 wt ratio, the count was about 900. In the CD19-52OP CAR T cells, the count per image was about 900. At 45 minutes, the count was about 0 in 3: 1 and 1:1 CAR^+:Nalm6 wt ratios. However, in the 1:10 CAR^+:Nalm6 wt ratio, the count was over 2000; and at 1:3 CAR^+:Nalm6 wt ratio, the count was about 1000.

[0549] These results showed that the optimized CD19 CAR T cells killed target cells within about 45 minutes at 1:1 and 3:1 CAR^+:Nalm6 wt ratios. At 1:10 and 1:3 ratios, there was an enhancement in the cell count rather than a decrease. The CAR T cells showed similar killing (e.g., cytotoxic) efficacy for CD19 42OP CAR T cells, CD19 44OP CAR T cells, CD19 45OP CAR T cells, and CD19 52OP CAR T cells.

[0550] *$CD8^+$ T cells expansion and $CD4^+$ T cells collapse.*

[0551] Concurrently, these same thawed ND528 CAR T cells were used to initiate the six rounds of restimulation stress tests to determine their ability to persist as effective therapeutics. In the first round, the CD19 CAR T cells were co-cultured with target cells, either Nalm6-wt or Nalm6-CD19KO. During the course of the six rounds of restimulation with Naplm6-wt, the percent increase in the population of $CD8^+$ and $CD4^+$ CAR T cells was evaluated.

[0552] Expansion of ND528 $CD4^+$ and $CD8^+$ CD19 CAR T cells over 6 re-stimulations was assessed (**Table 15**). At the end of stimulation 1 with Nalm6-CD19KO, the population of $CD4^+$ T cells in CAR T cells expressing 42OP, 44OP, 45OP, and 52OP were respectively, 53.6%, 40.1%, 47.6%, and 35.5%. A similar trend was observed in CAR T cells stimulated with Nalm6-WT. In particular, at the end of stimulation 1, the population of $CD4^+$ T cells in CAR T cells expressing 42OP, 44OP, 45OP, and 52OP were respectively, 53.6%, 40.1%, 47.6%, and 35.5%.

[0553] Over the course of the six rounds of stimulation, the population of $CD4^+$ T cells in ND528 CD19-42OP CAR T cells at the end of each round (R) was 53.6% (R1), 33.1% (R2), 15.7% (R3), 11.9%(R4), 9.47% (R5), and 8.89% (R6). The population of $CD4^+$ T cells in ND528 CD19-44OP CAR T cells at the end of each round (R) was 40.1% (R1), 18.6% (R2), 11.2% (R3), 9.11%(R4), 9.73% (R5), and 11.9% (R6). The population of $CD4^+$ T cells in ND528 CD19-45OP CAR T cells at the end of each round (R) was 47.6% (R1), 25.9% (R2), 14.8% (R3), 11.4%(R4), 9.84% (R5), and 9.68% (R6). The population of $CD4^+$ T cells in ND528 CD19-45OP CAR T cells at the end of each round (R) was 35.5% (R1), 19.0% (R2), 10.9% (R3), 7.48%(R4), 9.84% (R5), and 6.11% (R6). Generally, CD19 42OP CAR T cells, CD19 44OP CAR T Cells, CD19 45OP CAR T cells, CD19 52OP CAR T cells and a control CAR T cell comprising anti-CD19 scFv showed a similar population decrease trend.

[0554] At the end of Stimulation 1 with Nalm6-CD19KO, the population of $CD8^+$ T cells in CAR T cells expressing 42OP, 44OP, 45OP, and 52OP were respectively, 30.6%, 42.0%, 19.5%,54.3% and 48.0%. A similar trend was observed in CAR T cells stimulated with Nalm6-WT. In particular, at the end of stimulation 1, the population of $CD8^+$ T cells in CAR T cells expressing 42OP, 44OP, 45OP, and 52OP were respectively, 30.6%, 42.0%, 19.5%,54.3% and 48.0%.

[0555] Over the course of the six rounds of stimulation, the population of $CD8^+$ T cells in ND528 CD19-42OP CAR T cells at the end of each round (R) was 30.6%(R1), 41.2%(R2), 48%(R3), 54.3%(R4), 60.4%(R5), and 67.8% (R6). The population of $CD8^+$ T cells in ND528 CD19-44OP CAR T cells at the end of each round (R) was 42.0%(R1), 54.3%(R2), 56.6%(R3), 62.1%(R4), 62.0%(R5), and 62.4% (R6). The population of $CD8^+$ T cells in ND528 CD19-45OP CAR T cells at the end of each round (R) was 19.5%(R1), 27.7%(R2), 24.6%(R3), 33.0%(R4), 28.9%(R5), and 30.1% (R6). The population of $CD8^+$ T cells in ND528 CD19-52OP CAR T cells at the end of each round (R) was 48.0%(R1), 54.3%(R2), 55.2%(R3), 62.4%(R4), 64.4%(R5), and 70.2% (R6).

[0556] Thus, in ND528, the manufacturing expansion led to different levels of $CD8^+$ T cell population in control CD19 CAR, CD19 52OP CAR T cells, CD19 44OP CAR T cells, CD19 42OP CAR T cells, and CD19 45OPCAR T cells. For example, the $CD8^+$ T cell population were respectively about 51.9%, 48%, 42%, 30.6% and 19.5%. CD19 42OP CAR T cells,

CD19 44OP CAR T Cells, CD19 45OP CAR T cells, CD19 52OP CAR T cells and a control CAR T cell comprising anti-CD19 scFv showed a similar population increase trend.

[0557] The changes in $CD8^+$ T cell population were respectively 55% for CD19 42OP CAR T cells (30.6 to 67.8); 33% for CD19 44OP CAR T Cells (42 to 62.4); 35% for CD19 45OP CAR T cells (19.5 to 30.1); 32% for CD19 52OP (48 to 70.2); and 35% for the control CAR (51.9 to 79.7).

[0558] Relatively similar expansion was observed in all tested ND528 CD19 $CD4^+$ and $CD8^+$ CAR T cells over 6 re-stimulations. The percentages of $CD8^+$ and $CD4^+$ T cells at the beginning of the restimulation test (*e.g.*, end of simulation 1) were respectively (a) 30.6% and 53.6% for CD19 42OP CAR T cells, (b) 42% and 40.1% for CD19 44OP CAR T cells; (c) 19.5% and 47.6% for CD19 45OP CAR T cells, and (d) 48% and 35.5% for CD19 52OP CAR T cells. The percentages of $CD8^+$ and $CD4^+$ T cells at the end of the restimulation test (*e.g.*, end of simulation 6) were respectively (a) 67.8% and 8.9% for CD19 42OP CAR T cells, (b) 62.4% and 11.9% for CD19 44OP CAR T cells; (c) 30.1% and 9.7% for CD19 45OP CAR T cells; and (d) 70.2% and 6.1% for CD19 52OP CAR T cells. The loss of $CD8^+$ T cells in CAR T cells expressing CD19 45OP was unexpected.

[0559] CD19 42OP CAR T cells were an exception to this trend because the increase in $CD8^+$ T cell population was faster. For example, the initial $CD8^+$ T cell population in CD19 42OP CAR T cells was about 30.6%, yet it reached substantially similar final $CD8^+$ T cells levels as other tested CD19 CAR T cells (about 67.8%) within the same time frame. In contrast, an unusually high levels of $CD4^-CD8^-$ CAR T cells population were found in the CD19 45OP CAR T cells.

[0560] *Tumor clearance*

[0561] The ability of the novel CD19 CAR T cells to maintain tumor clearance during the stress test was analyzed by flow cytometry. Dead cells were identified based on the IRFP720 fluorescence exclusion. Table 16 shows raw data from flow cytometry analysis of the long term Nalm6 cells killing activity of CD19 CAR T cells expressing CARs comprising CD19 binders 42OP, 44OP, 45OP, and 52OP. As shown in **Table 16**, CD19 CAR T cells maintained long term cell killing activity over the course of the six restimulations. For the cytotoxicity assay, flow cytometry was gated for Nalm6 cells and viability was assessed

based on the exclusion of IRFP720 fluorescence. IRFP720 negative ($IRFP720^-$) cells were dead and IRFP720 positive ($IRFP720^+$) cells were alive.

[0562] At the end of Stimulation 1 with Nalm6-CD19KO, the population of $IRFP720^+$ cells exposed to CAR T cells expressing 42OP, 44OP, 45OP, and 52OP were respectively, 95.4%, 88.1%, 90.6% and 94.4%. While the population of $IRFP720^-$ cells was respectively 4.34%, 11.9%, 9.33%, and 5.46%.

[0563] Specifically, over the course of the restimulation, the population of $IRFP720^+$ Nalm6 cells exposed to CD19-42OP CAR T cells was 91.9%(R1), 5.92%(R2), 0.01%(R3), 4.53E-3%(R4), 5.69E-3%(R5), and 0%(R6). Conversely, the population of $IRFP720^-$ Nalm6 cells(dead cells) exposed to CD19-42OP CAR T cells was 7.99%(R1), 93.0% (R2), 99.9%(R3), 99.9%(R4), 99.9%(R5), and 99.9%(R6). The population of $IRFP720^+$ Nalm6 cells exposed to CD19-44OP CAR T cells was 22.0%(R1), 0.016%(R2),14.0%(R3), 0.02%(R4), 77.7%(R5), and 90.4%(R6). Conversely, the population of $IRFP720^-$ Nalm6 cells(dead cells) exposed to CD19-44OP CAR T cells was 77.9%(R1), 99.6% (R2), 85.9%(R3), 99.8%(R4), 22.1%(R5), and 7.83%(R6). The population of $IRFP720^+$ Nalm6 cells exposed to CD19-45OP CAR T cells was 71.5%(R1), 1.39%(R2), 1.79%(R3), 1.09%(R4), 73.3%(R5), and 82.6%(R6). Conversely, the population of $IRFP720^-$ Nalm6 cells(dead cells) exposed to CD19-45OP CAR T cells was 28.4%(R1), 98.0% (R2), 98.1%(R3), 98.5%(R4), 26.3%(R5), and 16.7%(R6). The population of $IRFP720^+$ Nalm6 cells exposed to CD19-52OP CAR T cells was 81.0%(R1), 0.04%(R2), 0.01%(R3), 0.01%(R4), 1.01%(R5), and 0.02%(R6). Conversely, the population of $IRFP720^-$ Nalm6 cells(dead cells) exposed to CD19-52OP CAR T cells was 18.9%(R1), 98.1% (R2), 99.9%(R3), 99.8%(R4), 98.8%(R5), and 99.9%(R6).

[0564] These results showed that CD19 CAR T cells expressing a CAR comprising CD19 binder 42OP or 52OP maintained long term cell killing over all six stimulation tests. At the end of the 6th stimulation 99.9% of Nalm6 cells remained dead in cells cocultured with CD19-42OP CAR T cells or CD19-52OP CAR T cells. CD19 binders 42OP, 52OP showed similar long-term ability in persistence and tumor clearance. CD19-44OP CAR T cells showed the greatest tumor clearance.

[0565] In contrast, at the end of the first four day rounds of re-stimulation between Nalm6-wt and Nalm6-CD19KO, 44OP and 45OP showed a greater number of dead Naml6 compared to 42OP, 52OP, but at the end of the 6th stimulation, the population of Naml6 had recovered with each having 90.4% (44OP) and 82.6% (45OP) IRFP720^{+} Nalm6 cells. Yet, in the absence of CD19 activation by Nalm6 (e.g., Nalm6-CD19KO co-culture), 44OP had the highest level of cell death (11.9%) suggesting a higher level of basal activation. By the end of the fifth and sixth rounds of re-stimulation, 44OP and 45OP had failed to control the tumor and showed a decline of surviving T cells, suggesting a failure in long term therapeutic persistence.

[0566] *Expression of Activation Markers CD137, PD1, CD69, and ICOS*

[0567] To determine the specificity of CD19 CAR T cells for the CD19 antigen, the expression of early activation markers, CD137, PD1, CD69, and ICOS in CAR T cells after the first round of stimulation with either Nalm6-WT cells or Nalm6-CD19KO cells was determined. **Table 17** shows raw data from flow cytometry analysis of the expression of these markers at the end of the first round of stimulation.

[0568] As shown in **Table 17**, the expression of CD137, PD1, CD69 was enhanced in CD19 CART cells co-cultured with Nalm6-WT when compared to CD19 CAR T cells co-cultured with CD19 knock-out(KO) Nalm6. In CD19-42OP CAR T cells, CD139 expression was 51.0% compared to 0.31% (CD19KO); PD1 expression was 80% compared to 32.7% (CD19KO); CD69 expression was 76.3% compared to 20.4%; ICOS expression was 3.72% compared to 1.77% (CD19KO). In In CD19-52OP CAR T cells, CD139 expression was 30.0% compared to 0.28% (CD19KO); PD1 expression was 74.8% compared to 45.1%(CD19KO); CD69 expression was 58.2% compared to 22.6%; ICOS expression was 4.19% compared to 14.5% (CD19KO). After one round of stimulation, these activation markers showed specificity to Nalm6-wt but not to Nalm6-CD19KO.

[0569] In addition, co-cultures with targeted CD19 expressed on Nalm6-wt cells led to enhanced CD137, PD1 and CD69 expression after the first round of stimulation **Table 17**.

Table 17: Expression of Activation Markers in $CD4^+$ T Cells at the End of Round 1 stimulation						
	Nalm6-Wt Stimulation			**Nalm6-CD19KO Stimulation**		
	CD137					
	CD19 CAR	**CD137**	**CAR CD137**	**CD19 CAR**	**CD137**	**CAR CD137**
42OP	0.450	51.000	14.800	55.800	0.310	0.670
44OP	1.770	15.200	4.820	35.100	0.230	0.430
45OP	3.650	17.300	9.190	53.600	0.210	0.770
52OP	2.000	30.000	14.90	49.300	0.280	0.340
Control	1.010	39.600	7.580	55.000	0.094	0.440
PD1						
	CD19 CAR	**PD1**	**CAR PD1**	**CD19 CAR**	**PD1**	**CAR PD1**
42OP	0.130	80.000	17.400	7.170	32.700	50.700
44OP	0.190	79.100	7.5400	6.700	39.500	30.000
45OP	0.160	78.300	14.400	5.800	35.000	49.500
52OP	0.240	74.800	18.900	1.730	45.100	49.500
Control	0.092	84.100	10.400	2.970	39.000	53.800
CD69						
	CD19 CAR	**CD69**	**CAR CD69**	**CD19 CAR**	**CD69**	**CAR CD69**
42OP	0.120	76.300	16.500	21.300	20.400	36.100
44OP	0.430	55.300	6.920	21.500	15.900	14.800
45OP	0.500	58.300	13.400	27.900	15.600	27.100
52OP	0.830	58.200	17.500	15.000	22.600	35.500
Control	0.170	76.300	9.720	24.800	19.300	31.600
ICOS						
	CD19 CAR	**ICOS**	**CAR ICOS**	**CD19 CAR**	**ICOS**	**CAR ICOS**
42OP	8.780	3.720	1.070	46.900	1.770	5.790
44OP	2.780	6.870	1.050	20.300	3.960	11.600
45OP	5.430	12.600	2.850	32.400	5.280	19.000
52OP	10.100	4.190	1.370	10.600	14.50	35.000
Control	3.780	3.360	0.720	33.600	8.510	18.000

[0570] These data show the specificity of CD19 CART cells for the CD19 antigen expressed on Nalm6-WT when compared to CD19 knock-out (KO) Nalm6 based on the activation of T cells markers (CD137, PD1, CD69 and ICOS). After one round of stimulation, these activation markers showed specificity to Nalm6-wt but not to Nalm6-CD19KO.

[0571] The expression of the activation markers were also assessed over the 6th rounds of stimulation (**Table 18**). **Table 18** shows data from flow cytometry that assessed the expression of activation receptor, CD137, on $CD4^+$ T cells over time. These results show general trend toward a decrease in CD137 expression.

[0572] Table 18 shows that the expression of the CD137 on CD4+ T cells may have been downregulated over the six round of stimulation (*e.g.*, high internalization or membrane turnover following activation). Alternatively, the cell surface or the epitope binding was blocked, for example after CD19 engagement or binding. This was because when T cells co-cultured with Nalm6wt were compared to those co-cultured with Nalm6-CD19KO, the expression of CD19 CARs was no longer detectable on CAR T cells co-cultured with nalm6-wt. However, their surface expression remained detectable following Nalm6-CD19KO co-culture. Accordingly, it is most likely that internalization or high membrane turnover following CAR activation is a unique feature of the novel CD19 binders described herein because most antigen targeting CARs showed lower decrease in surface detection.

Table 18: Expression of Activation Marker in $CD4^+$ T Cells over time

	Nalm6-CD19KO Stimulation		
	CD19 CAR	**CD137**	**CAR CD137**
42OP	55.800	0.310	0.670
44OP	35.100	0.230	0.430
45OP	53.600	0.210	0.770
52OP	49.300	0.280	0.340
Control	55.000	0.094	0.440
Nalm6-WT Stimulation R1			
42OP	0.450	51.000	14.800
44OP	1.770	15.200	4.820
45OP	3.650	17.300	9.190
52OP	2.00	30.000	14.900
Control	1.010	39.600	7.580
Nalm6-WT Stimulation R2			
42OP	0.810	35.300	5.070
44OP	0.750	37.400	23.600
45OP	0.810	9.220	0.910
52OP	4.810	10.600	10.100
Control	4.420	15.900	9.890
Nalm6-WT Stimulation R3			
42OP	2.250	12.60	2.740
44OP	0.810	9.880	0.280
45OP	1.940	6.570	1.270
52OP	4.430	7.390	2.710
Control	1.620	16.80	1.540
Nalm6-WT Stimulation R4			

Table 18: Expression of Activation Marker in $CD4^+$ T Cells over time			
42OP	14.800	3.280	7.670
44OP	6.170	3.790	3.450
45OP	7.110	1.640	2.920
52OP	10.200	2.020	8.480
Control	11.100	2.560	13.400
Nalm6-WT Stimulation R5			
42OP	7.930	9.440	3.610
44OP	6.120	5.760	1.580
45OP	3.060	6.640	1.280
52OP	10.700	8.690	5.030
Control	8.570	6.850	4.080

[0573] By the end of the third re-stimulation (R3), 4-1BB (CD137) expression was no longer observed in all CAR T cells tested (e.g., CD19CAR binder 42OP, 44OP, 45OP, and 52OP). 4-1BB (CD137) was used as an early activation marker, but its expression or lack thereof may not measure CART cells tumor control.

[0574] To determine whether CAR T cells were exhausted on both $CD4^+$ CAR T cells and $CD8^+$ CAR T, the expression of PD1, CD69, and ICOS was evaluated.

[0575] **Table 19** shows the quantification of the expression of the CD19 CAR, the activation markers, PD1, CD69, and ICOS on $CD4^+$ T cells at the end of the second and fifth re-stimulation. After five rounds of re-stimulation, the expression of these receptors (PD1, CD69, and ICOS) on the $CD4^+$ T cell surface was downregulated. No significant difference between 42OP, 44OP, 45OP, and 52OP CD19 CAR T cells was observed.

Table 19: Expression of Activation Markers in $CD4^+$ T Cells						
	End of Stim 2 Nalm6-Wt			**End of Stim 5 Nalm6-Wt**		
PD1						
	CD19 CAR	**PD1**	**CAR PD1**	**CD19 CAR**	**PD1**	**CAR PD1**
42OP	0.250	79.900	9.810	6.540	29.700	5.000
44OP	0.760	64.400	29.300	4.690	32.600	3.010
45OP	0.410	69.400	2.380	1.650	42.800	2.680
52OP	1.320	64.800	17.200	6.530	36.800	9.180
CD69						

Table 19: Expression of Activation Markers in $CD4^+$ T Cells						
	CD19 CAR	**CD69**	**CAR CD69**	**CD19 CAR**	**CD69**	**CAR CD69**
42OP	0.066	89.000	5.820	7.260	34.400	4.290
44OP	0.074	71.600	24.300	2.600	64.300	5.100
45OP	0.150	70.400	1.570	0.880	76.600	3.450
52OP	0.690	68.100	14.300	7.150	60.300	8.560
ICOS						
	CD19 CAR	**ICOS**	**CAR ICOS**	**CD19 CAR**	**ICOS**	**CAR ICOS**
42OP	7.410	12.400	4.140	9.120	6.600	0.900
44OP	25.500	8.790	6.960	5.320	4.140	1.720
45OP	2.330	8.970	0.900	2.950	16.300	0.900
52OP	12.600	10.800	7.320	11.200	9.900	2.420

[0576] Table 20 shows the quantification of the expression of the CD19 CAR, the activation markers, PD1, CD69, and ICOS on $CD8^+$ T cells at the end of the second and fifth re-stimulation. After five rounds of re-stimulation, the expression of these receptors (PD1, CD69, and ICOS) on the $CD8^+$ T cell surface was downregulated. No significant difference between 42OP, 44OP, 45OP, and 52OP CD19 CAR T cells was observed.

Table 20: Expression of Activation Markers in $CD8^+$ T Cells						
	End of Stim 2 Nalm6-Wt			**End of Stim 5 Nalm6-Wt**		
PD1						
	CD19 CAR	**PD1**	**CAR PD1**	**CD19 CAR**	**PD1**	**CAR PD1**
42OP	0.064	91.400	6.070	2.210	34.700	2.320
44OP	0.059	90.600	6.820	0.500	34.400	0.590
45OP	0.082	88.200	2.660	0.720	36.400	0.820
52OP	0.590	81.400	9.670	2.090	47.300	4.620
CD69						
	CD19 CAR	**CD69**	**CAR CD69**	**CD19 CAR**	**CD69**	**CAR CD69**
42OP	0.180	78.700	5.950	2.330	33.000	2.200
44OP	0.210	82.000	6.660	0.230	56.300	0.860
45OP	0.440	48.000	2.300	0.150	67.900	1.390
52OP	1.960	49.700	8.310	2.250	56.600	4.460
ICOS						
	CD19 CAR	**ICOS**	**CAR ICOS**	**CD19 CAR**	**ICOS**	**CAR ICOS**

42OP	5.190	1.910	0.950	3.320	2.890	0.540
44OP	6.300	1.460	0.580	0.840	0.520	0.086
45OP	2.800	0.810	0.150	1.250	0.880	0.110
52OP	9.520	1.160	0.750	4.810	1.790	1.110

[0577] Tables 19-20 show that the expression of activation receptors, such as *e.g.*, PD1, CD69, and ICOS, on $CD4^+$ and $CD8^+$ T cells decreased over time. These results suggested that CAR T cells expressing CARs comprising CD19 binders 42OP, 44OP, 45OP, and 52OP were not exhausted. Rather, CAR T cells expressing CAR comprising the tested novel CD19 binders were likely downregulated over time and/or that binding to CD19 protein was likely blocked. These data suggested that $CD4^+$ CAR T cells and $CD8^+$ CAR T cells expressing a CAR comprising a CD19 binder 42OP, 44OP, 45OP, or 52OP were not exhausted.

[0578] *Conclusion*

[0579] These functional assays suggested that the novel CD19 binders described herein behaved as expected. These novel CD19 binders may be more efficient than known CD19 binders. Tables 21 and 22 summarize the functional characteristics of CD19 CAR T cells expressing optimized CD19 binders 42OP, 43OP, 44OP, 45OP, 46OP, 51OP, and 52 OP. The illustrated functional characteristics include tonic signaling, kinetics of cell activation; CAR T cells doublings over time and long term tumor control.

Table 21: Summary

	NFAT activation		**Manufactured CAR19binders**					
CAR-OP CD19binder	**Tonic Signaling**	**Activation in Jurkat**	**ND518 Doublings**	**ND528 Doublings**	**CAR expression D7 to D11**	ND518 %CD8 during expansion	ND528 %CD8 during expansion	Cell killing of Nalm6
42OP	Lo	Fast/High	6.84	7.40	Robust, slight mfi decrease	Similar	Similar	Similar
43OP	Lo	Minimal	5.40	6.52	Poor	Similar	Similar	N/A
44OP	High	Lo	5.12	6.63	Okay, stable	Similar	Decrease	Similar
45OP	High	Medium	3.82	4.23	Robust, slight mfi decrease	Decrease	Decrease	Similar
46OP	High	Lo	5.52	5.10	Poor	Similar	Decrease	N/A
51OP	Lo	Minimal	6.75	6.47	Poor	Similar	Similar	N/A
52OP	Lo	Medium	6.79	7.77	Robust, slight increase	Similar	Similar	Similar

[0580] The data indicated that CAR T cells expressing a CAR comprising CD19 binders 42, 42OP, 52 and 52OP had the best attributes of low tonic signaling, strong activation rate (e.g., NFAT), similar doublings during manufacturing expansion phase (e.g., expansion profiles), robust and stable surface expression (*e.g.*, with good maintenance of mfi), enhanced killing, and long term persistence in therapeutic activity (**Table 21 and Table 22)**.

Table 22: Summary				
	Restim:Log term Tumor control			
CAR-OP CD19binder	%CD8/%CD4 ratio end of first and end of 6th	%CD8/%CD4 ratio end of first	%CD8/%CD4 ratio end of 6th	Killing ability after 6 rounds
42OP	(30.60/53.06) to (67.80/8.90)	0.57	7.61	Maintain
43OP	N/A	N/A	N/A	N/A
44OP	(42.00/40.10) to (62.40/11.90)	1.05	5.24	Failing
45OP	(19.50/47.60) to (30.10/9.70)	0.41	3.10	Failing
46OP	N/A	N/A	N/A	N/A
51OP	N/A	N/A	N/A	N/A
52OP	(48.00/35.50) to (70.20/6.10)	1.35	11.51	Maintain

Example 8: Effect of Optimization of the CD19 binders

[0581] To further evaluate the effect of optimization of the novel CD19 binders, CAR T cells expressing a CAR comprising original CD19 binder 42, 44, 45 or 52 were compared to CAR T cells expressing a CAR comprising their optimized CD19 binder counterparts. The CD19 CARs were expressed in ND608 T cells and their expansion profiles of growth and size over time were analyzed. As shown in **FIGs. 18A-B**, each of the paired CD19 binders (e.g., original vs optimized) showed similar expansion profiles over time. CAR T cells expressing CD19 CAR comprising original binder (e.g., 42) showed similar expansion numbers and size reduction.

[0582] In addition, original CD19 binders and their optimized counterparts were expressed at similar levels on CAR T cells (**Table 23**). **Table 23** shows data of the surface expression of CARs comprising optimized CD19 binders and original CD19 binders on ND608 CAR T cells.

[0583] On Day 6 after transduction into ND608 donor T cells, CARs expressing the novel CD19 binders has an expression levels that ranged from 74.3% (42OP), 53.1% (original 42), 58.6% (45 original), 57.9% (45OP), 55.6% (52OP), 44.2% (44 original) 37.5% (44OP), and 34.9% (52OP). At Day 9, the expression in all tested groups increased as shown in **Table 23**.

Table 23: Expression of CD19 CARs on ND608 CART Cells		
	Day 6	**Day 9**
Untransduced (NTD)	0.00	0.14
Opt42 (42OP)	74.30	77.30
Opt44(44OP)	37.50	54.60
Opt45(45OP)	57.90	68.30
Opt52(52OP)	34.90	54.30
Original 42	59.40	53.10
Original 44	44.20	52.00
Original 45	58.60	60.90
Original 52	55.60	65.00

[0584] CAR T cells expressing CD19 CAR with optimized 42 showed the highest surface expression (based on MFI) on day 6 after transduction (74.3%). However, the opt42 CAR T cells also had the highest transduction efficiency (mfi >9,000 counts) (**FIG. 19A**). The transduction efficiency of CD19 CAR comprising optimized 52 was around 4,000 counts (**FIG. 19A**). By day 9, the optimized 52 CAR T cells (54.3%), original 42 CAR T cells (53.1%), and original 52 CAR T cells (65.0%) showed similar surface expression (**Table 23**). The surface expression of CD19 CAR with optimized 42 binder (77.3%) remained higher than other tested binder.

[0585] *Cytokine production*

[0586] The ability for the original and optimized CD19 CART cells to respond to the antigen was evaluated using cytokine responses upon wild-type Nalm6 stimulation (**Table 24**). Table 24 shows data illustrating the cytokine response of ND608 CAR T cells expressing CARs comprising optimized CD19 binders or original CD19 binders in the absence of any stimulation (unst.), or following Nalm6-wt stimulation, CD19-KO Nalm6 stimulation; or PMA/ionomycin stimulation at Day 9 after transduction.

[0587] As shown in **Table 14**, each tested CAR T cell populations was capable of robust cytokine production when stimulated with PMA/Ionomycin. As expected, Nalm6- CD19KO cells stimulation did not induce a cytokine response even after 4hrs of stimulation. However,

activation with Nalm6-wt and the ensuing CD19 engagement induced the CD19 CAR T cells to produce cytokines to various extents. Generally, the cytokine response was CD19 CAR dependent.

Table 24: Cytokine response from Optimized CD19 ND608 CAR T Cells (Day 9)			
	No stimulation (Unstimulated)		
	IL-2	**TNF-α**	**IL-2 & TNF-α**
Untransduced (NTD)	0.690	0.050	0.820
42OP	1.580	0.180	0.890
44OP	0.870	0.370	0.850
45OP	1.440	1.180	1.600
52OP	0.740	0.420	0.640
42 original	0.450	0.140	0.510
44 Original	1.280	1.810	1.940
45 original	0.400	0.460	0.730
52 Original	0.520	1.730	1.150
	Nalm6 Stimulation		
	IL-2	**TNF-α**	**IL-2 & TNF-α**
Untransduced (NTD)	0.270	0.220	1.030
42OP	1.300	27.100	35.200
44OP	0.810	28.400	17.200
45OP	0.770	27.800	33.800
52OP	1.140	26.600	35.500
42 original	1.440	15.600	16.300
44 Original	1.310	31.800	18.300
45 original	1.160	25.200	28.100
52 Original	1.060	36.100	37.000
	CD19-KO Nalm6 Stimulation		
	IL-2	**TNF-α**	**IL-2 & TNF-α**
Untransduced (NTD)	0.320	0.160	1.280
42OP	0.300	4.170	1.150
44OP	0.160	2.060	0.590
45OP	0.310	7.800	2.290
52OP	0.190	2.420	0.870
42 original	0.310	3.030	1.490
44 Original	0.200	2.820	0.660
45 original	0.190	8.140	1.830
52 Original	0.170	4.320	0.920
	PMA/Ionomycin Stimulated		
	IL-2	**TNF-α**	**IL-2 &TNF-α**
Untransduced (NTD)	3.260	5.980	89.400
42OP	3.050	13.000	81.400
44OP	2.220	18.400	75.300
45OP	1.860	13.500	81.900

Table 24: Cytokine response from Optimized CD19 ND608 CAR T Cells (Day 9)			
52OP	3.180	11.400	83.600
42 original	4.510	9.550	83.000
44 Original	2.880	11.900	82.800
45 original	3.050	12.400	82.000
52 Original	3.620	12.900	81.100
Mean cell size (μm^3)			
Untransduced (NTD)	382		
42OP	474		
44OP	499		
45OP	512		
52OP	443		
42 original	390		
44 Original	550		
45 original	439		
52 Original	501		

[0588] Opt42, Opt52 and 52 CAR T cells produced the most robust cytokine response (**Table 24)**. The combination IL-2 and TNF-α produced by CD19 original 52 was about 37.0% when stimulated by Nalm6-Wt when compared to 0.92% when stimulated with CD19KONalm6 cells. The combination IL-2 and TNF-α produced by CD19 optimized 52 was about 35.5% when stimulated by Nalm6-Wt when compared to 0.87% when stimulated with CD19KONalm6 cells. The combination IL-2 and TNF-α produced by CD19 original 42 was about 16.3% when stimulated by Nalm6-Wt when compared to1.49% when stimulated with CD19KONalm6 cells. The combination IL-2 and TNF-α produced by CD19 optimized 42 was about 35.2% when stimulated by Nalm6-Wt when compared to 1.15% when stimulated with CD19KONalm6 cells. Cytokine production by original 42 CART cells was comparable to the production of a CD19 binder positive control (*e.g.*, FMC63). See also, **Table 6**, and **Table 14**.

[0589] Thus, Nalm6-CD19KO stimulation did not induce cytokine production while Nalm6-WT cells and PMA induced cytokine production in both original and optimized CD19 binders. The cell sizes (*i.e.*, contraction) were relatively similar in all groups tested (**Table 24**).

[0590] *T cell Activation markers*

[0591] Analysis of the expression of HLA-DR and 4-1BB, which are T cell activation state

markers, was performed by flow cytometry. The raw results are shown in **Table 25**. HLA-DR expression demonstrated the early activation state of the CD19 CAR T cells. The 4-1BB marker was also used as an early activation surface marker. Original and optimized CD19 CARs showed similar activation patterns on $CD4^+$ T cells or $CD8^+$ T cells.

Table 25: Expression of HLA-DR and 4-1BB on ND608 CD19 CAR T cells on Day 11

Novel CD19 binders-Clone #	HLA-DR expression		4-1BB expression	
	Gated on CD4+ T cells	Gated on CD8+ T cells	Gated on CD4+ T cells	Gated on CD8+ T cells
Untransduced (NTD)	2.75	2.40	0.37	0.71
42OP	27.80	33.60	1.14	1.60
44OP	52.50	60.00	3.56	6.99
45OP	50.60	59.40	2.58	6.03
52OP	27.80	31.20	1.26	1.23
42 Original	4.72	4.47	0.35	0.49
44 Original	56.00	64.80	4.99	8.80
45 Original	13.70	17.40	0.29	0.60
52 Original	51.00	58.40	0.88	2.12

[0592] **Table 25** shows that the expression of HLA-DR on $CD4^+$ and $CD8^+$ CAR T cells (42OP, 44OP, 45OP, 52OP, 44 original, 45 original, and 52 original) was still significantly higher at day 11 when compared to untransduced cells. The expression of 4-1BB on $CD4^+$ and $CD8^+$ CAR T cells (42OP, 44OP, 45OP, 52OP, 44 original, 45 original, and 52 original) was slightly higher at day 11 when compared to untransduced cells. These data suggested that CAR T cells expressing a CAR comprising original 42 CD19 binder were inactivated (*e.g.*, rested down) by day 11. However, CAR T cells expressing a CD19 CAR comprising binder Opt 42, Opt44, Opt45, Opt52, 44, 45 and 52 were still activated (*e.g.*, retained a more activated state) by Day 11. This enhanced activation state of the latter CAR T cells, which was in contrast to the behavior of original 42 CD19 CAR T cells, may be consistent with the less tonic signaling and a better contraction observed with CAR T cells expressing 42 original CARs described herein.

[0593] *In vivo Cytotoxicity Performance*

[0594] A comparison of the original and optimized versions of the 42 and 52 CD19 binders were also evaluated in the Jeko NSG mouse model to determine their killing properties. As shown in **FIG. 19B**, all tested CAR T cells cleared Jeko tumors at a comparable rate. Based

on 1E6 CAR^+ T cells for each group, **FIG. 19B** shows that tumor growth was suppressed at the greatest level by CD19 CAR T cells expressing a **C**D19 42 original CAR. CAR T cells expressing CD19 52OP and CD19 52 original CAR showed similar, but slightly lower cytotoxicity (tumor suppression) when compared to CD19 42 original CAR T cells. In contrast, CAR T cells expressing CD19 42OP CARs showed the least amount of tumor control, but by three weeks they had similar levels of tumor control as other CD19 CAR (**FIG. 19B**). These *in vivo* studies indicated that the original and optimized versions of 42 and 52 showed comparable tumor suppression.

[0595] These examples suggest that while all 12 CD19 binders have the requisite properties, CD19 CAR T cells expressing a CAR comprising CD19 binder 42 or 52 consistently had a low CAR-induced tonic signals; strong activation rates; healthy expansion profiles, robust and stable surface expressions; enhanced cytokine production; and tumor killing.

[0596] ***Conclusion***

[0597] Using real time PCR, the relative transcript levels of the novel CD19 binders were normalized to a control CD19 CAR (e.g., a FMC63-based CAR) on each day. The relative transcript levels for all CD19 binders were found to be comparable. The CD19 binder 42 had twice the transcript levels of all tested binders. Nonetheless, a substantially similar transcript pattern was observed for each day in all binders tested. When the transcript levels were normalized to a control CAR only on day 6, the same expression pattern was still observed though relative transcript numbers were decreased. The reduction was expected because T cell blast size was reduced. Together, the preliminary analysis showed that these CD19 binders produced similar transcriptional profiles. However, for efficiency reasons, CD19 binders 42, 43, 44, 45, 46, 50, 51, and 52 were pursued.

[0598] The translated products of the CD19 binders (e.g., total protein levels) were also evaluated by westerns. As shown for example in **FIGs. 11A-B,** two protein isoforms (e.g., species) were identified for each of the CD19 binders (except CD19 52). The Western blots results captured two protein bands with similar sizes but various expression levels. For example, CAR T cells expressing CD19 binder 52 had the larger molecular weight band. Two isoforms of the CD19 binder 42 were identified, and the smaller isoform was highly expressed. The two isoforms were also detected in CAR T cells expressing a control CD19

CAR. No correlation between the ratio of protein band sizes and cytokine production or tumor clearance was found.

[0599] Interestingly, the intensity of the two isoforms was affected by sequence optimization and the source of the T cells. For example, sequence optimization of the CD19 binders and switching T cells donor, changed the ratios of the expressed CAR isoforms. The isoform with the higher molecular weight became dominant when the CD19 binders were codon optimized and/or when the CD19 CAR was transduced in a different donor T cell. A low amount of CAR-CD3z protein was detected from CAR T cells expressing a CD19 CAR comprising CD19 binder 43, which correlated with the CAR's surface expression. CAR T cells expressing CD19 CAR comprising binder 46 or 51 had high protein levels by Western blot analysis, but flow cytometry indicated that these binders had a low cell surface levels. Despite the low surface expression, CAR T cells expressing the 46 binder produced cytokine upon stimulation.

[0600] An initial evaluation of epitope binding region of CD19 binders was also conducted as shown in FIGs. 12A-B and Tables 9-10. Specifically, a competitive assay was performed to determine if the novel CD19 binders bound to the anti-FMC63 antibody and if they shared the same binding site (e.g., epitope) or they bound to the same region. These data showed that the anti-FMC63 antibody was not an idiotype antibody for the novel CD19 binders. TFor example, the anti-FMC63 antibody did not bind to the novel CD19 binders, and it did not block the interaction between the novel CD19 binder and a CD19 protein.

[0601] In the second phase of evaluation, the scFv of the CD19 binders 42, 43, 44, 45, 46, 50, 51 and 52 were first optimized based on codon usage and GC content. Codon optimization was performed to determine if a more stable robust expression could be obtained. Optimization maintained or reduced CAR-induced tonic signaling of 42OP, 43OP, 51OP and 52OP CD19 CAR T cells. However, optimization enhanced the tonic signaling in CAR 44OP, 45OP, and 46OP CAR T cells.

Example 9: Novel CD19 binders binding Affinity

[0602] To determine if the CD19 binder disclosed herein can result in lower altered affinities and rate constants, the binders can be biotinylated, purified and tested *in vivo*. The 12 CD19 binders may be subjected to biolayer interferometry (BLI) using streptavidin biosensors and

recombinant CD19. It is expected that the affinities of the novel CD19 binders will range from about 175nM to >10,000 nM, but lower than the affinity of the FMC63 binder. In particular, the FMC63 binder is disclosed to have a K_{on}= 2.1 x 10^5 M^{-1} s^{-1}, K_{off} = 6.8 x 10^{-5} s^{-1}, and a K_D = 0.328 nM. In contrast, the present CD19 binders are expected to have between about 30-fold to about 60-fold lower affinity than the FMC63 binder. In particular, the novel CD19 binders may have K_{on}= 2.1 x 10^5 M^{-1} s^{-1}, K_{off} = bout 1.0 x10^{-3} s^{-1} to about 5.0 x10^{-3} s^{-1}, and a K_D = 1nM to about 175nM, or a K_D > 175nM. In addition, a very close correlation between the novel CD19 binders' affinity (K_D) and TRF binding.

Example 10: Generation and expansion of CD19 CAR T cells

[0603] The purpose of this example is to describe potential methods of making CAR-T cells described herein.

[0604] CAR T cells expressing the CD19 binders disclosed herein can be generated using any method known in the art for example as shown in WO 2014/153270. In particular, CD19 CAR disclosed herein can be expressed in a lentiviral vector. Primary human T lymphocytes can then be isolated and then stimulated with magnetic beads coated with anti-CD3/anti-CD28 antibodies (Miltenyi Biotec) at cell to bead ratio of 1 to 2. Approximately 0, 5, 10, 12, or 24 hours after activation, T cells may be transfected with a lentiviral vector encoding the CD19 CAR. Transfected T cells can be used immediately or expanded for up to 3 days.

[0605] In one example of the disclosure, CAR T cells expressing the CD19 binders of the present disclosure are expected to exhibit enhanced expansion rates when compared to CD19 CAR known in the art.

Example 11: Killing assays

[0606] The purpose of this example is to describe exemplary killing assays for the CAR-Ts described herein.

[0607] To determine the effectiveness of CAR T cells expressing the CD19 binders of the present disclosure (P1-P13), target cells, such as K562, K562-CD19, and NALM6 can be tagged with GFP and/or luciferase (GL), and incubated with GFP expressed T cells or CD19 CAR T cells of the present disclosure, at the desired ratios in triplicate wells in U-bottomed 96-well plates. Viability of target cells can be tested about 18 h later by adding 100 dl/well

substrate D-luciferin firefly (Life Sciences) at 150 μg/ml. Background luminescence may be negligible (< 1% than the signal from the wells with only target cells). The viability percentage can be calculated as experimental signal/maximal signal × 100%, and killing percentage was equal to 100 − viability percentage.

[0608] It is expected that the CAR T cells comprising any one of P1-P13 binders and generated from PBMC of ALL patient will lyse more than 75% CD19-expressing NALM-6 cells. The results of killing assay with K562 cells will reveal that CD19 CAR T cells will specifically kill the K562-CD19 cells but not the K562 cells without CD19 expression. In particular, it is expected that CD19 CAR T cells comprising any one of P1-P13 binders shown in Table 3, or CDRs shown in Table 2 will show either equal or enhanced anti-tumor activity when compared to a CD19 CAR known in the art. It is further expected that CAR comprising any one of the P1-P13 binders will be selective for tumor cells over wild-type cells when compared to CD19 known in the art.

Example 12: Shotgun Mutagenesis and epitope mapping of CD19 42og scFv

[0609] In order to map the epitopes of the CD19 42og scFv, an alanine-scan library of the CD19 protein (e.g., the extracellular domain) was performed using shotgun mutagenesis (Integral Molecular, Philadelphia, PA). The CD19 42og scFv was then screened for binding to each individual CD19 protein variant, which allowed the identification of the CD19 protein residues critical for CD19 42og scFv binding.

[0610] *Shotgun Mutagenesis*. Shotgun Mutagenesis epitope mapping services were provided by Integral Molecular (Philadelphia, PA) as described in Davidson and Doranz, Immunology *143*, 13-20 (2014). Briefly, a mutation library of the CD19 protein was created by high-throughput, site-directed mutagenesis. Each residue was individually mutated to alanine, with alanine codons mutated to serine. The mutant library was arrayed in 384-well microplates and transiently transfected into HEK-293T cells. Following transfection, cells were incubated with the indicated antibodies at concentrations pre-determined using an independent immunofluorescence titration curve on the CD19 wild type protein. MAbs were detected using an Alexa Fluor 488-conjugated secondary antibody and mean cellular fluorescence was determined using Intellicyt® iQue flow cytometry platform. Mutated residues were identified as being critical to the MAb epitope if they did not support the reactivity of the test MAb but

did support the reactivity of the reference MAb. This counterscreen strategy facilitated the exclusion of mutants that were locally misfolded or that had an expression defect.

[0611] *Assay setup*. Conditions for binding and screening of the CD19 42og scFv were initially assessed using high-throughput flow cytometry and optimized using wild-type CD19 protein cloned into a proprietary vector and expressed in HEK-293T cells. To optimize the CD19 42og scFv against the extracellular domain of CD19, HEK-293T cells were transfected with a CD19 wild-type (WT) construct or with an empty vector in 384-well format, followed by detection of cellular expression via high-throughput flow cytometry. GPC2-02 scFv-Fc, and two known anti-CD19 monoclonal antibodies, CB19 monoclonal antibody (CB19 Mab), and HIB19 monoclonal antibody (HIB19 Mab), were used as controls. Serial dilutions of each MAb and scFv were tested for immunoreactivity against cells expressing the WT CD19 or vector alone.

[0612] **Table 26** shows experimental parameters optimized for high-throughput flow cytometry.

Experimental Parameter	Test scFv-Fc	Control scFv-Fc	Control MAb	Control MAb
Cell Type	HEK-293T	HEK-293T	HEK-293T	HEK-293T
Fixative	None	None	None	None
Blocking Buffer	10% Goat Serum	10% Goat Serum	10% Goat Serum	10% Goat Serum
Primary Antibody				
Name	42og scFv-Fc	GPC2-02 scFv-Fc	CB19 (Novus. Cat no. NBP2-25196)	HIB19 (BioLegend. Cat no. 302202)
Target	CD19	CD19	CD19	CD19
Optimal Conc.(ug/mL)	0.25	N/A	0.25	0.25
Incubation (25°C)	60 min	60 min	60 min	60 min
Secondary Antibody				
Target	Human IgG. Fcγ	Human IgG. Fcγ	Mouse IgG	Mouse IgG
Optimal Conc.	1:400 (3.75 ug/ml)	1:400 (3.75 ug/ml)	1:400 (3.75 ug/ml)	1:400 (3.75 ug/ml)
Incubation (25°C)	30 min	30 min	30 min	30 min
Manufacturer	Jackson ImmunoResearch	Jackson ImmunoResearch	Jackson ImmunoResearch	Jackson ImmunoResearch
Cat #	109-545-008	109-545-008	115-545-003	115-545-003
Antibody ID	AlexaFluor® 488 AffiniPure Goat Anti-Human IgG. Fcγ fragment specific	AlexaFluor® 488 AffiniPure Goat Anti-Human IgG. Fcγ fragment specific	AlexaFluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L)	AlexaFluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L)
Wash Buffer	PBS (Ca2+. Mg2+ free)	PBS (Ca2+. Mg2+ free)	PBS (Ca2+. Mg2+ free)	PBS (Ca2+. Mg2+ free)
Signal:Background	45:1	N/A	40:1	50:1

[0613] The optimal screening concentration for each MAb was determined based on the raw signal values and signal-to-background calculations. Each data point shown in **Table 27** represents the mean of four replicates.

Table 27: Optimization of 42og scFv against CD19 target protein								
	42og scFv-Fc		**GPC2-02 scFv-Fc**		**CB19 MAb**		**HIB19 MAb**	
[[ug/ml]	CD19 [mfi]	Empty vector [mfi]	CD19 [mfi]	Empty vector [mfi]	CD19 [mfi]	Empty vector [mfi]	CD19 [mfi]	Empty vector [mfi]
4.000	2,250,000	0	50,000	35,000	2,000,000	0	2,000,000	0
2.000	2,500,000	0	30,000	30,000	1,750,000	0	1,750,000	0
1.000	2,000,000	0	20,000	20,000	1,500,000	0	1,750,000	0
0.500	1,500,000	0	20,000	19,000	1,250,000	0	1,250,000	0
0.250	1,000,000	0	19,000	19,000	1,000,000	0	750,000	0
0.125	500,000	0	19,000	19,000	500,000	0	500,000	0

[0614] As shown in **Tables 26** and **27**, the optimal concentration for 42og scFv-Fc was determined to be about 0.25 ug/ml, with a signal to background ratio of about 45:1. 42og scFv-Fc is a recombinant molecule comprising 42og scFv tagged to human Fc domain of IgG1. For the control monoclonal antibodies, the optimal concentration of CB19 was about 0.25 ug/ml with a signal to background ratio of about 40:1; and the optimal concentration of HIB19 was about 0.25 ug/ml with a signal to background ratio of about 50:1.

[0615] *Epitope Mapping*. To identify critical clones for 42og scFv binding, binding of 42og scFv and each control antibody to each mutant clone in the alanine scanning library was determined, in duplicate, by high-throughput flow cytometry. For each point, background fluorescence was subtracted from the raw data, which were then normalized to Ab reactivity with WT CD19 protein. For each mutant clone, the mean binding value was plotted as a function of expression (represented by control reactivity).

[0616] *High stringency conditions*. Library screens of very high-affinity antibodies sometimes fail to yield critical residues for antibody binding. For cases where antibody screens under standard conditions are insufficient to identify critical residues for binding, high stringency conditions are implemented. These conditions include combinations of increased pH, increased salinity, increased temperature, and/or increased wash time. Antibodies that required high stringency conditions are denoted “HS”. 42og scFv was determined to be a high affinity antibody.

[0617] To identify preliminary primary critical clones, a threshold anti-CD19 control Mab as percentage of WT mfi was set at >70% WT binding to control Ab (X-axis) and a threshold 42og scFv-Fc HS (high affinity or specificity) as a percentage of WT mfi was set at <20% WT binding to test Abs (Y-axis). From this selection, three primary critical clones were identified. In addition, two secondary clones were identified. These two secondary clones did not meet the set thresholds of >70% WT binding to control Ab (X-axis) and <20% WT binding to test Abs (Y-axis). However, their decreased binding activity and proximity to critical residues suggested that the mutated residue may be part of the antibody epitope. These five residues and their binding reactivity are shown in **Table 28**. In particular, the three primary critical clones were Q98A, E104A, and K105A. The two secondary residues were A106 and V207 based on the numbering of a WT CD19 sequence (SEQ ID NO: 217).

Table 28: Critical Residues for 42og scFv binding to CD19 extracellular domain

Binding Reactivity (% WT)			
Mutation	42og scFv-Fc HS	anti-CD19 control MAb	Clone types
Q98A	**5.1 (8)**	84.2 (12)	Primary
E104A	19.2 (1)	101.8 (35)	Primary
K105A	**1.1 (1)**	88.0 (23)	Primary
A106S	31.7 (18)	107.7 (17)	Secondary
V207A	29.9 (27)	82.3 (21)	Secondary

[0618] **Table 28** shows the mean binding reactivities for all identified critical residues. Ranges are listed in parentheses. A range is the difference between the highest and lowest mean binding values for each clone. Critical residues for Ab binding were residues whose mutations were negative for binding to test Abs, but positive for binding to control antibody. Additional secondary residues were identified that did not meet the threshold guidelines, but whose decreased binding activity and proximity to critical residues suggested that they may be part of the antibody epitope.

[0619] Based on these analyses, it was determined that the epitope of the CD19 42og binder most likely comprises QPGPPSEKA (SEQ ID NO: 223) and/or PPDSVSR (SEQ ID NO: 224). Critical residues whose mutation gave the lowest reactivities with specific antibodies were Q98 and K105. The highlighted residues were considered to likely be the major

energetic contributors to binding because validated critical residues represented amino acids whose side chains that made the highest energetic contributions to the antibody-epitope interaction (Bogan and Thorn, 1998; Lo Conte et al., 1999).

[0620] These results further demonstrate the unique functional characteristics of the novel CD19 binders described herein, in particular CD19 42og. The uniqueness is illustrated in FIG. 20, which shows that the epitope of CD19 42og does not overlap with epitopes from three well-characterized antibodies, namely, the FMC63, 4G7, and 3B10. Indeed, Klesmith et al. (Biochemistry 58:4869-4881 (2019)) characterized the conformational epitopes of FMC63, 4G7, and 3B10 (e.g., anti-CD19 clinical antibodies) using high-throughput screening strategies to comprehensively map the binding sequences of these antibodies to the extracellular domain of CD19 variant CD19.1. These extensive analyses showed that conformational epitope maps of FMC63 and 4G7 and the linear epitope map of 3B10. Klesmith et al. concluded that all three antibodies have partially overlapping epitopes near the published epitope of antibody B43 co-crystallized with CD19.

[0621] As shown in **FIG. 20**, two main regions were identified. The first region comprises amino acid sequence WAKDRPEIWEGEP (SEQ ID NO: 219) located at positions 159-171 of the wild-type CD19 protein (SEQ ID NO: 217). The second region comprises the amino acid sequence of PKGPKSLLSLE (SEQ ID NO: 220) and was located at positions 219-229 of SEQ ID NO: 217.

[0622] In contrast, the analyses described herein show that CD19 42og scFv bound to a completely region of the extracellular domain of CD19 that did not overlap with PKGPKSLLSLE (SEQ ID NO: 220) or WAKDRPEIWEGEP (SEQ ID NO: 219). In particular, CD19 42og scFv bound primarily to amino acid sequence of QPGPPSEKAWQP (SEQ ID NO: 221) located at 98-109 of SEQ ID NO: 217. CD19 42og scFv also interacted with another region comprising the amino acid sequence VPPDSVSRGPL (SEQ ID NO: 222) located at positions 202-212. Accordingly, CD19 42og did not and does not bind to the same epitope as FMC63, 4G7, or B43. The uniqueness of the epitope binding of 42og is further shown in **Table 29** and **FIG. 20** below.

Table 29: 42og scFv binds to a novel and non-overlapping epitope on CD19					
SEQ ID NO: 217 (FL CD19)		**Critical epitope residues for antibody binding**			
		FMC63	**4G7**	**3B10**	**42og scFv**
Region 1	***W***A**K**D***R***P***E***I**W**EG**EP (SEQ ID NO: 219)	W159 R163	W159 R163 E165	K161 E165 I166 W167 E168 G169	NO
Region 2	**P**K**GP**KSLLSLE (SEQ ID NO: 220)	G221 P222	P219 G221	NO	NO
Region 3	**Q**PGPPS**EKA**WQP (SEQ ID NO: 221)	NO	NO	NO	Q98 E104 K105 A106
Region 4	VPPDS**V**SRGPL (SEQ ID NO: 222)	NO	NO	NO	V207

Example 13: Cross-reactivity of CD19 42og scFv against human membrane proteins.

[0623] Assessing a biotherapeutic's cross-reactivity against human membrane proteins is important because unintended binding interactions can potentially lead to adverse clinical events. To determine potential interactions of 42OG scFv with non-target proteins, the novel CD19_42og scFv was fused to a Fc domain of human IgG1. This recombinant protein was tested using Integral Molecular's Membrane Proteome Array (MPA) (Integral Molecular, integralmolecular.com). The MPA developed and used by Integral Molecular was an array of 5,220 human membrane proteins and represented over 94% of the human membrane proteome. Each human membrane protein in the MPA was structurally intact and expressed in its native conformation within live cells. The MPA is an *in vitro* tool that enabled rapid and comprehensive specificity screening of candidate therapeutics, such as 42og scFv. The MPA screen evaluated 42og scFv-Fc binding interactions with structurally-intact (non-fixed) human membrane proteins.

[0624] *Method.* The MPA was a platform for profiling the specificity of antibodies and other ligands that target human membrane proteins. The MPA was based upon a library of 5,220 unique human membrane proteins, which includes 94% of all single-pass, multi-pass, and GPI-anchored proteins. The array of proteins was expressed in live, unfixed cells so was designed to exhibit native protein conformations and post-translational modifications. The MPA was screened in a 384-well format and assessed for reactivity with the test article using

high-throughput flow cytometry. Identified targets were validated in secondary titration screens to confirm reactivity.

[0625] HEK-293T cells (18,000 cells/well) and QT6 cells (18,000 cells/well) were transfected with plasmids encoding known binding partners (positive controls, described below) or vector alone (pUC; negative control) in 384-well cell-culture plates and incubated in media composed of Corning DMEM, 10% FBS, 2mM L-alanyl-L-glutamine, Pen-Strep, MEM NEAA and HEPES 10 mM. After incubation for 36 hours at 37°C and 5% CO2, four four-fold dilutions starting at 20 μg/mL of the test article (with 10% NGS in PBS with Ca2+, $Mg2^{+}$ as diluent) were added in quadruplicate to transfected cells and detected using a single dilution of secondary antibody in a high-throughput immunofluorescence flow cytometry assay. Average mean fluorescence intensity (MFI) values were determined for each article dilution using ForeCyt Software (Intellicyt) and plotted using Excel (Microsoft). In addition, MFI values for individual concentrations were converted to a signal-to-background ratio composed of target binding (signal) and negative control (background). Optimal screening concentrations were determined by the background signal (mean fluorescence intensity [MFI]), and high background rate in cells transfected with vector control. 42og scFv-Fc and controls were preferentially screened on HEK-293T cells at the highest concentration yielding an acceptably low background (<50,000 MFI) and minimal high background rate (<1%). If no acceptable screening conditions were identified on HEK-293T cells, the samples were screened at the highest acceptable concentration on QT6 cells.

[0626] *Controls and desired.* Protein A was used as a positive control, or assay Control. Protein A binds the Fc region of human antibodies. This control was transfected into cells to verify successful binding and detection. CD19 was used as a positive target Control (CD19). CD19 was included to confirm proper performance of the 42og scFv. An empty vector (pUC vector) was used as a negative Control. The pUC vector was transfected into cells as a negative control to ascertain background binding. Metrics used to select screening conditions were: (1) low background less than 50,000 mean fluorescence intensity (MFI) was used for cells transfected with pUC vector control; and (2) background less than 1% of negative control cells that displayed high background (250,000 MFI). The highest 42og scFv-Fc concentration that fulfilled the two criteria listed above was selected using HEK-293T cells when possible.

[0627] Serial dilutions of 42og scFv-Fc were assayed by incubation with the positive (Protein A, known binder CD19) and negative controls (pUC empty vector) expressed in HEK-293T and QT6 cells, and target binding was measured by flow cytometry. The optimal 42og scFv-Fc concentration for screening was chosen based on a joint assessment of binding strength and background signal and rate of high background events. 42og scFV-Fc showed strong binding to the positive controls in both cell types but MFI signal was higher in HEK-293T cells. Background binding to the negative control (empty vector) was within the acceptable range for the 42og scFv-Fc at all concentrations except 20μg/mL in HEK-293T cells. The cell highlighted in **Table 30** within the High Background Rate table indicates the concentration (5 μg/ml) and cell type (HEK-293T) chosen for further screening.

Table 30		High Background Rate (%)	
		HEK-293T	QT6
Concentration (μg/mL)	20	1.8	0.1
	5		<0.1
	1.25	<0.1	<0.1
	0.31	<0.1	<0.1

[0628] FIGs. 21A- C show results of the MPA screen (**FIG. 21A**) and validation assay (**FIGs. 21B-C**), which demonstrated no binding to non-target proteins. Thus, 42og scFv-Fc was not cross-reactive to any non-CD19 molecule included from the membrane proteome library.

[0629] The MPA was expressed in HEK-293T cells and 42og scFv-Fc binding was determined by flow cytometry. Each target was tested for binding in duplicate. 42og scFv-Fc showed strong binding to its known target CD19 (**FIG. 21A**). 42og scFv-Fc also showed reactivity to a Fc-binding positive control, a FCGR protein (FCGR1A). This binding was expected because FCGR proteins bind to IgG1 Fc domain. In addition, the Fc domain was tagged to 42og scFv to serve as positive controls in the assay. Any potential binding interactions identified on the MPA that did not validate in titration experiments were removed from the graph. There were no cross-reactive (non-target) binding interactions identified for the test article. Dotted line represents 3 Standard Deviation (SD) of the calculated background.

[0630] The 42OG scFv-Fc and the control article were further screened for binding against targets identified on the MPA screen (e.g., FCGR1A, S100A10, CLYBL, RRNAD1) as well as the positive controls (Protein A, known binder CD19), and the negative control (pUC empty Vector) expressed in HEK-293T cells. **FIGs. 21B-C** show the targets that the test or control article were screened against. 42og scFv-Fc showed strong binding to the positive controls Protein A and CD19, with MFI signals >750-fold and >400-fold above negative control, respectively, at the two highest concentrations. 42og scFv-Fc also bound FCGR1A with MFI signal >150-fold above negative control. Binding to S100A10, CLYBL, and RRNAD1 was seen as minimal or absent and did not pass validation criteria (binding in a concentration-dependent manner and with MFI signal 2-fold higher than the negative control at 20μg/mL and 5μg/mL). Thus, **FIG. 21B** shows that there were no cross-reactive non-target (non-CD19) binding interactions validated for 42OG scFv-Fc.

[0631] The isotype control showed strong binding to Protein A, as expected, with MFI signal >190-fold above negative control (**FIG. 21C**). The isotype control also bound FCGR1A with MFI signal >45-fold above negative control. There was no binding of the isotype control to CD19, S100A10, CLYBL, RRNAD1, empty vector, or any of the other targets tested.

[0632] The MPA screen and validation assay demonstrated no binding of 42og scFv-Fc to non-target proteins (i.e., non-CD19), and thus there were no cross-reactive targets identified from the membrane proteome library. As such, 42og scFv-Fc selectively and specifically bound to the extracellular domain of CD19 at the epitope described herein. The 42og scFv-Fc binding to one FCGR protein (FCGR1A) was expected because FCGR proteins are known to bind human Fc protein, which is fused to 42OG scFv. An isotype control article was included in the validation assay and showed binding to FCGR1A but no binding to CD19.

[0633] Table 1-Sequences

[0634] The novel 12 CD19-specific antibody identified clones are known alternatively as P1 (A2 or 42); P2 (A4 or 43); P3 (E4 or 44); P4 (E7 or 45); P5 (7 or 46); P6 (10 or 47); P7 (11 or 48); P8 (14 or 49); P9 (15 or 50); P10 (16 or 51); P11 (18 or 52); P12 (23 or 53). P13 (52 CO or 52 Opt) is a codon optimized version of P11 or (18 also known as clone 52).

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
1	42og CDR-L1	RASQTISNYLN
2	42og CDR-L2	AASSLQS
3	42og CDR-L3	QQSYSTPPT
4	42og CDR-H1	AASGFTFSNYAIS
5	42og CDR-H2	VSVITASGVDTYYADSV
6	42og CDR-H3	GGTPYFITTYDYYGFDV
7	CD19 42og V_L	DIQMTQSPSSLSASVGDRVTITCRASQTISNYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYSTPPTFGQGTKVEIK
8	CD19 42ogV_H	EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYAISW VRQAPGKGLEWVSVITASGVDTYYADSVKGRFTISR DNSKNTLYLQMNSLRAEDTAVYYCARGGTPYFITT YDYYGFDVWGQGTLVTVSS
9	scFv-CD19 42og human derived	DIQMTQSPSSLSASVGDRVTITCRASQTISNYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYSTPPTFGQGTKVEIKGGGGS GGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFSNYAISWVRQAPGKGLEWVSVITASGVDTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARGGTPYFITTYDYYGFDVWGQGTLVTVSS
10	A4 CDR-L1	RASQSVSSNYLA
11	A4 CDR-L2	GASSRAT
12	A4 CDR-L3	QQYESSPSWT
13	A4 CDR-H1	KASGGTFSNYYIS
14	A4 CDR-H2	MGGIIPLFGTTNYAQ
15	A4 CDR-H3	GTWYAGDI
16	A4 V_L	EIVLTQSPGTLSLSPGERATLSCRASQSVSSNYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYESSPSWTFGQGTKVEIK
17	A4 V_H	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSNYYIS WVRQAPGQGLEWMGGIIPLFGTTNYAQKFQGRVTI TADESTSTAYMELSSLRSEDTAVYYCARGTWYAGD IWGQGTLVTVSS
18	scFv-CD19 A4 human derived	EIVLTQSPGTLSLSPGERATLSCRASQSVSSNYLAWY QQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQYESSPSWTFGQGTKVEIKGG GGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSC KASGGTFSNYYISWVRQAPGQGLEWMGGIIPLFGTT NYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVY YCARGTWYAGDIWGQGTLVTVSS
19	Nucleic acid sequence CD19 42og V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagaccatctctaattatctgaattggtatcagcaaa aacccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccgga

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		gttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttct tttgcagcctgaagactttgcaacatattactgtcagcaatcttactctaccccaccaa cattcgggcaaggtaccaaggtcgaaattaag
20	Nucleic acid sequence CD19 42ogV$_H$	gaggttcagttgttagagagcgggggggggtctggttcagcctggtggcagcttaa gactgagttgtgccgcttctggttttactttctctaattatgcaatatcctgggtgaggc aagcccccggtaaaggcctggaatgggtttcagttatcaccgcttctggtgttgata cctactacgccgatagcgtgaaaggtagattcactatttccagggacaattcaaaga acactttgtacttacagatgaactctttgagagctgaggacaccgcagtgtattactg tgctagaggtggtaccccatactttatcaccacctacgattactacggttttgatgttg gggacaaggaactttggtcacagtgtcatct
21	Nucleic acid sequence scFv-CD19 42og	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagaccatctctaattatctgaattggtatcagcaaa aacccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccgga gttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttct tttgcagcctgaagactttgcaacatattactgtcagcaatcttactctaccccaccaa cattcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggc ggtagcggaggtggtggtagtgaggttcagttgttagagagcgggggggggtctg gttcagcctggtggcagcttaagactgagttgtgccgcttctggttttactttctctaat tatgcaatatcctgggtgaggcaagcccccggtaaaggcctggaatgggtttcagt tatcaccgcttctggtgttgatacctactacgccgatagcgtgaaaggtagattcact atttccagggacaattcaaagaacactttgtacttacagatgaactctttgagagctg aggacaccgcagtgtattactgtgctagaggtggtaccccatactttatcaccacct acgattactacggttttgatgtttggggacaaggaactttggtcacagtgtcatct
22	Nucleic acid sequence CD19 A4 V$_L$	gaaatcgtcttgacccagtccccaggtactctgtctttaagtcccggggaaagagct actctgtcctgtagggcaagtcagtctgtttcttctaattatctggcctggtatcagca aaaacccggtcaagctccaagactgttgatctatggtgcaagcagtagagccacc ggaatccctgataggttttccggttcaggcagcggaactgacttcactctgacaatc tcaagattggaacctgaggattttgccgtgtattactgtcagcagtacgaatcttctcc atcttggacattcgggcaaggtaccaaggtcgaaattaag
23	Nucleic acid sequence CD19 A4V$_H$	caggtccagttagttcaatcaggtgccgaggtcaaaaagccaggttcttccgtcaa agtgtcatgcaaggctagcggtggcacctttctaattactacatctcttgggtgaga caggcaccaggtcaaggactggaatggatgggaggtatcatcccactgtttggta ccaccaattatgcccagaagttccaaggcagggtgaccataactgctgatgagagt acatctaccgcatacatggaattaagttctctgagatccgaggacaccgcagtgtat tactgtgctagaggtacctggtacgctggtgatatctggggacaaggaactttggtc acagtgtcatct
24	Nucleic acid sequence scFv-CD19 A4	gaaatcgtcttgacccagtccccaggtactctgtctttaagtcccggggaaagagct actctgtcctgtagggcaagtcagtctgtttcttctaattatctggcctggtatcagca aaaacccggtcaagctccaagactgttgatctatggtgcaagcagtagagccacc ggaatccctgataggttttccggttcaggcagcggaactgacttcactctgacaatc tcaagattggaacctgaggattttgccgtgtattactgtcagcagtacgaatcttctcc atcttggacattcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctg gtggtggcggtagcggaggtggtggtagtcaggtccagttagttcaatcaggtgc cgaggtcaaaaagccaggttcttccgtcaaagtgtcatgcaaggctagcggtggc

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		acctttttctaattactacatctcttgggtgagacaggcaccaggtcaaggactggaat ggatgggaggtatcatcccactgtttggtaccaccaattatgcccagaagttccaag gcagggtgaccataactgctgatgagagtacatctaccgcatacatggaattaagtt ctctgagatccgaggacaccgcagtgtattactgtgctagaggtacctggtacgct ggtgatatctggggacaaggaactttggtcacagtgtcatct
25	CD8 Leader	MALPVTALLLPLALLLHAARP
26	CD8 Leader (nucleic acid)	atggccctgcctgtgacagccctgctgctgcctctggctctgctgctgcatgccgct agaccc
27	CD8 Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHT RGLDFACD
28	CD8 Hinge nucleic acid sequence	accacgacgccagcgccgcgaccaccaacaccggcgcccaccatcgcgtcgc agcccctgtccctgcgcccagaggcgtgccggccagcggcgggggggcgcagt gcacacgagggggctggacttcgcctgtgat
29	CD8 TM	IYIWAPLAGTCGVLLLSLVITLYC
30	CD8 TM nucleic acid sequence	atctacatctgggcgcccttggccgggacttgtggggtccttctcctgtcactggtta tcacccctttactgc
31	CD28 transmembrane domain amino acid sequence	FWVLVVVGGVLACYSLLVTVAFIIFWV
32	CD28 transmembrane domain nucleic acid sequence	ttttgggtgctggtggtggttggtggagtcctggcttgctatagcttgctagtaacagt ggcctttattattttctgggtg
33	ICOS transmembrane domain amino acid sequence	FWLPIGCAAFVVVCILGCILICWL
34	ICOS transmembrane domain nucleic acid sequence	ttctggttacccataggatgtgcagcctttgttgtagtct gcattttgggatgcatacttatttgttggctt
35	IgG4 Hinge amino acid sequence	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRT PEVTCWVDVSQEDPEVQFNWYVDGVEVHNAKTKP REEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHN HYTQKSLSLSLGKM
36	IgG4 Hinge nucleic acid sequence	gagagcaagtacggccctccctgccccccttgccctgccccgagttcctgggcg gacccagcgtgttcctgttcccccccaagcccaaggacaccctgatgatcagccg gacccccgaggtgacctgtgtggtggtggacgtgtcccaggaggaccccgaggt ccagttcaactggtacgtggacggcgtggaggtgcacaacgccaagaccaagcc

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		ccgggaggagcagttcaatagcacctaccgggtggtgtccgtgctgaccgtgctg caccaggactggctgaacggcaaggaatacaagtgtaaggtgtccaacaagggc ctgcccagcagcatcgagaaaaccatcagcaaggccaagggccagcctcggga gccccaggtgtacaccctgcccccctagccaagaggagatgaccaagaaccaggt gtccctgacctgcctggtgaagggcttctaccccagcgacatcgccgtggagtgg gagagcaacggccagcccgagaacaactacaagaccacccccccctgtgctgga cagcgacggcagcttcttcctgtacagccggctgaccgtggacaagagccggtg gcaggagggcaacgtctttagctgctccgtgatgcacgaggccctgcacaaccac tacacccagaagagcctgagcctgtccctgggcaagatg
37	4-1BB domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEE EGGCEL
38	4-1BB domain nucleic acid	aaacggggcagaaagaaactcctgtatatattcaaacaaccatttatgagaccagt acaaactactcaagaggaagatggctgtagctgccgatttccagaagaagaagaa ggaggatgtgaactg
39	CD28 intracellular domain amino acid sequence	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDF AAYRS
40	CD28 intracellular domain nucleic acid sequence	aggagtaagaggagcaggctcctgcacagtgactacatgaacatgactccccgc cgccccgggcccacccgcaagcattaccagccctatgccccaccacgcgacttc gcagcctatcgctcc
41	CD28 intracellular domain variant (YMFM) amino acid sequence	RSKRSRLLHSDYMFMTPRRPGPTRKHYQPYAPPRDF AAYRS
42	CD28 intracellular domain variant (YMFM) nucleic acid sequence	AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACT ACATGTTCATGACTCCCCGCCGCCCCGGGCCCACC CGCAAGCATTACCAGCCCTATGCCCCACCACGCG ACTTCGCAGCCTATCGCTCC
43	ICOS costimulatory domain amino acid sequence	TKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL
44	ICOS costimulatory domain nucleic acid sequence	acaaaaaagaagtattcatccagtgtgcacgaccctaacggtgaatacatgttcatg agagcagtgaacacagccaaaaaatc**t**agactcacagatgtgacccta
45	ICOS-1 costimulatory	acaaaaaagaagtattcatccagtgtgcacgaccctaacggtgaatacatgttcatg agagcagtgaacacagccaaaaaatc**c**agactcacagatgtgacccta

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
	domain nucleic acid sequence	
46	CD2 costimulatory domain amino acid sequence	TKRKKQRSRRNDEELETRAHRVATEERGRKPHQIPA STPQNPATSQHPPPPPGHRSQAPSHRPPPPGHRVQHQ PQKRPPAPSGTQVHQQKGPPLPRPRVQPKPPHGAAE NSLSPSSN
47	CD2 costimulatory domain nucleic acid sequence	accaaaaggaaaaaacagaggagtcggagaaatgatgaggagctggagacaa gagcccacagagtagctactgaagaaaggggccggaagccccaccaaattcca gcttcaacccctcagaatccagcaacttcccaacatcctcctccaccacctggtcat cgttcccaggcacctagtcatcgtcccccgcctcctggacaccgtgttcagcacca gcctcagaagaggcctcctgctccgtcgggcacacaagttcaccagcagaaagg cccgcccctccccagacctcgagttcagccaaaacctccccatggggcagcaga aaactcattgtccccttcctctaat
48	CD27 intracellular domain amino acid sequence	QRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQE DYRKPEPACSP
49	CD27 intracellular domain nucleic acid sequence	caacgaaggaaatatagatcaaacaaaggagaaagtcctgtggagcctgcagag ccttgtcgttacagctgccccagggaggaggagggcagcaccatccccatccag gaggattaccgaaaaccggagcctgcctgctccccc
50	OX40 intracellular domain amino acid sequence	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAH STLAKI
51	OX40 intracellular domain nucleic acid sequence	gccctgtacctgctccgcagggaccagaggctgccccccgatgcccacaagccc cctgggggaggcagtttcaggacccccatccaagaggagcaggccgacgccca ctccaccctggccaagatc
52	CD3 (Q14K)zeta domain	RVKFSRSADAPAY**K**QGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR
53	CD3 (Q14K) zeta domain nucleic acid	agagtgaagttcagcaggagcgcagacgcccccgcgtacaagcagggccaga accagctctataacgagctcaatctaggacgaagagaggagtacgatgttttggac aagagacgtggccgggaccctgagatgggggggaaagccgagaaggaagaacc ctcaggaaggcctgtacaatgaactgcagaaagataagatggcggaggcctaca gtgagattgggatgaaaggcgagcgccggaggggcaaggggcacgatggcctt taccagggtctcagtacagccaccaaggacacctacgacgcccttcacatgcagg ccctgcccccctcgc
54	CD3 zeta domain amino acid	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR
55	CD3 zeta domain nucleic acid	agagtgaagttcagcaggagcgcagacgcccccgcgtaccagcagggccaga accagctctataacgagctcaatctaggacgaagagaggagtacgatgttttggac aagagacgtggccgggaccctgagatgggggggaaagccgagaaggaagaacc ctcaggaaggcctgtacaatgaactgcagaaagataagatggcggaggcctaca gtgagattgggatgaaaggcgagcgccggaggggcaaggggcacgatggcctt taccagggtctcagtacagccaccaaggacacctacgacgcccttcacatgcagg ccctgcccccctcgc
92	T2A amino acid sequence	EGRGSLLTCGDVEENPGP
93	T2A nucleic acid sequence	gagggcagaggaagtcttctaacatgcggtgacgtggaggagaatcccggccct
94	T2A spacer amino acid sequence	SGRSGGG
95	T2A spacer nucleic acid sequence	tccggaagatctggcggcgga
96	F2A amino acid sequence	VKQTLNFDLLKLAGDVESNPGP
97	F2A nucleic acid sequence	gtgaaacagactttgaattttgaccttctcaagttggcgggagacgtggagtccaac ccagggccg
98	Furin-(G4S)2-T2A (F-GS2-T2A) linker nucleic acid sequence	cgtgcgaagaggggcggcgggggctccggcgggggaggcagtgagggccgc ggctccctgctgacctgcggagatgtagaagagaacccaggcccc
99	Furin-(G4S)2-T2A (F-GS2-T2A) linker amino acid sequence	RAKRGGGGSGGGGSEGRGSLLTCGDVEENPGP
100	WPRE	INLWITKFVKD*LVFLTMLLLLRYVDTLL*CLCIMLL LPVWLSFSPPCINPGCCLFMRSCGPLSGNVAWCALC LLTQPPLVGALPPPVSSFPGLSLSPSLLPRRNSSPPALP AAGQGLGCWALTIPWCCRGS*RPFLGCSPVLPPGFC AGRPSATSLRPSIQRTFLPAACCRLCGLFRVFAFALR RVGSPFGPPPRL
101	EF-1 alpha promoter	cgtgaggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccg agaagttgggggggaggggtcggcaattgaaccggtgcctagagaaggtggcgc ggggtaaactgggaaagtgatgtcgtgtactggctccgccttttcccgagggtgg gggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttcgcaacgggtt

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		tgccgccagaacacaggtaagtgccgtgtgtggttcccgcgggcctggcctcttta cgggttatggcccttgcgtgccttgaattacttccacctggctgcagtacgtgattctt gatcccgagcttcgggttggaagtgggtgggagagttcgaggccttgcgcttaag gagccccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggccgccg cgtgcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataagtctctagcc atttaaaatttttgatgacctgctgcgacgctttttttctggcaagatagtcttgtaaatg cgggccaagatctgcacactggtatttcggttttggggccgcgggcggcgacgg ggcccgtgcgtcccagcgcacatgttcggcgaggcggggcctgcgagcgcggc caccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctg gcctcgcgccgccgtgtatcgccccgccctgggcggcaaggctggcccggtcg gcaccagttgcgtgagcggaaagatggccgcttcccggccctgctgcagggagc tcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtcacccacac aaaggaaaagggcctttccgtcctcagccgtcgcttcatgtgactccacggagtac cgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtcttta ggttgggggagggttttatgcgatggagtttccccacactgagtgggtggaga ctgaagttaggccagcttggcacttgatgtaattctccttggaatttgccctttttgagt ttggatcttggttcattctcaagcctcagacagtggttcaaagttttttttcttccatttcag gtgtcgtga.
102	Nucleic acid sequence scFv-CD19 E4 P3 (43)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctcttcttatctgaattggtatcagcaaaa acccggcaaggctccaaaactgttgatctatggtgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaatcttacagaaccccagttac attcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggc ggtagcggaggtggtggtagtcaggtccagttagttcaatcaggtgccgaggtca aaaagccaggttcttccgtcaaagtgtcatgcaaggctagcggtggcacctttcta attacgctatcaattgggtgagacaggcaccaggtcaaggactggaatggatggg aagaatcgttccactgctgggtatcgctaattatgcccagaagttccaaggcagggt gaccataactgctgatgagagtacatctaccgcatacatggaattaagttctctgag atccgaggacaccgcagtgtattactgtgctagagaacatatcgcttacagaccaa cctctgctggttactactactacatggatatctggggacaaggaactttggtcacagt gtcatct
103	Nucleic acid sequence scFv-CD19 E7 (45)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccaggatatcaccagatatctgaattggtatcagcaa aaacccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccgg agttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagtt ctttgcagcctgaagactttgcaacatattactgtcagcaatcttactcttacccacca acattcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtgg cggtagcggaggtggtggtagtcaggttcagttagtcgagtctggtggcggtgtcg tccagcctggtagatccttaaggctgtcatgtgccgctagcggatttacctttagaga ttacggtatgcattgggtgagacaagcccccggtaaaggcctggaatgggtcgct gtgataagttacgaaggttctaacgaatactacgcagactccgttaagggtagattc actatttccagggataattcaaagaacactttgtatctgcagatgaactcattgagag

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		ctgaggacaccgcagtgtattactgtgctagagatagaggttttgctggttggtacg attacgcttttgatccatggggacaaggaactttggtcacagtgtcatct
104	Nucleic acid sequence scFv-CD19-7 (46)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctctaaatatctgaattggtatcagcaaa aacccggcaaggctccaaaactgttgatctatgatgcttcaagcctgcaatccgga gttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttct ttgcagcctgaagactttgcaacatattactgtcagcaatcttacaccatcccactga cattcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggc ggtagcggaggtggtggtagtcaggtccagttagttcaatcaggtgccgaggtca aaaagccaggttcttccgtcaaagtgtcatgcaaggctagcggtggcacctttttctt cttacgctttttcttgggtgagacaggcaccaggtcaaggactggaatggatgggga ggtatcgttccactgtttggtgctgttgaatatgcccagaagttccaaggcagggtg accataactgctgatgagagtacatctaccgcatacatggaattaagttctctgagat ccgaggacaccgcagtgtattactgtgctagagaaaaaggtttttacagatactttg atcattggggacaaggaactttggtcacagtgtcatct
105	hIL18 (e.g., GenBank Acc. No. AAK95950.1) leaderless	YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDS DCRDNAPRTIFIISMYKDSQPRGMAVTISVKCEKISTL SCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNK MQFESSSYEGYFLACEKERDLFKLILKKEDELGDRSI MFTVQNED
106	CD8leader +hIL18	MALPVTALLLPLALLLHAARPYFGKLESKLSVIRNL NDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMY KDSQPRGMAVTISVKCEKISTLSCENKIISFKEMNPP DNIKDTKSDIIFFQRSVPGHDNKMQFESSSYEGYFLA CEKERDLFKLILKKEDELGDRSIMFTVQNED
107	Human IL-18 (GenBank Acc. No. AAK95950.1)	MAAEPVEDNCINFVAMKFIDNTLYFIAEDDENLESD YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDS DCRDNAPRTIFIISMYKDSQPRGMAVTISVKCEKISTL SCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNK MQFESSSYEGYFLACEKERDLFKLILKKEDELGDRSI MFTVQNED
108	Human IL-2 signal sequence	MYRMQLLSCIALSLALVTNS
109	Murine IL-2 signal sequence	MYSMOLASCVTLTLVLLVNS
110	human kappa leader sequence	METPAQLLFLLLLWLPDTTG
111	Murine kappa leader sequence	METDTLLLWVLLLWVPGSTG
112	Human albumin signal sequence	MKWVTFISLLFSSAYS
113	Human prolactin signal sequence	MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
114	Nucleic acid sequence scFv-CD19 -14 (49)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctctaattatctgaattggtatcagcaaaa acccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaagcttactctgctccaatcaca ttcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggcg gtagcggaggtggtggtagtgaggttcagttgttagagagcgggggggggtctggt tcagcctggtggcagcttaagactgagttgtgccgcttctggttttactttcggtgatt atgcaatgtcctgggtgaggcaagcccccggtaaaggcctggaatgggtttcagc tatctctagaggtggtcatggtacctactatgccgatagcgtgaaaggtagattcact atttccagggacaattcaaagaacactttgtacttacagatgaactctttgagagctg aggacaccgcagtgtattactgtgctagactggttggttacggtctggattactggg gacaaggaactttggtcacagtgtcatct
115	Nucleic acid sequence scFv-CD19 -15 (50)	caaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtgactatta catgtagggccagccagcctatcagaccttatctgaattggtatcagcaaaaaccc ggcaaggctccaaaactgttgatctatgatgcttcaagcctgcaatccggagttccc tcaagattttctggttccggctcaggaactgatttcaccctgactataagttctttgcag cctgaagactttgcaacatattactgtcagcaatcttactctgctccatacacattcgg gcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggcggtagc ggaggtggtggtagtgaggttcagttgttagagagcgggggggggtctggttcagc ctggtggcagcttaagactgagttgtgccgcttctggttttactttctcttcttatgcaat gtcctgggtgaggcaagcccccggtaaaggcctggaatgggtttcagttatctctg gtggtggtgctaatacctactacgccgatagcgtgaaaggtagattcactatttcca gggacaattcaaagaacactttgtacttacagatgaactctttgagagctgaggaca ccgcagtgtattactgtgctagagattggagatactttgatcattggggacaaggaa ctttggtcacagtgtcatct
116	Nucleic acid sequence scFv-CD19 -18 (52)	cagtccgtgttgacccagcctccatcagtctcaggagccccaggccagagagtga ccatttcttgtactggatcttcctcaaatatcggggccggttacgatgttcattggtatc agcaactgcccggtacagctccaaaactgttgatctatggtaccaaaaacagacct agcggtgtgcccgacaggttctccggctcaaagagcggaacaagtgcttctttagc aattaccggcctgcaggctgaagatgaggcagactattactgtcaatcctacgatgt tagactgaaaggttgggtttttggtggcggtaccaaattgactgtcttaggtggtggt ggttctggtggtggcggtagcggaggtggtggtagtcagttacagttacaggaga gcggtccaggtttagtgaaaccatccgaaactttatcactgacctgtacagtgtctg gtggctccatcacctcttcttcttattactggggttggataagacagccacctggaaa agggctggagtggattggatctatctactacaccggtaccacctactacaatccttc attgaagagcagggtcacaataagcgtggataccagtaaaaaccagttttccttga agctgagttctgtcacagccgctgacaccgcagtgtattactgtgctagatacgttg gtctgtctggtggttttgattactggggacaaggaactttggtcacagtgtcatct
117	Nucleic acid sequence scFv-CD19 -23 (53)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctactcttatctgaattggtatcagcaaaa acgcggcaaggctccaaaactgttgatctatgatgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaatcttacaccgctccaccaac

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		attcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggc ggtagcggaggtggtggtagtgaggttcagttgttagagagcgggggggggtctg gttcagcctggtggcagcttaagactgagttgtgccgcttctggttttactttctctaat tatgcaatgtcctgggtgaggcaagcccccggtaaaggcctggaatgggtttcag ctatctctgaatctggtcatggtacctactacgccgatagcgtgaaaggtagattcac tatttccagggacaattcaaagaacactttgtacttacagatgaactctttgagagctg aggacaccgcagtgtattactgtgctagactggattgggctggttttgatgtttgggg acaaggaactttggtcacagtgtcatct
118	Nucleic acid sequence scFv-CD19 -#10 (47)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagaccatctctagatatctgaattggtatcagcaa aaacccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccgg agttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagtt ctttgcagcctgaagactttgcaacatattactgtcagcaatcttacagaccaccact gacattcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtg gcggtagcggaggtggtggtagtgaggttcagttgttagagagcgggggggggtc tggttcagcctggtggcagcttaagactgagttgtgccgcttctggttttactttctctt cttatgcaatgtcctgggtgaggcaagcccccggtaaaggcctggaatgggtttca accatctctgctggtggtcatggtacctactacgccgatagcgtgaaaggtagattc actatttccagggacaattcaaagaacactttgtacttacagatgaactctttgagag ctgaggacaccgcagtgtattactgtgctagaggtgctggttactttgattactgggg acaaggaactttggtcacagtgtcatct
119	Nucleic acid sequence scFv-CD19 -#11 (48)	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctcttcttatctgaattggtatcagcaaaa acccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaaaccggtgctgttccatacac attcgggcaaggtaccaaggtcgaaattaagggtggtggtggttctggtggtggc ggtagcggaggtggtggtagtgaggttcagttgttagagagcgggggggggtctg gttcagcctggtggcagcttaagactgagttgtgccgcttctggttttactttcagaga ttatgcaatgtcctgggtgaggcaagcccccggtaaaggcctggaatgggtttcag ctatctctgaatctggtatcgatacctactacgccgatagcgtgaaaggtagattcac tatttccagggacaattcaaagaacactttgtacttacagatgaactctttgagagctg aggacaccgcagtgtattactgtgctagagttgctggttacgattctgattcttctacc tactacgattacatggatgtttggggacaaggaactttggtcacagtgtcatct
120	Nucleic acid sequence scFv-CD19 -16 (51)	cagtccgtgttgacccagcctccatcagtctcaggagccccaggccagagagtga ccatttcttgtactggatcttcctcaaatatcggggccggttacgatgttcattggtatc agcaactgcccggtacagctccaaaactgttgatctatggtaataataacagaccta gcggtgtgcccgacaggttctccggctcaaagagcggaacaagtgcttctttagca attaccggcctgcaggctgaagatgaggcagactattactgtcaatcctacgatgtt tctctgggtgtttgggtttttggtggcggtaccaaattgactgtcttaggtggtggtgg ttctggtggtggcggtagcggaggtggtggtagtcagttacagttacaggagagc ggtccaggtttagtgaaaccatccgaaactttatcactgacctgtacagtgtctggtg gctccatctcttctccatcttattactggggttggataagacagccacctggaaaagg gctggagtggattggatctatctactacaccggtgctacctactacaatccttcattg

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		aagagcagggtcacaataagcgtggataccagtaaaaaccagttttccttgaagct gagttctgtcacagccgctgacaccgcagtgtattactgtgctagatacggtccagc tggtgttggttttgattactggggacaaggaactttggtcacagtgtcatct
121	linker	GGSG
122	linker	GGSGG
123	linker	GSGSG
124	linker	GSGGG
125	linker	GGGSG
126	linker	GSSSG
127	linker	GGGGS
128	linker	GGGGSGGGGSGGGGS
129	linker	GGTGGCGGTGGCTCGGGCGGTGGTGGGTCGGGTG GCGGCGGATCT
130	hinge	DKTHT
131	hinge	CPPC
132	hinge	CPEPKSCDTPPPCPR
133	hinge	ELKTPLGDTTHT
134	hinge	KSCDKTHTCP
135	hinge	KCCVDCP
136	hinge	KYGPPCP
137	human IgG1 hinge	EPKSCDKTHTCPPCP
138	human IgG2 hinge	ERKCCVECPPCP
139	human IgG3 hinge	ELKTPLGDTTHTCPRCP
140	human IgG4 hinge	SPNMVPHAHHAQ
56	E4 CDR-L1	RASQSISSYLN
57	E4 CDR-L2	GASSLQS
58	E4 CDR-L3	QQSYRTPVT
59	E4 CDR-H1	KASGGTFSNYAIN
60	E4 CDR-H2	MGRIVPLLGIANYAQ
61	E4 CDR-H3	EHIAYRPTSAGYYYYMDI
62	CD19 E4 V_L	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQ QKPGKAPKLLIYGASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYRTPVTFGQGTKVEIK

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
63	CD19 E4V_H	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSNYAIN WVRQAPGQGLEWMGRIVPLLGIANYAQKFQGRVTI TADESTSTAYMELSSLRSEDTAVYYCAREHIAYRPT SAGYYYYMDIWGQGTLVTVSS
64	scFv-CD19 E4 (44) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQ QKPGKAPKLLIYGASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYRTPVTFGQGTKVEIKGGGG SGGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSCKA SGGTFSNYAINWVRQAPGQGLEWMGRIVPLLGIAN YAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYY CAREHIAYRPTSAGYYYYMDIWGQGTLVTVSS
65	Nucleic acid sequence CD19 E4 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctcttcttatctgaattggtatcagcaaaa acccggcaaggctccaaaactgttgatctatggtgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaatcttacagaaccccagttac attcgggcaaggtaccaaggtcgaaattaag
66	Nucleic acid sequence CD19 E4V_H	caggtccagttagttcaatcaggtgccgaggtcaaaaagccaggttcttccgtcaa agtgtcatgcaaggctagcggtggcacctttctaattacgctatcaattgggtgaga caggcaccaggtcaaggactggaatggatgggaagaatcgttccactgctgggta tcgctaattatgcccagaagttccaaggcagggtgaccataactgctgatgagagt acatctaccgcatacatggaattaagttctctgagatccgaggacaccgcagtgtat tactgtgctagagaacatatcgcttacagaccaacctctgctggttactactacaca tggatatctggggacaaggaactttggtcacagtgtcatct
67	E7 CDR-L1	RASQDITRYLN
68	E7 CDR-L2	AASSLQS
69	E7 CDR-L3	QQSYSYPPT
70	E7 CDR-H1	AASGFTFRDYGMH
71	E7 CDR-H2	VAVISYEGSNEYYADSV
72	E7 CDR-H3	DRGFAGWYDYAFDP
73	CD19 E7 V_L	DIQMTQSPSSLSASVGDRVTITCRASQDITRYLNWY QQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQSYSYPPTFGQGTKVEIK
74	CD19 E7V_H	QVQLVESGGGVVQPGRSLRLSCAASGFTFRDYGMH WVRQAPGKGLEWVAVISYEGSNEYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCARDRGFAG WYDYAFDPWGQGTLVTVSS
75	scFv-CD19 E7 (45) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQDITRYLNWY QQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQSYSYPPTFGQGTKVEIKGGG GSGGGGSGGGGSQVQLVESGGGVVQPGRSLRLSCA ASGFTFRDYGMHWVRQAPGKGLEWVAVISYEGSNE

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAV YYCARDRGFAGWYDYAFDPWGQGTLVTVSS
76	Nucleic acid sequence CD19 E7 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccaggatatcaccagatatctgaattggtatcagcaa aaacccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccgg agttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagtt ctttgcagcctgaagactttgcaacatattactgtcagcaatcttactcttacccacca acattcgggcaaggtaccaaggtcgaaattaag
77	Nucleic acid sequence CD19 E7V_H	caggttcagttagtcgagtctggtggcggtgtcgtccagcctggtagatccttaagg ctgtcatgtgccgctagcggatttacctttagagattacggtatgcattgggtgagac aagcccccggtaaaggcctggaatgggtcgctgtgataagttacgaaggttctaac gaatactacgcagactccgttaagggtagattcactatttccagggataattcaaag aacactttgtatctgcagatgaactcattgagagctgaggacaccgcagtgtattact gtgctagagatagaggttttgctggttggtacgattacgcttttgatccatggggaca aggaactttggtcacagtgtcatct
78	7 CDR-L1	RASQSISKYLN
79	7 CDR-L2	DASSLQS
80	7 CDR-L3	QQSYTIPLT
81	7 CDR-H1	KASGGTFSSYAFS
82	7 CDR-H2	MGGIVPLFGAVEYAQ
83	7 CDR-H3	EKGFYRYFDH
84	CD19-7 V_L	DIQMTQSPSSLSASVGDRVTITCRASQSISKYLNWYQ QKPGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYTIPLTFGQGTKVEIK
85	CD19-7V_H	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAFS WVRQAPGQGLEWMGGIVPLFGAVEYAQKFQGRVTI TADESTSTAYMELSSLRSEDTAVYYCAREKGFYRYF DHWGQGTLVTVSS
86	scFv-CD19-7 (46) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQSISKYLNWYQ QKPGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYTIPLTFGQGTKVEIKGGGGS GGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSCKAS GGTFSSYAFSWVRQAPGQGLEWMGGIVPLFGAVEY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYC AREKGFYRYFDHWGQGTLVTVSS
87	Nucleic acid sequence CD19-7 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctctaaatatctgaattggtatcagcaaa aacccggcaaggctccaaaactgttgatctatgatgcttcaagcctgcaatccgga gttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttct ttgcagcctgaagactttgcaacatattactgtcagcaatcttacaccatcccactga cattcgggcaaggtaccaaggtcgaaattaag

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
88	Nucleic acid sequence CD19-7V_H	caggtccagttagttcaatcaggtgccgaggtcaaaaagccaggttcttccgtcaa agtgtcatgcaaggctagcggtggcacctttttcttcttacgctttttcttgggtgagac aggcaccaggtcaaggactggaatggatgggaggtatcgttccactgtttggtgct gttgaatatgcccagaagttccaaggcagggtgaccataactgctgatgagagtac atctaccgcatacatggaattaagttctctgagatccgaggacaccgcagtgtatta ctgtgctagagaaaaaggtttttacagatactttgatcattggggacaaggaactttg gtcacagtgtcatct
89	10 CDR-L1	RASQTISRYLN
90	10 CDR-L2	AASSLQS
91	10 CDR-L3	QQSYRPPLT
141	10 CDR-H1	AASGFTFSSYAMS
142	10 CDR-H2	VSTISAGGHGTYYADSV
143	10 CDR-H3	GAGYFDY
144	CD19 10 V_L	DIQMTQSPSSLSASVGDRVTITCRASQTISRYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYRPPLTFGQGTKVEIK
145	CD19 10V_H	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSW VRQAPGKGLEWVSTISAGGHGTYYADSVKGRFTISR DNSKNTLYLQMNSLRAEDTAVYYCARGAGYFDYW GQGTLVTVSS
146	scFv-CD19 10 (47) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQTISRYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYRPPLTFGQGTKVEIKGGGG SGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFSSYAMSWVRQAPGKGLEWVSTISAGGHGTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARGAGYFDYWGQGTLVTVSS
147	Nucleic acid sequence CD19 10 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagaccatctctagatatctgaattggtatcagcaa aaacccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccgg agttccctcaagattttctggttcggctcaggaactgatttcaccctgactataagtt ctttgcagcctgaagactttgcaacatattactgtcagcaatcttacagaccaccact gacattcgggcaaggtaccaaggtcgaaattaag
148	Nucleic acid sequence CD19 10V_H	gaggttcagttgttagagagcgggggggggtctggttcagcctggtggcagcttaa gactgagttgtgccgcttctggttttactttctcttcttatgcaatgtcctgggtgaggc aagcccccggtaaaggcctggaatgggtttcaaccatctctgctggtggtcatggt acctactacgccgatagcgtgaaaggtagattcactatttccagggacaattcaaag aacactttgtacttacagatgaactctttgagagctgaggacaccgcagtgtattact gtgctagaggtgctggttactttgattactggggacaaggaactttggtcacagtgtc atct
149	11 CDR-L1	RASQSISSYLN
150	11 CDR-L2	AASSLQS
151	11 CDR-L3	QQTGAVPYTF

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
152	11 CDR-H1	AASGFTFRDYAMS
153	11 CDR-H2	VSAISESGIDTYYADSV
154	11 CDR-H3	VAGYDSDSSTYYDYMDV
155	CD19 11 V_L	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQTGAVPYTFGQGTKVEIK
156	CD19 11V_H	EVQLLESGGGLVQPGGSLRLSCAASGFTFRDYAMS WVRQAPGKGLEWVSAISESGIDTYYADSVKGRFTIS RDNSKNTLYLQMNSLRAEDTAVYYCARVAGYDSD SSTYYDYMDVWGQGTLVTVSS
157	scFv-CD19 11 (48) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQTGAVPYTFGQGTKVEIKGGGG SGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFRDYAMSWVRQAPGKGLEWVSAISESGIDTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARVAGYDSDSSTYYDYMDVWGQGTLVTVSS
158	Nucleic acid sequence CD19 11 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctcttcttatctgaattggtatcagcaaaa acccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaaaccggtgctgttccatacac attcgggcaaggtaccaaggtcgaaattaag
159	Nucleic acid sequence CD19 11V_H	gaggttcagttgttagagagcgggggggtctggttcagcctggtggcagcttaa gactgagttgtgccgcttctggttttactttcagagattatgcaatgtcctgggtgagg caagcccccggtaaaggcctggaatgggtttcagctatctctgaatctggtatcgat acctactacgccgatagcgtgaaaggtagattcactatttccagggacaattcaaag aacactttgtacttacagatgaactctttgagagctgaggacaccgcagtgtattact gtgctagagttgctggttacgattctgattcttctacctactacgattacatggatgttt ggggacaaggaactttggtcacagtgtcatct
160	14 CDR-L1	RASQSISNYLN
161	14 CDR-L2	AASSLQS
162	14 CDR-L3	QQAYSAPIT
163	14 CDR-H1	AASGFTFGDYAMS
164	14 CDR-H2	VSAISRGGHGTYYADSV
165	14 CDR-H3	LVGYGLDY
166	CD19 14 V_L	DIQMTQSPSSLSASVGDRVTITCRASQSISNYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQAYSAPITFGQGTKVEIK
167	CD19 14V_H	EVQLLESGGGLVQPGGSLRLSCAASGFTFGDYAMS WVRQAPGKGLEWVSAISRGGHGTYYADSVKGRFTI

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		SRDNSKNTLYLQMNSLRAEDTAVYYCARLVGYGLD YWGQGTLVTVSS
168	scFv-CD19 14 (49) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQSISNYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQAYSAPITFGQGTKVEIKGGGG SGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFGDYAMSWVRQAPGKGLEWVSAISRGGHGTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY YCARLVGYGLDYWGQGTLVTVSS
169	Nucleic acid sequence CD19 14 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctctaattatctgaattggtatcagcaaaa acccggcaaggctccaaaactgttgatctatgctgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaagcttactctgctccaatcaca ttcgggcaaggtaccaaggtcgaaattaag
170	Nucleic acid sequence CD19 14V_H	gaggttcagttgttagagagcgggggggggtctggttcagcctggtggcagcttaa gactgagttgtgccgcttctggttttactttcggtgattatgcaatgtcctgggtgagg caagcccccggtaaaggcctggaatgggtttcagctatctctagaggtggtcatgg tacctactatgccgatagcgtgaaaggtagattcactatttccagggacaattcaaa gaacactttgtacttacagatgaactctttgagagctgaggacaccgcagtgtattac tgtgctagactggttggttacggtctggattactggggacaaggaactttggtcaca gtgtcatct
171	15 CDR-L1	RASQPIRPYLN
172	15 CDR-L2	DASSLQS
173	15 CDR-L3	QQSYSAPYT
174	15 CDR-H1	AASGFTFSSYAMS
175	15 CDR-H2	VSVISGGGANTYYADSVK
176	15 CDR-H3	DWRYFDH
177	CD19 15 V_L	DIQMTQSPSSLSASVGDRVTITCRASQPIRPYLNWYQ QKPGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYSAPYTFGQGTKVEIK
178	CD19 15V_H	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSW VRQAPGKGLEWVSVISGGGANTYYADSVKGRFTISR DNSKNTLYLQMNSLRAEDTAVYYCARDWRYFDHW GQGTLVTVSS
179	scFv-CD19 15 (50) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQPIRPYLNWYQ QKPGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYSAPYTFGQGTKVEIKGGGG SGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFSSYAMSWVRQAPGKGLEWVSVISGGGANTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARDWRYFDHWGQGTLVTVSS

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
180	Nucleic acid sequence CD19 15 V_L	caaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtgactatta catgtagggccagccagcctatcagaccttatctgaattggtatcagcaaaaaccc ggcaaggctccaaaactgttgatctatgatgcttcaagcctgcaatccggagttccc tcaagattttctggttccggctcaggaactgatttcaccctgactataagttctttgcag cctgaagactttgcaacatattactgtcagcaatcttactctgctccatacacattcgg gcaaggtaccaaggtcgaaattaag
181	Nucleic acid sequence CD19 15V_H	gaggttcagttgttagagagcgggggggtctggttcagcctggtggcagcttaa gactgagttgtgccgcttctggttttactttctcttcttatgcaatgtcctgggtgaggc aagcccccggtaaaggcctggaatgggtttcagttatctctggtggtggtgctaata cctactacgccgatagcgtgaaaggtagattcactatttccagggacaattcaaaga acactttgtacttacagatgaactctttgagagctgaggacaccgcagtgtattactg tgctagagattggagatactttgatcattggggacaaggaactttggtcacagtgtc atct
182	16 CDR-L1	TGSSSNIGAGYDVH
183	16 CDR-L2	GNNNRPS
184	16 CDR-L3	QSYDVSLGVWV
185	16 CDR-H1	TVSGGSISSPSYYWG
186	16 CDR-H2	IGSIYYTGATYYNPSL
187	16 CDR-H3	YGPAGVGFDY
188	CD19 16 V_L	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVH WYQQLPGTAPKLLIYGNNNRPSGVPDRFSGSKSGTS ASLAITGLQAEDEADYYCQSYDVSLGVWVFGGGTK LTVL
189	CD19 16V_H	QLQLQESGPGLVKPSETLSLTCTVSGGSISSPSYYWG WIRQPPGKGLEWIGSIYYTGATYYNPSLKSRVTISVD TSKNQFSLKLSSVTAADTAVYYCARYGPAGVGFDY WGQGTLVTVSS
190	scFv-CD19 16 (51) Human derived	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVH WYQQLPGTAPKLLIYGNNNRPSGVPDRFSGSKSGTS ASLAITGLQAEDEADYYCQSYDVSLGVWVFGGGTK LTVLGGGGSGGGGSGGGGSQLQLQESGPGLVKPSET LSLTCTVSGGSISSPSYYWGWIRQPPGKGLEWIGSIY YTGATYYNPSLKSRVTISVDTSKNQFSLKLSSVTAA DTAVYYCARYGPAGVGFDYWGQGTLVTVSS
191	Nucleic acid sequence CD19 16 V_L	cagtccgtgttgacccagcctccatcagtctcaggagccccaggccagagagtga ccatttcttgtactggatcttcctcaaatatcggggccggttacgatgttcattggtatc agcaactgcccggtacagctccaaaactgttgatctatggtaataataacagaccta gcggtgtgcccgacaggttctccggctcaaagagcggaacaagtgcttctttagca attaccggcctgcaggctgaagatgaggcagactattactgtcaatcctacgatgtt tctctgggtgtttgggttttggtggcggtaccaaattgactgtctta
192	Nucleic acid sequence CD19 16V_H	cagttacagttacaggagagcggtccaggtttagtgaaaccatccgaaactttatca ctgacctgtacagtgtctggtggctccatctcttctccatcttattactggggttggata agacagccacctggaaaagggctggagtggattggatctatctactacaccggtg

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
		ctacctactacaatccttcattgaagagcagggtcacaataagcgtggataccagta aaaaccagttttccttgaagctgagttctgtcacagccgctgacaccgcagtgtatta ctgtgctagatacggtccagctggtgttggttttgattactggggacaaggaactttg gtcacagtgtcatct
193	18 CDR-L1	TGSSSNIGAGYDVH
194	18 CDR-L2	GTKNRPS
195	18 CDR-L3	QSYDVRLKGWV
196	18 CDR-H1	TVSGGSITSSSYYWG
197	18 CDR-H2	IGSIYYTGTTYYNPSL
198	18 CDR-H3	YVGLSGGFDY
199	CD19 18 V_L	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVH WYQQLPGTAPKLLIYGTKNRPSGVPDRFSGSKSGTS ASLAITGLQAEDEADYYCQSYDVRLKGWVFGGGTK LTVL
200	CD19 18V_H	QLQLQESGPGLVKPSETLSLTCTVSGGSITSSSYYWG WIRQPPGKGLEWIGSIYYTGTTYYNPSLKSRVTISVD TSKNQFSLKLSSVTAADTAVYYCARYVGLSGGFDY WGQGTLVTVSS
201	scFv-CD19 18 (clone 52) Human derived	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVH WYQQLPGTAPKLLIYGTKNRPSGVPDRFSGSKSGTS ASLAITGLQAEDEADYYCQSYDVRLKGWVFGGGTK LTVLGGGGSGGGGSGGGGSQLQLQESGPGLVKPSET LSLTCTVSGGSITSSSYYWGWIRQPPGKGLEWIGSIY YTGTTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAAD TAVYYCARYVGLSGGFDYWGQGTLVTVSS
202	Nucleic acid sequence CD19 18 V_L	cagtccgtgttgacccagcctccatcagtctcaggagccccaggccagagagtga ccatttcttgtactggatcttcctcaaatatcggggccggttacgatgttcattggtatc agcaactgcccggtacagctccaaaactgttgatctatggtaccaaaaacagacct agcggtgtgcccgacaggttctccggctcaaagagcggaacaagtgcttctttagc aattaccggcctgcaggctgaagatgaggcagactattactgtcaatcctacgatgt tagactgaaaggttgggtttttggtggcggtaccaaattgactgtctta
203	Nucleic acid sequence CD19 18V_H	cagttacagttacaggagagcggtccaggtttagtgaaaccatccgaaactttatca ctgacctgtacagtgtctggtggctccatcacctcttcttcttattactggggttggata agacagccacctggaaaagggctggagtggattggatctatctactacaccggta ccacctactacaatccttcattgaagagcagggtcacaataagcgtggataccagt aaaaaccagttttccttgaagctgagttctgtcacagccgctgacaccgcagtgtatt actgtgctagatacgttggtctgtctggtggttttgattactggggacaaggaactttg gtcacagtgtcatct
204	23 CDR-L1	RASQSIYSYLN
205	23 CDR-L2	DASSLQS
206	23 CDR-L3	QQSYTAPPT
207	23 CDR-H1	AASGFTFSNYAMS
208	23 CDR-H2	VSAISESGHGTYYADSV

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
209	23 CDR-H3	LDWAGFDV
210	CD19 23 V_L	DIQMTQSPSSLSASVGDRVTITCRASQSIYSYLNWYQ QKRGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLT ISSLQPEDFATYYCQQSYTAPPTFGQGTKVEIK
211	CD19 23V_H	EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYAMS WVRQAPGKGLEWVSAISESGHGTYYADSVKGRFTIS RDNSKNTLYLQMNSLRAEDTAVYYCARLDWAGFD VWGQGTLVTVSS
212	scFv-CD19 23 (53) Human derived	DIQMTQSPSSLSASVGDRVTITCRASQSIYSYLNWYQ QKRGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLT ISSLQPEDFATYYCQQSYTAPPTFGQGTKVEIKGGGG SGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFSNYAMSWVRQAPGKGLEWVSAISESGHGTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARLDWAGFDVWGQGTLVTVSS
213	Nucleic acid sequence CD19 23 V_L	gacatacaaatgacccagtctccttcttctctgagcgcatctgtcggcgatagagtg actattacatgtagggccagccagtctatctactcttatctgaattggtatcagcaaaa acgcggcaaggctccaaaactgttgatctatgatgcttcaagcctgcaatccggag ttccctcaagattttctggttccggctcaggaactgatttcaccctgactataagttcttt gcagcctgaagactttgcaacatattactgtcagcaatcttacaccgctccaccaac attcgggcaaggtaccaaggtcgaaattaag
214	Nucleic acid sequence CD19 23V_H	gaggttcagttgttagagagcgggggggggtctggttcagcctggtggcagcttaa gactgagttgtgccgcttctggttttactttctctaattatgcaatgtcctgggtgaggc aagcccccggtaaaggcctggaatgggtttcagctatctctgaatctggtcatggta cctactacgccgatagcgtgaaaggtagattcactatttccagggacaattcaaaga acactttgtacttacagatgaactctttgagagctgaggacaccgcagtgtattactg tgctagactggattgggctggttttgatgtttggggacaaggaactttggtcacagtg tcatct
215	IL18(F170E)	YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDS DCRDNAPRTIFIISMYKDSQPRGMAVTISVKCEKISTL SCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNK MQFESSSYEGYFLACEKERDLEKLILKKEDELGDRSI MFTVQNED
216	Nucleic acid sequence CD19 52CO scFv (P13)	cagtctgtgctgacacagcctccatctgtgtctggcgctccaggccagagagtgac catcagctgtacaggcagcagcagcaatatcggagccggctatgacgtgcactg gtatcagcagctgcctggcacagcccctaaactgctgatctacggcaccaagaac agacccagcggcgtgcccgatagattcagcggctctaagtctggcacaagcgcc agcctggccattactggactgcaggccgaagatgaggccgactactactgccaga gctacgacgtgcggctgaaaggctgggttttcggcggaggcacaaagctgacag tgcttggaggcggaggatctggcggaggtggaagtggcggaggcggatctcaa ctgcagctccaagaatctggccctggcctggtcaagcctagcgagacactgagcc

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
	Codon optimized version of P11 (52 opt)	tgacctgtacagtgtccggcggcagcatcacaagcagcagctattactggggctg gatcagacagcctcctggcaaaggcctggaatggatcggctccatctactacacc ggcaccacctactacaaccccagcctgaagtcccgcgtgaccatctctgtggaca ccagcaagaaccagttctccctgaagctgagcagcgtgacagccgccgatacag ccgtgtactactgcgccagatacgtgggactgagcggcggctttgattattggggc cagggcacactggtcaccgtgtcatct
217	Full-length CD19 (UniProtKB accession: P15391-1)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVL QCLKGTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGI HMRPLAIWLFIFNVSQQMGGFYLCQPGPPSEKAWQP GWTVNVEGSGELFRWNVSDLGGLGCGLKNRSSEGP SSPSGKLMSPKLYVWAKDRPEIWEGEPPCLPPRDSL NQSLSQDLTMAPGSTLWLSCGVPPDSVSRGPLSWTH VHPKGPKSLLSLELKDDRPARDMWVMETGLLLPRA TAQDAGKYYCHRGNLTMSFHLEITARPVLWHWLLR TGGWK**VSAVTLAYLIFCLCSLVGILHL***QRALVLRRK RKRMTDPTRRFFKVTPPPGSGPQNQYGNVLSLPTPTSG LGRAQRWAAGLGGTAPSYGNPSSDVQADGALGSRSPP GVGPEEEEGEGYEEPDSEEDSEFYENDSNLGQDQLSQ DGSGYENPEDEPLGPEDEDSFSNAESYENEDEELTQPV ARTMDFLSPHGSAWDPSREATSLGSQSYEDMRGILYAA PQLRSIRGQPGPNHEEDADSYENMDNPDGPDPAWGG GGRMGTWSTR*
218	7URV_1|Chain A[auth C]|B-lymphocyte antigen CD19|Homo sapiens (9606)	EEPLVVKVEEGDNAVLQCLKGTSDGPTQQLTWSRE SPLKPFLKLSLGLPGLGIHVSPLAIWLFISNVSQQMG GFYLCQPGPPSEKAWQPGWTVNVEGSGELFRWNVS DLGGLGCGLKNRSSEGPSSPSGKLMSPKLYV**WAKD RPE**IWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWL SCGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDR PARDMWVMETGLLLPRATAQDAGKYYCHRGNLTM SFHLEITAR
219	FMC63, 4G7, 3B10 epitope region 1	***W***AKD***R***P***E***IWEGEP
220	FMC63, 4G7, 3B10 epitope region 2	PKGPKSLLSLE
221	CD19 42og epitope region 1	QPGPPSEKAWQP
222	CD19 42og epitope region 2	VPPDS**V**SRGPL
223	CD19 42og epitope region 1	**Q**PGPPSE**K**A

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
224	CD19 42og epitope region 2	PPDSVSR
225	Nucleic acid sequence CD19 42CO scFv (P14) Codon optimized version of P1 (42 opt) 42 scFv Codon Optimized-nucleotides	GACATCCAGATGACACAGAGCCCTAGCAGCCTGT CTGCCAGCGTGGGAGACAGAGTGACCATCACCTG TAGAGCCAGCCAGACCATCAGCAACTACCTGAAC TGGTATCAGCAGAAGCCCGGCAAGGCCCCTAAGC TGCTGATCTATGCTGCCAGCTCTCTGCAGTCTGGC GTGCCCAGCAGATTTTCTGGCAGCGGCTCTGGCAC CGACTTCACCCTGACCATATCTAGCCTGCAGCCTG AGGACTTCGCCACCTACTACTGCCAGCAGAGCTA CAGCACCCCTCCTACATTTGGCCAGGGCACCAAG GTGGAAATCAAAGGCGGCGGAGGATCTGGCGGAG GTGGAAGTGGCGGAGGCGGATCTGAAGTTCAGCT GCTTGAATCTGGCGGCGGACTGGTTCAACCTGGC GGATCTCTGAGACTGAGCTGTGCCGCCAGCGGCTT CACCTTCAGCAATTACGCCATCAGCTGGGTCCGAC AGGCCCCTGGAAAAGGCCTTGAATGGGTGTCCGT GATCACAGCCAGCGGCGTGGACACCTATTACGCC GATTCTGTGAAGGGCAGATTCACCATCAGCCGGG ACAACAGCAAGAACACCCTGTACCTGCAGATGAA CAGCCTGAGAGCCGAGGACACCGCCGTGTACTAT TGTGCCAGAGGCGGCACCCCTTACTTCATCACCAC CTACGACTACTACGGCTTCGACGTGTGGGGCCAG GGAACACTGGTTACCGTTAGCTCT
226	amino acid sequence CD19 42CO scFv (P14) Codon optimized version of P1 (42 opt)	DIQMTQSPSSLSASVGDRVTITCRASQTISNYLNWYQ QKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQSYSTPPTFGQGTKVEIKGGGGS GGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAAS GFTFSNYAISWVRQAPGKGLEWVSVITASGVDTYY

Table 1: All Sequences		
SEQ ID NO.	**Description**	**Sequences**
	42 scFv Codon Optimized-amino acids	ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARGGTPYFITTYDYYGFDVWGQGTLVTVSS

EQUIVALENTS

[0635] The present technology is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the present technology. Many modifications and variations of this present technology can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatuses within the scope of the present technology, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the present technology. It is to be understood that this present technology is not limited to particular methods, reagents, compounds compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0636] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0637] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, *etc.* As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, *etc.* As will also be understood by one skilled in the art all language such as “up to,”

"at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 cells refers to groups having 1, 2, or 3 cells. Similarly, a group having 1-5 cells refers to groups having 1, 2, 3, 4, or 5 cells, and so forth.

INCORPORATION BY REFERENCE

[0638] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

Table 15: Expansion of ND528 CD4$^+$ and CD8$^+$ CAR T cells over 6 re-stimulations														
End of	**Stim 1**		**Stim 1**		**Stim 2**		**Stim 3**		**Stim 4**		**Stim 5**		**Stim 6**	
Target cells	Nalm6-CD19KO		Nalm6-WT		Nalm6-WT		Nalm6-WT		Nalm6-WT		Nalm6-WT		Nalm6-WT	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
42OP	53.60	30.60	53.60	30.60	33.10	41.20	15.70	48.00	11.90	54.30	9.47	60.40	8.89	67.80
44OP	40.10	42.00	40.1	42.00	18.60	54.30	11.20	56.60	9.11	62.10	9.73	62.00	11.90	62.40
45OP	47.60	19.50	47.6	19.50	25.90	27.70	14.80	24.60	11.40	33.00	9.84	28.90	9.68	30.10
52OP	35.50	48.00	35.5	48.00	19.00	54.30	10.90	55.20	7.48	62.40	6.11	64.40	6.09	70.20

Table 16: ND528 CD19 CAR T cell Killing function over 6 re-stimulations														
End of	**Stim 1**		**Stim 1**		**Stim 2**		**Stim 3**		**Stim 4**		**Stim 5**		**Stim 6**	
Target cells	Nalm6-CD19KO		Nalm6-WT		Nalm6-WT		Nalm6-WT		Nalm6-WT		Nalm6-WT		Nalm6-WT	
IRFP720	-	+	-	+	-	+	-	+	-	+	-	+	-	+
42OP	4.340	95.400	7.990	91.900	93.000	5.920	99.900	0.010	99.900	0.005	99.900	0.006	99.900	0.000
44OP	11.900	88.100	77.900	22.000	99.600	0.016	85.900	14.000	99.800	0.020	22.100	77.700	7.830	90.400
45OP	9.330	90.600	28.400	71.500	98.000	1.390	98.100	1.790	98.500	1.090	26.300	73.300	16.700	82.600
52OP	5.460	94.400	18.900	81.000	98.100	0.049	99.900	0.013	99.800	0.013	98.800	1.010	99.900	0.022