

CHIMERIC ANTIGEN RECEPTORS TARGETING CD19 AND USE THEREOF**1. CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Patent Application
5 Serial No. 63/015,362, filed April 24, 2020, and U.S. Provisional Patent Application
Serial No. 63/073,133, filed September 1, 2020, the contents of each of which are
incorporated by reference in their entirety, and to each of which priority is claimed.

2. TECHNICAL FIELD

The presently disclosed subject matter provides methods for treating
10 neoplasm (e.g., cancer) using cells comprising a chimeric antigen receptor (CAR) that
specifically targets CD19.

3. BACKGROUND

Cell-based immunotherapy is a therapy with curative potential for the
treatment of cancer. T-cells and other immune cells may be modified to target tumor
15 antigens through the introduction of genetic material coding for artificial or synthetic
receptors for antigen, termed Chimeric Antigen Receptors (CARs), specific to selected
antigens. Targeted T-cell therapy using CARs has shown recent clinical success in
treating hematologic malignancies.

T-cells expressing CD19-specific CARs with binding domains from
20 murine monoclonal antibodies have been shown to treat B-cell malignancies. T-cell
mediated immune responses specific for murine scFv antigen-binding domain of the
CAR can develop in human patients and result in premature elimination of CAR T-cells
increasing the risk of tumor relapse. Thus, there are needs for improved CD19-targeted
CARs with reduced toxicity and immunogenicity and/or improved safety and efficacy.

4. SUMMARY OF THE INVENTION

The presently disclosed subject matter provides CD19-targeted chimeric
antigen receptors (CARs), cells comprising the CD19-targeted CARs, and uses of the
cells for treatments, e.g., for treating a neoplasm.

In certain embodiments, the CAR comprises an extracellular antigen-
30 binding domain that specifically binds to CD19, a transmembrane domain, and an
intracellular signaling domain. In certain embodiments, the extracellular antigen-binding
domain comprises a single-chain variable fragment (scFv), a Fab, or a F(ab)₂. In certain
embodiments, the extracellular antigen-binding domain comprises a scFv. In certain

embodiments, the scFv is a human scFv. In certain embodiments, the extracellular antigen-binding domain comprises (a) a heavy chain variable region comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and/or (b) a light chain variable region comprising:

i) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof;

ii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17 or a conservative modification thereof;

iii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof;

iv) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57 or a conservative modification thereof;

v) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59 or a conservative modification thereof;

vi) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence

set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61 or a conservative modification thereof;

5 vii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63 or a conservative modification thereof; or

10 viii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66 or a conservative modification thereof.

15 In certain embodiments, the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9.

20 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13.

25 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17.

30 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21.

 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57.

In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59.

5 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61.

10 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63.

15 In certain embodiments, the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66.

20 In certain embodiments, the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13.

25 In certain embodiments, the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3
30 comprising the amino acid sequence set forth in SEQ ID NO: 17.

In certain embodiments, the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region

comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21.

In certain embodiments, the heavy chain variable region comprises a
5 CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2
comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising
the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region
comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a
CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3
10 comprising the amino acid sequence set forth in SEQ ID NO: 57.

In certain embodiments, the heavy chain variable region comprises a
CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2
comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising
the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region
15 comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a
CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3
comprising the amino acid sequence set forth in SEQ ID NO: 59.

In certain embodiments, the heavy chain variable region comprises a
CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2
20 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising
the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region
comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a
CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3
comprising the amino acid sequence set forth in SEQ ID NO: 61.

In certain embodiments, the heavy chain variable region comprises a
25 CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2
comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising
the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region
comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a
30 CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3
comprising the amino acid sequence set forth in SEQ ID NO: 63.

In certain embodiments, the heavy chain variable region comprises a
CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2
comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising

the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66.

5 In certain embodiments, the heavy chain variable region comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO:
10 10. In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10.

 In certain embodiments, the light chain variable region comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%,
15 about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67. In certain embodiments, the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18,
20 SEQ ID NO: 22 SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67. In certain embodiments, the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, or SEQ ID NO: 22.

 In certain embodiments, the heavy chain variable region comprising an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about
25 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 10; and the light chain variable region comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%,
30 about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67. In certain embodiments, the heavy chain variable region comprises the

amino acid sequence set forth in SEQ ID NO: 10; and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67. In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10; and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, or SEQ ID NO: 22.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 18.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 22.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 58.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 60.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 62.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 64.

In certain embodiments, the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 67.

In certain embodiments, the extracellular antigen-binding domain comprises a linker between the heavy chain variable region and the light chain variable region. In certain embodiments, the linker consists of the amino acid sequence set forth

in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In certain embodiments, the heavy chain variable region and the light chain variable region are positioned from the N- to the C-terminus: V_L-V_H.

In certain embodiments, the extracellular antigen-binding domain
 5 comprises or is a scFv, which comprises or consists of the amino acid sequence set forth in SEQ ID NO: 15, SEQ ID NO: 19, or SEQ ID NO: 23.

In certain embodiments, the transmembrane domain comprises a CD8 polypeptide, a CD28 polypeptide, a CD3ζ polypeptide, a CD4 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a CTLA-4 polypeptide, a PD-1
 10 polypeptide, a LAG-3 polypeptide, a 2B4 polypeptide, or a BTLA polypeptide. In certain embodiments, the transmembrane domain comprises a CD28 polypeptide.

In certain embodiments, the intracellular signaling domain comprises a CD3ζ polypeptide. In certain embodiments, the CD3ζ polypeptide is a modified CD3ζ polypeptide. In certain embodiments, the modified CD3ζ polypeptide comprises a native
 15 ITAM1, an ITAM2 variant consisting of two loss-of-function mutations, and an ITAM3 consisting of two loss-of-function mutations. In certain embodiments, the native ITAM1 consists of the amino acid sequence set forth in SEQ ID NO: 31. In certain embodiments, the ITAM2 variant consists of the amino acid sequence set forth in SEQ ID NO: 37. In certain embodiments, the ITAM3 variant consists of the amino acid
 20 sequence set forth in SEQ ID NO: 41. In certain embodiments, the modified CD3ζ polypeptide comprising or consists of the amino acid sequence set forth in SEQ ID NO: 43.

In certain embodiments, the intracellular signaling domain further comprises at least one co-stimulatory signaling region. In certain embodiments, the at
 25 least one co-stimulatory signaling region comprises a CD28 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide, or a combination thereof. In certain embodiments, the at least one co-stimulatory signaling region comprises an intracellular domain of CD28 or a portion thereof, an intracellular domain of 4-1BB or a portion thereof, an intracellular domain of OX40 or a portion
 30 thereof, an intracellular domain of ICOS or a portion thereof, or an intracellular domain of DAP-10 or a portion thereof. In certain embodiments, the at least one co-stimulatory signaling region comprises a CD28 polypeptide.

In certain embodiments, the CAR is expressed from a vector. In certain embodiments, the vector is a retroviral vector.

The presently disclosed subject matter further provides cells comprising the CARs disclosed herein. In certain embodiments, the cell is transduced with the CAR. In certain embodiments, the CAR is constitutively expressed on the surface of the cell. In certain embodiments, the cell is an immunoresponsive cell. In certain embodiments, the cell is a cell of the lymphoid lineage or a cell of the myeloid lineage. In certain
5 embodiments, the cell is selected from the group consisting of a T cell, a Natural Killer (NK) cell, a stem cell from which lymphoid cells may be differentiated, and a stem cell from which myeloid cells may be differentiated. In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is selected from the group consisting of helper T
10 cells, cytotoxic T cells, memory T cells, regulatory T cells, tumor-infiltrating lymphocyte (TIL), Natural Killer T cells, mucosal associated invariant T cells, and $\gamma\delta$ T cells. In certain embodiments, the cell is a NK cell. In certain embodiments, the NK cell is derived from a stem cell. In certain embodiments, the stem cell is a pluripotent stem cell. In certain embodiments, the pluripotent stem cell is an embryoid stem cell or an induced
15 pluripotent stem cell.

Furthermore, the presently disclosed subject matter provides nucleic acid molecules encoding the CAR disclosed herein. In certain embodiments, the nucleic acid molecule further comprises a promoter that is operably linked to the CAR. The promoter can be endogenous or exogenous. In certain embodiments, the promoter is an exogenous
20 promoter. In certain embodiments, the exogenous promoter is selected from the group consisting of an elongation factor (EF)-1 promoter, a cytomegalovirus immediate-early promoter (CMV) promoter, a simian virus 40 early promoter (SV40) promoter, a phosphoglycerate kinase (PGK) promoter, a metallothionein promoter, and Ubiquitin C promoter. In certain embodiments, the promoter is an endogenous promoter. In certain
25 embodiments, the endogenous promoter is selected from a TCR alpha promoter, a TCR beta promoter, and a beta 2-microglobulin promoter. In certain embodiments, the promoter is an inducible promoter. In certain embodiments, the inducible promoter is selected from the group consisting of a NFAT transcriptional response element (TRE) promoter, a CD69 promoter, a CD25 promoter, an IL-2 promoter, a 4-1BB promoter, a
30 PD1 promoter, and a LAG3 promoter.

The presently disclosed subject matter also provides vectors comprising the nucleic acid molecule disclosed herein. In certain embodiments, the vector is a retroviral vector.

The presently disclosed subject matter further provides cells expressing the nucleic acid molecule disclosed herein. In certain embodiments, the cell is a T cell or Natural Killer (NK) cell.

5 The presently disclosed subject matter provides compositions comprising the cell disclosed herein. In certain embodiments, the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier. In certain embodiments, the composition comprises between about 1×10^6 and about 5×10^8 cells. In certain embodiments, the composition comprises between about 1×10^6 and about 1×10^8 cells. In certain embodiments, the composition comprises between about 1×10^6 and
10 about 5×10^7 cells. In certain embodiments, the composition comprises about 2.5×10^7 cells.

The presently disclosed subject matter further provides various methods of using the presently disclosed cells. The presently disclosed subject matter provides methods of reducing tumor burden in a subject. In certain embodiments, the method
15 comprises administering to the subject the cell or the composition disclosed herein. In certain embodiments, the method reduces the number of tumor cells, reduces tumor size, and/or eradicates the tumor in the subject.

The presently disclosed subject matter provides methods of increasing or lengthening survival of a subject having a neoplasm. In certain embodiments, the
20 method comprises administering to the subject the cell or the composition disclosed herein.

The presently disclosed subject matter provides methods of treating and/or preventing a neoplasm in a subject. In certain embodiments, the method comprises administering to the subject the cell or the composition disclosed herein.

25 The presently disclosed cells and compositions can be used in a therapy. In certain embodiments, the presently disclosed cells and compositions are used in treating and/or preventing a neoplasm in a subject. In certain embodiments, the presently disclosed cells and compositions are used in increasing or lengthening survival of a subject having a neoplasm. In certain embodiments, the presently disclosed cells and
30 compositions are used in treating and/or preventing a neoplasm in a subject.

In certain embodiments, the tumor and/or neoplasm is associated with CD19. In certain embodiments, the tumor and/or neoplasm is a blood cancer. In certain embodiments, the blood cancer is selected from the group consisting of multiple myeloma, leukemia, and lymphomas. In certain embodiments, the leukemia is selected

from the group consisting of include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute promyelocytic leukemia (APL), mixed-phenotype acute leukemia (MLL), hairy cell leukemia, and B cell prolymphocytic leukemia. In certain

5 lymphoma is Hodgkin's lymphoma or non-Hodgkin's lymphoma. In certain embodiments, the tumor and/or neoplasm is a B cell malignancy. In certain embodiments, the B cell malignancy is selected from the group consisting of B cell non-Hodgkin lymphomas (NHL), B cell Hodgkin's lymphomas, B cell acute lymphocytic leukemia (ALL), B cell chronic lymphocytic leukemia (CLL), multiple myeloma (MM), CLL with

10 Richter's transformation, and CNS lymphoma. In certain embodiments, the tumor and/or neoplasm is B cell lymphoma. In certain embodiments, the B cell lymphoma is relapsed or refractory (R/R) B cell lymphoma. In certain embodiments, the subject is a human subject.

Furthermore, the presently disclosed subject matter provides methods for

15 producing a cell comprising the CAR disclosed herein. In certain embodiments, the method comprises introducing into the cell a nucleic acid molecule that encodes the CAR disclosed herein.

Furthermore, the presently disclosed subject matter provides kits for reducing tumor burden in a subject, treating and/or preventing a neoplasm in a subject,

20 and/or increasing or lengthening survival of a subject having a neoplasm. In certain embodiments, the kit comprises the cell disclosed herein. In certain embodiments, the kit further comprises written instructions for using the cell for reducing tumor burden in a subject, treating and/or preventing a neoplasm in a subject, and/or increasing or lengthening survival of a subject having a neoplasm.

25 **5. BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A and 1B depict cytotoxicity of T cells comprising CD19-targeted CARs against CD19-expressing cells. Figure 1A shows the killing against CD19-expressing Raji cancer cell lines. Figure 1B shows the killing against CD19 knock-out Raji cells. Positive control is 1928z-1XX CAR.

30 Figures 2A and 2B depict cytotoxicity of T cells comprising CD19-targeted CARs against CD19-expressing cells measured by flow based killing assessment. Figure 2A shows the killing against CD19-expressing Raji cancer cell lines. Figure 2B shows the killing against CD19 knock-out Raji cells. Positive control is 1928z-1XX CAR.

Figures 3A and 3B depict IL-2 cytokine secretion by T cells comprising CD19-targeted CARs. Figure 3A shows IL-2 cytokine secretion by T cells comprising CD19-targeted CARs co-cultured with CD19-expressing Raji cancer cell lines. Figure 3B shows IL-2 cytokine secretion by T cells comprising CD19-targeted CARs co-cultured with CD19 knock-out Raji cells. Positive control is 1928z-1XX CAR.

Figures 4A and 4B depict IFN- γ cytokine secretion by T cells comprising CD19-targeted CARs. Figure 4A shows IFN- γ cytokine secretion by T cells comprising CD19-targeted CARs co-cultured with CD19-expressing Raji cancer cell lines. Figure 4B shows IFN- γ cytokine secretion by T cells comprising CD19-targeted CARs co-cultured with CD19 knock-out Raji cells. Positive control is 1928z-1XX CAR.

Figure 5 shows *in vivo* anti-tumor activity of T cells comprising CD19-targeted CARs. #17 is a negative control, which is a CD19-targeted CAR having no detectable *in vivo* activity.

Figure 6 shows the amino acid sequence of the CD19-HSA-His10.

Figure 7 shows binding data of #2 scFv, SJ25c1 scFv, and FMC63 scFv. The relative competition for a common epitope in the soluble CD19 antigen was evaluated in a Biacore surface plasmon resonance (SPR) assay. A mixture of soluble CD19-specific antibodies (FMC63 or SJ25c1) and CD19 antigen was flowed onto a chip containing #2 scFv to determine whether #2 scFv cross competes with the same epitope as SJ25c1 scFv, and FMC63 scFv. 19(T2) represents “#2 scFv”.

Figure 8 depicts the gamma-retroviral vector comprising the #2 CAR. “19(T2)” represents “#2”. The #2 CAR comprises the CD19-specific T2 (19(T2)) scFv (or “#2 scFv”), a CD8 alpha leader peptide, a CD28 gene fragment, and a modified CD3 ζ intracellular signaling domain. The CD28 gene fragment includes its extracellular hinge, transmembrane, and intracellular domains. The modified CD3 ζ chain has one functional wild-type and two mutated immunoreceptor tyrosine-based activation motifs (ITAMs), termed “1XX”. The amino acid sequence of the modified CD3 ζ intracellular signaling domain is set forth in SEQ ID NO: 43. The alignment of the amino acid sequence of the CD3 ζ -1XX to the CD3 ζ -wild type is presented in Figure 9. The amino acid differences between the two sequences are marked.

Figure 9 depicts the alignment of the amino acid sequence of the CD3 ζ -1XX to the CD3 ζ -wild type.

Figure 10 depicts *in vitro* cytotoxic activity of #2 CAR-T cells and 1928z-1XX CAR T cells. “19(T2)28z1xx” represents “#2 CAR”.

Figure 11 depicts tumor elimination by #2 CAR T cells and 1928z-1XX CAR T cells. “19(T2)28z1xx” represents “#2 CAR”.

Figure 12 depicts the gamma-retroviral vector comprising the 1928z CAR. The amino acid sequence for the wild-type hCD3 ζ is shown in Figure 9 as SEQ ID NO: 30.

Figure 13 depicts the CAR expression in the γ -retrovirally transduced CD4⁺ and CD8⁺ T cells. “19(T2)28z1XX” represents “#2 CAR”.

Figure 14 depicts the phenotypes 1928z CAR T cells and #2 CAR T cells. “19(T2)28z1XX” represents “#2 CAR”.

Figure 15 depicts kinetics of tumor eradication by #2 CAR T cells and 1928z CAR T cells. “19(T2)28z1XX” represents “#2 CAR”. Each curve represents the tumor burden in one mouse.

Figure 16 depicts the survival of NALM6 leukemia-bearing mice post i.v. infusion of #2 CAR T cells or 1928z CAR T cells. “19(T2)28z1XX” represents “#2 CAR”.

Figure 17 depicts quantification and phenotyping of CAR T cells in the bone marrow of mice treated with #2 CAR T cells or 1928z CAR T cells (Day 17). All data are mean $\pm$ s.e.m. and a two-tailed unpaired Student's *t*-test was used to calculate the *p*-values. “19(T2)28z1XX” represents “#2 CAR”.

Figure 18 depicts body weights of mice after treatment with #2 CAR T cells or 1928z CAR T cells. “19(T2)28z1XX” represents “#2 CAR”. An asterisk indicates the timepoints when unscheduled death occurred. Shorter curves represent the loss of all mice at the indicated timepoint.

Figure 19 depicts quantification of liver enzymes in the serum of mice treated with 1×10^6 CAR T cells at day 10 and day 27/28 post-infusion. Statistical comparisons were determined between two cohorts for the same day. All data are mean $\pm$ s.e.m.; a two-tailed unpaired Student's *t*-test was used to calculate for *p*-values. “19(T2)28z1XX” represents “#2 CAR”.

Figure 20 depicts quantification of human cytokine levels in mice treated with 1×10^6 #2 CAR T cells or 1928z CAR T cells on day 10 and day 27/28 post-infusion. All data are mean $\pm$ s.e.m.; a two-tailed unpaired Student's *t*-test was used to calculate the *p*-values. “19(T2)28z1XX” represents “#2 CAR”.

Figure 21 quantification of mouse cytokine levels in mice treated with 1×10^6 #2 CAR T cells or 1928z CAR T cells on day 10 and day 27/28 post-infusion. All

data are mean $\pm$ s.e.m.; a two-tailed unpaired Student's *t*-test was used to calculate the *p*-values. "19(T2)28z1XX" represents "#2 CAR".

Figure 22 depicts quantification of essential blood cells in mice treated with #2 CAR T cells and 1928z CAR T cells at day 27/28 post-infusion. All data are mean $\pm$ s.e.m.; a two-tailed unpaired Student's *t*-test was used to calculate the *p*-values. "19(T2)28z1XX" represents "#2 CAR".

Figure 23 depicts comparison of the body and critical organ weights of mice treated with #2 CAR T cells and 1928z CAR T cells at day 27/28 post-infusion. The number of mice per group is indicated in the graph legend. All data are mean $\pm$ s.e.m.; a two-tailed unpaired Student's *t*-test was used to calculate for the *p*-values. "19(T2)28z1XX" represents "#2 CAR".

Figure 24 depicts quantification of lymphocytes and tumor infiltration in the liver, spleen, and bone marrow of mice treated with CAR T cells. All data are mean $\pm$ s.e.m.; a two-tailed unpaired Student's *t*-test was used to calculate for the *p*-values. "19(T2)28z1XX" represents "#2 CAR".

Figure 25 depicts exemplary flow cytometry chromatograms of anti-CD19 antibodies binding to CD19 expressing Raji and NALM-6 cells wild type and CD19 knockout.

Figures 26A-26C depict exemplary binding curves of anti-CD19 antibodies binding CD19 on NALM-6 cells.

6. DETAILED DESCRIPTION

The presently disclosed subject matter provides chimeric antigen receptors (CARs) that specifically target CD19 and cells comprising such CD19-targeted CARs. The cells can be immunoresponsive cells, e.g., genetically modified immunoresponsive cells (e.g., T-cells or NK cells). The presently disclosed subject matter further provides uses of the cells and compositions comprising thereof for treatments, e.g., for treating a neoplasm. The presently disclosed CD19-targeted CARs shown to be safer and/or more potent than other CD19-targeted CARs, e.g., as evidenced by their greater persistence (*see* Examples 6 and 7). This greater persistence was also shown not to pose safety risk. Accordingly, the presently disclosed CD19-targeted CAR T cells may be used at a lower dose than most CD19-targeted CAR T cells that are used in current immunotherapy. The presently disclosed CD19-targeted CAR T cells are expected to be safer, e.g., and thus, fewer side effects, including, but not limited to, less incidence of cytokine release syndrome (CRS), and Neurotoxicity (NT).

Non-limiting embodiments of the present disclosure are described by the present specification and Examples.

For purposes of clarity of disclosure and not by way of limitation, the detailed description is divided into the following subsections:

- 5 5.1. Definitions;
- 5.2. CD19;
- 5.3. Chimeric Antigen Receptors (CARs);
- 5.4. Cells;
- 5.5. Nucleic Acid Molecules and Vectors;
- 10 5.6. Formulations and Administration; and
- 5.7. Methods of Treatment

5.1 Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which the
15 presently disclosed subject matter belongs.

As used herein, the term “about” or “approximately” refers to within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 3 or
20 more than 3 standard deviations, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value.

25 As used herein, the term “immunoresponsive cell” refers to a cell that functions in an immune response or a progenitor, or progeny thereof. In certain embodiments, the immunoresponsive cell is a cell of lymphoid lineage. Non-limiting examples of cells of lymphoid lineage include T-cells, Natural Killer (NK) cells, B cells, and stem cells from which lymphoid cells may be differentiated. In certain
30 embodiments, the immunoresponsive cell is a cell of myeloid lineage.

As used herein, the term “activates an immunoresponsive cell” refers to induction of signal transduction or changes in protein expression in the cell resulting in initiation of an immune response. For example, when CD3 chains cluster in response to ligand binding and immunoreceptor tyrosine-based inhibition motifs (ITAMs) a signal

transduction cascade is produced. In certain embodiments, when a CAR binds to an antigen, a formation of an immunological synapse occurs that includes clustering of many molecules near the bound receptor (e.g. CD4 or CD8, CD3 $\gamma/\delta/\epsilon/\zeta$, etc.). This clustering of membrane bound signaling molecules allows for ITAM motifs contained within the CD3 chains to become phosphorylated. This phosphorylation in turn initiates a T-cell activation pathway ultimately activating transcription factors, such as NF- κ B and AP-1. These transcription factors induce global gene expression of the T-cell to increase IL-2 production for proliferation and expression of master regulator T-cell proteins in order to initiate a T-cell mediated immune response.

As used herein, the term “stimulates an immunoresponsive cell” refers to a signal that results in a robust and sustained immune response. In certain embodiments, this occurs after immunoresponsive cell (e.g., T-cell) activation or concomitantly mediated through receptors including, but not limited to, CD28, 4-1BB, OX40, CD40 and ICOS. Receiving multiple stimulatory signals can be important to mount a robust and long-term T-cell mediated immune response. T-cells can quickly become inhibited and unresponsive to antigen. While the effects of these co-stimulatory signals may vary, they generally result in increased gene expression in order to generate long lived, proliferative, and anti-apoptotic T-cells that robustly respond to antigen for complete and sustained eradication.

As used herein, “complementarity determining regions” or “CDRs” are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. In certain embodiments, a “CDR” of a variable domain are amino acid residues within the variable region that are identified in accordance with the definitions of the Kabat, Chothia, the accumulation of both Kabat and Chothia, AbM, contact, and/or conformational definitions or any method of CDR determination well known in the art. Antibody CDRs may be identified as the hypervariable regions originally defined by Kabat et al. See, e.g., Kabat et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C. The positions of the CDRs may also be identified as the structural loop structures originally described by Chothia and others. See, e.g., Chothia et al., Nature 342:877-883, 1989. Other approaches to CDR identification include the “AbM definition,” which is a compromise between Kabat and Chothia and is derived using Oxford Molecular's AbM antibody

modeling software (now Accelrys®), or the “contact definition” of CDRs based on observed antigen contacts, set forth in MacCallum et al., J. Mol. Biol., 262:732-745, 1996. In another approach, referred to herein as the “conformational definition” of CDRs, the positions of the CDRs may be identified as the residues that make enthalpic contributions to antigen binding. See, e.g., Makabe et al., Journal of Biological Chemistry, 283: 1156-1166, 2008. Still other CDR boundary definitions may not strictly follow one of the above approaches, but will nonetheless overlap with at least a portion of the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. In some embodiments, a CDR may refer to CDRs defined by any approach known in the art, including combinations of approaches. The methods used herein may utilize CDRs defined according to any of these approaches. For any given embodiment containing more than one CDR, the CDRs may be defined in accordance with any of Kabat, Chothia, extended, AbM, contact, and/or conformational definitions. Generally, antibodies comprise three heavy chain and three light chain CDRs or CDR regions in the variable region. CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. In certain embodiments, the CDRs regions are delineated using the Kabat numbering system.

As used herein, the term “single-chain variable fragment” or “scFv” refers to a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of an immunoglobulin (e.g., mouse or human) covalently linked to form a $V_H::V_L$ heterodimer. The heavy (V_H) and light chains (V_L) are either joined directly or joined by a peptide-encoding linker (e.g., 10, 15, 20, 25 amino acids), which connects the N-terminus of the V_H with the C-terminus of the V_L , or the C-terminus of the V_H with the N-terminus of the V_L . The linker is usually 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. Non-limiting examples of linkers are disclosed in Shen et al., Anal. Chem. 80(6):1910-1917 (2008) and WO 2014/087010, the contents of which are hereby incorporated by reference in their entireties. In certain embodiments, the linker is a G4S linker.

In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1, which is provided below:

GGGGSGGGSGGGGS [SEQ ID NO: 1]

In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 2, which is provided below:

GGGGSGGGSGGGSGGGGS [SEQ ID NO: 2]

In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3, which is provided below:

GGGGSGGGSGGGSGGGSGGGGS [SEQ ID NO: 3]

In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 4, which is provided below:

GGGGSGGGSGGGSGGGSGGGSGGGGS [SEQ ID NO: 4]

As used herein, the term “substantially identical” or “substantially homologous” refers to a polypeptide or a nucleic acid molecule exhibiting at least about 50% identical or homologous to a reference amino acid sequence (for example, any of the amino acid sequences described herein) or a reference nucleic acid sequence (for example, any of the nucleic acid sequences described herein). In certain embodiments, such a sequence is at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99%, or at least about 100% identical or homologous to the amino acid sequence or the nucleic acid sequence used for comparison.

Sequence identity can be measured by using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

The percent homology between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent homology between two amino acid sequences can be determined

using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. Additionally or
5 alternatively, the amino acids sequences of the presently disclosed subject matter can further be used as a “query sequence” to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the XBLAST program (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST protein searches can be performed with the XBLAST program, score = 50,
10 wordlength = 3 to obtain amino acid sequences homologous to the specified sequences (*e.g.*, heavy and light chain variable region sequences of scFv703) disclosed herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective
15 programs (*e.g.*, XBLAST and NBLAST) can be used.

As used herein, the term “a conservative sequence modification” refers to an amino acid modification that does not significantly affect or alter the binding characteristics of the presently disclosed CD19-targeted CAR (*e.g.*, the extracellular antigen-binding domain of the CAR) comprising the amino acid sequence. Conservative
20 modifications can include amino acid substitutions, additions and deletions. Modifications can be introduced into the extracellular antigen-binding domain of the presently disclosed CAR by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Amino acids can be classified into groups according to their physicochemical properties such as charge and polarity. Conservative
25 amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid within the same group. For example, amino acids can be classified by charge: positively-charged amino acids include lysine, arginine, histidine, negatively-charged amino acids include aspartic acid, glutamic acid, neutral charge amino acids include alanine, asparagine, cysteine, glutamine, glycine, isoleucine, leucine, methionine,
30 phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In addition, amino acids can be classified by polarity: polar amino acids include arginine (basic polar), asparagine, aspartic acid (acidic polar), glutamic acid (acidic polar), glutamine, histidine (basic polar), lysine (basic polar), serine, threonine, and tyrosine; non-polar amino acids include alanine, cysteine, glycine, isoleucine, leucine, methionine,

phenylalanine, proline, tryptophan, and valine. Thus, one or more amino acid residues within a CDR region can be replaced with other amino acid residues from the same group and the altered antibody can be tested for retained function (*i.e.*, the functions set forth in (c) through (l) above) using the functional assays described herein. In certain
5 embodiments, no more than one, no more than two, no more than three, no more than four, no more than five residues within a specified sequence or a CDR region are altered.

As used herein, the term “effective amount” refers to an amount sufficient to affect a beneficial or desired clinical result upon treatment. An effective amount can be administered to a subject in one or more doses. In certain embodiments, an effective
10 amount can be an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the disease, or otherwise reduce the pathological consequences of the disease. The effective amount can be determined by a physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. These
15 factors include age, sex and weight of the subject, the condition being treated, the severity of the condition and the form and effective concentration of the cells administered.

As used herein, the term “neoplasm” refers to a disease characterized by the pathological proliferation of a cell or tissue and its subsequent migration to or
20 invasion of other tissues or organs. Neoplasia growth is typically uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells. Neoplasm can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group consisting of bladder, bone, brain, breast, cartilage, glia, esophagus, fallopian tube, gallbladder, heart,
25 intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and vagina, or a tissue or cell type thereof. Neoplasms include cancers, such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells). The neoplasm can a primary tumor or primary cancer.

30 As used herein, the term “signal sequence” or “leader sequence” refers to a peptide sequence (*e.g.*, 5, 10, 15, 20, 25 or 30 amino acids) present at the N-terminus of newly synthesized proteins that directs their entry to the secretory pathway.

The terms “comprises”, “comprising”, and are intended to have the broad meaning ascribed to them in U.S. Patent Law and can mean “includes”, “including” and the like.

As used herein, “treatment” refers to clinical intervention in an attempt to
5 alter the disease course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Therapeutic effects of treatment include, without limitation, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological
10 consequences of the disease, preventing metastases, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. By preventing progression of a disease or disorder, a treatment can prevent deterioration due to a disorder in an affected or diagnosed subject or a subject suspected of having the disorder, but also a treatment may prevent the onset of the disorder or a symptom of the disorder in a subject at risk for the disorder or suspected of having the
15 disorder.

An “individual” or “subject” herein is a vertebrate, such as a human or non-human animal, for example, a mammal. Mammals include, but are not limited to, humans, primates, farm animals, sport animals, rodents and pets. Non-limiting examples of non-human animal subjects include rodents such as mice, rats, hamsters, and guinea
20 pigs; rabbits; dogs; cats; sheep; pigs; goats; cattle; horses; and non-human primates such as apes and monkeys.

5.2. CD19

CD19 is a cell-surface 95 kDa glycoprotein present on normal B cells from early in their development until differentiation into plasma cells. CD19 is not
25 present in other normal tissues, including pluripotent blood stem cells. It is expressed in B cell lymphomas, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), hairy cell leukemias, and a subset of acute myelogenous leukemias making it an attractive target for immunotherapy with minimal risk of autoimmune disease (other than B cell aplasia) or irreversible bone marrow toxicity.

30 CD19 is a member of the immunoglobulin superfamily and a component of a cell surface signal transduction complex that includes Leu13, CD81, and CD21, which positively regulates signal transduction through the B cell receptor.

In certain embodiments, the presently disclosed CAR binds to human CD19. In certain embodiments, the human CD19 comprises or consists of the amino

acid sequence with a NCBI Reference No: NP_001171569.1 (SEQ ID NO: 5), or a fragment thereof.

SEQ ID NO: 5 is provided below:

5 MPPPRLLFFL LFLTPMEVRP EEPLVVKVEE GDNAVLQCLK GTSDGPTQQL TWSRESPLKP
 FLKLSLGLPG LGIHMRPLAI WLFIFNVSQQ MGGFYLCQPG PPSEKAWQPG WTVNVEGSGE
 LFRWNVSDLG GLGCGLKNRS SEGPPSSPSGK LMSPKLYVWA KDRPEIWEGE PPCLPPRDSL
 NQSLSQDLTM APGSTLWLSC GVPPDSVSRG PLSWTHVHPK GPKSLLSLEL KDDRPARDMW
 VMETGLLLP R ATAQDAGKYY CHRGNTMSF HLEITARPVL WHWLLRTGGW KVS AVT LAYL
 10 IFCLCSLVGI LHLQALVLR RKRKRMTDPT RRFFKVTPPP GSGPQNQYGN VLSLPTPTSG
 LGRAQRWAAG LGGTAPSYGN PSSDVQADGA LGSRSPPGVG PEEEEGEGYE EPDSEEDSEF
 YENDSNLQGD QLSQDGSQYE NPEDEPLGPE DEDSFSAES YENEDEELTQ PVARTMDFLS
 PHGSAWDPSR EATSLAGSQS YEDMRGILYA APQLRSIRGQ GPNHEEDAD SYENMDNPDG
 PDPAGGGGR MGTWSTR [SEQ ID NO: 5]

15 In certain embodiments, the human CD19 comprises or consists of the amino acid sequence with a NCBI Reference No: NP_001761.3 (SEQ ID NO: 6), or a fragment thereof.

SEQ ID NO: 6 is provided below:

20 MPPPRLLFFL LFLTPMEVRP EEPLVVKVEE GDNAVLQCLK GTSDGPTQQL TWSRESPLKP
 FLKLSLGLPG LGIHMRPLAI WLFIFNVSQQ MGGFYLCQPG PPSEKAWQPG WTVNVEGSGE
 LFRWNVSDLG GLGCGLKNRS SEGPPSSPSGK LMSPKLYVWA KDRPEIWEGE PPCLPPRDSL
 NQSLSQDLTM APGSTLWLSC GVPPDSVSRG PLSWTHVHPK GPKSLLSLEL KDDRPARDMW
 VMETGLLLP R ATAQDAGKYY CHRGNTMSF HLEITARPVL WHWLLRTGGW KVS AVT LAYL
 25 IFCLCSLVGI LHLQALVLR RKRKRMTDPT RRFFKVTPPP GSGPQNQYGN VLSLPTPTSG
 LGRAQRWAAG LGGTAPSYGN PSSDVQADGA LGSRSPPGVG PEEEEGEGYE EPDSEEDSEF
 YENDSNLQGD QLSQDGSQYE NPEDEPLGPE DEDSFSAES YENEDEELTQ PVARTMDFLS
 PHGSAWDPSR EATSLAGSQS EDMRGILYAA PQLRSIRGQP GPNHEEDADS YENMDNPDGP
 DPAWGGGGR GTWSTR [SEQ ID NO: 6]

In certain embodiments, the CAR binds to the extracellular domain of
 30 CD19. In certain embodiments, the CAR binds to the extracellular domain of human CD19. In certain embodiments, the extracellular domain of human CD19 comprises or consists of amino acids 20 to 291 of SEQ ID NO: 5. In certain embodiments, the extracellular domain of human CD19 comprises or consists of amino acids 20 to 291 of SEQ ID NO: 6.

35 In certain embodiments, the CD19 comprises or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identical to the amino acid sequence set forth in SEQ ID NO: 5 or a fragment thereof.

40 In certain embodiments, the CD19 comprises or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at

least about 100% identical to the amino acid sequence set forth in SEQ ID NO: 6 or a fragment thereof.

5.3. *Chimeric Antigen Receptors (CARs)*

CARs are engineered receptors, which graft or confer a specificity of interest onto an immune effector cell. CARs can be used to graft the specificity of a monoclonal antibody onto a cell, e.g., an immunoresponsive cell, e.g., a T cell or a NK cell; with transferring of their coding sequence facilitated by retroviral vectors.

There are three generations of CARs. “First generation” CARs are typically composed of an extracellular antigen-binding domain (e.g., a scFv), which is fused to a transmembrane domain, which is fused to an intracellular signaling domain. “First generation” CARs can provide *de novo* antigen recognition and cause activation of both CD4⁺ and CD8⁺ T-cells through their CD3ζ chain signaling domain in a single fusion molecule, independent of HLA-mediated antigen presentation. “Second generation” CARs add an intracellular signaling domain from a co-stimulatory molecule (including, but not limited to, CD28, 4-1BB, ICOS, OX40) to the cytoplasmic tail of the CAR to provide additional signals to the cell. “Second generation” CARs comprise those that provide both co-stimulation (e.g., CD28 or 4-1BB) and activation (CD3ζ). “Third generation” CARs comprise those that provide multiple co-stimulation (e.g., CD28 and 4-1BB) and activation (CD3ζ). In certain embodiments, the CD19-targeted CAR is a first-generation CAR. In certain embodiments, the CD19-targeted CAR does not comprise an intracellular signaling domain of a co-stimulatory molecule. In certain embodiments, the CD19-targeted CAR is a second-generation CAR. In certain embodiments, the CD19-targeted CAR comprises an intracellular signaling domain of a co-stimulatory molecule

In certain embodiments, the CAR comprises an extracellular antigen-binding domain that specifically binds to CD19, a transmembrane domain, and an intracellular signaling domain. In certain embodiments, the extracellular antigen-binding domain is fused to the transmembrane domain, which is fused to the intracellular signaling domain.

5.3.1. Extracellular Antigen-Binding Domain of A CAR

In certain embodiments, the extracellular antigen-binding domain of the CAR comprises or is a scFv. The scFv can be a human scFv, a humanized scFv, or a murine scFv. In certain embodiments, the scFv is a human scFv. The scFv can be derived from fusing the variable heavy and light regions of an antibody. Alternatively or

additionally, the scFv may be derived from Fab's (instead of from an antibody, *e.g.*, obtained from Fab libraries).

In certain embodiments, the extracellular antigen-binding domain of the CAR comprises or is a Fab. In certain embodiments, the Fab is crosslinked. In certain
5 embodiments, the extracellular antigen-binding domain of the CAR comprises or is a F(ab)₂.

Any of the foregoing molecules may be comprised in a fusion protein with a heterologous sequence to form the extracellular antigen-binding domain of the CAR.

10 In certain embodiments, the extracellular antigen-binding domain of the CAR binds to CD19 (*e.g.*, human CD19) with a dissociation constant (K_d) of at least about 1×10^{-6} M, at least about 1×10^{-7} M, at least about 1×10^{-8} M, at least about 1×10^{-9} M, or at least about 1×10^{-10} M. In certain embodiments, the extracellular antigen-binding domain of the CAR binds to CD19 (*e.g.*, human CD19) with a dissociation
15 constant (K_d) of at least about 2×10^{-8} M. In certain embodiments, the extracellular antigen-binding domain of the CAR binds to CD19 (*e.g.*, human CD19) with a dissociation constant (K_d) of between about 2×10^{-8} M and about 8×10^{-9} M.

In certain embodiments, the extracellular antigen-binding domain of the CAR binds to CD19 (*e.g.*, human CD19) with a dissociation constant (K_d) of between
20 about 1 nM and 50 nM, between about 5 nM and 30 nM, between about 5 nM and 25 nM, or between about 8 nM and 20 nM. In certain embodiments, the extracellular antigen-binding domain of the CAR binds to CD19 (*e.g.*, human CD19) with a dissociation constant (K_d) of at least about 50 nM, at least about 40 nM, at least about 35 nM, at least about 30 nM, at least about 25 nM, at least about 20 nM, at least about 19
25 nM, at least about 18 nM, at least about 17 nM, at least about 16 nM, at least about 15 nM, at least about 14 nM, at least about 13 nM, at least about 12 nM, at least about 11 nM, at least about 10 nM, at least about 9 nM, at least about 8 nM, at least about 7 nM, at least about 6 nM, or at least about 5 nM.

Binding of the extracellular antigen-binding domain of the CAR can be
30 confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (*e.g.*, growth inhibition), or Western Blot assay. Each of these assays generally detect the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody, or a scFv) specific for the complex of interest. For example, the scFv can be radioactively

labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography. In certain embodiments, the CD127-targeted extracellular antigen-binding domain is labeled with a fluorescent marker. Non-limiting examples of fluorescent markers include green fluorescent protein (GFP), blue fluorescent protein (e.g., EBFP, EBFP2, Azurite, and mKalamal), cyan fluorescent protein (e.g., ECFP, Cerulean, and CyPet), and yellow fluorescent protein (e.g., YFP, Citrine, Venus, and YPet). In certain embodiments, the CD19-targeted human scFv is labeled with a GFP.

In certain embodiments, the extracellular antigen-binding domain of the CAR comprises a heavy chain variable region (V_H) and a light chain variable region (V_L).

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof. In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9. SEQ ID NOs: 7-9 are provided below.

SYGMH [SEQ ID NO: 7]

LIWYDGSNKYYADSVKG [SEQ ID NO: 8]

PVEGLLRGFDY [SEQ ID NO: 9]

In certain embodiments, the V_H comprises an amino acid sequence that is at least about 80% (e.g., at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 10. For example, the V_H comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 10. In certain embodiments, the V_H comprises

the amino acid sequence set forth in SEQ ID NO: 10. SEQ ID NO: 10 is provided below.

QVQLVESGGGVVQPGRSLRLSCAASGFTTFSSYGMHVRQAPGKGLEWVALIWYDGSNKYYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYYCAKPFVEGLLRGFDYWGQGTLLTVSS [SEQ ID NO: 10]

5 In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof. In certain
10 embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13. SEQ ID NOs: 11-13 are provided below.

RASQSVSSSYLA [SEQ ID NO: 11]

15 GASSRAT [SEQ ID NO: 12]

QQAGAVPIT [SEQ ID NO: 13]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 14. For
20 example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 14. In certain embodiments, the V_L comprises
25 the amino acid sequence set forth in SEQ ID NO: 14. SEQ ID NO: 14 is provided below.

EIVLTQSPGTLISLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQAGAVPITFGGGTKVEIK [SEQ ID NO: 14]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification
30 thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a
35 thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a

conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof. In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11; a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13.

In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 14.

The V_H the V_L can be linked one after another, e.g., by a linker. The variable regions from the N- to the C-terminus can be V_L - V_H or V_L - V_H . In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, i.e., the V_H the V_L are positioned from the N- to the C-terminus as V_L - V_H . In certain embodiments, the extracellular antigen-binding domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 14. In certain embodiments, the scFv comprises or consists of the amino acid sequence set forth in SEQ ID NO: 15. SEQ ID NO: 15 is provided below. In certain embodiments, the anti-CD19 scFv is designated as “#2 scFv”.

EIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQAGAVPITFGGGTKVEIKGGGSGGGGSGGGGSGVQLVESGGGVVQPGRSR
LSCAASGFTFSSYGMHWVRQAPGKGLEWVALIWYDGSNKYYADSVKGRFTISRDN SKNTLYLQMNSLR
AEDTAVYYCAKPVGELLRGFDYWGQGLVTVSS [SEQ ID NO: 15]

In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a

conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17 or a conservative modification thereof. In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17. SEQ ID NOs: 16 and 17 are provided below.

RASQSVRSSYLA [SEQ ID NO: 16]

QQLFDSPYT [SEQ ID NO: 17]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 18. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 18. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 18. SEQ ID NO: 18 is provided below.

EIVLTQSPGTLSSLSPGERATLSCRASQSVRSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQLFDSPYTFGGGTKVEIK [SEQ ID NO: 18]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17 or a conservative modification thereof. In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a

CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17.

In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 18.

In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, i.e., the V_H the V_L are positioned from the N- to the C-terminus as V_L - V_H . In certain embodiments, the extracellular antigen-binding domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 18. In certain embodiments, the scFv comprises or consists of the amino acid sequence set forth in SEQ ID NO: 19. SEQ ID NO: 19 is provided below. In certain embodiments, the anti-CD19 scFv is designated as “#8 scFv”.

IVLTQSPGTLTSLSPGERATLSCRASQSVSRSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDF
TLTISRLEPEDFAVYYCQQLFDSPTFTGGGTKVEIKGGGSGGGGSGGGGSGVQLVESGGGVVQPGRSLRL
SCAASGFTFSSYGMHWVRQAPGKLEWVALIWYDGSNKYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDT
AVYYCAKPV EGLLRGFDYWGQGTLVTVSS [SEQ ID NO: 19]

In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof. In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21. SEQ ID NOs: 20 and 21 are provided below.

GASRRAT [SEQ ID NO: 20]

QQAGIPPYT [SEQ ID: 21]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 22. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 22. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 22. SEQ ID NO: 22 is provided below.

```
EIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASRRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQAGIPPYTFGGGTKVEIK [SEQ ID NO: 22]
```

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20 or a conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof. In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21.

In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 22.

In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, *i.e.*, the V_H the V_L are positioned from the N- to the C-terminus as V_L-V_H. In certain embodiments, the extracellular antigen-binding domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the

amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 22. In certain embodiments, the scFv comprises or consists of the amino acid sequence set forth in SEQ ID NO: 23. SEQ ID NO: 23 is provided below. In certain embodiments, the anti-CD19 scFv is designated as “#15 scFv”.

QVQLVESGGGVVQPGRSLRLSCAASGFTTFSSYGMHWVRQAPGKGLEWVALIWYDGSNKYYADSVKGRFTIS
RDNSKNTLYLQMNSLRAEDTAVYYCAKPFVEGLLRGFDYWGGTLVTVSSGGGGSGGGGSGGGGSEIVLTQS
PGTSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASRRATGIPDRFSGSGSGTDFTLTISR
LEPEDFAVYYCQQAGIPPYTFGGGTKVEIK [SEQ ID NO: 23]

In certain embodiments, the CDRs are identified according to the Kabat numbering system.

In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57 or a conservative modification thereof. In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57. SEQ ID NO: 57 is provided below.

QQVDSLHPFT [SEQ ID NO: 57]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 58. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino

acid sequence set forth in SEQ ID NO: 58. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 58. SEQ ID NO: 58 is provided below.

5 EIVLTQSPGTTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQVDSLHPFTFGGGTKVEIK [SEQ ID NO: 58]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57 or a conservative modification thereof. In certain
10
15
20
embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57.

In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 58.

In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, i.e., the V_H the V_L are positioned from the N- to the C-terminus as V_L-V_H. In certain embodiments, the extracellular antigen-binding domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid
25
30

sequence set forth in SEQ ID NO: 58. In certain embodiments, the anti-CD19 scFv is designated as “#4 scFv”.

In certain embodiments, the CDRs are identified according to the Kabat numbering system.

5 In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59 or a conservative modification thereof. In certain
10 embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59. SEQ ID NO: 59 is provided below.

QQAGGVPPLT [SEQ ID NO: 59]

15 In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 60. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%,
20 about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 60. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 60. SEQ ID NO: 60 is provided below.

25 EIVLTQSPGTTLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQAGGVPPLTFGGGTKVEIK [SEQ ID NO: 60]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative
30 modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification, and a CDR3 comprising the amino acid sequence

set forth in SEQ ID NO: 59 or a conservative modification thereof. In certain
embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in
SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8,
and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L
5 comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a
CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3
comprising the amino acid sequence set forth in SEQ ID NO: 59.

In certain embodiments, the V_H comprises the amino acid sequence set
forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ
10 ID NO: 60.

In certain embodiments, the V_L is positioned at the N-terminus of the
extracellular antigen-binding domain, i.e., the V_H the V_L are positioned from the N-
to the C-terminus as V_L - V_H . In certain embodiments, the extracellular antigen-binding
domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the
15 amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid
sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence
set forth in SEQ ID NO: 9; a V_L comprising a CDR1 comprising the amino acid
sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set
forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in
20 SEQ ID NO: 59. In certain embodiments, the scFv comprises a V_H comprising the
amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid
sequence set forth in SEQ ID NO: 60. In certain embodiments, the anti-CD19 scFv is
designated as “#5 scFv”.

In certain embodiments, the CDRs are identified according to the Kabat
25 numbering system.

In certain embodiments, the V_L comprises a CDR1 comprising the amino
acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a
CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a
conservative modification thereof, and a CDR3 comprising the amino acid sequence set
30 forth in SEQ ID NO: 61 or a conservative modification thereof. In certain
embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in
SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:
12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61. SEQ
ID NO: 61 is provided below.

QQAGVPPLT [SEQ ID NO: 61]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 62. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 62. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 62. SEQ ID NO: 62 is provided below.

EIVLTQSPGTLSSLSPGERATLSCRASQSVRSSLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQAGVPPLTFGGGTKVEIK [SEQ ID NO: 62]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61 or a conservative modification thereof. In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61.

In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 62.

In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, *i.e.*, the V_H the V_L are positioned from the N- to the C-terminus as V_L-V_H. In certain embodiments, the extracellular antigen-binding

domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 62. In certain embodiments, the anti-CD19 scFv is designated as “#6 scFv”.

In certain embodiments, the CDRs are identified according to the Kabat numbering system.

In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63 or a conservative modification thereof. In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63. SEQ ID NO: 63 is provided below.

QQAGGVPPFT [SEQ ID NO: 63]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 64. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 64. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 64. SEQ ID NO: 64 is provided below.

EIVMTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQAGGVPPFTFGGGTKVEIK [SEQ ID NO: 64]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63 or a conservative modification thereof. In certain

embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63.

In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 64.

In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, i.e., the V_H and the V_L are positioned from the N- to the C-terminus as V_L - V_H . In certain embodiments, the extracellular antigen-binding domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 64. In certain embodiments, the anti-CD19 scFv is designated as “#7 scFv”.

In certain embodiments, the CDRs are identified according to the Kabat numbering system.

In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66 or a conservative modification thereof. In certain embodiments, the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66. SEQ ID NOS: 66 and 67 are provided below.

10 GASNRAT [SEQ ID NO: 65]
 QQAGVFPFT [SEQ ID NO: 66]

In certain embodiments, the V_L comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 67. For example, the V_L comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 67. In certain embodiments, the V_L comprises the amino acid sequence set forth in SEQ ID NO: 67. SEQ ID NO: 67 is provided below.

EIVMTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASNRATGIPDRFSGSGSGTD
 FTLTISRLEPEDFAVYYCQQAGVFPFTFGGGTKVEIK [SEQ ID NO: 67]

In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65 or a conservative modification, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66 or a conservative modification thereof. In certain embodiments, the V_H comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8,

and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the V_L comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66.

5 In certain embodiments, the V_H comprises the amino acid sequence set forth in SEQ ID NO: 10, and the V_L comprises the amino acid sequence set forth in SEQ ID NO: 67.

In certain embodiments, the V_L is positioned at the N-terminus of the extracellular antigen-binding domain, i.e., the V_H the V_L are positioned from the N- to the C-terminus as V_L-V_H. In certain embodiments, the extracellular antigen-binding domain of the CAR is a scFv that comprises a V_H comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; a V_L comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66. In certain embodiments, the scFv comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 67. In certain embodiments, the anti-CD19 scFv is designated as “#1 scFv”.

In certain embodiments, the CDRs are identified according to the Kabat numbering system.

An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 10 is set forth in SEQ ID NO: 68. SEQ ID NO: 68 is provided below.

25 CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGC
GTCTGGATTACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGG
TGGCACTGATATGGTATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGCCGATTACCATCTCC
AGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCGGTGTACTA
CTGCGCCAAGCCAGTGGAAGGACTATTAAGAGGATTCGATTACTGGGGACAGGTACATTGGTCACCGTCT
30 CCTCA [SEQ ID NO: 68]

An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 14 is set forth in SEQ ID NO: 69. SEQ ID NO: 69 is provided below.

GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
35 TCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGAC

TTCACCTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGGCCGGAGCCGT
CCCTATCACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 69]

An exemplary nucleotide sequence encoding the amino acid sequence of
SEQ ID NO: 18 is set forth in SEQ ID NO: 70. SEQ ID NO: 70 is provided below.

5 GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGGAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
TCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGAC
TTCACCTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGCTCTTCGACAG
TCCTTACACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 70]

10 An exemplary nucleotide sequence encoding the amino acid sequence of
SEQ ID NO: 22 is set forth in SEQ ID NO: 71. SEQ ID NO: 71 is provided below.

GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
TCATCTATGGTGCATCCAGAAGGGCCACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGAC
15 TTCACCTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGGCCGGCATCCC
CCCTTACACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 71]

An exemplary nucleotide sequence encoding the amino acid sequence of
SEQ ID NO: 58 is set forth in SEQ ID NO: 72. SEQ ID NO: 72 is provided below.

GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
20 GGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
TCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGAC
TTCACCTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGGTCGACAGTCT
CCATCCTTTCACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 72]

An exemplary nucleotide sequence encoding the amino acid sequence of
25 SEQ ID NO: 60 is set forth in SEQ ID NO: 73. SEQ ID NO: 73 is provided below.

GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
TCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGAC
TTCACCTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGGCCGGAGGCGT
30 CCCTCCTCTCACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 73]

An exemplary nucleotide sequence encoding the amino acid sequence of
SEQ ID NO: 62 is set forth in SEQ ID NO: 74. SEQ ID NO: 74 is provided below.

GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGGAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
35 TCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGAC
TTCACCTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGGCCGGAGTCCC
CCCTCTCACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 74]

An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 64 is set forth in SEQ ID NO: 75. SEQ ID NO: 75 is provided below.

GAAATTGTGATGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
5 TCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGAC
TTCATCTCTACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTTCAGCAGGCCGGAGGCGT
CCCTCCTTTCACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 75]

An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 67 is set forth in SEQ ID NO: 76. SEQ ID NO: 76 is provided below.

10 GAAATTGTGATGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAG
GGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCC
TCATCTATGGTGCATCCAACAGGGCCACTGGCATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGAC
TTCATCTCTACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTTCAGCAGGCCGGAGTCTT
CCCTTTCACTTTTGGCGGAGGGACCAAGGTTGAGATCAAA [SEQ ID NO: 76]

15 In certain embodiments, the V_H and V_L are linked via a linker. In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1.

20 The V_H and/or V_L amino acid sequences consisting of at least about 80%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% (*e.g.*, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%) homology or identity to a
25 specific sequence (*e.g.*, SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67) may contain substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the specified sequence(s), but retain the ability to bind to a target antigen (*e.g.*, mesothelin). In certain embodiments, a total of 1 to 10 amino acids are substituted,
30 inserted and/or deleted in a specific sequence (*e.g.*, SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67). In certain embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (*e.g.*, in the FRs) of the extracellular antigen-binding domain. In certain embodiments, the extracellular antigen-binding
35 domain comprises V_H and/or V_L sequence selected from SEQ ID NOs: 10, 14, 18, 22,

59, 61, 63, 65, and 68, including post-translational modifications of SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67.

- In addition, the extracellular antigen-binding domain can comprise a
- 5 leader or a signal peptide that directs the nascent protein into the endoplasmic reticulum. Signal peptide or leader can be essential if the CAR is to be glycosylated and anchored in the cell membrane. The signal sequence or leader can be a peptide sequence (about 5, about 10, about 15, about 20, about 25, or about 30 amino acids long) present at the N-terminus of newly synthesized proteins that directs their entry to the secretory pathway.
- 10 In certain embodiments, the signal peptide is covalently joined to the 5' terminus (N-terminus) of the extracellular antigen-binding domain. In certain embodiments, the signal peptide comprises a CD8 polypeptide, e.g., the CAR comprises a truncated CD8 signal peptide. In certain embodiments, the signal peptide is generated from the antibody from which it is derived. In certain embodiments, the signal peptide for #2 scFv is set forth in SEQ ID
- 15 NO: 54. In certain embodiments, the signal peptide for #8 scFv is set forth in SEQ ID NO: 54. In certain embodiments, the signal peptide for #15 scFv is set forth in SEQ ID NO: 55.

MDMRVPAQLLGLLLWLPDTRC [SEQ ID NO: 54]

MEFGLSWVFLVALLRGVQC [SEQ ID NO: 55]

20 5.3.2. Transmembrane Domain of a CAR

- In certain embodiments, the transmembrane domain of the CAR comprises a hydrophobic alpha helix that spans at least a portion of the membrane. Different transmembrane domains result in different receptor stability. After antigen recognition, receptors cluster and a signal is transmitted to the cell. In certain
- 25 embodiments, the transmembrane domain of the CAR comprises a native or modified transmembrane domain of CD8, of CD28, of CD3 ζ , of CD4, of 4-1BB, of OX40, of ICOS, of CD84, of CD166, of CD8a, of CD8b, of ICAM-1, of CTLA-4, of CD27, of CD40, of NKGD2, or a combination thereof.

- In certain embodiments, the transmembrane domain of the CAR
- 30 comprises a CD28 polypeptide (e.g., a transmembrane domain of CD28 or a portion thereof). In certain embodiments, the transmembrane domain of the CAR comprises a transmembrane domain of human CD28 or a portion thereof. The CD28 polypeptide can comprise or consist of an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% identical or

homologous to the sequence having a NCBI Reference No: NP_006130 (SEQ ID NO: 24), or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 24, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, or at least about 60, and/or up to about 70, up to about 80, up to about 90, up to about 100, up to about 150, up to about 200, or up to about 220 amino acids in length. In certain embodiments, the CD28 polypeptide comprises or consists of the amino acid sequence of amino acids 1 to 220, 1 to 50, 50 to 100, 100 to 150, 154 to 179, 150 to 200, 153 to 179, or 200 to 220 of SEQ ID NO: 24. In certain embodiments, the transmembrane domain of the CAR comprises a CD28 polypeptide comprising or consisting of the amino acid sequence of amino acids 154 to 179 of SEQ ID NO: 24. SEQ ID NO: 24 is provided below.

MLRLLALNL FPSIQVTGNK ILVKQSPMLV AYDNAVNLSC KYSYNLFSRE FRASLHKGLD
 15 SAVEVCVVYG NYSQQLQVYS KTGFCNDGKL GNEVTFYLO NLYVNQTDIY FCKIEVMYPP
 PYLDNEKSNG TIIHVKGKHL CPSPLFPGPS KPFWVLVVVG GVLACYSLLV TVAFIIFWVR
 SKRSRLHSD YNMTPRRPG PTRKHYQPYA PPRDFAAYRS [SEQ ID NO: 24]

An exemplary nucleotide sequence encoding the amino acid sequence of amino acids 154 to 179 of SEQ ID NO: 24 is set forth in SEQ ID NO: 25, which is provided below.

TGGGTGCTGGTGGTGGTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATTATTTT
 CTGGGTG [SEQ ID NO: 25]

In certain embodiments, the transmembrane domain of the CAR comprises a transmembrane domain of mouse CD28 or a portion thereof. The CD28 polypeptide can consist of an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% identical or homologous to the sequence having a NCBI Reference No: NP_031668.3 (SEQ ID No: 26), or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 26, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to about 218 amino acids in length. In certain embodiments, the CD28 polypeptide comprises or consists of an amino acid sequence of amino acids 1 to 218, 1 to 50, 50 to 100, 100 to 150, 150 to 200, 151 to 177, or 200 to 218 of SEQ ID NO: 26. In certain embodiments, the transmembrane domain of the CAR

comprises a CD28 polypeptide comprising or consisting of the amino acid sequence of amino acids 151 to 177 of SEQ ID NO: 26. SEQ ID NO: 26 is provided below:

5 MTLRLFLAL NFFSVQVTEN KILVKQSPLL VVDSNEVSLS CRYSYNLLAK EFRASLYKGV
NSDVEVCVGN GNFTYQPQFR SNAEFNCDGD FDNETVTFRL WNLHVNHDTI YFCKIEFMYP
PPYLDNERSN GTIIHIKEKH LCHTQSSPKL FWALVVVAGV LFCYGLLVTV ALCVIWTNSR
RNRLLQSDYM NMTPRRPGLT RKPYPYAPA RDFAAYRP [SEQ ID NO: 26]

In certain embodiments, the transmembrane domain of the CAR comprises a CD8 polypeptide (e.g., a transmembrane domain of CD8 or a portion thereof). In certain embodiments, the transmembrane domain of the CAR comprises a transmembrane domain of human CD8 or a portion thereof. In certain embodiments, the CD8 polypeptide comprises or has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the sequence having a NCBI Reference No: NP_001139345.1 (SEQ ID NO: 27) or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD8 polypeptide comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 27, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to about 235 amino acids in length. Alternatively or additionally, in certain embodiments, the CD8 polypeptide comprises or consists of an amino acid sequence of amino acids 1 to 235, 1 to 50, 50 to 100, 100 to 150, 150 to 200, 137 to 209 or 200 to 235 of SEQ ID NO: 27. In certain embodiments, the transmembrane domain of the CAR comprises a CD8 polypeptide comprising or consisting of the amino acid sequence of amino acids 137 to 209 of SEQ ID NO: 27. SEQ ID NO: 27 is provided below.

25 MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYFCSALSNSIMYFSHFVFPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV [SEQ ID NO: 27]

30 In certain embodiments, the transmembrane domain of the CAR comprises a transmembrane domain of mouse CD8 or a portion thereof. In certain embodiments, the CD8 polypeptide comprises or has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the sequence having a NCBI Reference No: AAA92533.1 (SEQ ID NO: 28) or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD8 polypeptide comprises or has an amino acid sequence that is a

consecutive portion of SEQ ID NO: 28, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, or at least about 60, or at least about 70, or at least about 100, or at least about 200, and up to 247 amino acids in length. Alternatively or additionally, in certain embodiments, the CD8 polypeptide comprises or consists of an amino acid sequence of amino acids 1 to 247, 1 to 50, 50 to 100, 100 to 150, 150 to 200, 151 to 219, or 200 to 247 of SEQ ID NO: 28. In certain embodiments, the transmembrane domain of the CAR comprises a CD8 polypeptide comprising or consisting of the amino acid sequence of amino acids 151 to 219 of SEQ ID NO: 28.

SEQ ID NO: 28 is provided below.

```

10 MASPLTRFLS LNLLLMGESI ILGSGEAKPQ APELRIFPKK MDAELGQKVD LVCEVLGGSVS
   QGCSWLFQNS SSKLPQPTFV VYMASSHNKI TWDEKLNSSK LFSAVRDTNN KYVLTNLKFS
   KENEGYYFCS VISNSVMYFS SVVPVLQKVN STTTKPVLRT PSPVHPTGTS QPQRPEDCRP
   RGSVKGTGLD FACDIYIWAP LAGICVAPLL SLIITLICYP RSRKRVCKCP RPLVRQEGKP
   RPSEKIV [SEQ ID NO: 28]

```

15 In certain non-limiting embodiments, the CAR further comprise a spacer region that links the extracellular antigen-binding domain to the transmembrane domain. The spacer region can be flexible enough to allow the antigen binding domain to orient in different directions to facilitate antigen recognition while preserving the activating activity of the CAR.

20 In certain embodiments, the hinge/spacer region of the CAR comprises a native or modified hinge region of CD8, of CD28, of CD3 ζ , of CD40, of 4-1BB, of OX40, of CD84, of CD166, of CD8a, of CD8b, of ICOS, of ICAM-1, of CTLA-4, of CD27, of CD40, of NKGD2, or a combination thereof. The hinge/spacer region can be the hinge region from IgG1, or the CH₂CH₃ region of immunoglobulin and portions of CD3, a portion of a CD28 polypeptide (e.g., a portion of SEQ ID NO: 24 or SEQ ID NO: 26), a portion of a CD8 polypeptide (e.g., a portion of SEQ ID NO: 27 or SEQ ID NO: 28), a variation of any of the foregoing which is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% identical or homologous thereto, or a synthetic spacer sequence.

30 In certain embodiments, the hinge domain of the CAR comprises a native or modified hinge region of CD28. In certain embodiments, the hinge domain of the CAR comprises a native hinge region of CD28. In certain embodiments, the hinge domain of the CAR comprises the amino acid sequence of amino acids 114 to 153 of SEQ ID NO: 24. An exemplary nucleotide sequence encoding the amino acid sequence of amino acids 114 to 153 of SEQ ID NO: 24 is set forth in SEQ ID NO: 56, which is provided below.

ATTGAAGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAGAGCAATGGAACCATTATCCATGTGAAAGG
GAAACACCTTTGTCCAAGTCCCCTATTTCCCGGACCTTCTAAGCCCTTT [SEQ ID NO: 56]

5.3.3. Intracellular Signaling Domain of a CAR

In certain embodiments, the CAR comprises an intracellular signaling domain. In certain embodiments, the intracellular signaling domain of the CAR comprises a CD3 ζ polypeptide. CD3 ζ can activate or stimulate a cell (*e.g.*, a cell of the lymphoid lineage, *e.g.*, a T-cell). Wild type (“native”) CD3 ζ comprises three functional immunoreceptor tyrosine-based activation motifs (ITAMs), three functional basic-rich stretch (BRS) regions (BRS1, BRS2 and BRS3). CD3 ζ transmits an activation signal to the cell (*e.g.*, a cell of the lymphoid lineage, *e.g.*, a T-cell) after antigen is bound. The intracellular signaling domain of the CD3 ζ -chain is the primary transmitter of signals from endogenous TCRs.

In certain embodiments, the intracellular signaling domain of the CAR comprises a native CD3 ζ . In certain embodiments, the native CD3 ζ comprises or consists of an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence having a NCBI Reference No: NP_932170 (SEQ ID NO: 29), or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD3 ζ polypeptide comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 29, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to about 164 amino acids in length. In certain embodiments, the native CD3 ζ comprises or consists of the amino acid sequence of amino acids 1 to 164, 1 to 50, 50 to 100, 52 to 164, 100 to 150, or 150 to 164 of SEQ ID NO: 29. In certain embodiments, the intracellular signaling domain of the CAR comprises a native CD3 ζ comprising or consisting of the amino acid sequence of amino acids 52 to 164 of SEQ ID NO: 29. SEQ ID NO: 29 is provided below:

MKWKALFTAA ILQAQLPITE AQSFGLLDPK LCYLLDGILF IYGVILTALF LRVKFSSRAD
APAYQQGQNG LYNELNLGRR EEYDVLDKRR GRDPEMGGKP QRRKNPQEG LYNELQKDKMA
EAYSEIGMKG ERRRGKGGHDG LYQGLSTATK DTYDALHMQA LPPR [SEQ ID NO: 29]

In certain embodiments, the native CD3 ζ comprises or consists of an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 30. SEQ ID NO: 30 is provided below:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAY
SEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR [SEQ ID NO: 30]

In certain embodiments, the intracellular signaling domain of the CAR comprises a modified CD3 ζ polypeptide. In certain embodiments, the modified CD3 ζ polypeptide comprises one, two or three ITAMs. In certain embodiments, the modified CD3 ζ polypeptide comprises a native ITAM1. In certain embodiments, the native ITAM1 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 31.

QNQLYNELNLGRREEYDVLDKR [SEQ ID NO: 31]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 31 is set forth in SEQ ID NO: 32, which is provided below.

CAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGA [SEQ ID NO: 32]

In certain embodiments, the modified CD3 ζ polypeptide comprises an ITAM1 variant comprising one or more loss-of-function mutations. In certain embodiments, the ITAM1 variant comprises or consists of two loss-of-function mutations. In certain embodiments, each of the one or more (e.g., two) loss of function mutations comprises a mutation of a tyrosine residue in ITAM1. In certain embodiments, the ITAM1 variant consists of two loss-of-function mutations. In certain embodiments, the ITAM1 variant comprises or consists of the amino acid sequence set forth in SEQ ID NO: 33, which is provided below.

QNQLFNLNLGRREEFDVLDKR [SEQ ID NO: 33]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 33 is set forth in SEQ ID NO: 34, which is provided below.

CAGAACCAGCTCTTTAACGAGCTCAATCTAGGACGAAGAGAGGAGTTCGATGTTTTGGACAAGAGA [SEQ ID NO: 34]

In certain embodiments, the modified CD3 ζ polypeptide comprises a native ITAM2. In certain embodiments, the native ITAM2 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 35, which is provided below.

QEGLYNELQKDKMAEAYSEIGMK [SEQ ID NO: 35]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 35 is set forth in SEQ ID NO: 36, which is provided below.

CAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAA [SEQ ID NO: 36]

In certain embodiments, the modified CD3 ζ polypeptide comprises an ITAM2 variant. In certain embodiments, the ITAM2 variant comprises or consists of one or more loss-of-function mutations. In certain embodiments, the ITAM2 variant

comprises or consists of two loss-of-function mutations. In certain embodiments, each of the one or more (e.g., two) the loss of function mutations comprises a mutation of a tyrosine residue in ITAM2. In certain embodiments, the ITAM1 variant consists of two loss-of-function mutations. In certain embodiments, the ITAM2 variant comprises or
 5 consists of the amino acid sequence set forth in SEQ ID NO: 37, which is provided below.

QEGLFNEQLQKDKMAEAFSEIGMK [SEQ ID NO: 37]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 37 is set forth in SEQ ID NO: 38, which is provided below.
 10 CAGGAAGGCCTGTTCAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTTCAGTGAGATTGGGATGAAA
 [SEQ ID NO: 38]

In certain embodiments, the modified CD3 ζ polypeptide comprises a native ITAM3. In certain embodiments, the native ITAM3 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 39, which is provided below.

15 HDGLYQGLSTATKDTYDALHMQ [SEQ ID NO: 39]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 39 is set forth in SEQ ID NO: 40, which is provided below.

CACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAG [SEQ ID NO: 40]

20 In certain embodiments, the modified CD3 ζ polypeptide comprises an ITAM3 variant. In certain embodiments, the ITAM3 variant comprises or consists of two loss-of-function mutations. In certain embodiments, each of the one or more (e.g., two) the loss of function mutations comprises a mutation of a tyrosine residue in ITAM3. In certain embodiments, the ITAM3 variant comprises or consists of two loss-of-function
 25 mutations. In certain embodiments, the ITAM3 variant comprises or consists of the amino acid sequence set forth in SEQ ID NO: 41, which is provided below.

HDGLFQGLSTATKDTFDALHMQ [SEQ ID NO: 41]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 41 is set forth in SEQ ID NO: 42, which is provided below.

30 CACGATGGCCTTTTCCAGGGGCTCAGTACAGCCACCAAGGACACCTTCGACGCCCTTCACATGCAG [SEQ ID NO: 42]

Various modified CD3 ζ polypeptides and CARs comprising modified CD3 ζ polypeptides are disclosed in International Patent Application Publication No. WO2019/133969, which is incorporated by reference hereby in its entirety.

In certain embodiments, the intracellular signaling domain of the CAR comprises a modified CD3 ζ polypeptide comprising a native ITAM1, an ITAM2 variant comprising or consisting of one or more (e.g., two) loss-of-function mutations, and an ITAM3 variant comprising or consisting of one or more (e.g., two) loss-of-function mutations. In certain embodiments, the intracellular signaling domain of the CAR comprises a modified CD3 ζ polypeptide comprising a native ITAM1, an ITAM2 variant consisting of two loss-of-function mutations, and an ITAM3 variant consisting of two loss-of-function mutations. In certain embodiments, the intracellular signaling domain of the CAR comprises a modified CD3 ζ polypeptide comprising a native ITAM1 consisting of the amino acid sequence set forth in SEQ ID NO: 31, an ITAM2 variant consisting of the amino acid sequence set forth in SEQ ID NO: 37, and an ITAM3 variant consisting of the amino acid sequence set forth in SEQ ID NO: 41. In certain embodiments, the CAR is designated as "1XX". In certain embodiments, the modified CD3 ζ polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 43. SEQ ID NO: 43 is provided below:

```
RVKFSRSADA PAYQQGQNQL YNELNLGRRE EYDVLDKRRG RDPENGGKPR RKNPQEGLEFN
ELQKDKMAEA FSEIGMKGER RRGKGDGLF QGLSTATKDT FDLHMQALP PR [SEQ ID NO:
43]
```

In certain embodiments, the intracellular signaling domain of the CAR comprises a modified CD3 ζ polypeptide comprising or consisting of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, at least about 100% identical to SEQ ID NO: 43 or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 43 is set forth in SEQ ID NO: 44, which is provided below.

```
AGAGTGAAGTTT CAGCAGGAGCGCAGACGCCCCCGCTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCT
CAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAA
AGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTTCAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTTC
AGTGAGATTGGGATGAAAGGCGAGCGCCGAGGGGCAAGGGGCACGATGGCCTTTTCAGGGGCTCAGTAC
AGCCACCAAGGACACCTTCGACGCCCTTCACATGCAGGCCCTGCCCCCTCGC [SEQ ID NO: 44]
```

Another exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 43 is set forth in SEQ ID NO: 45, which is provided below.

AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCT
 CAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAA
 AGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTTCAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTTC
 AGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTTCCAGGGTCTCAGTAC
 5 AGCCACCAAGGACACCTTCGACGCCCTTCACATGCAGGCCCTGCCCCCTCGC [SEQ ID NO: 45]

In certain embodiments, the intracellular signaling domain of the CAR further comprises at least one co-stimulatory signaling region. In certain embodiments, the at least one co-stimulatory region comprises a co-stimulatory molecule or a portion thereof. In certain embodiments, the at least one co-stimulatory region comprises an
 10 intracellular domain of at least one co-stimulatory molecule or a portion thereof.

As used herein, a “co-stimulatory molecule” refers to a cell surface molecule other than antigen receptor or its ligand that can provide an efficient response of lymphocytes to an antigen. In certain embodiments, a co-stimulatory molecule can provide optimal lymphocyte activation. Non-limiting examples of co-stimulatory
 15 molecules include CD28, 4-1BB, OX40, ICOS, DAP-10, and combinations thereof. The co-stimulatory molecule can bind to a co-stimulatory ligand, which is a protein expressed on cell surface that upon binding to its receptor produces a co-stimulatory response, *i.e.*, an intracellular response that effects the stimulation provided when an antigen-recognizing receptor (e.g., a chimeric antigen receptor (CAR)) binds to its target antigen.
 20 As one example, a 4-1BB ligand (*i.e.*, 4-1BBL) may bind to 4-1BB for providing an intracellular signal that in combination with a CAR signal induces an effector cell function of the CAR⁺ T-cell. In certain embodiments, the at least one co-stimulatory signaling region comprises an intracellular signaling domain of CD28 or a portion thereof, an intracellular domain of 4-1BB or a portion thereof, an intracellular domain of
 25 OX40 or a portion thereof, an intracellular domain of ICOS or a portion thereof, or an intracellular domain of DAP-10 or a portion thereof.

In certain embodiments, the intracellular signaling domain of the CAR comprises a co-stimulatory signaling region that comprises a CD28 polypeptide, e.g., an intracellular domain of CD28 or a portion thereof. In certain embodiments, the
 30 intracellular signaling domain of the CAR comprises a co-stimulatory signaling region that comprises an intracellular domain of human CD28 or a portion thereof.

In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region of the CAR comprise or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at

least about 96%, at least about 97%, at least about 98%, or at least about 99%, at least about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 24, or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consist of an amino acid sequence that is a consecutive portion of SEQ ID NO: 24, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to about 220 amino acids in length. Alternatively or additionally, in certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consists of amino acids 1 to 220, 1 to 50, 50 to 100, 100 to 150, 114 to 220, 150 to 200, 180 to 220, or 200 to 220 of SEQ ID NO: 24. In certain embodiments, the intracellular signaling domain of the CAR comprises a co-stimulatory signaling region that comprises a CD28 polypeptide comprising or consisting of the amino acid sequence of amino acids 180 to 220 of SEQ ID NO: 24.

An exemplary nucleic acid sequence encoding the amino acid sequence of amino acids 180 to 220 of SEQ ID NO: 24 is set forth in SEQ ID NO: 46, which is provided below.

AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCGCCCGGGCCACCCG
CAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCC [SEQ ID NO: 46]

In certain embodiments, the intracellular signaling domain of the CAR comprises a co-stimulatory signaling region that comprises an intracellular domain of mouse CD28 or a portion thereof. In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, at least about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 26, or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 26, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to 218 amino acids in length. In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consists of the amino acid sequence of amino acids 1 to 218, 1 to 50, 50 to 100, 100 to 150, 150 to 218, 178 to 218, or 200 to

218 of SEQ ID NO: 26. In certain embodiments, the co-stimulatory signaling region of a presently disclosed CAR comprises a CD28 polypeptide that comprises or consists of the amino acid sequence of amino acids 178 to 218 of SEQ ID NO: 26.

In certain embodiments, the intracellular signaling domain of the CAR comprises a co-stimulatory signaling region that comprises a 4-1BB polypeptide, e.g., an intracellular domain of 4-1BB or a portion thereof. In certain embodiments, the co-stimulatory signaling region comprises an intracellular domain of human 4-1BB or a portion thereof. In certain embodiments, the 4-1BB comprised in the co-stimulatory signaling region comprises or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, at least about 100% identical or homologous to the sequence having a NCBI Ref. No.: NP_001552 (SEQ ID NO: 47) or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the 4-1BB comprised in the co-stimulatory signaling region comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 47, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and/or up to about 50, up to about 60, up to about 70, up to about 80, up to about 90, up to about 100, up to about 200, or up to about 255 amino acids in length. In certain embodiments, the 4-1BB polypeptide comprised in the co-stimulatory signaling region comprises or consists of the amino acid sequence of amino acids 1 to 255, 1 to 50, 50 to 100, 100 to 150, 150 to 200, or 200 to 255 of SEQ ID NO: 47. In certain embodiments, the co-stimulatory signaling region comprises a 4-1BB polypeptide comprising or consisting of the amino acid sequence of amino acids 214 to 255 of SEQ ID NO: 46. SEQ ID NO: 47 is provided below.

MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR
 TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC
 CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPFPAPARE
 PGHSPQIISF FLALTSTALL FLLFFLTFRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG
 CSCRFPEEEE GGCEL [SEQ ID NO: 47]

In certain embodiments, the intracellular signaling domain of the CAR comprises a co-stimulatory signaling region that comprises intracellular domains of two or more co-stimulatory molecules or portions thereof, e.g., an intracellular domain of CD28 or a portion thereof and an intracellular domain of 4-1BB or a portion thereof, or

an intracellular domains of CD28 or a portion thereof and an intracellular domain of OX40 or a portion thereof.

5.3.4. Exemplified CARs

In certain embodiments, the CAR comprises (a) an extracellular antigen-binding domain comprising (i) a V_H that comprises a CDR1 consisting of the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 consisting of the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 consisting of the amino acid sequence set forth in SEQ ID NO: 9, and (ii) a V_L that comprises a CDR1 consisting of the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 consisting of the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 consisting of the amino acid sequence set forth in SEQ ID NO: 13, (b) a hinge domain comprising a CD28 polypeptide (e.g., a hinge domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino acid sequence of amino acids 114 to 153 of SEQ ID NO: 24); (c) a transmembrane domain comprising a CD28 polypeptide (e.g., a transmembrane domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino acid sequence of amino acids 154 to 179 of SEQ ID NO: 24), and (d) an intracellular signaling domain comprising (i) a CD3 ζ polypeptide (e.g., a modified human CD3 ζ polypeptide, e.g., one consisting of the amino acid sequence set forth in SEQ ID NO: 43), and (ii) a co-stimulatory signaling region comprising a CD28 polypeptide (e.g., an intracellular domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino acid sequence of amino acids 180 to 220 of SEQ ID NO: 24). In certain embodiments, the V_H and V_L are linked via a linker consisting of the amino acid sequence set forth in SEQ ID NO: 1. In certain embodiments, the V_H and V_L are positioned from the N- to the C-terminus: V_L - V_H . In certain embodiments, the CAR is designed as "CAR#2". In certain embodiments, the CAR comprises the amino acid sequence set forth in SEQ ID NO: 48, which is provided below.

MDMRVPAQLLGLLLWLPDTRCEIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLI
YGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQAGAVPITFGGGTKVEIKGGGSGGGGSGG
GGSQVQLVESGGGVVQPGRLRLSCAASGFTFSSYGMHWVRQAPGKLEWVALIWYDGSNKYYADSVKGRF
TISRDNKNTLYLQMNSLRAEDTAVYYCAKPVEGLLRGFDYWGQGTLLTVSSRAAAIEVMYPPPYLDNEKS
NGTIIHVKGKHLCPSPFLPGPSKPFWVLVVGGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRP
GPTRKHYPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPPEMGGKPR
RKNPQEGFLFNEQLQDKMAEAFSEIGMKGERRRGKGHDGLFQGLSTATKDTFDALHMQLPFR [SEQ ID
NO: 48]

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 48 is set forth in SEQ ID NO: 49, which is provided below.

ATGGATATGAGAGTACCAGCTCAGCTGCTGGGCTGCTGCTTTTGTGGTTGCCGGACACACGCTGTGAGAT
 TGCTCTGACTCAGTCTCCCGGACTCTGTCCCTCAGCCCCGGTGAACGCGCTACCCCTTCATGCAGAGCCT
 5 CTCAGTCTGTGTCCAGCAGCTACCTCGCATGGTATCAGCAGAAGCCCGACAGGCTCCAGGCTGTTGATC
 TATGGAGCTAGTAGTCGAGCAACAGGCATCCAGATCGCTTCTCAGGGAGCGGTTAGGTACAGACTTCAC
 GCTGACGATTTCAAGGCTGGAACCCGAAGATTTTGCCGTCTATTATTGTCAACAGGCAGGGGCTGTGCCAA
 TCACTTTTCGGGGCGGGACCAAGGTGGAATCAAAGGAGCGGAGGAAGTGGAGGAGGAGGGAGCGGTGGA
 GGAGGGTCACAGGTGCAGCTGGTAGAATCTGGCGAGGGGTCGTTCAACCAGGGAGGTCATTGCGGTTGAG
 10 CTGCGCAGCGAGTGGTTTTACCTTCAGCAGTTATGGAATGCATTGGGTGAGACAAGCACCAGGAAAAGGTC
 TGGAGTGGGTGGCTTTGATTTGGTACGACGGCAGTAATAAATACTACGCCGATTCTGTTAAGGGCAGATTT
 ACTATTTCTCGGCAACAGCAAGAACACGCTGTACCTGCAGATGAACTCTCTGAGAGCCGAAGATACAGC
 AGTGTACTATTGTGCTAAGCCCGTAGAAGGGCTCCTGAGGGGATTTCGATTATTGGGGCCAGGGTACGCTTG
 TGACAGTGTCTAGTCGGGCGGCGCAATTGAAGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAGAGC
 15 AATGGAACCATTATCCATGTGAAAGGGAACACCTTTGTCCAAGTCCCTATTTCCCGACCTTCTAAGCC
 CTTTTGGGTGCTGGTGGTGGTGGTGGTGGAGTCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATTA
 TTTTCTGGGTGAGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCGCC
 GGGCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCCAGAGTGAA
 GTTCAGCAGGAGCGCAGACGCCCCCGCTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAG
 20 GACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGA
 AGGAAGAACCCTCAGGAAGGCCTGTTCAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTTCAGTGAGAT
 TGGGATGAAAGGCGAGCGCCGAGGGGCAAGGGGCACGATGGCCTTTTCCAGGGTCTCAGTACAGCCACCA
 AGGACACCTTCGACGCCCTTCACATGCAGGCCCTGCCCCCTCGC [SEQ ID NO: 49]

In certain embodiments, the CAR comprises (a) an extracellular antigen-
 25 binding domain comprising (i) a V_H that comprises a CDR1 consisting of the amino acid
 sequence set forth in SEQ ID NO: 7, a CDR2 consisting of the amino acid sequence set
 forth in SEQ ID NO: 8, and a CDR3 consisting of the amino acid sequence set forth in
 SEQ ID NO: 9, and (ii) a V_L that comprises a CDR1 consisting of the amino acid
 sequence set forth in SEQ ID NO: 16, a CDR2 consisting of the amino acid sequence set
 30 forth in SEQ ID NO: 12, and a CDR3 consisting of the amino acid sequence set forth in
 SEQ ID NO: 17, (b) a hinge domain comprising a CD28 polypeptide (e.g., a hinge
 domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the
 amino acid sequence of amino acids 114 to 153 of SEQ ID NO: 24); (c) a
 transmembrane domain comprising a CD28 polypeptide (e.g., a transmembrane domain
 35 of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino
 acid sequence of amino acids 114 to 179 of SEQ ID NO: 24), and (c) an intracellular
 signaling domain comprising (i) a CD3ζ polypeptide (e.g., a modified human CD3ζ
 polypeptide, e.g., one consisting of the amino acid sequence set forth in SEQ ID NO:
 43), and (ii) a co-stimulatory signaling region comprising a CD28 polypeptide (e.g., an
 40 intracellular domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide
 consisting of the amino acid sequence of amino acids 180 to 220 of SEQ ID NO: 24). In
 certain embodiments, the V_H and V_L are linked via a linker consisting of the amino acid
 sequence set forth in SEQ ID NO: 1. In certain embodiments, the V_H and V_L are

positioned from the N- to the C-terminus: V_L-V_H. In certain embodiments, the CAR is designed as “CAR#8”. In certain embodiments, the CAR comprises the amino acid sequence set forth in SEQ ID NO: 50, which is provided below.

```
MDMRVPAQLLGLLLWLPDTRCEIVLTQSPGTLSSLSPGERATLSCRASQSVRSYLAWYQ
5 QKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQLFDSFYT
FGGGTKVEIKGGGSGGGSGGGGSQVQLVESGGGVVQPGRSRLRLSCAASGFTFSSYGMH
WVRQAPGKGLEWVALIWYDGSNKYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYY
CAKPEVEGLLRGFDYWGGTTLTVSSRAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSP
LFPGPSKPFVWLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRK
10 HYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPE
MGGKPRRKNPQEGLFNELQKDKMAEAFSEIGMKGERRRGKGDGLFQGLSTATKDTFDAL
HMQALPPR [SEQ ID NO: 50]
```

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 50 is set forth in SEQ ID NO: 51, which is provided below.

```
15 atggatatgagagtaccagctcagctgctgggctgctgtttgtggttgccggacacacgctgtgagat
tgtgctgacacagtctccaggcacactctcccttagcccgaggagagggccactctgagctgtcgggcta
gtcagtcagtaaggagctcttatctggcctggatcagcagaaaccagggcaggctccaaggctgcttacc
tacggtgcaagttcccggaacaggcatccagatcgcttttagcggtagcgggagtgaggaccgatttcac
tctgaccatctcccgcttgagcccgaggatttcgctgtctattattgccagcaactgtttgactaccct
20 atacgttcggtggagggaacaaagtggagatcaaggaggcggaggaaagtggaggaggaggaggcgggtgga
ggagggtcacaggtgcagctggtagaatctggcggagggtcggttcaaccaggaggtcattgcggttgag
ctgcgcagcgagtggttttacctcagcagttatggaatgcattgggtgagacaagcaccaggaaaaggctc
tgagtggggtggtttgatttggtacgacggcagtaataataactacgccgattctgttaagggcagattt
actatctctcgacacaacagcaagaacacgctgtacctgcagatgaactctctgagagccgaagatacagc
25 agtggtactattgtgctaagcccgtagaagggtcctgaggggattcgattattggggccagggtacgcttg
tgacagtgtctagtccggcgccgcaattgaagttatgtatcctcctccttacctagacaatgagaagagc
aatggaaccattatccatgtgaaagggaacacacctttgtccaagtcacctatttcccgaccttctaagcc
cttttggtgctggtggtggttggtggagtcctggcttgctatagcttgctagtaacagtggcctttatta
ttttctgggtgaggagtaagaggagcaggctcctgcacagtgactacatgaacatgactccccgcgcccc
30 gggcccaaccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgctccagagtga
gttcagcaggagcgcagacgccccgcgtaccagcaggggccagaaccagctctataacgagctcaatctag
gacgaagagaggagtagatgttttgacaagagacgtggcgggaccctgagatgggggaaagccgaga
aggaagaaccctcaggaaggcctgttcaatgaactgcagaaagataagatggcggaggccttcagttagat
tgggatgaaaggcgagcgccggaggggcaaggggcacgatggccttttccaggggctcagtagcaccacca
35 aggacaccttcgacgcccttcacatgcaggccctgccccctgcg [SEQ ID NO: 51]
```

In certain embodiments, the CAR comprises (a) an extracellular antigen-binding domain comprising (i) a V_H that comprises a CDR1 consisting of the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 consisting of the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 consisting of the amino acid sequence set forth in

SEQ ID NO: 9, and (ii) a V_L that comprises a CDR1 consisting of the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 consisting of the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 consisting of the amino acid sequence set forth in SEQ ID NO: 21, (b) a hinge domain comprising a CD28 polypeptide (e.g., a hinge domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino acid sequence of amino acids 114 to 153 of SEQ ID NO: 24); (c) a transmembrane domain comprising a CD28 polypeptide (e.g., a transmembrane domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino acid sequence of amino acids 114 to 179 of SEQ ID NO: 24), and (c) an intracellular signaling domain comprising (i) a CD3ζ polypeptide (e.g., a modified human CD3ζ polypeptide, e.g., one consisting of the amino acid sequence set forth in SEQ ID NO: 43), and (ii) a co-stimulatory signaling region comprising a CD28 polypeptide (e.g., an intracellular domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of the amino acid sequence of amino acids 180 to 220 of SEQ ID NO: 24). In certain embodiments, the V_H and V_L are linked via a linker consisting of the amino acid sequence set forth in SEQ ID NO: 1. In certain embodiments, the V_H and V_L are positioned from the N- to the C-terminus: V_L-V_H. In certain embodiments, the CAR is designed as "CAR#15". In certain embodiments, the CAR comprises the amino acid sequence set forth in SEQ ID NO: 51, which is provided below.

20 MEFGLSWVFLVALLRGVQCQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAP
GKGLEWVALIWYDGSNKYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPVE
GLLRGFDYWGQGLTVTVSSGGGGSGGGSGGGGSEIVLTQSPGTLSTLSPGERATLSCRAS
QSVSSSYLAWYQQKPGQAPRLLIYGASRRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV
YYCQQAGIPPYTFGGGTKEIKRAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFLFP
25 GPSKPFVWLVVGGVLACYSLLVTVAFIIFWVRSKRSLRLHSDYMNMTFRRPGPTRKHYQ
PYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGG
KPRRKNPQEGFLNELQDKDMAEAFSEIGMKGERRRGKGHDGLFQGLSTATKDTFDALHMQ
ALPPR [SEQ ID NO: 52]

An exemplary nucleic acid sequence encoding the amino acid sequence of
30 SEQ ID NO: 52 is set forth in SEQ ID NO: 53, which is provided below.

atggaattcggcttgatgcatgggtgttcctcgtcgcgctgctgcgcggttcagtgccaggtgcagctggt
agaatctggcggagggtcgttcaaccagggaaggtcattgcggttgagctgcgcagcgagtggttttacct
tcagcagttatggaatgcattgggtgagacaagcaccaggaaggtctggagtggtggctttgatttgg
tacgacggcagtaataaatactacgccgattctgttaagggcagatttactatttctcgcgacaacagcaa
gaacacgctgtacctgcagatgaactctctgagagccgaagatacagcagtggtactattgtgctaagcccg
tagaaggqctcctgaggggattcgattattggggccagggtacgcttgtgacagtgctagtggaggcgga

ggaagtggaggaggaggagcggtggaggagggtcagaaatcgtgctcaccagtcctcccggaacactgag
tctctctccaggggaaagagcaacattgtcctgcagagcatcccagagcgtgagctccagctacctcgctt
ggatcagcagaaaccaggccaggcaccgccctgcttatctacggtgcatccaggagagccactgggatc
cccgatcgattctctggatcaggggtctggcactgactttacattgacgatctcacggttggaaccggagga
5 tttcgcggtgtattactgccaacaggccggaattccaccgtataccttcggaggaggtactaaagtagaga
ttaaaccgggcgccgcaattgaagtattgtatcctcctccttacctagacaatgagaagagcaatggaacc
attatccatgtgaaagggaaacacctttgtccaagtccctatttcccggaaccttctaagcccttttgggt
gctggtggtggttggagggtcctggcttgctatagcttgctagtaacagtggtgctttattatcttctggg
tgaggagtaagaggagcagggtcctgcacagtgactacatgaacatgactccccgcccggggccacc
10 cgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgctccagagtgaagttcagcag
gagcgcagacgcccccgctaccagcagggccagaaccagctctataacgagctcaatctaggacgaagag
aggagtacgatgttttgacaagagacgtggccgggacctgagatggggggaaagccgagaaggaagaac
cctcaggaaggcctgttcaatgaactgcagaagataagatggcggaggcccttcagtgagattgggatgaa
aggcgagcgccggagggggcaaggggcacgatggccttttccagggggtcagtagcaccaccaaggacacct
15 tcgacgcccttcacatgcagggcctgccccctcgc [SEO ID NO: 53]

5.4. Cells

The presently disclosed subject matter provides cells comprising a presently disclosed CD19-targeted CAR (e.g., one disclosed in Section 5.3). In certain embodiments, the cell is selected from the group consisting of cells of lymphoid lineage and cells of myeloid lineage. In certain embodiments, the cell is an immunoresponsive cell. In certain embodiments, the immunoresponsive cell is a cell of lymphoid lineage.

In certain embodiments, the cell is a cell of the lymphoid lineage. Cells of the lymphoid lineage can provide production of antibodies, regulation of cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. Non-limiting examples of cells of the lymphoid lineage include T-cells, Natural Killer (NK) cells, B cells, dendritic cells, and stem cells from which lymphoid cells may be differentiated. In certain embodiments, the stem cell is a pluripotent stem cell (e.g., embryonic stem cell or an induced pluripotent stem cell).

In certain embodiments, the cell is a T cell. T cells can be lymphocytes that mature in the thymus and are chiefly responsible for cell-mediated immunity. T cells are involved in the adaptive immune system. The T cells of the presently disclosed subject matter can be any type of T cells, including, but not limited to, helper T cells, cytotoxic T cells, memory T cells (including central memory T cells, stem-cell-like memory T cells (or stem-like memory T cells), and two types of effector memory T cells: *e.g.*, TEM cells and TEMRA cells), Regulatory T cells (also known as suppressor T cells), tumor-infiltrating lymphocyte (TIL), Natural killer T cells, Mucosal associated

invariant T cells, and $\gamma\delta$ T cells. Cytotoxic T-cells (CTL or killer T cells) are a subset of T lymphocytes capable of inducing the death of infected somatic or tumor cells. A patient's own T cells may be genetically modified to target specific antigens through the introduction of a CAR. In certain embodiments, the immunoresponsive cell is a T cell.

- 5 The T cell can be a $CD4^+$ T-cell or a $CD8^+$ T cell. In certain embodiments, the T cell is a $CD4^+$ T cell. In certain embodiments, the T cell is a $CD8^+$ T cell. In certain embodiments, the cell is a T cell, and the presently disclosed CD19-targeted CAR is integrated at a locus within the genome of the T cell. Non-limiting examples of the loci include a TRAC locus, a TRBC locus, a TRDC locus, and a TRGC locus. In certain
- 10 embodiments, the locus is a TRAC locus or a TRBC locus. Methods of targeting a CAR to a site within the genome of T cell are disclosed in WO2017180989 and Eyquem *et al.*, *Nature*. (2017 Mar 2); 543(7643): 113–117, both of which are incorporated by reference in their entireties.

- In certain embodiments, the cell is a NK cell. Natural killer (NK) cells
- 15 can be lymphocytes that are part of cell-mediated immunity and act during the innate immune response. NK cells do not require prior activation in order to perform their cytotoxic effect on target cells. In certain embodiments, the cell is a genetically modified NK cell. In certain embodiments, the cell is an edited NK cell. In certain embodiments, the cell is a NK cell derived from a stem cell. In certain embodiments, the
- 20 cell is a NK cell derived from a pluripotent stem cell. In certain embodiments, the cell is an induced pluripotent stem cell (iPSC)-derived NK cell.

The cells (*e.g.*, T cells or NK cells) can be autologous, non-autologous (*e.g.*, allogeneic), or derived *in vitro* from engineered progenitor or stem cells.

- The cells of the presently disclosed subject matter can be cells of the
- 25 myeloid lineage. Non-limiting examples of cells of the myeloid lineage include monocytes, macrophages, neutrophils, dendritic cells, basophils, neutrophils, eosinophils, megakaryocytes, mast cell, erythrocyte, thrombocytes, and stem cells from which myeloid cells may be differentiated. In certain embodiments, the stem cell is a pluripotent stem cell (*e.g.*, an embryonic stem cell or an induced pluripotent stem cell).

- 30 In certain embodiments, the cells can be transduced with the presently disclosed CD19-targeted CAR such that the cells express the CD19-targeted CAR.

5.5. Nucleic Acid Molecules and Vectors

The presently disclosed subject matter provides nucleic acid molecules encoding the presently disclosed CD19-targeted CARs (e.g., those disclosed in Section 5.3). Also provided are cells comprising such nucleic acid molecules.

5 In certain embodiments, the nucleic acid molecule further comprises a promoter that is operably linked to the presently disclosed CD19-targeted CAR.

In certain embodiments, the promoter is endogenous or exogenous. In certain embodiments, the exogenous promoter is selected from the group consisting of an elongation factor (EF)-1 promoter, a cytomegalovirus immediate-early promoter (CMV) promoter, a simian virus 40 early promoter (SV40) promoter, a phosphoglycerate kinase
10 (PGK) promoter, a metallothionein promoter, and Ubiquitin C promoter. In certain embodiments, the endogenous promoter is selected from a TCR alpha promoter, a TCR beta promoter, and a beta 2-microglobulin promoter. In certain embodiments, the promoter is an inducible promoter. In certain embodiment, the inducible promoter is selected from the group consisting of a NFAT transcriptional response element (TRE)
15 promoter, a CD69 promoter, a CD25 promoter, an IL-2 promoter, a 4-1BB promoter, a PD1 promoter, and a LAG3 promoter.

The presently disclosed subject matter also provides vectors comprising the presently disclosed nucleic acid molecules.

The nucleic acid molecules can be delivered into cells by art-known
20 methods or as described herein. Genetic modification of a cell can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA construct. In certain embodiments, a retroviral vector (e.g., gammaretroviral vector or lentiviral vector) is employed for the introduction of the DNA construct into the cell. For example, a polynucleotide encoding a CAR can be cloned into a retroviral vector and
25 expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for a target cell type of interest. Non-viral vectors may be used as well.

For initial genetic modification of a cell to include a presently disclosed CD19-targeted CAR, a retroviral vector can be employed for transduction, however any
30 other suitable viral vector or non-viral delivery system can be used. The CAR can be constructed in a single, multicistronic expression cassette, in multiple expression cassettes of a single vector, or in multiple vectors. Examples of elements that create polycistronic expression cassette include, but is not limited to, various viral and non-viral Internal Ribosome Entry Sites (IRES, *e.g.*, FGF-1 IRES, FGF-2 IRES, VEGF IRES,

IGF-II IRES, NF- κ B IRES, RUNX1 IRES, p53 IRES, hepatitis A IRES, hepatitis C IRES, pestivirus IRES, aphthovirus IRES, picornavirus IRES, poliovirus IRES and encephalomyocarditis virus IRES) and cleavable linkers (*e.g.*, 2A peptides, *e.g.*, P2A, T2A, E2A and F2A peptides). Combinations of retroviral vector and an appropriate
 5 packaging line are also suitable, where the capsid proteins will be functional for infecting human cells. Various amphotropic virus-producing cell lines are known, including, but not limited to, PA12 (Miller *et al.*, (1985) *Mol Cell Biol* (1985);5:431-437); PA317 (Miller, *et al.*, *Mol Cell Biol* (1986); 6:2895-2902); and CRIP (Danos *et al.*, *Proc Natl Acad Sci USA* (1988);85:6460-6464). Non-amphotropic particles are suitable too, *e.g.*,
 10 particles pseudotyped with VSVG, RD114 or GALV envelope and any other known in the art.

Possible methods of transduction also include direct co-culture of the cells with producer cells (Bregni *et al.*, *Blood* (1992);80:1418-1422), or culturing with viral supernatant alone or concentrated vector stocks with or without appropriate growth
 15 factors and polycations (Xu *et al.*, *Exp Hemat* (1994); 22:223-230; and Hughes *et al.* *J Clin Invest* (1992); 89:1817).

Other transducing viral vectors can be used to modify a cell. In certain embodiments, the chosen vector exhibits high efficiency of infection and stable integration and expression (*see, e.g.*, Cayouette *et al.*, *Human Gene Therapy* 8:423-430,
 20 1997; Kido *et al.*, *Current Eye Research* 15:833-844, 1996; Bloomer *et al.*, *Journal of Virology* 71:6641-6649, 1997; Naldini *et al.*, *Science* 272:263-267, 1996; and Miyoshi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:10319, 1997). Other viral vectors that can be used include, for example, adenoviral, lentiviral, and adena-associated viral vectors, vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also *see*,
 25 for example, the vectors of Miller, *Human Gene Thera* (1990);15-14; Friedman, *Science* 244:1275-1281, 1989; Eglitis *et al.*, *BioTechniques* (1988);6:608-614; Tolstoshev *et al.*, *Cur Opin Biotechnol* (1990); 1:55-61; Sharp, *The Lancet* (1991);337:1277-78; Cornetta *et al.*, *Nucleic Acid Research and Molecular Biology* 36:311-22, 1987; Anderson, *Science* (1984);226:401-409; Moen, *Blood Cells* 17:407-16, 1991; Miller *et al.*,
 30 *Biotechnol* (1989);7:980-90; LeGal La Salle *et al.*, *Science* (1993);259:988-90; and Johnson, *Chest* (1995)107:77S- 83S). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg *et al.*, *N Engl J Med* (1990);323:370, 1990; Anderson *et al.*, U.S. Patent. No. 5,399,346).

Non-viral approaches can also be employed for genetic modification of a cell. For example, a nucleic acid molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner et al., *Proc Natl Acad Sci U.S.A.* (1987);84:7413; Ono et al., *Neurosci Lett* (1990);17:259; Brigham et al., *Am J Med Sci* (1989);298:278; Staubinger et al., *Methods in Enzymol* (1983);101:512, Wu et al., *J Biol Chem* (1988);263:14621; Wu et al., *J Biol Chem* (1989);264:16985), or by micro-injection under surgical conditions (Wolff et al., *Science* (1990);247:1465). Other non-viral means for gene transfer include transfection *in vitro* using calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type *ex vivo* (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue or are injected systemically. Recombinant receptors can also be derived or obtained using transposases or targeted nucleases (e.g. Zinc finger nucleases, meganucleases, or TALEN, CRISPR). Transient expression may be obtained by RNA electroporation.

Any targeted genome editing methods can also be used to deliver a presently disclosed CD19-targeted CAR to a cell. In certain embodiments, a CRISPR system is used to deliver a presently disclosed CD19-targeted CAR. In certain embodiments, zinc-finger nucleases are used to deliver a presently disclosed CD19-targeted CAR. In certain embodiments, a TALEN system is used to deliver a presently disclosed CD19-targeted CAR.

Clustered regularly-interspaced short palindromic repeats (CRISPR) system is a genome editing tool discovered in prokaryotic cells. When utilized for genome editing, the system includes Cas9 (a protein able to modify DNA utilizing crRNA as its guide), CRISPR RNA (crRNA, contains the RNA used by Cas9 to guide it to the correct section of host DNA along with a region that binds to tracrRNA (generally in a hairpin loop form) forming an active complex with Cas9), trans-activating crRNA (tracrRNA, binds to crRNA and forms an active complex with Cas9), and an optional section of DNA repair template (DNA that guides the cellular repair process allowing insertion of a specific DNA sequence). CRISPR/Cas9 often employs a plasmid to transfect the target cells. The crRNA needs to be designed for each application as this is the sequence that Cas9 uses to identify and directly bind to the target DNA in a cell. The

repair template carrying CAR expression cassette need also be designed for each application, as it must overlap with the sequences on either side of the cut and code for the insertion sequence. Multiple crRNA's and the tracrRNA can be packaged together to form a single-guide RNA (sgRNA). This sgRNA can be joined together with the Cas9 gene and made into a plasmid in order to be transfected into cells.

A zinc-finger nuclease (ZFN) is an artificial restriction enzyme, which is generated by combining a zinc finger DNA-binding domain with a DNA-cleavage domain. A zinc finger domain can be engineered to target specific DNA sequences which allows a zinc-finger nuclease to target desired sequences within genomes. The DNA-binding domains of individual ZFNs typically contain a plurality of individual zinc finger repeats and can each recognize a plurality of base pairs. The most common method to generate new zinc-finger domain is to combine smaller zinc-finger "modules" of known specificity. The most common cleavage domain in ZFNs is the non-specific cleavage domain from the type IIs restriction endonuclease FokI. Using the endogenous homologous recombination (HR) machinery and a homologous DNA template carrying CAR expression cassette, ZFNs can be used to insert the CAR expression cassette into genome. When the targeted sequence is cleaved by ZFNs, the HR machinery searches for homology between the damaged chromosome and the homologous DNA template, and then copies the sequence of the template between the two broken ends of the chromosome, whereby the homologous DNA template is integrated into the genome.

Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. TALEN system operates on almost the same principle as ZFNs. They are generated by combining a transcription activator-like effectors DNA-binding domain with a DNA cleavage domain. Transcription activator-like effectors (TALEs) are composed of 33-34 amino acid repeating motifs with two variable positions that have a strong recognition for specific nucleotides. By assembling arrays of these TALEs, the TALE DNA-binding domain can be engineered to bind desired DNA sequence, and thereby guide the nuclease to cut at specific locations in genome. cDNA expression for use in polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), metallothionein promoters, or Ubiquitin C promoter), and regulated by any appropriate mammalian regulatory element or intron (e.g. the elongation factor 1a enhancer/promoter/intron structure). For example, if desired, enhancers known to preferentially direct gene expression in specific cell types

can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences
5 derived from a heterologous source, including any of the promoters or regulatory elements described above.

Methods for delivering the genome editing agents/systems can vary depending on the need. In certain embodiments, the components of a selected genome editing method are delivered as DNA constructs in one or more plasmids. In certain
10 embodiments, the components are delivered via viral vectors. Common delivery methods include but is not limited to, electroporation, microinjection, gene gun, impalefection, hydrostatic pressure, continuous infusion, sonication, magnetofection, adeno-associated viruses, envelope protein pseudotyping of viral vectors, replication-competent vectors cis and trans-acting elements, herpes simplex virus, and chemical
15 vehicles (e.g., oligonucleotides, lipoplexes, polymersomes, polyplexes, dendrimers, inorganic Nanoparticles, and cell-penetrating peptides).

5.6. Formulations and Administration

The presently disclosed subject matter also provides compositions comprising the presently disclosed cells comprising a presently disclosed CD19-targeted
20 CAR. In certain embodiments, the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable carrier.

Compositions comprising the presently disclosed cells can be conveniently provided as sterile liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may be buffered to
25 a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can
30 comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof.

Compositions comprising the presently disclosed cells can be provided systemically or directly to a subject for inducing and/or enhancing an immune response

to an antigen and/or treating and/or preventing a neoplasm. In certain embodiments, the presently disclosed cells or compositions comprising thereof are directly injected into an organ of interest (*e.g.*, an organ affected by a neoplasm). Alternatively, the presently disclosed cells or compositions comprising thereof are provided indirectly to the organ of interest, for example, by administration into the circulatory system (*e.g.*, the tumor vasculature). Expansion and differentiation agents can be provided prior to, during or after administration of the cells or compositions to increase production of cells *in vitro* or *in vivo*.

The quantity of cells to be administered can vary for the subject being treated. In certain embodiments, between about 10^4 and about 10^{10} , between about 10^4 and about 10^7 , between about 10^5 and about 10^7 , between about 10^5 and about 10^9 , or between about 10^6 and about 10^8 of the presently disclosed cells are administered to a subject. In certain embodiments, between about 1×10^6 and about 5×10^8 of the presently disclosed cells are administered to a subject. More effective cells may be administered in even smaller numbers. Usually, at least about 1×10^5 cells will be administered, eventually reaching about 1×10^{10} or more. In certain embodiments, at least about 1×10^5 , 5×10^5 , 1×10^6 , about 5×10^6 , about 1×10^7 , about 2.5×10^7 , about 5×10^7 , about 1×10^8 , about 1.5×10^8 , about 2×10^8 , or about 5×10^8 of the presently disclosed cells are administered to a subject. In certain embodiments, about 1×10^6 of the presently disclosed cells are administered to a subject. The precise determination of what would be considered an effective dose can be based on factors individual to each subject, including their size, age, sex, weight, and condition of the particular subject. Dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art.

The presently disclosed cells and compositions can be administered by any method known in the art including, but not limited to, intravenous administration, subcutaneous administration, intranodal administration, intratumoral administration, intrathecal administration, intrapleural administration, intraosseous administration, intraperitoneal administration, pleural administration, and direct administration to the subject. The presently disclosed cells can be administered in any physiologically acceptable vehicle, normally intravascularly, although they may also be introduced into bone or other convenient site where the cells may find an appropriate site for regeneration and differentiation (*e.g.*, thymus).

5.7. *Methods of Treatment*

The presently disclosed subject matter provides various methods of using the presently disclosed cells or compositions comprising thereof. The presently disclosed cells and compositions comprising thereof can be used in a therapy or medicament. For example, the presently disclosed subject matter provides methods for

5 inducing and/or increasing an immune response in a subject in need thereof. The presently disclosed cells and compositions comprising thereof can be used for reducing tumor burden in a subject. The presently disclosed cells and compositions comprising thereof can reduce the number of tumor cells, reduce tumor size, and/or eradicate the tumor in the subject. The presently disclosed cells and compositions comprising thereof

10 can be used for treating and/or preventing a neoplasm in a subject. The presently disclosed cells and compositions comprising thereof can be used for prolonging the survival of a subject suffering from a neoplasm. In certain embodiments, each of the above-noted method comprises administering the presently disclosed cells or a composition (*e.g.*, a pharmaceutical composition) comprising thereof to achieve the

15 desired effect, *e.g.*, palliation of an existing condition or prevention of recurrence. For treatment, the amount administered is an amount effective in producing the desired effect. An effective amount can be provided in one or a series of administrations. An effective amount can be provided in a bolus or by continuous perfusion.

In certain embodiments, the tumor and/or neoplasm is associated with

20 CD19. In certain embodiments, the tumor and/or neoplasm expresses CD19. In certain embodiments, the tumor and/or neoplasm overexpresses CD19. In certain embodiments, the tumor and/or neoplasm that can be treated by the presently disclosed cells and compositions is a blood cancer. Non-limiting examples of blood cancer include multiple myeloma, leukemia, and lymphomas. Non-limiting examples of leukemia include acute

25 myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute promyelocytic leukemia (APL), mixed-phenotype acute leukemia (MLL), hairy cell leukemia, and B cell prolymphocytic leukemia. The lymphoma can be Hodgkin's lymphoma or non-Hodgkin's lymphoma. In certain embodiments, the lymphoma is B cell lymphoma

30 (BCL).

In certain embodiments, the tumor and/or neoplasm is a B cell malignancy. Non-limiting examples of B cell malignancy include B cell lymphoma (BCL), B cell acute lymphocytic leukemia (ALL), B cell chronic lymphocytic leukemia (CLL), multiple myeloma (MM), CLL with Richter's transformation, and CNS

lymphoma. B cell lymphoma includes B cell non-Hodgkin lymphoma (NHL) and B cell Hodgkin's lymphoma. In certain embodiments, the tumor and/or neoplasm is B cell lymphoma. In certain embodiments, the B cell lymphoma is relapsed or refractory (R/R) B cell lymphoma.

5 In certain embodiments, the subject is a human subject. The subjects can have an advanced form of disease, in which case the treatment objective can include mitigation or reversal of disease progression, and/or amelioration of side effects. The subjects can have a history of the condition, for which they have already been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk
10 of recurrence.

 As a consequence of surface expression of a presently disclosed CD19-targeted CAR, adoptively transferred cells are endowed with augmented and selective cytolytic activity at the tumor site. Furthermore, subsequent to their localization to tumor and their proliferation, the cells turn the tumor site into a highly conductive
15 environment for a wide range of cells involved in the physiological anti-tumor response.

 Further modification can be introduced to the presently disclosed cells to avert or minimize the risks of immunological complications (known as “malignant T-cell transformation”), *e.g.*, graft versus-host disease (GvHD), or when healthy tissues express the same target antigens as the tumor cells, leading to outcomes similar to GvHD. A
20 potential solution to this problem is engineering a suicide gene into the presently disclosed cells. Suitable suicide genes include, but are not limited to, Herpes simplex virus thymidine kinase (hsv-tk), inducible Caspase 9 Suicide gene (iCasp-9), and a truncated human epidermal growth factor receptor (EGFRt) polypeptide. In certain embodiments, the suicide gene is an EGFRt polypeptide. The EGFRt polypeptide can
25 enable T-cell elimination by administering anti-EGFR monoclonal antibody (*e.g.*, cetuximab). EGFRt can be covalently joined to the upstream of the CD19-targeted CAR. The suicide gene can be included within the vector comprising nucleic acids encoding a presently disclosed CD19-targeted CAR. In this way, administration of a prodrug designed to activate the suicide gene (*e.g.*, a prodrug (*e.g.*, AP1903 that can activate
30 iCasp-9) during malignant T-cell transformation (*e.g.*, GVHD) triggers apoptosis in the suicide gene-activated cells expressing the CD19-targeted CAR. The incorporation of a suicide gene into the a presently disclosed CD19-targeted CAR gives an added level of safety with the ability to eliminate the majority of receptor-expressing cells within a very short time period. A presently disclosed cell incorporated with a suicide gene can be

pre-emptively eliminated at a given timepoint post the cell infusion, or eradicated at the earliest signs of toxicity.

7. EXAMPLE

The presently disclosed subject matter will be better understood by
5 reference to the following Example, which is provided as exemplary of the presently disclosed subject matter, and not by way of limitation.

Example 1 – Identification And Characterization Of Anti-CD19 Antibodies

This Example demonstrates characterization of anti-CD19 antibodies
comprising the V_H and V_L of the scFvs disclosed herein. Human anti-CD19 antibodies
10 were derived from and produced in Adimab yeast. Antigens were biotinylated using the EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Scientific, Cat #21425). The antigens were concentrated to about 1 mg/mL and buffer exchanged into PBS before addition of 1:7.5 molar ratio of biotinylation reagent. The mixture was held at 4°C overnight prior to another buffer exchange to remove free biotin in the solution. Biotinylation was
15 confirmed through streptavidin sensor binding of the labeled proteins on a ForteBio.

Eight naïve human synthetic yeast libraries each of $\sim 10^9$ diversity were propagated as previously described (see, e.g., Y. Xu et al, PEDS 26(10), 663-70 (2013); WO2009036379; WO2010105256; and WO2012009568.)

For the first two rounds of selection, a magnetic bead sorting technique
20 utilizing the Miltenyi MACS system was performed, as previously described (see, e.g., Siegel et al, J Immunol Methods 286(1-2), 141-153 (2004).) Briefly, yeast cells ($\sim 10^{10}$ cells/library) were incubated with biotinylated antigen for 30 min at 30°C in wash buffer (phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA)). After washing once with 40 mL ice-cold wash buffer, the cell pellet was resuspended in 20 mL wash
25 buffer, and Streptavidin MicroBeads (500 μ L) were added to the yeast and incubated for 15 min at 4°C. Next the yeast were pelleted, resuspended in 5 mL wash buffer, and loaded onto a Miltenyi LS column. After the 5 mL were loaded, the column was washed 3 times with 3 mL wash buffer. The column was then removed from the magnetic field, and the yeast were eluted with 5 mL of growth media and then grown overnight.

30 The third round of selection was performed using flow cytometry (FACS). Approximately 2×10^7 yeast were pelleted, washed three times with wash buffer, and incubated at 30°C with 100-200 nM biotinylated antigen under equilibrium conditions. The fourth and fifth rounds of selections were performed by incubating

biotinylated NALM-6 and Raji cells with the selected yeast output from the round 3 FACS. After incubation, pre-washed M-280 Streptavidin Dynabeads (Cat #60210) were added to the yeast/mammalian cell complexes and incubated. Next, the complexes were separated using a DynaMag-2 magnet and the non-binding supernatants were removed.

5 The bead/cell complexes were washed three times with 1 mL of selection buffer. The captured complexes were then transferred into flasks containing yeast growth media for propagation. In the sixth round of selection, propagated yeast were subjected to either and additional round of NALM-6/Raji cell selection, selection with 100 nM recombinant CD19 antigen, or negative selection with a polyspecificity reagent (PSR) to remove non-

10 specific antibodies.

For the PSR depletion, the libraries were incubated with a 1:10 dilution of biotinylated PSR reagent as previously described (see, e.g., Y. Xu et al, PEDS 26(10), 663-70 (2013).) Yeast were then washed twice with wash buffer and stained with goat F(ab')₂ anti-human kappa-FITC (LC-FITC) diluted 1:100 (Southern Biotech, Cat #

15 2062-02) and either Streptavidin-AF633 (SA-633) diluted 1:500 (Life Technologies, Cat # S21375) or Extravidin- phycoerythrin (EA-PE) diluted 1:50 (Sigma-Aldrich, Cat # E4011), secondary reagents for 15 min at 4°C. After washing twice with ice-cold wash buffer, the cell pellets were resuspended in 0.3 mL wash buffer and transferred to strainer-capped sort tubes. Sorting was performed using a FACS ARIA sorter (BD

20 Biosciences) and sort gates were determined to select for antibodies with desired characteristics. Selection rounds were repeated until a population with all of the desired characteristics was obtained. After the final round of sorting, yeast were plated and individual colonies were picked for characterization.

Light chain diversification

25 Heavy chains from the naïve output were used to prepare light chain diversification libraries used for additional selection rounds. Heavy chain plasmids were extracted from the yeast, propagated in and subsequently purified from *E. coli*, and transformed into a light chain library with a diversity of 5×10^6 . Selections were performed on these libraries as described above, i.e., with one round of MACS, two

30 rounds of cell selection with either Raji or NALM-6 cells, followed by a fourth round FACS selection using recombinant CD19 antigen. Specific to the light chain diversification, the Raji and NALM-6 cells selections incorporated an initial negative selection with engineered Raji and NALM-6 cells that had undergone targeted genetic knockout of the CD19 gene. Following the depletion with the CD19 knockout cells, a

positive selection was performed using engineered Raji and NALM-6 cells that expressed both endogenous CD19 and overexpressed. In the different FACS selection rounds, the libraries were evaluated for (Poly-Specificity Reagent) PSR binding, species cross-reactivity, and affinity pressure by antigen titration. Sorting was performed in order to obtain a population with the desired characteristics. Individual colonies from each FACS selection round described above were picked for sequencing and characterization.

Antibody production and purification

Yeast clones were grown to saturation and then induced for 48 h at 30°C with shaking. After induction, yeast cells were pelleted and the supernatants were harvested for purification. IgGs were purified using a Protein A column and eluted with acetic acid, pH 2.0.

ForteBio K_D measurements

These anti-CD19 antibodies were tested for their binding affinity on soluble CD19 in a ForteBio Octet system (Octet RED384 generally as previously described (see, e.g., Estep et al, Mabs 5(2), 270-278 (2013)). Briefly, ForteBio affinity measurements were performed by loading IgGs on-line onto AHC sensors. The antibodies were immobilized on anti-human IgG pins and bound to soluble CD19-HSA fusion protein (CD19 extracellular domains fused to human serum albumin). Sensors were equilibrated off-line in assay buffer for 30 min and then monitored on-line for 60 seconds for baseline establishment. Sensors with loaded IgGs were exposed to 100 nM soluble CD19-HSA fusion protein antigen for 3 minutes, and afterwards were transferred to assay buffer for 3 min for off-rate measurement. All kinetics were analyzed using the 1:1 binding model. A summary of the binding kinetics are provided in Table 1. The affinities of exemplary antibodies ranged between 8 nM and 20 nM. All the anti-CD19 antibodies shown in Table 1 comprise a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10.

Table 1. Binding kinetics of anti-CD19 antibodies

CD19 Antibody	SEQ ID NO. of the V_L amino acid sequence	IgG K_D (M) monovalent	k_{on} (1/Ms)	k_{off} (1/s)
Antibody 2	14	9.01E-09	6.15E+04	5.54E-04
Antibody 4	58	9.27E-09	2.35E+04	2.18E-04
Antibody 5	60	1.04E-08	7.56E+04	7.85E-04
Antibody 8	18	8.22E-09	5.52E+04	4.54E-04
Antibody 6	62	6.40E-09	7.56E+04	4.84E-04

Antibody 7	64	1.49E-08	7.00E+04	1.05E-03
Antibody 1	67	3.29E-08	5.34E+04	1.76E-03
Antibody 15	22	2.08E-08	6.98E+04	1.45E-03

Example 2 – Generation of CD19-targeted CARs and CAR Expression

Three CD19-targeted CARs “CAR#2”, “CAR #8”, and “CAR#15” were generated. The expression of CAR molecules on the cell surface was examined using PE conjugated anti-human LNGFR antibody followed by flow cytometry analysis. LNGFR was co-expressed with CAR through via a self-cleaving linker 2A sequence, thus the expression of LNGFR were used as indication of CAR expression. The results are shown in Table 2.

Table 2

CAR-T #	LNGFR+ %
CAR #2	53.7 %
CAR #8	64.1 %
CAR #15	18.5 %
Positive control	31.6 %
UTD	0.35 %

UTD: un-transduced T cells

Positive control is a CD19-targeted CAR comprising a murine scFv SJ25c1 and CD3ζ1XX, termed “1928z1XX CAR”.

Example 3 – In Vitro Activities of CD19-targeted CARs

The cytotoxicity of these CD19-targeted CARs against CD19-expressing Raji cancer cell lines and CD19 knock-out Raji cells was examined. Each target cell line was seeded in a 384-well plate, and CD19 CAR-T or untransduced T cells (UTD) were added at 4 effector-to-target (E:T) ratios. Wells with target cells only and wells with effector cells only were included as controls. After 24 hours, cell viability was measured using CellTiter-Glo® One Solution Assay (Promega, G8462). Percent viability of target cells was calculated from the luminescence signals of the co-culture wells, after first subtracting the signals of the effector-cells-only wells, then dividing by the signals of the target-cell-only wells. Percent killing was calculated by subtracting the percent viability of target cells from 100%. As shown in Figures 1A and 1B, T cells comprising CAR#2, T cells comprising CAR#8, and T cells comprising CAR#15 all demonstrated cell killing activity against Raji cells, in contrast to CD19 knock-out Raji cells, indicating the killing activity of T cells comprising these CD19-targeted CARs was antigen-dependent.

To confirm antigen dependent cytotoxicity, T cells comprising these CD19-targeted CARs were co-cultured with CD19-expressing Raji cancer cell lines or CD19 knock-out Raji cells. Each target cell line was labelled with cell trace violet dye and then seeded in a 96 well plate, and CD19 CAR-T or untransduced T cells (UTD) were added at 2 effector-to-target (E:T) ratios. Wells with target cells only and wells with effector cells only were included as controls. After 20 hours, cell viability was measured using live/dead dye staining followed by flow cytometry analysis. Percent viability of target cells was calculated from live cells measured by flow cytometry, then dividing by the signals of the target-cell-only wells. Percent killing was calculated by subtracting the percent viability of target cells from 100%. As shown in Figures 2A and 2B, T cells comprising CAR#2, T cells comprising CAR#8, and T cells comprising CAR#15 all demonstrated cell killing activity against Raji cells, in contrast to CD19 knock-out Raji cells, confirming the killing activity of T cells comprising these CD19-targeted CARs was antigen-dependent.

Antigen independent cytokines (IL-2 and IFN- γ) secretion was evaluated with T cells comprising CAR#2, T cells comprising CAR#8, and T cells comprising CAR#15 co-culture with CD19-expressing Raji cancer cell lines and CD19 knock-out Raji cells. Each target cell line was seeded in a 384-well plate, and CD19 CAR-T or untransduced T cells (UTD) were added at 4 effector-to-target (E:T) ratios. Wells with target cells only and wells with effector cells only were included as controls. After 24 hours, supernatant was and secreted IFN- γ and IL2 in the supernatant were detected using the Intellicyt QBeads Human PlexScreen kit (Sartorius, 90702). The IL-2 secretion results are shown in Figures 3A and 3B, and the IFN- γ secretion results are shown in Figures 4A and 4B. T cells comprising these CD19-targeted CARs showed IL-2 and IFN- γ secretion when co-culture with Raji cells, in contrast to CD19 knock-out Raji cells, indicating antigen-dependent cytokine release of T cells comprising these CD19-targeted CARs.

Example 4 – In Vivo Activities of CD19-targeted CARs

The *in vivo* activity of T cells comprising these CD19-targeted CARs was next investigated.

NOD-scid IL2Rg^{null} (NSG) mice were intravenously (iv) injected with 0.5×10^6 firefly luciferase expressing NALM6 cells (B-ALL cell line) with either wild type CD19 levels (CD19-WT) or reduced CD19 levels (CD19-Low, a Nalm6 clone generated by CRISPR-Cas9-editing of the CD19 gene, followed by CD19 re-expression

using a lentiviral vector). CD19-WT (blue, red) and CD19^{Low} (green) NALM6 bearing mice were treated with a single intravenous injection of fresh 5×10^4 (red) or 2×10^5 (blue, green) #17, #8, #15 or #2 CAR T cells 4 days later. CAR T cell treated-mice were imaged repeatedly over 60-100 days to assess tumor response and potential relapse for a rigorous assessment of CAR T cell potency. Tumor burden was analyzed by bioluminescence imaging until day 30 post T cell infusions and is demonstrated for each mouse at indicated time points in Figure 5. #2 CAR T cells demonstrated rapid and effective antitumor activity, resulting in complete tumor eradication of NALM6 WT and NALM6 CD19-Low cells at all tested T cell doses. #17 showed insufficient tumor control, leading to rapid tumor progression and served as control.

Example 5 – Binding Epitope of #2 scFv

Anti-CD19 antibody binding to CD19 expressing NALM6 cell lines was determined by serial dilution and flow cytometry. CD19-T2 binder, SJ25c1 (from the 19-28z CAR), and FMC63 (from the Kymriah's "Tisagenlecleucel" and Yescarta's "Axicabtagene ciloleucel") scFvs competitive binding to CD19 was evaluated by Biacore SPR. A mixture of 100 nM CD19-HSA-His (Takeda, Cambridge) and increasing concentration of soluble CD19-T2 SJ25c1 or FMC63 scFv (0, 50 nM, 100 nM, 200 nM, 400 nM, and 800 nM) were flowed through a Biacore CMS chip with immobilized CD19-T2 scFv for 3 minutes. The dissociation of the complexes bound on the chip was evaluated by flowing HBS-EP buffer (300 mM NaCl) for 5 minutes. The CD19-HSA-His was purified from HEK293 that was transiently transfected with a pcDNA3.4 plasmid (ThermoFisher) comprising a gene expressing the CD19 extracellular domain fused to 10 decahistine tag, TEV cleavage site, GS linker, and HSA. The recombinant CD19-HSA-His10 was purified by using a nickel-affinity chromatography. The amino sequence of the CD19-HSA-His10 is shown in Figure 6.

The epitope binding data of SJ25c, FMC63, and #2 scFv are shown in Figure 7. As shown in Figure 7, #2 scFv competed for binding to CD19 with FMC63 and SJ25C1. These findings establish that the #2 scFv, FMC63, and SJ25C1 scFv's bind to an overlapping epitope on CD19. An unrelated antibody (anti-CTLA4 VHH), which does not bind to CD19, failed to compete with #2 scFv for binding to CD19.

Example 6 – Pharmacology Studies

Summary

In support of a first-in-human clinical trial, a series of studies were conducted to demonstrate the improved efficacy of #2 CAR T cells (represented as

“19(T2)28z1XX CAR” in this Example) relative to 1928z CAR T cells (which have been extensively administered in prior clinical trials), as measured by increased anti-tumor potency at low T cell doses and increased durability of response. CAR T cells engineered to express either CAR displayed comparable cytolytic activity, cytokine secretion, and proliferation *in vitro*, but the #2 CAR T cells achieved greater potency *in vivo* owing to their greater functional persistence (Feucht, *et al. Nature Medicine* (2019);25(1):82-88).

Results

The *in vitro* cytotoxic activity of #2 CAR T cells and 1928z-1XX CAR T cells were measured using an 18-h bioluminescence assay. These two CARs are identical, but for the different scFv (#2 vs. SJ25c1). The retroviral vector comprising the #2 CAR is shown in Figure 8. Recombinant retroviral particles were generated from HEK293 GalV9 packaging cells, as previously described (Przybylowski *et al.*, (2006);13(1):95-100). Firefly luciferase (FFL)-expressing NALM6 cells were used as target cells. The T cells were isolated by negative selection from human PMBCs, activated with CD3/CD28 antibodies bound to magnetic beads for 48hrs, and after bead removal, was transduced with γ -retroviral vectors expressing the CARs. CAR+ population was determined by LNGFR detection, which was co-expressed with the CAR gene. The CAR+ T cells were incubated with target NALM6 cells at different effector (E) to target (T) ratios for 18h, followed by quantification of FFL activity. The cytotoxic activity of the #2 CAR T cells was compared to that of the 1928z-1xx CAR T cells, which were previously shown to kill CD19⁺NALM6 cells *in vitro* effectively (Feucht *et al.*, *Nature Medicine* (2019);25(1):82-88). Untransduced (UT) T cells were used as negative controls. NALM6-CD19KO is a cell line generated by CRISPR-Cas9-editing of the CD19 gene (Hamieh *et al.*, *Nature* (2019);568(7750):112-116) and was used to demonstrate the specificity of the CAR T cells cytotoxic activity. A representative experiment is shown (n = 2 independent experiments on 2 healthy donors). As shown in Figure 10, the #2 CAR T cells and 1928z-1XX CAR T cells showed comparable cytotoxicity.

Next, the *in vivo* activities of #2 CAR T cells (represented as “19(T2)28z1XX CAR”) and 1928z-1XX CAR T cells were measured. NALM6, a pre-B acute lymphoblastic leukemia human tumor cell line expressing normal (wt) or low (low) levels of CD19 antigen, were transduced with firefly luciferase and green fluorescent protein (GFP) (Zhao *et al.*, *Cancer Cell* (2015);28:415-428). CD19⁺ wt (blue) and CD19⁺ low (red) NALM6 bearing mice were treated with a single intravenous injection

of fresh 2×10^5 untransduced T cells, #2 CAR T cells, or 1928z-1XX CAR T cells. The tumor burden in each mouse was monitored by bioluminescence imaging (BLI) for 85 days. There were five mice in each group, and the response of each mouse is shown in Figure 11. As shown in Figure 11, #2 CAR T cells and 1928z1XX CAR T cells had similar tumor eradication kinetics. The CAR cDNA was co-expressed with the LNGFR reporter (to monitor T cell transduction). “19(T2)28z1XX CAR” represents “#2 CAR”.

The activities of #2 CAR T cells and 1928z CAR T cells were studied and compared. First, CAR expression and phenotypes of 1928z CAR T cells and #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were measured. A vector comprising 1928z CAR is shown in Figure 12. CD4⁺ and CD8⁺ T cells were transduced with γ -retroviral vectors, which express the 1928z CAR or #2 CAR (represented as “19(T2)28z1XX CAR”) and produced using a process representative of the clinical manufacturing. Because the SJ25c1 scFv in the SGF-1928z is derived from mouse and the #2 scFv (represented as “19(T2) scFv”) is humanized, two different goat antibodies specific to either mouse (GAM) or human (GAH) Fc portion of the IgG heavy chain were used to detect CAR surface expression. As shown in Figure 13, more than 50% of the CD4⁺ and CD8⁺ T cells expressed the CARs.

Next, the phenotypes of CAR T cells were determined by flow cytometry using antibodies that recognize markers for naïve, central memory, effector memory, and effector T cells as well as for exhaustion markers (LAG3, PD1, and Tim3). “SFG-T2-1XX-GAH” refers to CD4/CD8 T cells transduced with γ -retroviral vector expressing the 19(T2)28z1XX CAR (representing “#2 CAR”), while “SFG-1928z-GAM” were from T cells transduced with γ -retroviral vector expressing the 1928z CAR. As shown in Figure 14, the phenotypes of the 1928z CAR T cells and #2 CAR T cells were similar. The ratio of CD4/CD8 T cells was similar in 1928z and #2 CAR T cells, and T cell differentiation states were comparable between both CAR groups (for CD4 and CD8 T cells) as determined by CD62L/CD45RA and CD45RA/CCR7 staining. Expression of exhaustion markers PD1, LAG3, and TIM3 did not reveal major differences between both CAR T cell groups.

Subsequently, the *in vivo* activities of #2 CAR T cells and 1928z CAR T Cells were assessed and compared. Four days after 5×10^5 CD19⁺ NALM6 -FFLuc/GFP cells were injected in the tail vein of the mice, thawed cryopreserved untransduced or CAR T cells were given. As shown in Figure 15, #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) provided superior tumor control relative to 1928z CAR

T cells. The survival of the CD19⁺ NALM6 bearing mice that were treated with untransduced or CAR T cells is plotted in a Kaplan-Meier curve plot as shown in Figure 16. As shown in Figure 16, CD19⁺ NALM6 bearing mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) survived longer than mice treated with 1928z CAR T cells at all CAR T cell doses below 1×10^6 cells per mouse. All 1928z CAR T cell recipients died by day 25 while only one mouse died in the study groups that received #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”). At the highest dose (1×10^6), one mouse died in both control and test groups.

In vivo persistence of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) and 1928z CAR T cells in the CD19⁺ NALM6 leukemia mouse model was also measured. Persistence of CAR T cells after 17 days post-treatment, and the numbers of CAR T cells in the bone marrow ($n=5$ per group) are shown in Figure 17. PD1, LAG3, and TIM3 expression percentage of CAR T cells, and T_n, T_{cm}, T_{eff}, and T_{em} percentages of CAR T cells are presented in Figure 17.

As shown in Figure 17, #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were readily detected in bone marrow by day 17, comprising both CD4⁺ and CD8⁺ CAR T cells. 1928z CAR T cells were few, consistent with their limited persistence (Zhao *et al.*, *Cancer Cell* (2015);28:415-428). The respective phenotypes of persisting T cells detected on day 17 were overall similar (T_n, T_{cm}, and T_{em}), showing a slightly higher fraction of effector T cells (T_{eff}) in the #2 CAR (represented as “19(T2)28z1XX CAR”) group. The latter expressed more PD1 and comparable levels of LAG3 and TIM3. This observation corroborates the greater persistence of CAR T cells expressing the CD28/CD3z-1XX signaling motif (Feucht *et al.*, *Nature Medicine* (2019);25(1):82-88).

These results confirm the greater potency of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) relative to 1928z CAR T cells (higher tumor eradication at 4 dose levels). This study further establishes that the phenotype of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) is overall similar to that of 1928z CAR T cells and is consistent with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) achieving better tumor control owing to their greater persistence relative to the shorter-lived 1928z CAR T cells.

Methods and Materials

Cytolytic assay

Luciferase-based assays were used to measure the cytotoxicity of T cells transduced with different CAR constructs. NALM6- expressing FFLuc-GFP served as the target cells. The effector and tumor target cells were co-cultured in triplicates at the indicated effector/target ratio in black-walled 96- well plate. 5×10^4 target cells were seeded in a total volume of 100 μ L per well with X-VIVO15 supplemented with 5% of human AB Serum (Gemini), 10 U/ml IL7, and 10 U/mL IL15 (PeproTech, Inc). Target cell alone plated at the same cell density was used to determine maximum luciferase expression (relative light units (RLU)). 100 μ L luciferase substrate (Bright-Glo; Promega) was directly added to each well 18 hrs post-co-culture. Emitted light was determined using a luminescence plate reader. Tumor cell lysis was calculated as $(1 - (RLU_{\text{sample}})/(RLU_{\text{max}})) \times 100$.

Isolation, transduction, and expansion of genetically modified human T cells

Buffly coats from de-identified healthy donors were purchased from the New York Blood Center (exempted from Institutional Review Board). All blood samples were handled in compliance with the required ethical and safety procedures. Isolation of peripheral blood mononuclear cells was carried out by density gradient centrifugation and activated with Dynabeads® ClinExVivo™ CD3/CD28 antibodies beads before transduction with γ -retroviral vector by centrifugation on RetroNectin-coated plates (Takara) as described previously (Zhao *et al.*, *Cancer Cell* (2015);28:415-428). After two days, the activated T cells were debeaded and transduced by spinoculation, as previously described (Hollyman et al., *J. Immunotherapy* (2009);32(2):160-180). Then, the transduced cells were expanded in a G-Rex 6M-well plate as per the manufacture's instruction (Wilson Wolf Corporation). Transduction efficiency was determined 5 days posttransduction. The CAR-T cells were used fresh or thawed from cryopreserved in 50% CS10 and 5% HSA in Plasmalyte.

Mouse systemic tumor model

6- to 12-week-old NOD/SCID/IL-2R γ null male mice were used under a protocol approved by the Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee. All relevant animal use guidelines and ethical regulations were followed. In general, mice were inoculated with 0.5×10^6 NALM6 (CD19⁺ wt or low expression)-FFLuc-GFP tumor cells through tail vein injection, followed by treatment of a specific dose CAR-T cells per mouse 4 days after tumor injection. NALM6 cells provided even tumor burdens, and no mice were excluded prior to CAR-T cell treatment. No randomization or blinding methods were used.

Bioluminescent imaging

Tumor burden was evaluated as previously described (Gade et al., Cancer Research (2005);65(16):9080-9088). Briefly, mice that were under 2% isoflurane anesthesia for 10 minutes were intraperitoneally injected with D-luciferin (Xenogen, 3 mg per mouse) resuspended in PBS and imaged. IVIS Imaging System (PerkinElmer) was used to image the bioluminescence of the injected tumor. Data were analyzed using the Living Image software (PerkinElmer).

Antibodies used in flow cytometry

The expression of the surface markers from CAR T cells generated *ex vivo* or isolated from mouse bone marrow and spleen after 17 days of 1×10^6 CAR T treatments were detected using the following fluorophore-conjugated antibodies: APC-Cy7 anti-human CD8 (SK1), APC-Cy7 mouse anti-human CD45 (2D1), BV395 mouse anti-human CD4 (SK3), BV421 mouse anti-human CD62L (DREG-56), BV650 mouse anti-human CD45RA (HI100), BV480 or BV510 mouse anti-human PD-1 (EH12.1) and BUV737 mouse anti-human CD19 (SJ25C1) (BD Bioscience); PE-Cy7 anti-human CD8 (SK1) and PerCP-eFluor 710 anti-human LAG-3 (3DS223H) (eBioscience); PerCP anti-human CD45RA (HI100), Brilliant Violet 785 anti-human Tim-3 (F38-2E2) and PE anti-human CD127 (IL7Ra) (Biolegend). The expression of the 1928z CAR was detected by using Alexa Fluor 647 goat anti-mouse AffiniPure IgG, F(ab')₂ fragment. To detect #2 CAR (represented as “19(T2)28z1XX CAR”), Alexa Fluor 647-goat anti-human IgG, F(ab')₂ fragment was used. 7-AAD (Beckman Coulter) was used as a viability dye. Flow cytometry was performed on a Cytex Aurora instrument (Cytex Biosciences). Data were analyzed with FlowJo software v.10.1 (FlowJo LLC).

Statistical analyses

All statistical analyses were performed using Prism 8 (GraphPad) software. Statistical comparisons between two groups were determined by two-tailed unpaired t-test. For *in vivo* studies, the overall survival was presented using a Kaplan-Meier curve. Statistical significance is defined as a p-value <0.05.

Example 7 – Toxicity Assessment**Summary**

The toxicity of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) was assessed *in vivo* in NSG mice bearing NALM6 leukemia. Mice injected *i.v.* with 1928z CAR T cells at the same doses served as controls. The recorded parameters included whole body weight, tumor burden, and survival for all mice. More extensive

toxicology investigation focused on mice treated at the highest dose (1×10^6 CAR T cells/recipient), a dose that exceeds the minimum therapeutic (curative) dose in this mouse model (10x) and also exceeds the CAR T cell doses used clinically in proportion to recipient body weight (10-100x). These studies included enumeration and phenotyping of T cells at the main tumor site (bone marrow), serum cytokine measurements (both human, i.e., T cell-derived, and murine, i.e., host-derived), basic serum biochemistry including liver enzymes, peripheral blood cell counts and organ weights and comprehensive tissue immuno-histochemistry at the end of study (Days 27-28). The infused CAR T cells were produced in clinically relevant fashion and comparable between the two CAR T cell products.

Whole-body weights and survival did not significantly differ between the test and control group. Serum cytokine levels were also comparable and did not suggest the occurrence of CRS in either group. ALT and AST enzyme levels were comparable between both study groups on day 10. The enzyme levels of mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were lower than mice treated with 1928z CAR T cells on day 27/28.

As anticipated, #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) outlasted 1928z CAR T cells. Mice in the #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) treated group had higher lymphocytes and reticulocyte counts than the 1928z CAR T cell group. Mice that received 1928z CAR T cells showed diffuse tumor infiltrates but not recipients of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”). Mice with persisting #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) showed mild to moderate xenogeneic GVHD and hepatocellular necrosis.

Whereas the GVHD is to be expected in NSG mice treated with a high dose of human CAR T cells, the latter's mechanism is uncertain. Given the xenogeneic nature of the study (prone to xenoreactivity of human T cells against mouse tissues) and its conduct using a single donor (unknown underlying T cell receptor repertoire), the significance of this adverse event for an autologous therapy in humans is uncertain.

30 Justification of Animal Model

The NALM6 model in NSG mice has been extensively used to assess the activity of CD19-specific CAR T cells in the Sadelain laboratory (Zhao *et al.*, *Cancer Cell* (2015);28:415-428, Feucht, *et al.* *Nature Medicine* (2019);25(1):82-88, Hamieh *et al.*, *Nature* (2019);568(7750):112-116, Eyquem *et al.*, *Nature* (2017);543(7643):113-

117) and others. NALM6 is a human pre-B acute lymphocytic leukemia (ALL) that expresses CD19, but neither CD80, CD86, 4-1BBL, or other major costimulatory ligands.

Justification of Dose and Schedule

5 A single intravenous injection of 1×10^6 viable CAR T cells, which was based on % transduction efficiency and total viable cells, was the selected dose given that it corresponds to 10x the minimum effective treatment (1×10^5 CAR T cells) in this animal model (see Figure 15). The CAR T cell dosage is approximately 10 – 100x higher than the approved CD19 CAR T cell doses for adults (e.g., approved dose of $0.1 - 1.5 \times 10^8$ viable CAR T cells) when converted by bodyweight from mouse to human. The proposed starting flat-dose in the human clinical trial protocol for #2 CAR T cells is 25×10^6 CAR T cells.

Methods

Test Article and Vehicle

15 The test article is 1928z CAR T cells, #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells” in this Example), and untransduced T cells prepared by Cell Therapy and Cell Engineering Facility (CTCEF) at MSKCC. The cells were frozen in liquid nitrogen until the start of the study. On the day of T cell injection, cells were thawed at 37°C and washed with 1% HSA in Plasmalyte. Cells were then formulated into doses of 1×10^6 , 5×10^5 , 2×10^5 , and 1×10^5 CAR-T cells in a volume of 200 μL per mice using 1% HSA in Plasmalyte. The numbers of CAR T cells were determined by multiplying the total cell numbers by the CAR⁺ percentage, which has been predetermined using flow cytometry.

Test System

25 Experiments were conducted using 6- to 12-week-old NOD/SCID/IL-2R γ null male mice injected with 0.5×10^6 FFLuc-GFP NALM6 cancer cells. Thawed cryopreserved 1×10^6 CD4/CD8 selected T cells transduced a γ -retroviral vector for either 1928z CAR or #2 CAR (represented as “19(T2)28z1XX CAR”) were infused into the mice four days after the injection of the tumor cells. Untreated mice were expected to develop hindlimb paralysis due to bone marrow-infiltrating leukemia by Day 16-18.

30 Wild type NALM6 human tumor cell line is a well-characterized model for pre-B acute lymphoblastic leukemia, which the Sadelain laboratory has used in numerous peer-reviewed publications (Zhao *et al.*, *Cancer Cell* (2015);28:415-428,

Feucht, *et al. Nature Medicine* (2019);25(1):82-88, Hamieh *et al., Nature* (2019);568(7750):112-116, Eyquem *et al., Nature* (2017);543(7643):113-117).

Study Design

- 115 Mice were divided into 1928z CAR, #2 CAR (represented as “19(T2)28z1XX CAR”), and untransduced groups. All mice received 0.5×10^6 FFLuc-GFP NALM6 tumor cells via tail vein injection 4 days prior to T cell treatment. On Study Day 0, mice were assigned into treatment groups. Each group received one single dose (200 μ l) of the test article via intravenous administration. The group assignments are outlined in Table 3.

Table 3 – Experimental design and group assignments

Group number	CAR T dose (x10 ⁶)	Number of mice/ Group	Sex	Mice ID#	BLI*	Blood/ Tissue Collection	Complete Necropsy
Blood Collection at Day 10 and Day 27 (or 28) and Final Sacrifice at Day 27 (or 28)							
1. NALM6 + 1928z CAR T cells	1.0	10	M	6 - 15	Imaged	- Whole Blood (day 10 and day 27/28) - CBC/Chem	Day 27/28
2. NALM6 + 19(T2)28z1XX CAR T cells	1.0	10	M	66-75	Imaged	- Whole Blood (day 10 and day 27/28) - CBC/Chem	Day 27/28
Interim Sacrifice at Day 17							
3. NALM6 + 1928z CAR T cells	1.0	5	M	21-25	Imaged	Bone Marrow	No
4. NALM6 + 19(T2)28z1XX CAR T cells	1.0	5	M	76 – 80	Imaged	Bone Marrow	No
Imaging every 7 days							
5. NALM6 + UNTRANSDUCED T cells	1.0*	5	M	1 -5	Imaged	None	No
6. NALM6 + 1928z CAR T cells	1.0	10	M	16 – 20 26 – 30	Imaged	None	No
7. NALM6 + 1928z CAR T cells	0.5	10	M	31-40	Imaged	None	No
8. NALM6 + 1928z CAR T cells	0.2	10	M	41-50	Imaged	None	No
9. NALM6 + 1928z CAR T cells	0.1	10	M	51-60	Imaged	None	No
10. NALM6 +19(T)28z1XX CAR T cells	1.0	10	M	61-65 81-85	Imaged	None	No
11. NALM6 + 19(T)28z1XX CAR T cells	0.5	10	M	86 -95	Imaged	None	No
12. NALM6 + 19(T)28z1XX CAR T cells	0.2	10	M	96 – 105	Imaged	None	No

13. NALM6 + 19(T)28z1XX CAR T cells	0.1	10	M	106 - 115	Imaged	None	No
---	-----	----	---	--------------	--------	------	----

*BLI – bioluminescence imaging once every week starting at Day 0 (infusion of the CAR T cells)

‡ - Untransduced CD4/CD8 T cells.

** - All mice were weighed on day 7, day 11, day 21, and day 27 until the animal's interim or final sacrifice.

Toxicology parameters

Toxicity was monitored by changes in body weights following test article administration. After 10 days and 28 days of test article administration, blood samples from the 1×10^6 dose groups of 1928z CAR T cells and #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were collected from the orbital venous plexus following anesthesia under 2% isoflurane. Blood samples were collected into tubes containing a serum separator and were used for measuring the levels of cytokines and liver enzymes. On day 27 and day 28, mice from the 1×10^6 dose groups of 1928z CAR and #2 CAR (represented as “19(T2)28z1XX CAR”) were submitted for full necropsy analyses on each indicated day.

Cytokine analysis

Sera were collected from mice on day 10 and day 28 post CAR T treatment from both 1928z CAR and #2 CAR (represented as “19(T2)28z1XX CAR”) groups that received the 1×10^6 CAR T cells dose. The samples were stored at -80°C until analysis. ProcartaPlex assay was used to quantify the levels of mouse cytokines (MCP-1, IL-6, and G-CSF) and human cytokines (IL2, IL3, IFN- γ , GM-CSF, Granzyme B, and TNF- α). All ProcartaPlex related reagents in this study were purchased from ThermoFisher Scientific. Serum collected from a mouse model with severe cytokine release syndrome were used as positive controls (Giavridis *et al.*, *Nature Medicine* (2018);24(6):731-738). 60 μL of beads from the simplex kits of individual cytokines were pooled, and 50 μL of bead mixtures were dispensed into each well of a 96-well plate according to manufacturer's protocol. Standards, quality controls, and samples diluted 1:4 using Universal Assay Buffer were added to the wells. The plates were sealed, shaken at 500rpm for 30 minutes at room temperature, and transferred to 4°C . After overnight incubation, the plates were shaken for an additional 30 minutes at room temperature at 500 rpm. Plates were washed, and the detection antibody was added to each well. The plates were sealed and shaken at 500 rpm for 30 minutes at room

temperature. After washing, a Streptavidin RPE solution was added to each well. The plates were sealed and shaken at 500 rpm for 30 minutes at room temperature. After a final wash step, the beads were resuspended in reading buffer and the assay signals acquired on a Luminex 200 instrument. Standard curves for individual cytokines were generated using a 5-parameter logistic equation, and the concentration of individual cytokines in each sample was calculated by the xPONENT software (version 4.2.1509.0).

Statistics

All statistical analyses were done using Prism 8 (GraphPad) software. Statistical comparisons between two groups were determined by two-tailed unpaired t-test. Statistical significance was defined as a p-value <0.05.

Results

Changes in mice bodyweight during 1928z CAR T cells and #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) treatments were measured. NALM6 tumor-bearing mice were treated with 1×10^6 , 5×10^5 , 2×10^5 , or 1×10^5 of 1928z CAR T cells or #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) (n=10 per group), whereas untransduced T cell treatment served as the control group (n=5). The results are shown in Figure 18. As shown in Figure 18, the change of body weight between the two CAR T cell treatments was similar.

Next, liver enzymes in serum of mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) and 1928z CAR T cells were measured. Quantification of liver enzyme levels from the two CAR T treated mice on day 10 and day 27/28 (dose level= 1×10^6 CAR T, n=10 per group) is presented in Figure 19. NALM6 tumor-bearing mice were treated with 1×10^6 of 1928z CAR T cells or #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were evaluated for ALT and AST levels on Day 10 and Day 27/28. As shown in Figure 19, ALT and AST enzyme levels were comparable between both study groups on day 10. The enzyme levels of mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were lower than mice treated with 1928z CAR T cells on day 27/28.

Human and mouse cytokine levels in mice treated with 1×10^6 #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) or 1928z CAR T cells on day 10 and day 27/29 post-infusion were measured. Figure 20 shows the quantification of human cytokine levels from sera obtained from two groups of different CAR T treated mice on day 10 and day 27/28 (dose level= 1×10^6 CAR T, n=10 per group). Sera

collected from a mouse model with severe cytokine release syndrome were used as positive controls ($n=2$) (Giavridis *et al.*, *Nature Medicine* (2018);24(6):731-738). IL2, IL3, GM-CSF, Granzyme B, and TNF alpha levels are comparable between two CAR T cell groups on day10 and day 27/28. IFN- γ level from 19(T2)28z1XX CAR T treated group was higher than the levels in mice that received 1928z CAR T cells on day 27/28.

Figure 21 shows the results of the quantification of mouse cytokine levels from mice sera treated with either 1928z CAR T cells or #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) (dose level= 1×10^6 CAR T, $n=10$ per group) on day 10 and day 27/28. Sera collected from a mouse model with severe cytokine release syndrome was used as positive controls ($n=2$) (Giavridis *et al.*, *Nature Medicine* (2018);24(6):731-738). On day10, mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) had slightly higher levels of MCP-1 and G-CSF than mice infused with 1928z CAR T cells, whereas IL6 level was comparable for both CAR T cell groups. On day27/28, mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) had similar levels of MCP-1, IL-6, and G-CSF as the levels in the 1928z CAR T group. These observations suggest that there was no evidence of cytokine release syndrome (CRS) in either group.

Blood cell counts of mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) and 1928z CAR T cells at day 27/28 post-infusion were measured. Quantification of essential blood cells from two different CAR T treatment groups on day 27/28 (dose level= 1×10^6 CAR T, $n=10$ per group) is presented in Figure 22. Comparisons of neutrophil, lymphocyte, and monocyte counts, and reticulocyte counts are shown. As shown in Figure 22, neutrophil and monocytes were comparable between the two CAR T groups (1×10^6 dose level). The mice in the 19(T2)28z1XX CAR T cell treated group had higher lymphocytes and reticulocyte counts than the 1928z CAR T cell group.

In addition, body and organ weights of mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) and 1928z CAR T cells at necropsy were measured. Comparison of body and essential organ weights from two different CAR T treatment groups on day 27/28 (dose level= 1×10^6 CAR T). The results are shown in Figure 23. As shown in Figure 23, bodyweight, spleen, and kidney weights were comparable between the two treatment Groups 1 and 2. The mice in 19(T2)28z1XX CAR T treated group (labeled “SFG-19(T2)28z-1XX”) had lighter liver weight than the

mice in the 1928z CAR T group (labeled “SFG-1928z”). The differences in the heart weight were ascribed to uneven technical handling.

CD3⁺ and CD19⁺ immunohistochemistry of liver, bone marrow, and spleen of mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) and 1928z CAR T cells was measured. Evaluations of lymphocytes and tumor infiltration levels in liver, spleen, and bone marrow from mice on 27/28 days post CAR T treatment (dose level= 1×10^6 CAR T, $n=6$) are presented in Figure 24. As shown in Figure 24, the mice in 19(T2)28z1XX CAR T treatment group (labeled “SFG-19(T2)28z-1XX”) had a higher level of CD3⁺ cells in all evaluated organs than the animals treated with 1928z CAR T cells (labeled “SFG-1928z”). Tumor infiltration was observed in all tested organs from 1928z CAR T group, but not in 19(T2)28z1XX CAR T group.

Conclusions

The toxicity profile of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) was compared to that of 1928z CAR T cells at the high dose of 1×10^6 CAR T cells per recipient. No difference in whole-body weight or survival was found. Serum cytokine levels measured on day 10 were comparable between the two groups, suggesting that #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) were not more likely than 1928z CAR T cells to induce a cytokine release syndrome (CRS). Hepatic enzymes were elevated in both groups on days 10 and 17, but less so in recipients of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) than in those given 1928z CAR T cells. Peripheral blood cell counts showed higher lymphocyte counts in recipients of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”), consistent with the latter’s greater persistence documented by FACs analysis (Day 17) and tissue IHC (Days 27/28). An isolated reticulocytosis was observed, without anemia, polycythemia or erythroid hyperplasia, which is therefore not regarded as an adverse event.

Organ weights were similar between the two groups, except for liver, which was heavier in mice treated with 1928z CAR T cells, owing to substantial tumor progression (BLI and IHC). In contrast, mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) showed the absence of tumors by IHC, corroborating the BLI findings in these same mice. Multi-organ lymphocytic infiltrates were observed in the #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) recipient mice, consistent with xenogeneic GVHD. The latter was corroborated by the finding of

epithelial necrosis in those tissues. Mild to moderate hepatocellular necrosis was also observed in #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) infused mice. This microscopic finding is not typically associated with GVHD and thus represents a possible adverse event. Its impact on liver function is limited given the modest elevation of liver enzymes (AST only, not ALT) and the normal bilirubin values. Other pathology findings were minor, equally represented between the two treatment groups, and thus were not deemed to represent significant findings. T cell flow cytometry profiles were similar between the two treatment groups, consistent with the expected finding that #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) show increased therapeutic potency owing to the greater persistence of CAR T cells.

The toxicity studies, assessing #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) in comparison to the clinically tested 1928z CAR T cells (Park *et al.*, *N. Engl. J. Med.* (2018);378(5):449-459), uncovered mild to moderate GVHD as well as mild to moderate hepatocellular necrosis in mice treated with #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”).

It is concluded from the data shown in this Example and Example 6 that #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) are more potent, owing to their greater persistence relative to 1928z CAR T cells, but without posing greater risks. The relatively increased potency of #2 CAR T cells (represented as “19(T2)28z1XX CAR T cells”) supports a human clinical trial design starting with the dose of 25×10^6 CAR T cells, a relatively low dose in comparison to most CAR T cell trials targeting CD19.

Example 8 – Pharmacology Studies

On-cell binding of anti-CD19 antibodies

The on-cell binding for the anti-CD19 antibodies comprising the V_H and V_L of the presently disclosed scFvs were assessed by flow cytometry on the endogenous CD19 expressing Raji and NALM-6 cell lines. Each antibody was tested on both the CD19 positive parental lines and corresponding CD19 knock-out Raji and NALM-6 lines to confirm on-cell target specific binding. The flow cytometry chromatograms shown in Figure 25 demonstrate 1-2 log shifts on the CD19 positive lines over the background binding on the corresponding knockout cell lines.

The serial dilution flow cytometry results shown in Figure 26A-26C demonstrate saturation binding on NALM-6 cells. The EC₅₀ values calculated from the curves, are provided in Table 4. Exemplary anti-CD19 antibodies bind CD19 positive

NALM-6 cells at high affinities from of at least 0.2 nM. The higher affinities observed on CD19 positive cells versus the soluble protein suggests that these antibodies bind an epitope that may be more natively presented on cells than in the CD19-HSA fusion protein. All the anti-CD19 antibodies shown in Table 4 comprise a V_H comprising the amino acid sequence set forth in SEQ ID NO: 10.

Table 4. EC50 of anti-CD19 antibodies on NALM6 cells

CD19 Antibody	SEQ ID NO. of the V _L amino acid sequence	NALM-6 on-cell binding affinity EC50 (nM)
Antibody 2	14	0.3136
Antibody 4	58	0.5195
Antibody 5	60	0.2192
Antibody 8	18	0.2097
Antibody 6	62	0.1798
Antibody 7	64	0.2104
Antibody 1	67	0.5879
Antibody 15	1	0.2698

Example 9 – Phase I Study of CD19-Targeted 19(T2)28z1XX CAR T Cells in Adult Patients with Relapsed or Refractory B-Cell Malignancies

This Example describes a Phase I Study of CD19-Targeted 19(T2)28z1XX CAR T Cells in Adult Patients with Relapsed or Refractory B-Cell Malignancies. “19(T2)28z1XX” and “19(T2)28z1XX” are used Interchangeably herein. Autologous CAR T cell therapy targeting the B-cell specific surface antigen CD19 has demonstrated favorable clinical responses in relapsed or refractory (R/R) B-cell lymphomas (BCL). However, despite 40-60% initial complete response (CR) rates, only a subset of patients experiences durable remissions, and there is a need to further improve the efficacy of CAR therapies by preventing relapse and attaining a deeper clinical response (CR).

Study Design and Methods: This study is a single center Phase I clinical trial of 19(T2)28z1XX in patients with R/R B-cell malignancies. Key disease eligibility criteria include R/R diffuse large B cell lymphoma (DLBCL), high grade BCL, primary mediastinal BCL, indolent BCL and chronic lymphocytic leukemia (CLL). Patients with prior CD19 CAR therapies are eligible as long as expression of CD19 is confirmed. Key exclusion criteria include ongoing immunosuppression such as systemic GvHD therapy and active CNS disease.

The study uses a 3+3 dose-escalation design to identify the maximum tolerated dose for BCL. There are 5 planned flat-dose levels: 25×10^6 , 50×10^6 , 100×10^6 , 150×10^6 , and 200×10^6 CAR T cells. Patients receive conditioning chemotherapy consisting of 3 days of fludarabine and cyclophosphamide followed by a single infusion of 19(T2)28z1XX CAR T cells. In the dose-escalation phase, patients with DLBCL, high grade BCL, and primary mediastinal BCL are eligible to participate. Once the recommended phase 2 dose (RP2D) is determined, the study is open to dose expansion phase with two cohorts. Cohort 1 includes DLBCL, high grade BCL and primary mediastinal BCL (*i.e.* same eligibility criteria as the dose-escalation phase). Cohort 2 includes patients with indolent BCL, CLL, and Richter's transformation. The dose-expansion part of the trial is designed to further characterize the safety, efficacy, and pharmacokinetics of 19(T2)28z1XX CAR in multiple indications. Up to a maximum of 60 patients are enrolled.

The primary objective of the trial is to evaluate safety and tolerability and determine the recommended Phase 2 dose of 19(T2)28z1XX. Key secondary objectives include evaluation of the efficacy and pharmacokinetics of 19(T2)28z1XX. Exploratory objectives include assessment of B cell aplasia and measurable residual disease (MRD), characterization of 19(T2)28z1XX CAR T cell phenotypes pre- and post-infusion, and analysis of serum cytokines.

20

Although the presently disclosed subject matter and certain of its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the disclosure. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, and composition of matter, and methods described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the presently disclosed subject matter, processes, machines, manufacture, compositions of matter, or methods, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the presently disclosed subject matter. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, or methods.

30

Various patents, patent applications, publications, product descriptions, protocols, and sequence accession numbers are cited throughout this application, the disclosure of which are incorporated herein by reference in their entireties for all purposes.

5

WHAT IS CLAIMED IS:

1. A chimeric antigen receptor (CAR), comprising an extracellular antigen-binding domain that specifically binds to CD19, a transmembrane domain, and an intracellular signaling domain, wherein the extracellular antigen-binding domain comprises:
 - 5 a) a heavy chain variable region comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof; and/or
 - 10 b) a light chain variable region comprising:
 - i) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof;
 - 15 ii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17 or a conservative modification thereof;
 - 20 iii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof;
 - 25 iv) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57 or a conservative modification thereof;
 - v) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59 or a conservative modification thereof;
 - 30 vi) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the

amino acid sequence set forth in SEQ ID NO: 61 or a conservative modification thereof;

vii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63 or a conservative modification thereof;

or

viii) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65 or a conservative modification thereof, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66 or a conservative modification thereof.

2. The CAR of claim 1, wherein the extracellular antigen-binding domain comprises a single-chain variable fragment (scFv), a Fab, or a F(ab)₂.

3. The CAR of claim 2, wherein the extracellular antigen-binding domain comprises a scFv.

4. The CAR of claim 3, wherein the scFv is a human scFv.

5. The CAR of any one of claims 1-4, wherein the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9.

6. The CAR of any one of claims 1-5, wherein the light chain variable region comprises:

(a) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13;

(b) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17;

(c) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21

(d) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57;

5 (e) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59;

(f) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61;

10 (g) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63; or

(h) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3
15 comprising the amino acid sequence set forth in SEQ ID NO: 66.

7. The CAR of any one of claims 1-6, wherein the light chain variable region comprises:

(a) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a
20 CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13;

(b) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17; or

25 (c) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21.

8. The CAR of any one of claims 1-7, wherein:

30 (a) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the

amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13;

(b) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17;

(c) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21;

(d) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 57;

(e) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 59;

(f) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in

SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 61;

- 5 (g) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid
10 sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 63; or

- (h) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in
15 SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 65, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 66.

- 20 9. The CAR of any one of claims 1-8, wherein:

- (a) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the
25 amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13;

- (b) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set
30 forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 12, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 17; or

(c) the heavy chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 7, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 9; and the light chain variable region comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 11, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 20, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 21

10. The CAR of any one of claims 1-9, wherein the heavy chain variable region comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 10.

11. The CAR of any one of claims 1-10, wherein the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10.

12. The CAR of any one of claims 1-11, wherein the light chain variable region comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67.

13. The CAR of any one of claims 1-12, wherein the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67.

14. The CAR of any one of claims 1-13, wherein the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, or SEQ ID NO: 22.

15. The CAR of any one of claims 1-14, wherein the heavy chain variable region comprising an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 10; and (b) the light chain variable region comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67.

16. The CAR of any one of claims 1-15, wherein the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10; and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, or SEQ ID NO: 67.

17. The CAR of any one of claims 1-16, wherein the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10; and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14, SEQ ID NO: 18, or SEQ ID NO: 22.

18. The CAR of any one of claims 1-17, wherein:

(a) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14;

(b) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 18;

(c) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 22;

(d) the heavy chain variable region comprises the amino acid sequence set forth

in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 58;

- (e) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 60;

(f) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 62;

- (g) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 64; or

(h) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 67.

19. The CAR of any one of claims 1-18, wherein:

(a) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 14;

- (b) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 18; or

(c) the heavy chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 10, and the light chain variable region comprises the amino acid sequence set forth in SEQ ID NO: 22.

21. The CAR of any one of claims 1-20, wherein the extracellular antigen-binding domain comprises a linker between the heavy chain variable region and the light chain variable region.

22. The CAR of claim 21, wherein the linker consists of the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

23. The CAR of any one of claims 1-22, wherein the heavy chain variable region and the light chain variable region are positioned from the N- to the C-terminus: V_L-V_H.

24. The CAR of any one of claims 1-23, wherein the extracellular antigen-binding domain comprises or is a scFv, which comprises or consists of the amino acid sequence set forth in SEQ ID NO: 15, SEQ ID NO: 19, or SEQ ID NO: 23.
25. The CAR of any one of claims 1-24, wherein the transmembrane domain
5 comprises a CD8 polypeptide, a CD28 polypeptide, a CD3 ζ polypeptide, a CD4 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a CTLA-4 polypeptide, a PD-1 polypeptide, a LAG-3 polypeptide, a 2B4 polypeptide, or a BTLA polypeptide.
26. The CAR of claim 25, wherein the transmembrane domain comprises a CD28
10 polypeptide.
27. The CAR of any one of claims 1-26, wherein the intracellular signaling domain comprises a CD3 ζ polypeptide.
28. The CAR of claim 27, wherein the CD3 ζ polypeptide is a modified CD3 ζ polypeptide.
- 15 29. The CAR of claim 28, wherein the modified CD3 ζ polypeptide comprises a native ITAM1, an ITAM2 variant consisting of two loss-of-function mutations, and an ITAM3 consisting of two loss-of-function mutations.
30. The CAR of claim 29, wherein the native ITAM1 consists of the amino acid sequence set forth in SEQ ID NO: 31.
- 20 31. The CAR of claim 29, wherein the ITAM2 variant consists of the amino acid sequence set forth in SEQ ID NO: 37.
32. The CAR of claim 29, wherein the ITAM3 variant consists of the amino acid sequence set forth in SEQ ID NO: 41.
33. The CAR of any one of claims 28-32, wherein the modified CD3 ζ polypeptide
25 comprising or consists of the amino acid sequence set forth in SEQ ID NO: 43.
34. The CAR of any one of claims 1-33, wherein the intracellular signaling domain further comprises at least one co-stimulatory signaling region.

35. The CAR of claim 34, wherein the at least one co-stimulatory signaling region comprises a CD28 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide, or a combination thereof.
36. The CAR of claim 35, wherein the at least one co-stimulatory signaling region
5 comprises a CD28 polypeptide.
37. The CAR of any one of claims 1-36, wherein the CAR is expressed from a vector.
38. The CAR of claim 37, wherein the vector is a retroviral vector.
39. A cell comprising the CAR of any one of claims 1-38.
- 10 40. The cell of claim 39, wherein the cell is transduced with the CAR.
41. The cell of claim 39 or claim 40, wherein the CAR is constitutively expressed on the surface of the cell.
42. The cell of any one of claims 39-41, wherein the cell is an immunoresponsive cell.
- 15 43. The cell of any one of claims 39-42, wherein the cell is a cell of the lymphoid lineage or a cell of the myeloid lineage.
44. The cell of any one of claims 39-43, wherein the cell is selected from the group consisting of a T cell, a Natural Killer (NK) cell, a stem cell from which lymphoid cells may be differentiated, and a stem cell from which myeloid cells may be differentiated.
- 20 45. The cell of any one of claims 39-44, wherein the cell is a T cell.
46. The cell of claim 38 or claim 45, wherein the T cell is selected from the group consisting of helper T cells, cytotoxic T cells, memory T cells, regulatory T cells, tumor-infiltrating lymphocyte (TIL), Natural Killer T cells, mucosal associated invariant T cells, and $\gamma\delta$ T cells.
- 25 47. The cell of any one of claims 39-44, wherein the cell is a NK cell.
48. The cell of claim 47, wherein the NK cell is derived from a stem cell.

49. The cell of claim 44 or 48, wherein the stem cell is a pluripotent stem cell.
50. The cell of claim 49, wherein the pluripotent stem cell is an embryoid stem cell or an induced pluripotent stem cell.
51. A nucleic acid molecule encoding the CAR of any one of claims 1-38.
- 5 52. The nucleic acid molecule of claim 51, further comprising a promoter that is operably linked to the CAR.
53. The nucleic acid molecule of claim 52, wherein the promoter is endogenous or exogenous.
54. The nucleic acid molecule of claim 53, wherein the exogenous promoter is
10 selected from the group consisting of an elongation factor (EF)-1 promoter, a cytomegalovirus immediate-early promoter (CMV) promoter, a simian virus 40 early promoter (SV40) promoter, a phosphoglycerate kinase (PGK) promoter, a metallothionein promoter, and Ubiquitin C promoter.
55. The nucleic acid molecule of claim 51, wherein the promoter is an inducible
15 promoter.
56. The nucleic acid molecule of claim 55, wherein the inducible promoter is selected from the group consisting of a NFAT transcriptional response element (TRE) promoter, a CD69 promoter, a CD25 promoter, an IL-2 promoter, a 4-1BB promoter, a PD1 promoter, and a LAG3 promoter.
- 20 57. The nucleic acid molecule of claim 53, wherein the promoter is an endogenous promoter.
58. The nucleic acid molecule of claim 57, wherein the endogenous promoter is selected from a TCR alpha promoter, a TCR beta promoter, and a beta 2-microglobulin promoter.
- 25 59. A vector comprising the nucleic acid molecule of any one of claims 51-58.
60. The vector of claim 59, wherein the vector is a retroviral vector.
61. A cell expressing the nucleic acid molecule of any one of claims 51-58.

62. The cell of claim 61, wherein the cell is a T cell or a Natural Killer (NK) cell.
63. A composition comprising the cell of any one of claims 39-50, 61, and 62.
64. The composition of claim 63, which is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.
- 5 65. The composition of claim 63 or claim 64, comprising between about 1×10^6 and about 5×10^8 cells.
66. The composition of claim 63 or claim 64, comprising between about 1×10^6 and about 1×10^8 cells.
67. The composition of claim 63 or claim 64, comprising between about 1×10^6 and
10 about 5×10^7 cells.
68. The composition of any one of claims 63-67, comprising about 2.5×10^7 cells.
69. A method of reducing tumor burden in a subject, comprising administering to the subject the cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68.
- 15 70. The method of claim 69, wherein the method reduces the number of tumor cells, reduces tumor size, and/or eradicates the tumor in the subject.
71. A method of increasing or lengthening survival of a subject having a neoplasm, comprising administering to the subject the cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68.
- 20 72. A method of treating and/or preventing a neoplasm in a subject, comprising administering to the subject the cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68.
73. The method of any one of claims 69-72, wherein the tumor and/or neoplasm is associated with CD19.
- 25 74. The method of any one of claims 69-73, wherein the tumor and/or neoplasm is a blood cancer.

75. The method of claim 74, wherein the blood cancer is selected from the group consisting of multiple myeloma, leukemia, and lymphomas.
76. The method of claim 75, wherein the leukemia is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute promyelocytic leukemia (APL), mixed-phenotype acute leukemia (MLL), hairy cell leukemia, and B cell prolymphocytic leukemia.
77. The method of claim 75, wherein the lymphoma is Hodgkin's lymphoma or non-Hodgkin's lymphoma.
78. The method of any one of claims 69-73, wherein the tumor and/or neoplasm is a B cell malignancy.
79. The method of claim 78, wherein the B cell malignancy is selected from the group consisting of B cell non-Hodgkin lymphomas (NHL), B cell Hodgkin's lymphomas, B cell acute lymphocytic leukemia (ALL), B cell chronic lymphocytic leukemia (CLL), multiple myeloma (MM), CLL with Richter's transformation, and CNS lymphoma.
80. The method of any one of claims 69-79, wherein the tumor and/or neoplasm is B cell lymphoma.
81. The method of claim 80, wherein the B cell lymphoma is relapsed or refractory (R/R) B cell lymphoma.
82. The method of any one of claims 69-81, wherein the subject is a human subject.
83. The cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68 for use in a therapy.
84. The cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68 for use in treating and/or preventing a neoplasm in a subject.
85. The cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68 for use in increasing or lengthening survival of a subject having a neoplasm.

86. The cell of any one of claims 39-50, 61, and 62 or the composition of any one of claims 63-68 for use in treating and/or preventing a neoplasm in a subject.
87. The cell or composition for use of any one of claims 84-86, wherein the tumor and/or neoplasm is associated with CD19.
- 5 88. The cell or composition for use of any one of claims 84-87, wherein the tumor and/or neoplasm is a blood cancer.
89. The cell or composition for use of claim 88, wherein the blood cancer is selected from the group consisting of multiple myeloma, leukemia, and lymphomas.
90. The cell or composition for use of claim 89, wherein the leukemia is selected
10 from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute promyelocytic leukemia (APL), mixed-phenotype acute leukemia (MLL), hairy cell leukemia, and B cell prolymphocytic leukemia.
91. The cell or composition for use of claim 89, wherein the lymphoma is Hodgkin's
15 lymphoma or non-Hodgkin's lymphoma.
92. The cell or composition for use of any one of claims 84-87, wherein the tumor and/or neoplasm is a B cell malignancy.
93. The cell or composition for use of claim 92, wherein the B cell malignancy is selected from the group consisting of B cell non-Hodgkin lymphomas (NHL), B cell
20 Hodgkin's lymphomas, B cell acute lymphocytic leukemia (ALL), B cell chronic lymphocytic leukemia (CLL), multiple myeloma (MM), CLL with Richter's transformation, and CNS lymphoma.
94. The cell or composition for use of any one of claims 84-93, wherein the tumor and/or neoplasm is B cell lymphoma.
- 25 95. The cell or composition for use of claim 94, wherein the B cell lymphoma is relapsed or refractory (R/R) B cell lymphoma.
96. The cell or composition for use of any one of claims 84-95, wherein the subject is a human subject.

97. A method for producing a cell comprising the CAR of any one of claims 1-38, comprising introducing into the cell a nucleic acid molecule that encodes the CAR of any one of claims 1-38.

5 98. A kit for reducing tumor burden in a subject, treating and/or preventing a neoplasm in a subject, and/or increasing or lengthening survival of a subject having a neoplasm, comprising the cell of any one of claims 39-50, 61, and 62.

99. The kit of claim 98, wherein the kit further comprises written instructions for using the cell for reducing tumor burden in a subject, treating and/or preventing a neoplasm in a subject, and/or increasing or lengthening survival of a subject having a
10 neoplasm.