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(54) Title: CHIMERIC ANTIGEN RECEPTORS BASED ON SINGLE-DOMAIN ANTIBODIES AND METHODS OF USE THEREOF

(57) Abrégé/Abstract:
The present application provides single-domain antibodies, and chimeric antigen receptors comprising one or more antigen binding domains each comprising a single-domain antibody. Further provided are engineered immune effector cells (such as T cells) comprising the chimeric antigen receptors. Pharmaceutical compositions, kits and methods of treating cancer are also provided.

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(54) Title: CHIMERIC ANTIGEN RECEPTORS BASED ON SINGLE-DOMAIN ANTIBODIES AND METHODS OF USE THEREOF

(57) Abstract: The present application provides single-domain antibodies, and chimeric antigen receptors comprising one or more antigen binding domains each comprising a single-domain antibody. Further provided are engineered immune effector cells (such as T cells) comprising the chimeric antigen receptors. Pharmaceutical compositions, kits and methods of treating cancer are also provided.

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JUMBO APPLICATIONS/PATENTS

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CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefits of Chinese Patent Application No. CN201510490002.8 filed August 11, 2015, and Chinese Patent Application No. CN201510733585.2 filed November 2, 2015.

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

[0002] The contents of the following submission on ASCII text file: a computer readable form (CRF) of the Sequence Listing (file name: 761422000340SEQLISTING.txt, date recorded: August 9, 2016, size: 355 KB).

FIELD OF THE PRESENT APPLICATION

[0003] The present invention relates to single-domain antibodies, chimeric antigen receptors, engineered immune effector cells, and methods of use thereof. The present invention further relates to activation and expansion of cells for therapeutic uses, especially for chimeric antigen receptor-based T cell immunotherapy.

BACKGROUND OF THE PRESENT APPLICATION

[0004] With the development of tumor immunotherapy and clinical technology, chimeric antigen receptor T cell (CAR-T) immunotherapy is now one of the most promising tumor immunotherapy approaches. Generally, a chimeric antigen receptor (CAR) comprises an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain. The extracellular antigen binding domain may comprise a single chain variable fragment (scFv) targeting an identified tumor antigen. CARs can be expressed on the surface of T cells using gene transfection techniques. Upon binding to the target tumor antigen, the CARs can activate the T cells to launch specific anti-tumor response in an antigen-dependent manner without being limited by the availability of major histocompatibility complexes (MHC) specific to the target tumor antigen.

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[0005] Single-domain antibodies (sdAbs) are different from conventional 4-chain antibodies by having a single monomeric antibody variable domain. For example, camelids and sharks produce single-domain antibodies named heavy chain-only antibodies (HcAbs), which naturally lack light chains. The antigen-binding fragment in each arm of the camelid heavy-chain only antibodies has a single heavy chain variable domain (V_HH), which can have high affinity to an antigen without the aid of a light chain. Camelid V_HH is known as the smallest functional antigen-binding fragment with a molecular weight of approximately 15 kD.

[0006] (Blank).

BRIEF SUMMARY OF THE PRESENT APPLICATION

[0007] The present application provides single-domain antibodies, chimeric antigen receptors (CARs) based on single-domain antibodies (such as V_HH fragments), engineered immune effector cells, and methods of use thereof in cancer immunotherapy.

[0008] One aspect of the present application provides an anti-CD19 sdAb comprising the CDR regions of SEQ ID NO: 76. In some embodiments, the anti-CD19 sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the anti-CD19 sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 76.

[0009] In some embodiments, there is provided an anti-CD19 heavy-chain only antibody (HcAb) or an antigen binding protein comprising any one of the anti-CD19 sdAbs described above.

[0010] One aspect of the present application provides a CD19 chimeric antigen receptor comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 sdAb (such as any one of the anti-CD19 sdAbs described above); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent (such as bivalent or trivalent). In some embodiments, the CAR is multispecific (such as bispecific).

[0011] One aspect of the present application provides an anti-CD20 sdAb comprising the CDR regions of SEQ ID NO: 77. In some embodiments, the anti-CD20 sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:4, a CDR2 comprising the amino acid

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sequence of SEQ ID NO:5, and a CDR3 comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, the anti-CD20 sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 77.

[0012] In some embodiments, there is provided an anti-CD20 heavy-chain only antibody (HCAB) or an antigen binding protein comprising any one of the anti-CD20 sdAbs described above.

[0013] One aspect of the present application provides a CD20 chimeric antigen receptor comprising: (a) an extracellular antigen binding domain comprising an anti-CD20 sdAb (such as any one of the anti-CD20 sdAbs described above); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent (such as bivalent or trivalent). In some embodiments, the CAR is multispecific (such as bispecific).

[0014] One aspect of the present application provides an anti-BCMA sdAb comprising the CDR regions of any one of SEQ ID NOs: 78-88. In some embodiments, the anti-BCMA sdAb comprises any one of the following:

(1) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:18; and a CDR3 comprising the amino acid sequence of SEQ ID NO:29;

(2) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:19; and a CDR3 comprising the amino acid sequence of SEQ ID NO:30;

(3) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:20; and a CDR3 comprising the amino acid sequence of SEQ ID NO:31;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:21; and a CDR3 comprising the amino acid sequence of SEQ ID NO:32;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:22; and a CDR3 comprising the amino acid sequence of SEQ ID NO:33;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:23; and a CDR3 comprising the amino acid sequence of SEQ ID NO:34;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:24; and a CDR3 comprising the amino acid sequence of SEQ ID NO:35;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:25; and a CDR3 comprising the amino acid sequence of SEQ ID NO:36;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:26; and a CDR3 comprising the amino acid sequence of SEQ ID NO:37;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:27; and a CDR3 comprising the amino acid sequence of SEQ ID NO:38; or

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:28; and a CDR3 comprising the amino acid sequence of SEQ ID NO:39.

In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO:78-88.

[0015] In some embodiments, there is provided an anti-BCMA heavy-chain only antibody (HCAB) or an antigen binding protein comprising any one of the anti-BCMA sdAbs described above.

[0016] One aspect of the present application provides a BCMA chimeric antigen receptor comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb (such as any one of the anti-BCMA sdAbs described above); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent (such as bivalent or trivalent). In some embodiments, the CAR is multispecific (such as bispecific).

[0017] One aspect of the present application provides an anti-CD38 sdAb comprising the CDR regions of any one of SEQ ID NOs: 89-100. In some embodiments, the anti-CD38 sdAb comprises any one of the following:

(1) a CDR1 comprising the amino acid sequence of SEQ ID NO:40; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:64;

(2) a CDR1 comprising the amino acid sequence of SEQ ID NO:41; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:65;

(3) a CDR1 comprising the amino acid sequence of SEQ ID NO:42; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:66;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO:43; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:67;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO:44; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:68;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO:45; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:69;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO:46; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:70;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO:47; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:71;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO:48; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:72;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO:49; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:73;

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO:50; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:74; or

(12) a CDR1 comprising the amino acid sequence of SEQ ID NO:51; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:75.

In some embodiments, the anti-CD38 sdAb comprises a V_HH domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO:89-100.

[0018] In some embodiments, there is provided an anti-BCMA heavy-chain only antibody (HCAB) or an antigen binding protein comprising any one of the anti-BCMA sdAbs described above.

[0019] One aspect of the present application provides a CD38 chimeric antigen receptor comprising: (a) an extracellular antigen binding domain comprising an anti-CD38 sdAb (such as any one of the anti-CD38 sdAbs described above); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent (such as bivalent or trivalent). In some embodiments, the CAR is multispecific (such as bispecific).

[0020] One aspect of the present application provides a CD22 chimeric antigen receptor comprising: (a) an extracellular antigen binding domain comprising an anti-CD22 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent (such as bivalent or trivalent). In some embodiments, the CAR is multispecific (such as bispecific).

[0021] One aspect of the present application provides a chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody (sdAb) specifically binding to a first antigen and a second single-domain antibody (sdAb) specifically binding to a second antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the first sdAb is located at the

N-terminus of the second sdAb. In some embodiments, the first sdAb is located at the C-terminus of the second sdAb.

[0022] In some embodiments according to any one of the CARs provided above, the first antigen and the second antigen are selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the first sdAb is an anti-BCMA sdAb, such as any one of the BCMA sdAbs described above. In some embodiments, the CAR comprises an extracellular antigen binding domain comprising at least two copies (such as 2, 3, or more copies) of an anti-BCMA sdAb. In some embodiments, the first sdAb is an anti-CD19 sdAb, such as any one of the anti-CD19 sdAbs described above. In some embodiments, the first sdAb is an anti-CD20 sdAb, such as any one of the anti-CD20 sdAbs described above. In some embodiments, the first sdAb is an anti-CD38 sdAb, such as any one of the anti-CD38 sdAbs described above. In some embodiments, the CAR comprises an extracellular antigen binding domain comprising at least two copies (such as 2, 3, or more copies) of an anti-CD38 sdAb. In some embodiments, the first sdAb is an anti-CD22 sdAb.

[0023] In some embodiments according to any one of the CARs provided above, the first antigen is different from the second antigen. In some embodiments, the CAR is multispecific, such as bispecific. In some embodiments, the first sdAb is an anti-BCMA sdAb, and the second sdAb is an anti-CD38 sdAb. In some embodiments, the first sdAb is an anti-BCMA sdAb, and the second sdAb is an anti-CD19 sdAb. In some embodiments, the first sdAb is an anti-CD19 sdAb, and the second sdAb is an anti-CD20 sdAb. In some embodiments, the first sdAb is an anti-CD19 sdAb, and the second sdAb is an anti-CD22 sdAb.

[0024] In some embodiments according to any one of the monospecific CARs provided above, the first antigen is the same as the second antigen. In some embodiments, the CAR is bivalent or trivalent. In some embodiments, the first sdAb and the second sdAb specifically bind to the same epitope. In some embodiments, the first sdAb is the same as the second sdAb. In some embodiments, the first sdAb and the second sdAb specifically bind to different epitopes.

[0025] In some embodiments according to any one of the CARs provided above, the first sdAb and/or the second sdAb are camelid, chimeric, human, or humanized.

[0026] In some embodiments according to any one of the CARs provided above, the first sdAb and the second sdAb are directly fused to each other via a peptide bond. In some embodiments,

the first sdAb and the second sdAb are fused to each other via a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from SEQ ID NOs: 144-151.

[0027] In some embodiments according to any one of the CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) provided above, the transmembrane domain is derived from a molecule selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the transmembrane domain is derived from CD8 or CD28. In some embodiments, the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 132 or SEQ ID NO: 133.

[0028] In some embodiments according to any one of the CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) provided above, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as a T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the primary intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 140 or SEQ ID NO: 141.

[0029] In some embodiments according to any one of the CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) provided above, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the co-stimulatory signaling domain comprises a cytoplasmic domain of CD28 and/or a cytoplasmic domain of CD137. In some embodiments, the co-stimulatory signaling domain comprises the amino acid sequence of SEQ ID NO: 136 and/or SEQ ID NO: 137.

[0030] In some embodiments according to any one of the CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) provided above, the CAR further comprises a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the hinge domain is derived from CD8α. In some embodiments, the hinge domain comprises the amino acid sequence of SEQ ID NO: 130.

[0031] In some embodiments, the CAR further comprises a signal peptide located at the N-terminus of the polypeptide. In some embodiments, the signal peptide is derived from a molecule selected from the group consisting of CD8α, GM-CSF receptor α, and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8α. In some embodiments, the signal peptide comprises the amino acid sequence of SEQ ID NO: 127.

[0032] One aspect of the present application provides a chimeric antigen receptor of any one listed in Tables 4, 5, and 6. In some embodiments, the CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 152-174, 198-201, 206-216, 248-249, 257-260, and 265-270.

[0033] One aspect of the present application provides a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 76-100, 152-174, 198-201, 206-216, 248-249, 257-260, and 265-270.

[0034] One aspect of the present application provides an isolated nucleic acid comprising a nucleic acid sequence encoding any one of the CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) provided above. In some embodiments, the nucleic acid sequence is selected from the group consisting of SEQ ID NOs: 175-197, 202-205, 217-227, 250-251, 261-264, and 271-276. In some embodiments, the isolated nucleic acid further comprises a second nucleic acid sequence encoding a second CAR, wherein the nucleic acid sequence encoding the CAR is operably linked to the second nucleic acid sequence via a third nucleic acid sequence encoding a self-cleaving peptide, such as a T2A, P2A, or F2A peptide. In some embodiments, the third nucleic acid sequence is SEQ ID NO: 256. In some embodiments, the isolated nucleic acid is a DNA molecule. In some embodiments, the isolated nucleic acid is an RNA molecule.

[0035] One aspect of the present application provides a vector comprising any one of the isolated nucleic acids described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector. In some embodiments, the vector is a lentiviral vector.

[0036] One aspect of the present application provides an engineered immune effector cell, comprising any one of the CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) provided above, or any one of the isolated nucleic acids described above, or any one of the vectors described above. In some embodiments, the engineered immune

effector cell comprises or expresses two or more CARs (including CD19 CARs, CD20 CARs, BCMA CARs, CD38 CARs, and CD22 CARs) described above, wherein the two or more CARs specifically bind to different antigens. In some embodiments, the immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the immune effector cell is a T cell.

[0037] One aspect of the present application provides a pharmaceutical composition comprising any one of the engineered immune effector cells described above and a pharmaceutically acceptable carrier. Further provided is a method of treating cancer in an individual, comprising administering to the individual an effective amount of any one of the pharmaceutical compositions described above. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic. In some embodiments, the cancer is a liquid cancer. In some embodiments, the cancer is multiple myeloma, acute lymphoblastic leukemia, or chronic lymphocytic leukemia. In some embodiments, the cancer is a solid cancer, such as glioblastoma.

[0038] One aspect of the present application provides a pharmaceutical composition comprising any one of the anti-CD19 sdAbs, anti-CD20 sdAbs, anti-CD38 sdAbs or anti-BCMA sdAbs described above and a pharmaceutically acceptable carrier. In some embodiments, there is provided a method of treating a disease (such as cancer) in an individual, comprising administering to the individual an effective amount of the pharmaceutical composition.

[0039] Also provided are methods of use, kits, and articles of manufacture comprising any one of the single-domain antibodies, CARs, engineered immune effector cells, isolated nucleic acids, or vectors described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] FIG. 1A compares the structures of a V_HH-based CAR and a conventional scFv-based CAR. The schematic structure on the left shows an exemplary monospecific monovalent CAR having an extracellular antigen binding domain comprising a V_HH domain. The schematic structure on the right shows an exemplary monospecific monovalent CAR having an extracellular antigen binding domain comprising a scFv domain.

[0041] FIG. 1B compares the structures of a V_HH-based CAR having two antigen binding sites and a conventional scFv-based CAR having two antigen binding sites. The schematic structure on the left is an exemplary CAR having an extracellular antigen binding domain comprising two V_HH domains. The two V_HH domains may be the same or different. The schematic structure on the right shows an exemplary CAR having an extracellular antigen binding domain comprising two scFv domains. The two scFv domains may be the same or different.

[0042] FIG. 1C shows schematic structures of exemplary bivalent and bispecific V_HH-based CARs. The schematic structure in the top left panel shows an exemplary monospecific, bivalent CAR having an extracellular antigen binding domain comprising two identical V_HH domains, each of which specifically binds to epitope 1 of antigen A. The schematic structure in the top right panel shows an exemplary monospecific, bivalent CAR having an extracellular antigen binding domain comprising a first V_HH domain specifically binding to epitope 1 of antigen A, and a second V_HH domain specifically binding to epitope 2 of antigen A. Epitope 1 and epitope 2 of antigen A may be different in their structures and/or sequences. The schematic structure in the bottom left panel shows an exemplary bispecific CAR having an extracellular antigen binding domain comprising a first V_HH domain specifically binding to antigen A, and a second V_HH domain specifically binding to antigen B. Antigen A and antigen B are different antigens.

[0043] FIG. 1D shows schematic structures of exemplary V_HH-based CARs having three or more V_HH domains. The CARs may have a plurality of V_HH domains fused to each other directly or via peptide linkers. The V_HH domains may be the same or different. Different V_HH domains may specifically bind to different epitopes on the same antigen or different antigens.

[0044] FIG. 1E shows exemplary engineered immune effector cells co-expressing two different V_HH-based CARs. The exemplary engineered immune effector cell in the left panel co-expresses two different monospecific, monovalent CARs. The exemplary engineered immune effector cell in the middle panel co-expresses a monospecific, monovalent CAR and a bispecific or bivalent CAR. The exemplary engineered immune effector cell in the right panel co-expresses two different bispecific or bivalent CARs. The CARs may recognize different antigens.

[0045] FIG. 2A shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary monospecific CARs comprising various anti-BCMA (*i.e.*, anti-CD269) or anti-CD38 single-domain antibodies against multiple myeloma cell line RPMI8226.Luc.

[0046] FIG. 2B shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary monospecific CARs comprising various anti-BCMA (*i.e.*, anti-CD269) or anti-CD38 single-domain antibodies against glioblastoma cell line U87MG.Luc.

[0047] FIG. 3A shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary bispecific CARs against multiple myeloma cell line RPMI8226.Luc.

[0048] FIG. 3B shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary bispecific CARs against multiple myeloma cell line RPMI8226.Luc.

[0049] FIG. 4 shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary bispecific CARs against multiple myeloma cell line RPMI8226.Luc.

[0050] FIG. 5 shows constructs of an exemplary bispecific CAR targeting CD19 and CD20, an exemplary monospecific CAR targeting CD19, and an exemplary monospecific CAR targeting CD20.

[0051] FIG. 6 shows results of an *in vitro* cytotoxicity assay for various T cells. Top left panel shows results of untransduced control T cells. Top right panel shows results of T cells expressing an exemplary CD19 CAR. Bottom left panel shows results of T cells expressing an exemplary CD20 CAR. Bottom right panel shows results of T cells expressing an exemplary bispecific CD19 × CD20 CAR.

[0052] FIG. 7 shows results of an *in vivo* antitumor assay of T cells expressing an exemplary bispecific CAR targeting CD19 and CD20.

[0053] FIG. 8A shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary monospecific, bivalent CARs against multiple myeloma cell line RPMI8226.Luc. The CARs each comprise an extracellular antigen binding domain comprising two different anti-BCMA (*i.e.*, anti-CD269) sdAbs.

[0054] FIG. 8B shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary monospecific, bivalent CARs against glioblastoma cell line U87MG.Luc. The CARs each comprise an extracellular antigen binding domain comprising two different anti-BCMA (*i.e.*, anti-CD269) sdAbs.

DETAILED DESCRIPTION OF THE PRESENT APPLICATION

[0055] The present application provides monospecific, multispecific (such as bispecific), and multivalent (such as bivalent or trivalent) chimeric antigen receptors comprising single-domain antibody (sdAb) based extracellular antigen binding domains. Unlike antigen binding fragments

derived from conventional four-chain antibodies, sdAbs only contain a single variable domain, such as V_HH. Thus, sdAbs are much smaller in size than antigen binding fragments such as scFvs that are currently used as extracellular antigen binding domains in CARs. Also, as there is no need for pairing of the heavy chain and light chain during folding of the sdAbs, misfolding of the extracellular antigen binding domain can be reduced in engineered immune cells expressing CARs based on sdAbs. CARs having extracellular antigen binding domains comprising multiple copies of an sdAb or multiple sdAbs targeting different epitopes or antigens can be conveniently constructed and produced recombinantly, thereby providing an efficient platform for preparation and screening of multivalent and multispecific CARs. Additionally, the small footprint of sdAbs may allow access of the CARs to hidden antigen targets and epitopes in tumor tissues.

[0056] Multispecific and multivalent CARs may have improved efficacy over monospecific monovalent CARs for cancer immunotherapy. Cancer cells are unstable genetically, which allows them to escape from targeted therapies by mutating or losing genes encoding the target antigens. By targeting two or more different epitopes or antigens on cancer cells, multivalent or multispecific CARs can make it more difficult for cancer cells to completely escape from targeting by engineered immune effector cells (such as T cells) expressing the CARs. Owing to their small size, tandemly fused single-domain antibodies, which are used as extracellular antigen binding domains in the multivalent or multispecific CARs of the present application, can preserve their individual structural integrity and binding affinity to target antigens, thereby allowing effective targeting of each epitope or antigen by the CARs. Engineered immune effector cells expressing the multivalent or multispecific CARs or co-expressing two or more chimeric antigen receptors that target different tumor antigens may overcome tumor immune escape mechanisms that are due to abnormalities in protein-antigen processing and presentation.

[0057] Accordingly, one aspect of the present application provides a multispecific (such as bispecific) chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody (sdAb) specifically binding to a first antigen and a second single-domain antibody (sdAb) specifically binding to a second antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first antigen is different from the second antigen. In some embodiments, the first antigen is BCMA, and the second antigen is CD38. In some embodiments, the first antigen is CD19, and the second antigen is BCMA. In some embodiments, the first antigen is CD19, and

the second antigen is CD20. In some embodiments, the first antigen is CD19, and the second antigen is CD22.

[0058] In another aspect, there is provided a multivalent chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a plurality of a single-domain antibody (sdAb) specifically binding to an antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain.

[0059] In another aspect, there is provided a multivalent chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody specifically binding to a first epitope of an antigen, and a second single-domain antibody specifically binds a second epitope of the antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope is different from the second epitope.

[0060] Further provided are novel anti-CD19, anti-CD20, anti-BCMA, and anti-CD38 single-domain antibodies and chimeric antigen receptors comprising any one of the sdAbs.

[0061] Engineered immune effector cells (such as T cells) comprising the CARs, pharmaceutical compositions, kits, articles of manufacture and methods of treating cancer using the engineered immune effectors cells or the single-domain antibodies are also described herein.

I. Definitions

[0062] The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Current Protocols in Molecular Biology or Current Protocols in Immunology, John Wiley & Sons, New York, N.Y.(2009); Ausubel *et al*, Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995; Sambrook and Russell, Molecular Cloning: A Laboratory Manual (3rd Edition, 2001); Maniatis *et al.* Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., 1984); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1985); Transcription and Translation (B. Hames & S. Higgins, eds., 1984); Animal Cell Culture (R. Freshney, ed., 1986); Perbal, A Practical Guide to Molecular Cloning (1984) and other like references.

[0063] The term "antibody" includes monoclonal antibodies (including full length 4-chain antibodies or full length heavy-chain only antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, multispecific antibodies (*e.g.*, bispecific antibodies, diabodies, and single-chain molecules), as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv). The term "immunoglobulin" (Ig) is used interchangeably with "antibody" herein. Antibodies contemplated herein include single-domain antibodies, such as heavy chain only antibodies.

[0064] The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains. An IgM antibody consists of 5 of the basic heterotetramer units along with an additional polypeptide called a J chain, and contains 10 antigen binding sites, while IgA antibodies comprise from 2-5 of the basic 4-chain units which can polymerize to form polyvalent assemblages in combination with the J chain. In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to an H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (V_H) followed by three constant domains (C_H) for each of the α and γ chains and four C_H domains for μ and ε isotypes. Each L chain has at the N-terminus, a variable domain (V_L) followed by a constant domain at its other end. The V_L is aligned with the V_H and the C_L is aligned with the first constant domain of the heavy chain (C_H1). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a V_H and V_L together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see *e.g.*, *Basic and Clinical Immunology*, 8th Edition, Daniel P. Sties, Abba I. Terr and Tristram G. Parsolw (eds), Appleton & Lange, Norwalk, Conn., 1994, page 71 and Chapter 6. The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains (C_H), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, having heavy chains designated α, δ, ε, γ and μ, respectively. The γ and α classes are further divided into subclasses on the basis of relatively

minor differences in the C_H sequence and function, *e.g.*, humans express the following subclasses: IgG1, IgG2A, IgG2B, IgG3, IgG4, IgA1 and IgA2.

[0065] The term "heavy chain-only antibody" or "HCAb" refers to a functional antibody, which comprises heavy chains, but lacks the light chains usually found in 4-chain antibodies. Camelid animals (such as camels, llamas, or alpacas) are known to produce HCAbs.

[0066] The term "single-domain antibody" or "sdAb" refers to a single antigen-binding polypeptide having three complementary determining regions (CDRs). The sdAb alone is capable of binding to the antigen without pairing with a corresponding CDR-containing polypeptide. In some cases, single-domain antibodies are engineered from camelid HCAbs, and their heavy chain variable domains are referred herein as "V_HHs". Some V_HHs may also be known as Nanobodies. Camelid sdAb is one of the smallest known antigen-binding antibody fragments (see, *e.g.*, Hamers-Casterman *et al.*, Nature 363:446-8 (1993); Greenberg *et al.*, Nature 374:168-73 (1995); Hassanzadeh-Ghassabeh *et al.*, Nanomedicine (Lond), 8:1013-26 (2013)). A basic V_HH has the following structure from the N-terminus to the C-terminus: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3.

[0067] An "isolated" antibody is one that has been identified, separated and/or recovered from a component of its production environment (*e.g.*, natural or recombinant). Preferably, the isolated polypeptide is free of association with all other components from its production environment. Contaminant components of its production environment, such as that resulting from recombinant transfected cells, are materials that would typically interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified: (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, an isolated polypeptide or antibody will be prepared by at least one purification step.

[0068] The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domains of the heavy chain and light chain may be referred to as "V_H" and "V_L", respectively. These domains are generally the most variable parts of the antibody (relative to other antibodies of the same class) and contain the antigen binding sites. Heavy-chain only antibodies from the Camelid species have a single heavy chain variable region, which is referred to as "V_HH". V_HH is thus a special type of V_H.

[0069] The term "variable" refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and defines the specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the entire span of the variable domains. Instead, it is concentrated in three segments called hypervariable regions (HVRs) both in the light-chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs in each chain are held together in close proximity by the FR regions and, with the HVRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat *et al.*, *Sequences of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in the binding of antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

[0070] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (*e.g.*, isomerizations, amidations) that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture or recombinantly, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character

of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present application may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler and Milstein., *Nature,* 256:495-97 (1975); Hongo *et al.*, *Hybridoma,* 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual,* (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567), phage-display technologies (see, *e.g.*, Clackson *et al.*, *Nature,* 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222: 581-597 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.*, WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits *et al.*, *Nature* 362: 255-258 (1993); Bruggemann *et al.*, *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al.*, *Bio/Technology* 10: 779-783 (1992); Lonberg *et al.*, *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

[0071] The term "naked antibody" refers to an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

[0072] The terms "full-length antibody," "intact antibody" or "whole antibody" are used interchangeably to refer to an antibody in its substantially intact form, as opposed to an antibody fragment. Specifically, full-length 4-chain antibodies include those with heavy and light chains including an Fc region. Full-length heavy-chain only antibodies include the heavy chain (such as V_HH) and an Fc region. The constant domains may be native sequence constant domains (*e.g.*, human native sequence constant domains) or amino acid sequence variants thereof. In some cases, the intact antibody may have one or more effector functions.

[0073] An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding and/or the variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments; diabodies; linear antibodies (see U.S. Pat. No. 5,641,870, Example 2; Zapata *et al.*, *Protein Eng.* 8(10): 1057-1062 [1995]); single-chain antibody molecules; single-domain antibodies (such as V_HH), and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produced two identical antigen-binding fragments, called "Fab" fragments, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (V_H), and the first constant domain of one heavy chain (C_H1). Each Fab fragment is monovalent with respect to antigen binding, *i.e.*, it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large $F(ab')_2$ fragment which roughly corresponds to two disulfide linked Fab fragments having different antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having a few additional residues at the carboxy terminus of the C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0074] The Fc fragment comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, the region which is also recognized by Fc receptors (FcR) found on certain types of cells.

[0075] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0076] "Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the V_H and V_L antibody domains connected into a single polypeptide chain. Preferably, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of the sFv, see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0077] "Functional fragments" of the antibodies described herein comprise a portion of an intact antibody, generally including the antigen binding or variable region of the intact antibody or the Fc region of an antibody which retains or has modified FcR binding capability. Examples of antibody fragments include linear antibody, single-chain antibody molecules and multispecific antibodies formed from antibody fragments.

[0078] The term "diabodies" refers to small antibody fragments prepared by constructing sFv fragments (see preceding paragraph) with short linkers (about 5-10) residues) between the V_H and V_L domains such that inter-chain but not intra-chain pairing of the V domains is achieved, thereby resulting in a bivalent fragment, *i.e.*, a fragment having two antigen-binding sites. Bispecific diabodies are heterodimers of two "crossover" sFv fragments in which the V_H and V_L domains of the two antibodies are present on different polypeptide chains. Diabodies are described in greater detail in, for example, EP 404,097; WO 93/11161; Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993).

[0079] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is(are) identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include PRIMATTZFD® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, *e.g.*, immunizing macaque monkeys with an antigen of interest. As used herein, "humanized antibody" is used a subset of "chimeric antibodies."

[0080] "Humanized" forms of non-human (*e.g.*, camelid) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In some embodiments, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from an HVR (hereinafter defined) of the recipient are replaced by residues from an HVR of a non-human species (donor antibody) such as mouse, rat, rabbit or non-human primate having the desired specificity, affinity, and/or capacity. In some instances, framework ("FR") residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance, such as binding affinity. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin sequence, and all or substantially all of the FR regions are those of a human immunoglobulin sequence, although the FR regions may include one or more individual FR residue substitutions that improve antibody performance, such as binding affinity, isomerization, immunogenicity, etc. The number of these amino acid substitutions in the FR is typically no more than 6 in the H chain, and in the L chain, no more than 3. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, *e.g.*, Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, for example, Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

[0081] A "human antibody" is an antibody that possesses an amino-acid sequence corresponding to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, *Monoclonal Antibodies and Cancer*

Therapy, Alan R. Liss, p. 77 (1985); Boerner *et al.*, *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.*, 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, immunized xenomice (see, *e.g.*, U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li *et al.*, *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

[0082] The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, single-domain antibodies comprise three HVRs (or CDRs): HVR1 (or CDR1), HVR2 (or CDR2), and HVR3 (or CDR3). HVR3 displays the most diversity of the three HVRs, and is believed to play a unique role in conferring fine specificity to antibodies. See, *e.g.*, Hamers-Casterman *et al.*, *Nature* 363:446-448 (1993); Sheriff *et al.*, *Nature Struct. Biol.* 3:733-736 (1996).

[0083] The term "Complementarity Determining Region" or "CDR" are used to refer to hypervariable regions as defined by the Kabat system. See Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)

[0084] A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987)). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below in Table 1.

Table 1. HVR delineations.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36

Loop	Kabat	AbM	Chothia	Contact
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B
		(Kabat Numbering)		
H1	H31-H35	H26-H35	H26-H32	H30-H35
		(Chothia Numbering)		
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

[0085] HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the V_L and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the V_H. The variable domain residues are numbered according to Kabat *et al.*, supra, for each of these definitions.

[0086] The amino acid residues of a single-domain antibody (such as V_HH) are numbered according to the general numbering for V_H domains given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, Md., Publication No. 91), as applied to V_HH domains from Camelids in the article of Riechmann and Muyldermans, J. Immunol. Methods 2000 Jun. 23; 240 (1-2): 185-195. According to this numbering, FR1 of a V_HH comprises the amino acid residues at positions 1-30, CDR1 of a V_HH comprises the amino acid residues at positions 31-35, FR2 of a V_HH comprises the amino acids at positions 36-49, CDR2 of a V_HH comprises the amino acid residues at positions 50-65, FR3 of a V_HH comprises the amino acid residues at positions 66-94, CDR3 of a V_HH comprises the amino acid residues at positions 95-102, and FR4 of a V_HH comprises the amino acid residues at positions 103-113. In this respect, it should be noted that—as is well known in the art for V_H domains and for V_HH domains—the total number of amino acid residues in each of the CDR's may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering).

[0087] The expression "variable-domain residue-numbering as in Kabat" or "amino-acid-position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy-chain variable domains or light-chain variable domains of the compilation of antibodies in

Kabat *et al.*, supra. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy-chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (*e.g.* residues 82a, 82b, and 82c, etc. according to Kabat) after heavy-chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

[0088] Unless indicated otherwise herein, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat *et al.*, supra. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

[0089] "Framework" or "FR" residues are those variable-domain residues other than the HVR residues as herein defined.

[0090] A "human consensus framework" or "acceptor human framework" is a framework that represents the most commonly occurring amino acid residues in a selection of human immunoglobulin V_L or V_H framework sequences. Generally, the selection of human immunoglobulin V_L or V_H sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Examples include for the V_L, the subgroup may be subgroup kappa I, kappa II, kappa III or kappa IV as in Kabat *et al.*, supra. Additionally, for the VH, the subgroup may be subgroup I, subgroup II, or subgroup III as in Kabat *et al.* Alternatively, a human consensus framework can be derived from the above in which particular residues, such as when a human framework residue is selected based on its homology to the donor framework by aligning the donor framework sequence with a collection of various human framework sequences. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain pre-existing amino acid sequence changes. In some embodiments, the number of pre-existing amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less.

[0091] An "amino-acid modification" at a specified position, *e.g.* of the Fc region, refers to the substitution or deletion of the specified residue, or the insertion of at least one amino acid residue adjacent the specified residue. Insertion "adjacent" to a specified residue means insertion within one to two residues thereof. The insertion may be N-terminal or C-terminal to the specified residue. The preferred amino acid modification herein is a substitution.

[0092] An "affinity-matured" antibody is one with one or more alterations in one or more HVRs thereof that result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody that does not possess those alteration(s). In some embodiments, an affinity-matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity-matured antibodies are produced by procedures known in the art. For example, Marks *et al.*, *Bio/Technology* 10:779-783 (1992) describes affinity maturation by V_H- and V_L -domain shuffling. Random mutagenesis of HVR and/or framework residues is described by, for example: Barbas *et al. Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier *et al. Gene* 169:147-155 (1995); Yelton *et al. J. Immunol.* 155:1994-2004 (1995); Jackson *et al., J. Immunol.* 154(7):3310-9 (1995); and Hawkins *et al, J. Mol. Biol.* 226:889-896 (1992).

[0093] As use herein, the term "specifically binds," "specifically recognizes," or is "specific for" refers to measurable and reproducible interactions such as binding between a target and an antigen binding protein (such as a CAR or an sdAb), which is determinative of the presence of the target in the presence of a heterogeneous population of molecules including biological molecules. For example, an antigen binding protein (such as a CAR or an sdAb) that specifically binds a target (which can be an epitope) is an antigen binding protein (such as a CAR or an sdAb) that binds this target with greater affinity, avidity, more readily, and/or with greater duration than it binds other targets. In some embodiments, the extent of binding of an antigen binding protein (such as a CAR or an sdAb) to an unrelated target is less than about 10% of the binding of the antigen binding protein (such as a CAR or an sdAb) to the target as measured, *e.g.*, by a radioimmunoassay (RIA). In some embodiments, an antigen binding protein (such as a CAR or an sdAb) that specifically binds a target has a dissociation constant (Kd) of $\leqq$1 μM, $\leqq$100 nM, $\leqq$10 nM, $\leqq$1 nM, or $\leqq$0.1 nM. In some embodiments, an antigen binding protein (such as a CAR or an sdAb) specifically binds an epitope on a protein that is conserved among the protein from different species. In some embodiments, specific binding can include, but does not require exclusive binding.

[0094] The term "specificity" refers to selective recognition of an antigen binding protein (such as a CAR or an sdAb) for a particular epitope of an antigen. Natural antibodies, for example, are monospecific. The term "multispecific" as used herein denotes that an antigen binding protein (such as a CAR or an sdAb) has two or more antigen-binding sites of which at least two bind a different antigen or a different epitope of the same antigen. "Bispecific" as used herein denotes that an antigen binding protein (such as a CAR or an sdAb) has two different antigen-binding specificities. The term "monospecific" CAR as used herein denotes an antigen binding protein (such as a CAR or an sdAb) that has one or more binding sites each of which bind the same epitope of the same antigen.

[0095] The term "valent" as used herein denotes the presence of a specified number of binding sites in an antigen binding protein (such as a CAR or an sdAb). A natural antibody for example or a full length antibody has two binding sites and is bivalent. As such, the terms "trivalent", "tetravalent", "pentavalent" and "hexavalent" denote the presence of two binding site, three binding sites, four binding sites, five binding sites, and six binding sites, respectively, in an antigen binding protein (such as a CAR or an sdAb).

[0096] "Antibody effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody—dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.*, B cell receptors); and B cell activation. "Reduced or minimized" antibody effector function means that which is reduced by at least 50% (alternatively 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) from the wild type or unmodified antibody. The determination of antibody effector function is readily determinable and measurable by one of ordinary skill in the art. In a preferred embodiment, the antibody effector functions of complement binding, complement dependent cytotoxicity and antibody dependent cytotoxicity are affected. In some embodiments, effector function is eliminated through a mutation in the constant region that eliminated glycosylation, *e.g.*, "effector-less mutation." In one aspect, the effector-less mutation is an N297A or DANA mutation (D265A+N297A) in the C_H2 region. Shields *et al.*, *J. Biol. Chem.* 276 (9): 6591-6604 (2001). Alternatively, additional mutations resulting in reduced or eliminated effector function include: K322A and L234A/L235A (LALA). Alternatively, effector

function can be reduced or eliminated through production techniques, such as expression in host cells that do not glycosylate (*e.g.*, *E. coli.*) or in which result in an altered glycosylation pattern that is ineffective or less effective at promoting effector function (*e.g.*, Shinkawa *et al.*, *J. Biol. Chem.* 278(5): 3466-3473 (2003).

[0097] "Antibody-dependent cell-mediated cytotoxicity" or ADCC refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (*e.g.*, natural killer (NK) cells, neutrophils and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are required for killing of the target cell by this mechanism. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. Fc expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9: 457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes *et al.*, *PNAS USA* 95:652-656 (1998).

[0098] The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native-sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy-chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. Suitable native-sequence Fc regions for use in the antibodies described herein include human IgG1, IgG2 (IgG2A, IgG2B), IgG3 and IgG4.

[0099] "Binding affinity" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an antibody or a CAR) and its

binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity that reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen, or CAR and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present application. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

[0100] A "blocking" antibody or an "antagonist" antibody is one that inhibits or reduces a biological activity of the antigen it binds. In some embodiments, blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

[0101] An "agonist" or activating antibody is one that enhances or initiates signaling by the antigen to which it binds. In some embodiments, agonist antibodies cause or activate signaling without the presence of the natural ligand.

[0102] "Percent (%) amino acid sequence identity" and "homology" with respect to a peptide, polypeptide or antibody sequence are defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific peptide or polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or MEGALIGN™ (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0103] "Chimeric antigen receptor" or "CAR" as used herein refers to genetically engineered receptors, which can be used to graft one or more antigen specificity onto immune effector cells, such as T cells. Some CARs are also known as "artificial T-cell receptors," "chimeric T cell receptors," or "chimeric immune receptors." In some embodiments, the CAR comprises an

extracellular antigen binding domain specific for one or more antigens (such as tumor antigens), a transmembrane domain, and an intracellular signaling domain of a T cell and/or other receptors. "CAR-T" refers to a T cell that expresses a CAR.

[0104] An "isolated" nucleic acid molecule encoding a CAR or an sdAb described herein is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the environment in which it was produced. Preferably, the isolated nucleic acid is free of association with all components associated with the production environment. The isolated nucleic acid molecules encoding the polypeptides and antibodies herein is in a form other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from nucleic acid encoding the polypeptides and antibodies herein existing naturally in cells.

[0105] The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

[0106] Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[0107] The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of

nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors."

[0108] As used herein, the term "autologous" is meant to refer to any material derived from the same individual to whom it is later to be re-introduced into the individual.

[0109] "Allogeneic" refers to a graft derived from a different individual of the same species.

[0110] The term "transfected" or "transformed" or "transduced" as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

[0111] As used herein, the expressions "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transfectants" and "transfected cells" include the primary subject cell and cultures derived there from without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

[0112] The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

[0113] As used herein, "treatment" or "treating" is an approach for obtaining beneficial or desired results including clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, one or more of the following: alleviating one or more symptoms resulting from the disease, diminishing the extent of the disease, stabilizing the disease (*e.g.*, preventing or delaying the worsening of the disease), preventing or delaying the spread (*e.g.*, metastasis) of the disease, preventing or delaying the recurrence of the disease, delay or slowing the progression of the disease, ameliorating the disease state, providing a

remission (partial or total) of the disease, decreasing the dose of one or more other medications required to treat the disease, delaying the progression of the disease, increasing the quality of life, and/or prolonging survival. Also encompassed by "treatment" is a reduction of pathological consequence of cancer. The methods of the present application contemplate any one or more of these aspects of treatment.

[0114] As used herein, an "individual" or a "subject" refers to a mammal, including, but not limited to, human, bovine, horse, feline, canine, rodent, or primate. In some embodiments, the individual is a human.

[0115] The term "effective amount" used herein refers to an amount of an agent, such as a single-domain antibody, an engineered immune effector cell, or a pharmaceutical composition thereof, sufficient to treat a specified disorder, condition or disease such as ameliorate, palliate, lessen, and/or delay one or more of its symptoms. In reference to cancer, an effective amount comprises an amount sufficient to cause a tumor to shrink and/or to decrease the growth rate of the tumor (such as to suppress tumor growth) or to prevent or delay other unwanted cell proliferation. In some embodiments, an effective amount is an amount sufficient to delay development. In some embodiments, an effective amount is an amount sufficient to prevent or delay recurrence. An effective amount can be administered in one or more administrations. The effective amount of the drug or composition may: (i) reduce the number of cancer cells; (ii) reduce tumor size; (iii) inhibit, retard, slow to some extent and preferably stop cancer cell infiltration into peripheral organs; (iv) inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; (v) inhibit tumor growth; (vi) prevent or delay occurrence and/or recurrence of tumor; and/or (vii) relieve to some extent one or more of the symptoms associated with the cancer.

[0116] "Adjuvant setting" refers to a clinical setting in which an individual has had a history of cancer, and generally (but not necessarily) been responsive to therapy, which includes, but is not limited to, surgery (*e.g.*, surgery resection), radiotherapy, and chemotherapy. However, because of their history of cancer, these individuals are considered at risk of development of the disease. Treatment or administration in the "adjuvant setting" refers to a subsequent mode of treatment. The degree of risk (*e.g.*, when an individual in the adjuvant setting is considered as "high risk" or "low risk") depends upon several factors, most usually the extent of disease when first treated.

[0117] "Neoadjuvant setting" refers to a clinical setting in which the method is carried out before the primary/definitive therapy.

[0118] As used herein, "delaying" the development of cancer means to defer, hinder, slow, retard, stabilize, and/or postpone development of the disease. This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease. A method that "delays" development of cancer is a method that reduces probability of disease development in a given time frame and/or reduces the extent of the disease in a given time frame, when compared to not using the method. Such comparisons are typically based on clinical studies, using a statistically significant number of individuals. Cancer development can be detectable using standard methods, including, but not limited to, computerized axial tomography (CAT Scan), Magnetic Resonance Imaging (MRI), abdominal ultrasound, clotting tests, arteriography, or biopsy. Development may also refer to cancer progression that may be initially undetectable and includes occurrence, recurrence, and onset.

[0119] The term "pharmaceutical formulation" refers to a preparation that is in such form as to permit the biological activity of the active ingredient to be effective, and that contains no additional components that are unacceptably toxic to a subject to which the formulation would be administered. Such formulations are sterile. A "sterile" formulation is aseptic or free from all living microorganisms and their spores.

[0120] "Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and

other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counterions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™ or polyethylene glycol (PEG).

[0121] The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, such as a formulation reconstituted after lyophilization. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (*e.g.* phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. In an alternative embodiment, diluents can include aqueous solutions of salts and/or buffers.

[0122] A "preservative" is a compound which can be added to the formulations herein to reduce bacterial activity. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-dose) formulation. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

[0123] A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40° C. for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2-8° C., generally the formulation should be stable at 30° C. or 40° C. for at least 1 month and/or stable at 2-8° C. for at least 2 years. Where the formulation is to be stored at 30° C., generally the formulation should be stable for at least 2 years at 30° C. and/or stable at 40° C. for at least 6 months. For example, the extent of aggregation during storage can be used as an indicator of protein stability. Thus, a "stable" formulation may be one wherein less than about 10% and

preferably less than about 5% of the protein are present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation during storage of the formulation can be determined.

[0124] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein or antibody formulation in a diluent such that the protein is dispersed throughout. The reconstituted formulation is suitable for administration (*e.g.* subcutaneous administration) to a patient to be treated with the protein of interest and, In some embodiments of the present application, may be one which is suitable for parenteral or intravenous administration.

[0125] An "isotonic" formulation is one which has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. The term "hypotonic" describes a formulation with an osmotic pressure below that of human blood. Correspondingly, the term "hypertonic" is used to describe a formulation with an osmotic pressure above that of human blood. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example. The formulations of the present invention are hypertonic as a result of the addition of salt and/or buffer.

[0126] It is understood that embodiments of the present application described herein include "consisting" and/or "consisting essentially of" embodiments.

[0127] Reference to "about" a value or parameter herein includes (and describes) variations that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X".

[0128] As used herein, reference to "not" a value or parameter generally means and describes "other than" a value or parameter. For example, the method is not used to treat cancer of type X means the method is used to treat cancer of types other than X.

[0129] The term "about X-Y" used herein has the same meaning as "about X to about Y."

[0130] As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise.

II. Single-Domain Antibodies

[0131] The present application in one aspect provides single-domain antibodies, antigen-binding fragments thereof, and antigen binding proteins comprising any one of the single-domain antibodies. Exemplary single-domain antibodies are listed in Table 2 below.

Table 2. Exemplary single-domain antibodies.

Ab	Ex. AA SEQ ID	Ex. NA SEQ ID	CDR1	CDR2	CDR3
Exemplary Anti-CD19 single-domain antibodies					
CD19 V_HH	76	101	INRMG (SEQ ID NO: 1)	SITVRGITNYADSVKG (SEQ ID NO: 2)	VSSNRDPDY (SEQ ID NO: 3)
Exemplary Anti-CD20 single-domain antibodies					
CD20 V_HH	77	102	IGTMG (SEQ ID NO: 4)	AIRWSTGGTRYADSVKG (SEQ ID NO: 5)	DRLSLDLSGRYHYNPAVYDY (SEQ ID NO: 6)
Exemplary Anti-BCMA single-domain antibodies					
269A37346	78	103	SGFTLDYYAIG (SEQ ID NO: 7)	CISRSDGSTYYADSVKG (SEQ ID NO: 18)	AGADCSGYLRDYEF (SEQ ID NO: 29)
269A37348	79	104	SGRTFSTYGMA (SEQ ID NO: 8)	SKASMNYSGRTYYADSVKG (SEQ ID NO: 19)	AGTGCSTYGCFDAQIIDY (SEQ ID NO: 30)
269A37917	80	105	SGRTFTMG (SEQ ID NO: 9)	AISLSPTLAYYAESVKG (SEQ ID NO: 20)	ADRKSVMSIRPDY (SEQ ID NO: 31)
269A37355	81	106	SGGIFVINAMG (SEQ ID NO: 10)	SIRGLGRTNYDDSVKG (SEQ ID NO: 21)	VYVTLLGGVNRDY (SEQ ID NO: 32)
269A37915	82	107	SGRTFSSIVMG (SEQ ID NO: 11)	AIMWNDGITYLQDSVKG (SEQ ID NO: 22)	ASKGRYSEYEY (SEQ ID NO: 33)
269A37936	83	108	SGFTFDRAVIV (SEQ ID NO: 12)	FIKPSDGTIYYIDSLKG (SEQ ID NO: 23)	ASPEDWYTDWIDWSIYR (SEQ ID NO: 34)
269A37953	84	109	STYTVNSDVMG (SEQ ID NO: 13)	AIMWNDGITYLQDSVKG (SEQ ID NO: 24)	ASKGRYSEYEY (SEQ ID NO: 35)
269A37965	85	110	SGATLTNDHMA (SEQ ID NO: 14)	AIDWSGRTTNYADPVEG (SEQ ID NO: 25)	VLRAWISYDNDY (SEQ ID NO: 36)
269A37972	86	111	SGGTLSKNTVA (SEQ ID NO: 15)	SITWDGRTTYYADSVKG (SEQ ID NO: 26)	DLGKWPAGPADY (SEQ ID NO: 37)
269A37353	87	112	SEHTFSSHVMG (SEQ ID NO: 16)	VIGWRDISTSYADSVKG (SEQ ID NO: 27)	ARRIDAADFDS (SEQ ID NO: 38)
269A3	88	113	SGRAFSTYFMA	GIAWSGGSTAYADS	SRGIEVEEFGA

7948			(SEQ ID NO: 17)	VKG (SEQ ID NO: 28)	(SEQ ID NO: 39)
Exemplary Anti-CD38 single-domain antibodies					
38A37333	89	114	SGLTFSSYPMM (SEQ ID NO: 40)	RISDSGGYTNYDDSVKG (SEQ ID NO: 52)	ILGLPT (SEQ ID NO: 64)
38A37336	90	115	SGFTFSSNWMY (SEQ ID NO: 41)	TISTDGRGTYYKDSVKG (SEQ ID NO: 53)	KEPRVLMAYLRNLGDFGS (SEQ ID NO: 65)
38A37699	91	116	SGRIFSINAMG (SEQ ID NO: 42)	AISTAGSTNYGDSVKG (SEQ ID NO: 54)	LNFPPYVY (SEQ ID NO: 66)
38A37331	92	117	SGSIFKVFRVFAMS (SEQ ID NO: 43)	SISSGETTTYADSVKG (SEQ ID NO: 55)	ADHTFTGDF (SEQ ID NO: 67)
38A37717	93	118	TGKVFSIYDMG (SEQ ID NO: 44)	EITSSGTTHYDDFVSG (SEQ ID NO: 56)	NHVFGGSY (SEQ ID NO: 68)
38A37719	94	119	SASIFTRLPMG (SEQ ID NO: 45)	GIVPSGRINYADSVKG (SEQ ID NO: 57)	ADTFPLPT (SEQ ID NO: 69)
38A37330	95	120	SGRAYATMA (SEQ ID NO: 46)	HLRVSGDTTYYTDSVKG (SEQ ID NO: 58)	GPYGILAAARVSNPGNYDY (SEQ ID NO: 70)
38A37334	96	121	SGLTFSSYIMG (SEQ ID NO: 47)	EISSGGMTSYADSVKG (SEQ ID NO: 59)	APERGSIWYSRYEYKY (SEQ ID NO: 71)
38A37730	97	122	SQGIFTINAMG (SEQ ID NO: 48)	EVSSGGRTDYADSVKG (SEQ ID NO: 60)	VSGWHVFVGDRIV (SEQ ID NO: 72)
38A37340	98	123	SGRTFSSYAMA (SEQ ID NO: 49)	SISTSGGITDYADSVKG (SEQ ID NO: 61)	ARTWYLRTSLQYDY (SEQ ID NO: 73)
38A37731	99	124	SGTIVSISTMG (SEQ ID NO: 50)	TITRRGRTNYTDSVKG (SEQ ID NO: 62)	AEVQLDIWASAYDY (SEQ ID NO: 74)
38A37326	100	125	SGRTYAMG (SEQ ID NO: 51)	TISGAGNTKYADSVKG (SEQ ID NO: 63)	AGKWFPAANEY (SEQ ID NO: 75)

Anti-CD19 single-domain antibodies

[0132] In one aspect, the present application provides isolated single-domain antibodies that specifically bind to CD19, such as human CD19. In some embodiments, the anti-CD19 single-

domain antibody modulates CD19 activity. In some embodiments, the anti-CD19 single-domain antibody is an antagonist antibody.

[0133] In some embodiments, there is provided an anti-CD19 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 76. In some embodiments, the anti-CD19 single-domain antibody is camelid. In some embodiments, the anti-CD19 single-domain antibody is humanized. In some embodiments, the anti-CD19 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0134] In some embodiments, there is provided an anti-CD19 single-domain antibody comprising at least one, at least two, or all three CDRs selected from (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 1; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 2; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 3. In some embodiments, the anti-CD19 single-domain antibody is camelid. In some embodiments, the anti-CD19 single-domain antibody is humanized. In some embodiments, the anti-CD19 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0135] In some embodiments, there is provided an anti-CD19 single-domain antibody comprising three CDRs comprising: (a) a CDR1 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:1; (b) a CDR2 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:2; and (c) a CDR3 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:3. In some embodiments, the anti-CD19 single-domain antibody is camelid. In some embodiments, the anti-CD19 antibody is humanized. In some embodiments, a CDR having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-CD19 single-domain antibody comprising that sequence retains the ability to bind to CD19. In some embodiments, the anti-CD19 antibody is affinity matured. In some embodiments, the anti-CD19 single-domain antibody is camelid. In some

embodiments, the anti-CD19 single-domain antibody is humanized. In some embodiments, the anti-CD19 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0136] In some embodiments, there is provided an anti-CD19 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 1; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 2; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 3. In some embodiments, the anti-CD19 single-domain antibody is camelid. In some embodiments, the anti-CD19 antibody is humanized. In some embodiments, the anti-CD19 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0137] In some embodiments, there is provided an anti-CD19 single-domain antibody comprising a V_HH domain having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 76. In some embodiments, a V_HH sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-CD19 single-domain antibody comprising that sequence retains the ability to bind to CD19. In some embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in the amino acid sequence of SEQ ID NO: 76. In some embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (i.e., in the FRs). Optionally, the anti-CD19 single-domain antibody comprises the amino acid sequence of SEQ ID NO: 76, including post-translational modifications of that sequence. In some embodiments, the anti-CD19 single-domain antibody is camelid. In some embodiments, the anti-CD19 antibody is humanized. In some embodiments, the anti-CD19 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0138] In some embodiments, there is provided an anti-CD19 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 76. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 76.

[0139] In some embodiments, functional epitopes can be mapped by combinatorial alanine scanning. In this process, a combinatorial alanine-scanning strategy can be used to identify amino acids in the CD19 protein that are necessary for interaction with the anti-CD19 single-domain antibodies. In some embodiments, the epitope is conformational and crystal structure of the anti-CD19 single-domain antibody bound to CD19 may be employed to identify the epitopes. In some embodiments, the present application provides an antibody that specifically binds to the same epitope as any one of the anti-CD19 single-domain antibodies provided herein. For example, in some embodiments, an antibody is provided that binds to the same epitope as an anti-CD19 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 76.

[0140] In some embodiments, the present application provides an anti-CD19 antibody, or antigen binding fragment thereof, that specifically binds to CD19 competitively with any one of the anti-CD19 single-domain antibodies described herein. In some embodiments, competitive binding may be determined using an ELISA assay. For example, in some embodiments, an antibody is provided that specifically binds to CD19 competitively with an anti-CD19 single-domain antibody comprising the amino acid sequence of SEQ ID NO:76.

[0141] In some embodiments, there is provided an anti-CD19 antibody or antigen binding protein comprising any one of the anti-CD19 single-domain antibodies described above. In some embodiments, the anti-CD19 antibody is a monoclonal antibody, including a camelid, chimeric, humanized or human antibody. In some embodiments, the anti-CD19 antibody is an antibody fragment, *e.g.*, a V_HH fragment. In some embodiments, the anti-CD19 antibody is a full-length heavy-chain only antibody comprising an Fc region of any antibody class or isotype, such as IgG1 or IgG4. In some embodiments, the Fc region has reduced or minimized effector function.

[0142] In some embodiments, the anti-CD19 antibody (such as anti-CD19 single-domain antibody) or antigen binding protein according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 of "Features of antibodies" below.

[0143] In some embodiments, there is provided an isolated nucleic acid encoding any one of the anti-CD19 antibodies (such as anti-CD19 single-domain antibodies) described above. In some embodiments, an isolated nucleic acid encoding an anti-CD19 single-domain antibody is provided wherein the nucleic acid comprises a sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%

sequence identity to the nucleic acid sequence of SEQ ID NO:101. In some embodiments, there is provided an isolated nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 101. In some embodiments, a vector (*e.g.*, expression vector) comprising such nucleic acid are provided. In some embodiments, a host cell comprising such nucleic acid is provided. In some embodiments, a method of making an anti-CD19 antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the anti-CD19 antibody, as provided above, under conditions suitable for expression of the anti-CD19 antibody, and optionally recovering the anti-CD19 antibody from the host cell (or host cell culture medium).

Anti-CD20 single-domain antibodies

[0144] In one aspect, the present application provides isolated single-domain antibodies that specifically bind to CD20, such as human CD20. In some embodiments, the anti-CD20 single-domain antibody modulates CD20 activity. In some embodiments, the anti-CD20 single-domain antibody is an antagonist antibody.

[0145] In some embodiments, there is provided an anti-CD20 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 77. In some embodiments, the anti-CD20 single-domain antibody is camelid. In some embodiments, the anti-CD20 single-domain antibody is humanized. In some embodiments, the anti-CD20 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0146] In some embodiments, there is provided an anti-CD20 single-domain antibody comprising at least one, at least two, or all three CDRs selected from (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 4; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 5; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 6. In some embodiments, the anti-CD20 single-domain antibody is camelid. In some embodiments, the anti-CD20 single-domain antibody is humanized. In some embodiments, the anti-CD20 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0147] In some embodiments, there is provided an anti-CD20 single-domain antibody comprising three CDRs comprising: (a) a CDR1 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:4; (b) a CDR2 having at least about any one

of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:5; and (c) a CDR3 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:6. In some embodiments, the anti-CD20 single-domain antibody is camelid. In some embodiments, the anti-CD20 antibody is humanized. In some embodiments, a CDR having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-CD20 single-domain antibody comprising that sequence retains the ability to bind to CD20. In some embodiments, the anti-CD20 single-domain antibody is affinity matured. In some embodiments, the anti-CD20 single-domain antibody is camelid. In some embodiments, the anti-CD20 single-domain antibody is humanized. In some embodiments, the anti-CD20 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0148] In some embodiments, there is provided an anti-CD20 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 4; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 5; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 6. In some embodiments, the anti-CD20 single-domain antibody is camelid. In some embodiments, the anti-CD20 single-domain antibody is humanized. In some embodiments, the anti-CD20 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0149] In some embodiments, there is provided an anti-CD20 single-domain antibody comprising a V_HH domain having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 77. In some embodiments, a V_HH sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-CD20 single-domain antibody comprising that sequence retains the ability to bind to CD20. In some embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in the amino acid sequence of SEQ ID NO: 77. In some

embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (i.e., in the FRs). Optionally, the anti-CD20 single-domain antibody comprises the amino acid sequence of SEQ ID NO: 77, including post-translational modifications of that sequence.

[0150] In some embodiments, there is provided an isolated anti-CD20 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 77. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 77.

[0151] In some embodiments, functional epitopes can be mapped by combinatorial alanine scanning. In this process, a combinatorial alanine-scanning strategy can be used to identify amino acids in the CD20 protein that are necessary for interaction with anti-CD20 single-domain antibodies. In some embodiments, the epitope is conformational and crystal structure of the anti-CD20 single-domain antibody bound to CD20 may be employed to identify the epitopes. In some embodiments, the present application provides an antibody that specifically binds to the same epitope as any one of the anti-CD20 single-domain antibodies provided herein. For example, in some embodiments, an antibody is provided that binds to the same epitope as an anti-CD20 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 77.

[0152] In some embodiments, the present application provides an anti-CD20 antibody, or antigen binding fragment thereof, that specifically binds to CD20 competitively with any one of the anti-CD20 single-domain antibodies described herein. In some embodiments, competitive binding may be determined using an ELISA assay. For example, in some embodiments, an antibody is provided that specifically binds to CD20 competitively with an anti-CD20 single-domain antibody comprising the amino acid sequence of SEQ ID NO:77.

[0153] In some embodiments, there is provided an anti-CD20 antibody or antigen binding protein comprising any one of the anti-CD20 single-domain antibodies described above. In some embodiments, the anti-CD20 antibody is a monoclonal antibody, including a camelid, chimeric, humanized or human antibody. In some embodiments, the anti-CD20 antibody is an antibody fragment, *e.g.*, a V_HH fragment. In some embodiments, the anti-CD20 antibody is a full-length heavy-chain only antibody comprising an Fc region of any antibody class or isotype, such as IgG1 or IgG4. In some embodiments, the Fc region has reduced or minimized effector function.

[0154] In some embodiments, the anti-CD20 antibody (such as anti-CD20 single-domain antibody) or antigen binding protein according to any of the above embodiments may

incorporate any of the features, singly or in combination, as described in Sections 1-7 of "Features of antibodies" below.

[0155] In some embodiments, there is provided an isolated nucleic acid encoding any one of the anti-CD20 antibodies (such as anti-CD20 single-domain antibodies) described above. In some embodiments, an isolated nucleic acid encoding an anti-CD20 single-domain antibody is provided wherein the nucleic acid comprises a sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the nucleic acid sequence of SEQ ID NO:102. In some embodiments, there is provided an isolated nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 102. In some embodiments, a vector (*e.g.*, expression vector) comprising such nucleic acid are provided. In some embodiments, a host cell comprising such nucleic acid is provided. In some embodiments, a method of making an anti-CD20 antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the anti-CD20 antibody, as provided above, under conditions suitable for expression of the anti-CD20 antibody, and optionally recovering the anti-CD20 antibody from the host cell (or host cell culture medium).

Anti-BCMA single-domain antibodies

[0156] In one aspect, the present application provides isolated single-domain antibodies that specifically bind to BCMA, such as human BCMA. In some embodiments, the anti-BCMA single-domain antibody modulates BCMA activity. In some embodiments, the anti-BCMA single-domain antibody is an antagonist antibody.

[0157] B cell mature antigen (BCMA), also known as CD269, is a member of the tumor necrosis factor receptor superfamily, namely TNFRSF17 (Thompson et al., J. Exp. Medicine, 192 (1):129-135, 2000). Human BCMA is almost exclusively expressed in plasma cells and multiple myelomia cells (see *e.g.* Novak et al., Blood, 103(2): 689-694, 2004; Neri et al., Clinical Cancer Research, 73(19):5903-5909; Felix et al., Mol. Oncology, 9(7):1348-58, 2015). BCMA can bind B-cell activating factor (BAFF) and a proliferation including ligand (APRIL) (*e.g.* Mackay et al., 2003 and Kalled et al., Immunological Review, 204: 43-54, 2005). BCMA can be a suitable tumor antigen target for immunotherapeutic agents against multiple myelomia. Antibodies of high affinity can block the binding between BCMA and its native ligands BAFF and APRIL. The anti-BCMA single-domain antibodies can be used in combination with cell immunotherapy using CAR-T cells, for example, to enhance cytotoxic effects against tumor cells.

[0158] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 78. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 79. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 80. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 81. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 82. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 83. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 84. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 85. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 86. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 87. In some embodiments, there is provided an anti-BCMA single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 88. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0159] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising at least one, at least two, or all three CDRs selected from (a) a CDR1 comprising an amino acid sequence selected from SEQ ID NO: 7-17; (b) a CDR2 comprising an amino acid sequence selected from SEQ ID NO: 18-28; and (c) a CDR3 comprising an amino acid sequence selected from SEQ ID NO: 29-39. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an

acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0160] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO:7-17; (b) a CDR2 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO:18-28; and (c) a CDR3 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO:29-39. In some embodiments, a CDR having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-BCMA single-domain antibody comprising that sequence retains the ability to bind to BCMA. In some embodiments, the anti-BCMA single-domain antibody is affinity matured. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0161] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 7; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 18; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 29. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0162] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 8; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 19; and (c) a CDR3

comprising the amino acid sequence of SEQ ID NO: 30. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0163] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 9; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 20; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 31. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0164] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 10; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 21; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 32. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0165] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 11; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 22; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 33. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0166] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID

NO: 12; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 23; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 34. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0167] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 13; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 24; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 35. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0168] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 14; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 25; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 36. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0169] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 15; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 26; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 37. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0170] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 16; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 27; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 38. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0171] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 28; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 39. In some embodiments, the anti-BCMA single-domain antibody is camelid. In some embodiments, the anti-BCMA single-domain antibody is humanized. In some embodiments, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0172] In some embodiments, there is provided an anti-BCMA single-domain antibody comprising a V_HH domain having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO: 78-88. In some embodiments, a V_HH sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-BCMA single-domain antibody comprising that sequence retains the ability to bind to BCMA. In some embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in an amino acid sequence selected from SEQ ID NO: 78-88. In some embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (i.e., in the FRs). Optionally, the anti-BCMA single-domain antibody comprises an amino acid sequence selected from SEQ ID NO: 78-88, including post-translational modifications of that sequence.

[0173] In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 78. In some

embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 78. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 79. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 79. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 80. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 80. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 81. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 81. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 82. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 82. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 83. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 83. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 84. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 84. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 85. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 85. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 86. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 86. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 87. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 87. In some embodiments, there is provided an isolated anti-BCMA single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 88. In some

embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 88.

[0174] In some embodiments, functional epitopes can be mapped by combinatorial alanine scanning. In this process, a combinatorial alanine-scanning strategy can be used to identify amino acids in the BCMA protein that are necessary for interaction with anti-BCMA single-domain antibodies. In some embodiments, the epitope is conformational and crystal structure of anti-BCMA single-domain antibody bound to BCMA may be employed to identify the epitopes. In some embodiments, the present application provides an antibody that specifically binds to the same epitope as any of the anti-BCMA single-domain antibodies provided herein. For example, in some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 78. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 79. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 80. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 81. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 82. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 83. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 84. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 85. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 86. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 87. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO: 88.

[0175] In some embodiments, the present application provides an anti-BCMA antibody, or antigen binding fragment thereof, that specifically binds to BCMA competitively with any one of the anti-BCMA single-domain antibodies described herein. In some embodiments, competitive binding may be determined using an ELISA assay. For example, in some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:78. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:79. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:80. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:81. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:82. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:83. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:84. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:85. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:86. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:87. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising the amino acid sequence of SEQ ID NO:88.

[0176] In some embodiments, there is provided an anti-BCMA antibody or antigen binding protein comprising any one of the anti-BCMA single-domain antibodies described above. In some embodiments, the anti-BCMA antibody is a monoclonal antibody, including a camelid, chimeric, humanized or human antibody. In some embodiments, the anti-BCMA antibody is an

antibody fragment, *e.g.*, a V_HH fragment. In some embodiments, the anti-BCMA antibody is a full-length heavy-chain only antibody comprising an Fc region of any antibody class or isotype, such as IgG1 or IgG4. In some embodiments, the Fc region has reduced or minimized effector function.

[0177] In some embodiments, the anti-BCMA antibody (such as anti-BCMA single-domain antibody) or antigen binding protein according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 of "Features of antibodies" below.

[0178] In some embodiments, there is provided an isolated nucleic acid encoding any one of the anti-BCMA antibodies (such as anti-BCMA single-domain antibodies) described above. In some embodiments, an isolated nucleic acid encoding an anti-BCMA single-domain antibody is provided wherein the nucleic acid comprises a sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 103-113. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 103-113. In some embodiments, a vector (*e.g.*, expression vector) comprising such nucleic acid are provided. In some embodiments, a host cell comprising such nucleic acid is provided. In some embodiments, a method of making an anti-BCMA antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the anti-BCMA antibody, as provided above, under conditions suitable for expression of the anti-BCMA antibody, and optionally recovering the anti-BCMA antibody from the host cell (or host cell culture medium).

Anti-CD38 single-domain antibodies

[0179] In one aspect, the present application provides isolated single-domain antibodies that specifically bind to CD38, such as human CD38. In some embodiments, the anti-CD38 single-domain antibody modulates CD38 activity. In some embodiments, the anti-CD38 single-domain antibody is an antagonist antibody.

[0180] CD38 is a type II transmembrane glycoprotein that associates with cell-surface receptors, regulates cytoplasmic Ca^{2+} flux, and mediates signal transduction in lymphoid and myeloid (Konopleva et al., J Immunol, 161:4702-8, 1998; Deaglio et al., Blood, 109:5390-8,

2007). Human CD38 is highly and uniformly expressed on myeloma cells and is expressed at relatively low levels on normal lymphoid and myeloid cells and in some tissues of non-hematopoietic origin, which makes it a potential target in the treatment of myeloma (See, for example, Lin *et al.*, *Am J Clin Pathol*, 2004, 121:482; H. M. Lokhorst *et al.*, *New Eng. J. Med.*, 2015, 373:13).

[0181] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 89. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 90. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 91. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 92. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 93. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 94. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 95. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 96. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 97. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 98. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 99. In some embodiments, there is provided an anti-CD38 single-domain antibody comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 100. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0182] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising at least one, at least two, or all three CDRs selected from (a) a CDR1 comprising an amino acid sequence selected from SEQ ID NO: 40-51; (b) a CDR2 comprising an amino acid sequence selected from SEQ ID NO: 52-63; and (c) a CDR3 comprising an amino acid sequence selected from SEQ ID NO: 64-75. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0183] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO:40-51; (b) a CDR2 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO:52-63; and (c) a CDR3 having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO:64-75. In some embodiments, a CDR having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-CD38 single-domain antibody comprising that sequence retains the ability to bind to CD38. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework. In some embodiments, the anti-CD38 single-domain antibody is affinity matured.

[0184] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 40; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 52; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 64. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises

an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0185] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 41; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 53; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 65. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0186] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 42; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 54; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 66. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0187] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 43; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 55; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 67. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0188] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 44; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 56; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 68. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain

antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0189] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 45; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 57; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 69. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0190] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 46; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 58; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 70. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0191] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 47; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 59; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 71. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0192] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 48; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 60; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 72. In some embodiments, the anti-CD38

single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0193] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 49; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 61; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 73. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0194] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 50; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 62; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 74. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0195] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 51; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 63; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 75. In some embodiments, the anti-CD38 single-domain antibody is camelid. In some embodiments, the anti-CD38 single-domain antibody is humanized. In some embodiments, the anti-CD38 single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0196] In some embodiments, there is provided an anti-CD38 single-domain antibody comprising a V_HH domain having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid

sequence selected from SEQ ID NO: 89-100. In some embodiments, a V_HH sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-CD38 single-domain antibody comprising that sequence retains the ability to bind to CD38. In some embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in an amino acid sequence selected from SEQ ID NO: 89-100. In some embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (i.e., in the FRs). Optionally, the anti-CD38 single-domain antibody comprises an amino acid sequence selected from SEQ ID NO: 89-100, including post-translational modifications of that sequence.

[0197] In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 89. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 89. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 90. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 90. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 91. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 91. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 92. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 92. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 93. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 93. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 94. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 94. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 95. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID

NO: 95. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 96. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 96. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 97. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 97. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 98. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 98. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 99. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 99. In some embodiments, there is provided an isolated anti-CD38 single-domain antibody comprising a V_HH domain having the amino acid sequence of SEQ ID NO: 100. In some embodiments, there is provided a polypeptide comprising the amino acid sequence of SEQ ID NO: 100.

[0198] In some embodiments, functional epitopes can be mapped by combinatorial alanine scanning. In this process, a combinatorial alanine-scanning strategy can be used to identify amino acids in the CD38 protein that are necessary for interaction with the anti-CD38 single-domain antibodies. In some embodiments, the epitope is conformational and crystal structure of anti-CD38 single-domain antibody bound to CD38 may be employed to identify the epitopes. In some embodiments, the present application provides an antibody that specifically binds to the same epitope as any of the anti-CD38 single-domain antibodies provided herein. For example, in some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 89. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 90. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 91. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 92. In some embodiments, an antibody is provided that

binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 93. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 94. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 95. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 96. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 97. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 98. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 99. In some embodiments, an antibody is provided that binds to the same epitope as an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO: 100.

[0199] In some embodiments, the present application provides an anti-CD38 antibody, or antigen binding fragment thereof, that specifically binds to CD38 competitively with any one of the anti-CD38 single-domain antibodies described herein. In some embodiments, competitive binding may be determined using an ELISA assay. For example, in some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:89. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:90. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:91. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:92. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:93. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:94. In some embodiments,

an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:95. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:96. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:97. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:98. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:99. In some embodiments, an antibody is provided that specifically binds to CD38 competitively with an anti-CD38 single-domain antibody comprising the amino acid sequence of SEQ ID NO:100.

[0200] In some embodiments, there is provided an anti-CD38 antibody or antigen binding protein comprising any one of the anti-CD38 single-domain antibodies described above. In some embodiments, the anti-CD38 antibody is a monoclonal antibody, including a camelid, chimeric, humanized or human antibody. In some embodiments, the anti-CD38 antibody is an antibody fragment, *e.g.*, a V_HH fragment. In some embodiments, the anti-CD38 antibody is a full-length heavy-chain only antibody comprising an Fc region of any antibody class or isotype, such as IgG1 or IgG4. In some embodiments, the Fc region has reduced or minimized effector function.

[0201] In some embodiments, the anti-CD38 antibody (such as anti-CD38 single-domain antibody) or antigen binding protein according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 of "Features of antibodies" below.

[0202] In some embodiments, there is provided an isolated nucleic acid encoding any one of the anti-CD38 antibodies (such as anti-CD38 single-domain antibodies) described above. In some embodiments, an isolated nucleic acid encoding an anti-CD38 single-domain antibody is provided wherein the nucleic acid comprises a sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 114-125. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 114-125. In some

embodiments, a vector (*e.g.*, expression vector) comprising such nucleic acid are provided. In some embodiments, a host cell comprising such nucleic acid is provided. In some embodiments, a method of making an anti-CD38 antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the anti-CD38 antibody, as provided above, under conditions suitable for expression of the anti-CD38 antibody, and optionally recovering the anti-CD38 antibody from the host cell (or host cell culture medium).

Features of antibodies

1. Antibody Affinity

[0203] In some embodiments, an antibody provided herein has a dissociation constant (Kd) of $\leq 1\mu M$, ≤ 100 nM, ≤ 10 nM, ≤ 1 nM, ≤ 0.1 nM, ≤ 0.01 nM, or ≤ 0.001 nM (*e.g.* 10^{-8} M or less, *e.g.* from 10^{-8} M to 10^{-13} M, *e.g.*, from 10^{-9} M to 10^{-13} M).

[0204] In some embodiments, Kd is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version or V_HH fragment of an antibody of interest and its antigen as described by the following assay. For example, solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, *e.g.*, Chen et al., *J. Mol. Biol.* 293:865-881(1999)). To establish conditions for the assay, MICROTITER® multi-well plates (Thermo Scientific) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (*e.g.*, consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta et al., *Cancer Res.* 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (*e.g.*, about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (*e.g.*, for one hour). The solution is then removed and the plate washed eight times with 0.1% polysorbate 20 (TWEEN-20®) in PBS. When the plates have dried, 150 μl/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

[0205] In some embodiments, Kd is measured using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab or V_HH of the antibody of interest (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, *e.g.*, Chen et al., *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

2. Antibody Fragments

[0206] In some embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, $F(ab')_2$, Fv, and scFv fragments, V_HH, and other fragments described below. For a review of certain antibody fragments, see Hudson et al. *Nat. Med.* 9:129-134 (2003). For a review of scFv fragments, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

[0207] Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., *Nat. Med.* 9:129-134 (2003); and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., *Nat. Med.* 9:129-134 (2003).

[0208] Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (*e.g. E. coli* or phage), as described herein.

3. Chimeric and Humanized Antibodies

[0209] In some embodiments, the antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, *e.g.*, in U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (*e.g.*, a variable region derived from a camelid species, such as llama) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

[0210] In some embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, *e.g.*, CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (*e.g.*, the antibody from which the HVR residues are derived), *e.g.*, to restore or improve antibody specificity or affinity.

[0211] Humanized antibodies and methods of making them are reviewed, *e.g.*, in Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008), and are further described, *e.g.*, in Riechmann et al., *Nature* 332:323-329 (1988); Queen et al., *Proc. Nat'l Acad. Sci. USA* 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri *et al.*, *Methods* 36:25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol. Immunol.* 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36:43-60 (2005) (describing "FR

shuffling"); and Osbourn et al., *Methods* 36:61-68 (2005) and Klimka et al., *Br. J. Cancer*, 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling).

[0212] Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (*see, e.g.*, Sims et al. *J. Immunol.* 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, *e.g.*, Carter et al. *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); and Presta et al. *J. Immunol.*, 151:2623 (1993)); human mature (somatically mutated) framework regions or human germline framework regions (see, *e.g.*, Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, *e.g.*, Baca et al., *J. Biol. Chem.* 272:10678-10684 (1997) and Rosok et al., *J. Biol. Chem.* 271:22611-22618 (1996)).

[0213] In some embodiments, the single-domain antibodies are modified, such as humanized, without diminishing the native affinity of the domain for antigen and while reducing its immunogenicity with respect to a heterologous species. For example, the amino acid residues of the antibody variable domain (V_HH) of an llama antibody can be determined, and one or more of the Camelid amino acids, for example, in the framework regions, are replaced by their human counterpart as found in the human consensus sequence, without that polypeptide losing its typical character, *i.e.* the humanization does not significantly affect the antigen binding capacity of the resulting polypeptide. Humanization of Camelid single-domain antibodies requires the introduction and mutagenesis of a limited amount of amino acids in a single polypeptide chain. This is in contrast to humanization of scFv, Fab', (Fab')2 and IgG, which requires the introduction of amino acid changes in two chains, the light and the heavy chain and the preservation of the assembly of both chains.

[0214] Single-domain antibodies comprising a V_HH domain can be humanized to have human-like sequences. In some embodiments, the FR regions of the V_HH domain used herein comprise at least about any one of 50%, 60%, 70%, 80%, 90%, 95% or more of amino acid sequence homology to human V_H framework regions. One exemplary class of humanized V_HH domains is characterized in that the V_HHs carry an amino acid from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, or glutamine at position 45, such as, for example, L45 and a tryptophan at position 103, according to the Kabat numbering. As such, polypeptides

belonging to this class show a high amino acid sequence homology to human V_H framework regions and said polypeptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanization.

[0215] Another exemplary class of humanized Camelid single-domain antibodies has been described in WO 03/035694 and contains hydrophobic FR2 residues typically found in conventional antibodies of human origin or from other species, but compensating this loss in hydrophilicity by the charged arginine residue on position 103 that substitutes the conserved tryptophan residue present in V_H from double-chain antibodies. As such, peptides belonging to these two classes show a high amino acid sequence homology to human V_H framework regions and said peptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanization.

4. Human Antibodies

[0216] In some embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr. Opin. Pharmacol.* 5: 368-74 (2001) and Lonberg, *Curr. Opin. Immunol.* 20:450-459 (2008). Transgenic mice or rats capable of producing fully human single-domain antibodies are known in the art. See, *e.g.*, US20090307787A1, U.S. Pat. No. 8,754,287, US20150289489A1, US20100122358A1, and WO2004049794.

[0217] Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, *Nat. Biotech.* 23:1117-1125 (2005). *See also, e.g.*, U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™ technology; U.S. Patent No. 5,770,429 describing HUMAB® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing

VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, *e.g.*, by combining with a different human constant region.

[0218] Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (*See, e.g.*, Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human antibodies generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

[0219] Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.

[0220] One technique for obtaining V_HH sequences directed against a particular antigen or target involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (i.e. so as to raise an immune response and/or heavy chain antibodies directed against said antigen or target), obtaining a suitable biological sample from said transgenic mammal that contains (nucleic acid sequences encoding) said V_HH sequences (such as a blood sample, serum sample or sample of B-cells), and then generating V_HH sequences directed against said antigen or target, starting from said sample, using any suitable technique known per se (such as any of the methods described herein or a hybridoma technique). For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO 02/085945, WO 04/049794 and WO 06/008548 and Janssens et al., Proc. Natl. Acad. Sci. USA. 2006 Oct. 10; 103(41):15130-5 can be used. For example, such heavy chain antibody expressing mice can express heavy chain antibodies with any suitable (single) variable domain,

such as (single) variable domains from natural sources (*e.g.* human (single) variable domains, Camelid (single) variable domains or shark (single) variable domains), as well as for example synthetic or semi-synthetic (single) variable domains.

5. Library-Derived Antibodies

[0221] Antibodies of the present application may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, *e.g.*, in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, *e.g.*, in the McCafferty et al., *Nature* 348:552-554; Clackson et al., *Nature* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Marks and Bradbury, in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132(2004). Methods for constructing single-domain antibody libraries have been described, for example, see U.S. Pat. NO. 7371849.

[0222] In certain phage display methods, repertoires of V_H and V_L genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (*e.g.*, from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J,* 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

[0223] Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

6. Multispecific Antibodies

[0224] In some embodiments, an antibody provided herein is a multispecific antibody, *e.g.* a bispecific antibody. Multispecific antibodies are antibodies that have binding specificities for at least two different sites. In some embodiments, one of the binding specificities is for an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38, and the other is for any other antigen. In some embodiments, bispecific antibodies may bind to two different epitopes of an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38.

[0225] Bispecific antibodies can be prepared as full length antibodies or antibody fragments. Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)), WO 93/08829, and Traunecker et al., *EMBO J.* 10: 3655 (1991)), and "knob-in-hole" engineering (*see, e.g.*, U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (*see, e.g.*, US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (*see, e.g.*, Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992)); using "diabody" technology for making bispecific antibody fragments (see, *e.g.*, Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (*see,e.g.*, Gruber et al., *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, *e.g.*, in Tutt et al. *J. Immunol.* 147: 60 (1991); and creating polypeptides comprising tandem single-domain antibodies (*see,* e.g, U.S. Patent Application No. 20110028695; and Conrath et al. J. Biol. Chem., 2001; 276(10):7346-50). Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (*see, e.g.*, US 2006/0025576A1).

7. Antibody Variants

[0226] In some embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleic acid sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, *e.g.*, antigen-binding.

a) Substitution, Insertion, and Deletion Variants

[0227] In some embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in Table 3 under the heading of "Preferred substitutions." More substantial changes are provided in Table 3 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, *e.g.*, retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE 3. Amino Acid Substitutions

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

[0228] Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

[0229] Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0230] One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (*e.g.*, improvements) in certain biological properties (*e.g.*, increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, *e.g.*, using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant

antibodies displayed on phage and screened for a particular biological activity (*e.g.* binding affinity).

[0231] Alterations (*e.g.*, substitutions) may be made in HVRs, *e.g.*, to improve antibody affinity. Such alterations may be made in HVR "hotspots," *i.e.*, residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (*see, e.g.*, Chowdhury, *Methods Mol. Biol.* 207:179-196 (2008)), and/or SDRs (a-CDRs), with the resulting variant V_H or V_L being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, *e.g.*, in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (*e.g.*, error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (*e.g.*, 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, *e.g.*, using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

[0232] In some embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (*e.g.*, conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or CDRs. In some embodiments of the variant V_HH sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

[0233] A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (*e.g.*, charged residues such as Arg, Asp, His, Lys, and Glu) are identified and replaced by a neutral or negatively charged amino acid (*e.g.*, alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions.

Alternatively, or additionally, a crystal structure of an antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

[0234] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (*e.g.*, for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

b) Glycosylation variants

[0235] In some embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

[0236] Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. *See, e.g.*, Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, *e.g.*, mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the present application may be made in order to create antibody variants with certain improved properties.

[0237] In some embodiments, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (*e.g.*, complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located

at about position 297 in the Fc region (EU numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, *i.e.*, between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. *See, e.g.*, US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); US Patent Application No. US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8*, knockout CHO cells (*see, e.g.*, Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

[0238] Antibody variants are further provided with bisected oligosaccharides, *e.g.*, in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, *e.g.*, in WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana *et al.*). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, *e.g.*, in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

c) Fc region variants

[0239] In some embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or

IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions.

[0240] In some embodiments, the present application contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express Fc(RI, Fc(RII and Fc(RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (*see, e.g.* Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (*see* Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (*see*, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes et al. *Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. *See, e.g.*, C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (*see*, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. et al., *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (*see, e.g.*, Petkova, S.B. et al., *Int'l. Immunol.* 18(12):1759-1769 (2006)).

[0241] Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

[0242] Certain antibody variants with improved or diminished binding to FcRs are described. (*See, e.g.*, U.S. Patent No. 6,737,056; WO 2004/056312, and Shields et al., *J. Biol. Chem.* 9(2): 6591-6604 (2001).)

[0243] In some embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, *e.g.*, substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

[0244] In some embodiments, alterations are made in the Fc region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), *e.g.*, as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

[0245] Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, *e.g.*, substitution of Fc region residue 434 (US Patent No. 7,371,826).

[0246] See also Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

d) Cysteine engineered antibody variants

[0247] In some embodiments, it may be desirable to create cysteine engineered antibodies, *e.g.*, "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties,

such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In some embodiments, any one or more of the following residues may be substituted with cysteine: A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, *e.g.*, in U.S. Patent No. 7,521,541.

e) Antibody Derivatives

[0248] In some embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, *etc.*

[0249] In some embodiments, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In some embodiments, the nonproteinaceous moiety is a carbon nanotube (Kam et al., *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

Methods of preparation

[0250] The antibodies (such as single-domain antibodies) described herein may be prepared using any methods known in the art or as described herein.

[0251] Methods of preparing single-domain antibodies have been described. See, for example, Els Pardon et al, *Nature Protocol*, 2014; 9(3): 674. Single-domain antibodies (such as V_HHs) may be obtained using methods known in the art such as by immunizing a *Camelid* species (such as camel or llama) and obtaining hybridomas therefrom, or by cloning a library of single-domain antibodies using molecular biology techniques known in the art and subsequent selection by ELISA with individual clones of unselected libraries or by using phage display.

[0252] For recombinant production of the single-domain antibodies, the nucleic acids encoding the single-domain antibodies are isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the single-domain antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The choice of vector depends in part on the host cell to be used. Generally, preferred host cells are of either prokaryotic or eukaryotic (generally mammalian) origin.

1. Polyclonal Antibodies

[0253] Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, *e.g.*, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are independently lower alkyl groups. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

[0254] The animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg or the protein or conjugate (for rabbits or mice, respectively)

with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to fourteen days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitable to enhance the immune response.

2. Monoclonal Antibodies

[0255] Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translational modifications (*e.g.*, isomerizations, amidations) that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

[0256] For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

[0257] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986).

[0258] The immunizing agent will typically include the antigenic protein or a fusion variant thereof. Generally either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press (1986), pp. 59-103.

[0259] Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed.

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which are substances that prevent the growth of HGPRT-deficient cells.

[0260] Preferred immortalized myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 cells (and derivatives thereof, *e.g.*, X63-Ag8-653) available from the American Type Culture Collection, Manassas, Va. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0261] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

[0262] The culture medium in which the hybridoma cells are cultured can be assayed for the presence of monoclonal antibodies directed against the desired antigen. Preferably, the binding affinity and specificity of the monoclonal antibody can be determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked assay (ELISA). Such techniques and assays are known in the in art. For example, binding affinity may be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

[0263] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, supra). Suitable culture media for this purpose include, for

example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as tumors in a mammal.

[0264] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0265] Monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567, and as described above. DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, in order to synthesize monoclonal antibodies in such recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Pliickthun, *Immunol. Revs.* 130:151-188 (1992).

[0266] In a further embodiment, antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nucl. Acids Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[0267] The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for

a non-immunoglobulin polypeptide. Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

[0268] The monoclonal antibodies described herein may by monovalent, the preparation of which is well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and a modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues may be substituted with another amino acid residue or are deleted so as to prevent crosslinking. In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly Fab fragments, can be accomplished using routine techniques known in the art.

[0269] Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

3. Recombinant production in Prokaryotic Cells

a) Vector Construction

[0270] Polynucleic acid sequences encoding the antibodies of the present application can be obtained using standard recombinant techniques. Desired polynucleic acid sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once obtained, sequences encoding the polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides. The

vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

[0271] In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter *et al.*, U.S. Pat. No. 5,648,237.

[0272] In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as GEM™-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as *E. coli* LE392.

[0273] The expression vector of the present application may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5′) to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, *e.g.* the presence or absence of a nutrient or a change in temperature.

[0274] A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the present application. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized,

as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

[0275] Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the -galactamase and lactose promoter systems, a tryptophan (trp) promoter system and hybrid promoters such as the tac or the trc promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleic acid sequences have been published, thereby enabling a skilled worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist *et al.* (1980) *Cell* 20: 269) using linkers or adaptors to supply any required restriction sites.

[0276] In one aspect, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (*i.e.* cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In some embodiments of the present application, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

[0277] In some embodiments, the production of the antibodies according to the present application can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In some embodiments, polypeptide components, such as the polypeptide encoding the V_H domain of the first antigen binding portion optionally fused to the second antigen binding portion, and the polypeptide encoding the V_L domain of the first antigen binding portion optionally fused to the second antigen binding portion, are expressed, folded and assembled to form functional antibodies within the cytoplasm. Certain host strains (*e.g.*, the *E. coli* trxB$^-$ strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun *Gene,* 159:203 (1995).

[0278] The present invention provides an expression system in which the quantitative ratio of expressed polypeptide components can be modulated in order to maximize the yield of secreted and properly assembled the antibodies of the present application. Such modulation is accomplished at least in part by simultaneously modulating translational strengths for the polypeptide components. One technique for modulating translational strength is disclosed in Simmons *et al.*, U.S. Pat. No. 5,840,523. It utilizes variants of the translational initiation region (TIR) within a cistron. For a given TIR, a series of amino acid or nucleic acid sequence variants can be created with a range of translational strengths, thereby providing a convenient means by which to adjust this factor for the desired expression level of the specific chain. TIR variants can be generated by conventional mutagenesis techniques that result in codon changes which can alter the amino acid sequence, although silent changes in the nucleic acid sequence are preferred. Alterations in the TIR can include, for example, alterations in the number or spacing of Shine-Dalgarno sequences, along with alterations in the signal sequence. One method for generating mutant signal sequences is the generation of a "codon bank" at the beginning of a coding sequence that does not change the amino acid sequence of the signal sequence (*i.e.*, the changes are silent). This can be accomplished by changing the third nucleotide position of each codon; additionally, some amino acids, such as leucine, serine, and arginine, have multiple first and second positions that can add complexity in making the bank. This method of mutagenesis is described in detail in Yansura *et al.* (1992) *METHODS: A Companion to Methods in Enzymol.* 4:151-158.

[0279] Preferably, a set of vectors is generated with a range of TIR strengths for each cistron therein. This limited set provides a comparison of expression levels of each chain as well as the yield of the desired protein products under various TIR strength combinations. TIR strengths can be determined by quantifying the expression level of a reporter gene as described in detail in Simmons *et al.* U.S. Pat. No. 5,840,523. Based on the translational strength comparison, the desired individual TIRs are selected to be combined in the expression vector constructs of the present application.

b) Prokaryotic Host Cells.

[0280] Prokaryotic host cells suitable for expressing the antibodies of the present application include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (*e.g.*, *E. coli*), *Bacilli* (*e.g.*, *B. subtilis*),

Enterobacteria, *Pseudomonas* species (*e.g.*, *P. aeruginosa*), *Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla*, or *Paracoccus*. In some embodiments, gram-negative cells are used. In one embodiment, *E. coli* cells are used as hosts for the invention. Examples of *E. coli* strains include strain W3110 (Bachmann, *Cellular and Molecular Biology*, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 AfhuA (AtonA) ptr3 lac Iq lacL8 AompT A(nmpc-fepE) degP41 kan^R (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308(ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass *et al.*, *Proteins*, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli, Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon.

[0281] Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

c) Protein Production

[0282] Host cells are transformed with the above-described expression vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transformation means introducing DNA into the prokaryotic host so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO. Yet another technique used is electroporation.

[0283] Prokaryotic cells used to produce the antibodies of the present application are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include luria broth (LB) plus necessary nutrient supplements. In some embodiments, the

media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

[0284] Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

[0285] The prokaryotic host cells are cultured at suitable temperatures. For *E. coli* growth, for example, the preferred temperature ranges from about 20°C to about 39°C, more preferably from about 25°C to about 37°C, even more preferably at about 30°C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For *E. coli*, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

[0286] If an inducible promoter is used in the expression vector of the present application, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the present application, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium (see, *e.g.*, Simmons *et al.*, *J. Immunol. Methods* (2002), 263:133-147). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

[0287] The expressed antibodies of the present application are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

[0288] Alternatively, protein production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

[0289] During the fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, *e.g.*, an OD_{550} of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

[0290] To improve the production yield and quality of the antibodies of the present application, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen *et al.* (1999) *J Bio Chem* 274:19601-19605; Georgiou *et al.*, U.S. Pat. No. 6,083,715; Georgiou *et al.*, U.S. Pat. No. 6,027,888; Bothmann and Pluckthun (2000) J. Biol. Chem. 275:17100-17105; Ramm and Pluckthun (2000) J. Biol. Chem. 275:17106-17113; Arie *et al.* (2001) *Mol. Microbiol.* 39:199-210.

[0291] To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain host strains deficient for proteolytic enzymes can be used for the present invention. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI and combinations thereof. Some *E. coli* protease-deficient strains are available and described in, for example, Joly *et al.* (1998), supra;

Georgiou *et al.*, U.S. Pat. No. 5,264,365; Georgiou *et al.*, U.S. Pat. No. 5,508,192; Hara *et al.*, *Microbial Drug Resistance,* 2:63-72 (1996).

[0292] *E. coli* strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins may be used as host cells in the expression system encoding the antibodies of the present application.

d) Protein Purification

[0293] The antibodies produced herein are further purified to obtain preparations that are substantially homogeneous for further assays and uses. Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using, for example, Sephadex G-75.

[0294] In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the antibodies comprising an Fc region of the present application. Protein A is a 41kD cell wall protein from *Staphylococcus aureas* which binds with a high affinity to the Fc region of antibodies. Lindmark *et al* (1983) *J. Immunol. Meth.* 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants. The solid phase is then washed to remove contaminants non-specifically bound to the solid phase. Finally the antibodies of interest is recovered from the solid phase by elution.

4. Recombinant Production in Eukaryotic Cells

[0295] For Eukaryotic expression, the vector components generally include, but are not limited to, one or more of the following, a signal sequence, an origin of replication, one or more marker genes, and enhancer element, a promoter, and a transcription termination sequence.

a) Signal Sequence Component

[0296] A vector for use in a eukaryotic host may also an insert that encodes a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or

polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

[0297] The DNA for such precursor region is ligated in reading frame to DNA encoding the antibodies of the present application.

b) Origin of Replication

[0298] Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

c) Selection Gene Component

[0299] Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli.

[0300] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[0301] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up nucleic acid encoding the antibodies of the present application, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

[0302] For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity (*e.g.*, ATCC CRL-9096).

[0303] Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with the polypeptide encoding-DNA sequences, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3′-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such

as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

d) Promoter Component

[0304] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the desired polypeptide sequences. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 based upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of the transcription of many genes is a CNCAAT region where N may be any nucleotide. A the 3′ end of most eukaryotic is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3′ end of the coding sequence. All of these sequences may be inserted into eukaryotic expression vectors.

[0305] Other promoters suitable for use with prokaryotic hosts include the phoA promoter, -lactamase and lactose promoter systems, alkaline phosphatase promoter, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antibodies.

[0306] Polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[0307] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from

herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

e) Enhancer Element Component

[0308] Transcription of a DNA encoding the antibodies of the present application by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5′ or 3′ to the polypeptide encoding sequence, but is preferably located at a site 5′ from the promoter.

f) Transcription Termination Component

[0309] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5′ and, occasionally 3′, untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the polypeptide-encoding mRNA. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

g) Selection and Transformation of Host Cells

[0310] Suitable host cells for cloning or expressing the DNA in the vectors herein include higher eukaryote cells described herein, including vertebrate host cells. Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/−DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216

(1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather *et al.*, *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[0311] Host cells are transformed with the above-described expression or cloning vectors for antibodies production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

h) Culturing the Host Cells

[0312] The host cells used to produce the antibodies of the present application may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.* 58:44 (1979), Barnes *et al.*, *Anal. Biochem.* 102:255 (1980), U.S. Pat. No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

i) Protein Purification

[0313] When using recombinant techniques, the antibodies can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced

intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0314] The protein composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify the antibodies that are based on human immunoglobulins containing 1, 2, or 4 heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human 3 (Guss *et al.*, *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABXTMresin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[0315] Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*, from about 0-0.25M salt).

Immunoconjugates

[0316] In some embodiments, the present application also provides immunoconjugates comprising any of the antibodies (such as single-domain antibodies) described herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (*e.g.*, protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

[0317] In some embodiments, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (*see* U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1); an auristatin such as monomethylauristatin drug moieties DE and DF (MMAE and MMAF) (*see* U.S. Patent Nos. 5,635,483 and 5,780,588, and 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see U.S. Patent Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, and 5,877,296; Hinman et al., *Cancer Res.* 53:3336-3342 (1993); and Lode et al., *Cancer Res.* 58:2925-2928 (1998)); an anthracycline such as daunomycin or doxorubicin (*see* Kratz et al., *Current Med. Chem.* 13:477-523 (2006); Jeffrey et al., *Bioorganic & Med. Chem. Letters* 16:358-362 (2006); Torgov et al., *Bioconj. Chem.* 16:717-721 (2005); Nagy et al., *Proc. Natl. Acad. Sci. USA* 97:829-834 (2000); Dubowchik et al., *Bioorg. & Med. Chem. Letters* 12:1529-1532 (2002); King et al., *J. Med. Chem.* 45:4336-4343 (2002); and U.S. Patent No. 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

[0318] In some embodiments, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[0319] In some embodiments, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186},

Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

[0320] Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., *Cancer Res.* 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

[0321] The immunuoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (*e.g.*, from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

Methods and Compositions for Diagnostics and Detection

[0322] In some embodiments, any of the antibodies (such as single-domain antibodies) provided herein is useful for detecting the presence of the corresponding antigen (such as CD19, CD20, BCMA, or CD38) in a biological sample. The term "detecting" as used herein

encompasses quantitative or qualitative detection. In certain embodiments, a biological sample is blood, serum or other liquid samples of biological origin. In some embodiments, a biological sample comprises a cell or tissue.

[0323] In some embodiments, an anti-CD19 antibody (such as any one of the anti-CD19 single-domain antibodies described herein) for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of CD19 in a biological sample is provided. In certain embodiments, the method comprises detecting the presence of CD19 protein in a biological sample. In certain embodiments, CD19 is human CD19. In certain embodiments, the method comprises contacting the biological sample with an anti-CD19 antibody as described herein under conditions permissive for binding of the anti-CD19 antibody to CD19, and detecting whether a complex is formed between the anti-CD19 antibody and CD19. Such method may be an *in vitro* or *in vivo* method. In some embodiments, an anti-CD19 antibody is used to select subjects eligible for therapy with an anti-CD19 antibody, *e.g.* where CD19 is a biomarker for selection of patients.

[0324] In some embodiments, an anti-CD20 antibody (such as any one of the anti-CD20 single-domain antibodies described herein) for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of CD20 in a biological sample is provided. In certain embodiments, the method comprises detecting the presence of CD20 protein in a biological sample. In certain embodiments, CD20 is human CD20. In certain embodiments, the method comprises contacting the biological sample with an anti-CD20 antibody as described herein under conditions permissive for binding of the anti-CD20 antibody to CD20, and detecting whether a complex is formed between the anti-CD20 antibody and CD20. Such method may be an *in vitro* or *in vivo* method. In some embodiments, an anti-CD20 antibody is used to select subjects eligible for therapy with an anti-CD20 antibody, *e.g.* where CD20 is a biomarker for selection of patients.

[0325] In some embodiments, an anti-BCMA antibody (such as any one of the anti-BCMA single-domain antibodies described herein) for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of BCMA in a biological sample is provided. In certain embodiments, the method comprises detecting the presence of BCMA protein in a biological sample. In certain embodiments, BCMA is human BCMA. In certain embodiments, the method comprises contacting the biological sample with an anti-

BCMA antibody as described herein under conditions permissive for binding of the anti-BCMA antibody to BCMA, and detecting whether a complex is formed between the anti-BCMA antibody and BCMA. Such method may be an *in vitro* or *in vivo* method. In some embodiments, an anti-BCMA antibody is used to select subjects eligible for therapy with an anti-BCMA antibody, *e.g.* where BCMA is a biomarker for selection of patients.

[0326] In some embodiments, an anti-CD38 antibody (such as any one of the anti-CD38 single-domain antibodies described herein) for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of CD38 in a biological sample is provided. In certain embodiments, the method comprises detecting the presence of CD38 protein in a biological sample. In certain embodiments, CD38 is human CD38. In certain embodiments, the method comprises contacting the biological sample with an anti-CD38 antibody as described herein under conditions permissive for binding of the anti-CD38 antibody to CD38, and detecting whether a complex is formed between the anti-CD38 antibody and CD38. Such method may be an *in vitro* or *in vivo* method. In some embodiments, an anti-CD38 antibody is used to select subjects eligible for therapy with an anti-CD38 antibody, *e.g.* where CD38 is a biomarker for selection of patients.

[0327] In certain embodiments, labeled antibodies (such as anti-CD19, anti-CD20, anti-BCMA, or anti-CD38 single-domain antibodies) are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, *e.g.*, through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, *e.g.*, firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, *e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

III. Chimeric antigen receptors

[0328] One aspect of the present application provides a chimeric antigen receptor (CAR) comprising an extracellular antigen binding domain comprising one or more single-domain antibodies (such as V_HHs). Any one of the single-domain antibodies described in Section II can be used in the CARs described herein. Exemplary CARs comprising one or more V_HH domains (*i.e.*, V_HH-based CARs) are illustrated and compared with conventional CARs comprising scFvs (*i.e.*, scFv-based CARs) in FIGs. 1A-1D. One of skill in the art would recognize that the V_HH domains in the exemplary CARs of FIGs. 1A-1D may be substituted with other sdAbs.

[0329] In some embodiments, there is provided a chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a single-domain antibody (sdAb) specifically binding to an antigen (such as a tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the sdAb is camelid, chimeric, human, or humanized. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the

polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent, such as bivalent or trivalent. In some embodiments, the CAR is multispecific, such as bispecific.

Chimeric antigen receptors of specific targets

[0330] In some embodiments, the present application provides CARs comprising an extracellular antigen binding domain comprising any one of the anti-CD19, anti-CD20, anti-BCMA, or anti-CD38 single-domain antibodies described herein. The CARs can be monospecific or multispecific (such as bispecific or of higher number of specificities), and the CARs can be monovalent or multivalent (such as bivalent, trivalent, or of higher number of valencies). A list of exemplary monospecific chimeric antigen receptors, exemplary sequences, constructs and vectors thereof are shown in Table 4.

[0331] Tables 4, 5, and 6 listed in the "III. Chimeric antigen receptor" section use the following abbreviations: Ex.: exemplary; Vec.: vector; AA: amino acid sequence of CAR; NA: nucleic acid sequence of CAR; SP: signal peptide; Extracellular: extracellular antigen binding domain; sdAb: single-domain antibody; TM: transmembrane domain; CO1: co-stimulatory signaling domain 1; CO2: co-stimulatory signaling domain 2; Prim.: primary intracellular signaling domain. Domains are listed from the left to the right of each row that corresponds to the order of the domains from the N-terminus to the C-terminus of the CAR polypeptide.

1. CD19 CAR

[0332] In some embodiments, there is provided a CAR targeting CD19 (also referred herein as "CD19 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling

domain. In some embodiments, the anti-CD19 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD19 CAR is monospecific. In some embodiments, the CD19 CAR is monovalent. In some embodiments, the CD19 CAR is multispecific, such as bispecific. In some embodiments, the CD19 CAR is multivalent, such as bivalent or trivalent.

[0333] In some embodiments, there is provided a CD19 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-CD19 sdAb comprises the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the anti-CD19 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD19 sdAb further comprises an FR1 comprising the amino acid sequence of SEQ ID NO: 240, an FR2 comprising the amino acid sequence of SEQ ID NO: 241, an FR3 comprising the amino acid sequence of SEQ ID NO: 242, and/or an FR4 comprising the amino acid sequence of SEQ ID NO: 243. In some embodiments, the anti-CD19 sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 76. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an

immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD19 CAR is monospecific. In some embodiments, the CD19 CAR is monovalent. In some embodiments, the CD19 CAR is multispecific, such as bispecific. In some embodiments, the CD19 CAR is multivalent, such as bivalent or trivalent.

[0334] In some embodiments, there is provided a CD19 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 248. In some embodiments, there is provided a CD19 CAR comprising the amino acid sequence of SEQ ID NO: 248. Also provided is a polypeptide comprising the amino acid sequence of SEQ ID NO: 248.

[0335] In some embodiments, there is provided an isolated nucleic acid encoding any of the CD19 CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the nucleic acid sequence of SEQ ID NO: 250. In some embodiments, there is provided an isolated nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 250. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is a RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the CD19 CARs described above. In

some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

2. CD20 CAR

[0336] In some embodiments, there is provided a CAR targeting CD20 (also referred herein as "CD20 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD20 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD20 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD20 CAR is monospecific. In some embodiments, the CD20 CAR is monovalent. In some embodiments, the CD20 CAR comprises the amino acid sequence of SEQ ID NO: 249. In some embodiments, the CAR is multispecific, such as bispecific. In some embodiments, the CD20 CAR is multivalent, such as bivalent or trivalent.

[0337] In some embodiments, there is provided a CD20 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD20 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-CD20 sdAb comprises the amino acid sequence of SEQ ID NO:4, a CDR2 comprising the amino acid

sequence of SEQ ID NO:5, and a CDR3 comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, the anti-CD20 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD20 sdAb further comprises an FR1 comprising the amino acid sequence of SEQ ID NO: 244, an FR2 comprising the amino acid sequence of SEQ ID NO: 245, an FR3 comprising the amino acid sequence of SEQ ID NO: 246, and/or an FR4 comprising the amino acid sequence of SEQ ID NO: 247. In some embodiments, the anti-CD20 sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 77. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD20 CAR is monospecific. In some embodiments, the CD20 CAR is monovalent. In some embodiments, the CD20 CAR comprises the amino acid sequence of SEQ ID NO: 249. In some embodiments, the CAR is multispecific, such as bispecific. In some embodiments, the CD20 CAR is multivalent, such as bivalent or trivalent.

[0338] In some embodiments, there is provided a CD20 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 249. In some embodiments, there is provided a CD20 CAR comprising the amino acid sequence of SEQ ID NO: 249. Also provided is a polypeptide comprising the amino acid sequence of SEQ ID NO: 249.

[0339] In some embodiments, there is provided an isolated nucleic acid encoding any of the CD20 CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the nucleic acid sequence of SEQ ID NO: 251. In some embodiments, there is provided an isolated nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 251. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is a RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the CD20 CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

3. BCMA CAR

[0340] In some embodiments, there is provided a CAR targeting BCMA (also referred herein as "BCMA CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling

domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the BCMA CAR is monospecific. In some embodiments, the BCMA CAR is monovalent.

[0341] In some embodiments, there is provided a BCMA CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises any one of the following:

(1) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:18; and a CDR3 comprising the amino acid sequence of SEQ ID NO:29;

(2) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:19; and a CDR3 comprising the amino acid sequence of SEQ ID NO:30;

(3) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:20; and a CDR3 comprising the amino acid sequence of SEQ ID NO:31;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:21; and a CDR3 comprising the amino acid sequence of SEQ ID NO:32;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:22; and a CDR3 comprising the amino acid sequence of SEQ ID NO:33;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:23; and a CDR3 comprising the amino acid sequence of SEQ ID NO:34;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:24; and a CDR3 comprising the amino acid sequence of SEQ ID NO:35;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:25; and a CDR3 comprising the amino acid sequence of SEQ ID NO:36;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:26; and a CDR3 comprising the amino acid sequence of SEQ ID NO:37;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:27; and a CDR3 comprising the amino acid sequence of SEQ ID NO:38; or

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:28; and a CDR3 comprising the amino acid sequence of SEQ ID NO:39.

In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence from the group consisting of SEQ ID NO:78-88. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal

peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the BCMA CAR is monospecific. In some embodiments, the BCMA CAR is monovalent.

[0342] In some embodiments, there is provided a BCMA CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 152-162, and 257-259. In some embodiments, there is provided a BCMA CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 152-162, and 257-259. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 152-162, and 257-259.

[0343] In some embodiments, there is provided an isolated nucleic acid encoding any of the BCMA CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 175-185, and 261-263. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 175-185, and 261-263. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the BCMA CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

4. CD38 CAR

[0344] In some embodiments, there is provided a CAR targeting CD38 (also referred herein as "CD38 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD38 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD38 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-

stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD38 CAR is monospecific. In some embodiments, the CD38 CAR is monovalent.

[0345] In some embodiments, there is provided a CD38 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD38 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-CD38 sdAb comprises any one of the following:

(1) a CDR1 comprising the amino acid sequence of SEQ ID NO:40; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:64;

(2) a CDR1 comprising the amino acid sequence of SEQ ID NO:41; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:65;

(3) a CDR1 comprising the amino acid sequence of SEQ ID NO:42; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:66;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO:43; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:67;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO:44; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:68;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO:45; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:69;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO:46; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:70;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO:47; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:71;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO:48; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:72;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO:49; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:73;

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO:50; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:74; or

(12) a CDR1 comprising the amino acid sequence of SEQ ID NO:51; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:75.

In some embodiments, the anti-CD38 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD38 sdAb comprises a V_HH domain comprising an amino acid sequence from the group consisting of SEQ ID NO:89-100. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell

(such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD38 CAR is monospecific. In some embodiments, the CD38 CAR is monovalent.

[0346] In some embodiments, there is provided a CD38 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 163-174, and 260. In some embodiments, there is provided a CD38 CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 163-174, and 260. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 163-174, and 260.

[0347] In some embodiments, there is provided an isolated nucleic acid encoding any of the CD38 CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 186-197, and 264. In some embodiments, there is provided an

isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 186-197, and 264. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the CD38 CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

5. CD22 CAR

[0348] In some embodiments, there is provided a CAR targeting CD22 (also referred herein as "CD22 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD22 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD22 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD22CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD22 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some

embodiments, the CD22 CAR is monospecific. In some embodiments, the CD22 CAR is monovalent.

Table 4. Exemplary monospecific, monovalent CARs.

Ex. Vector or CAR name	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extra-cellular. sdAb	Hinge	TM	Intracellular signaling		
							CO1	CO2	Prim.
BCMA CAR									
PLVX-hEF1a-269A37346	152	175	CD8α	269A37346	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37348	153	176	CD8α	269A37348	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37917	154	177	CD8α	269A37917	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37355	155	178	CD8α	269A37355	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37915	156	179	CD8α	269A37915	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37936	157	180	CD8α	269A37936	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37953	158	181	CD8α	269A37953	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37965	159	182	CD8α	269A37965	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37972	160	183	CD8α	269A37972	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37353	161	184	CD8α	269A37353	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-269A37948	162	185	CD8α	269A37948	CD8α	CD28	CD28	CD137	CD3ζ
GSI5011 CAR	257	261	CD8α	269A37346	CD8α	CD8α	CD137	NA	CD3ζ
GSI5019 CAR	258	262	CD8α	269A37353	CD8α	CD8α	CD137	NA	CD3ζ
GSI5020 CAR	259	263	CD8α	269A37917	CD8α	CD8α	CD137	NA	CD3ζ
CD38 CAR									
PLVX-hEF1a-38A37333	163	186	CD8α	38A37333	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37336	164	187	CD8α	38A37336	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37699	165	188	CD8α	38A37699	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-	166	189	CD8α	38A373	CD8α	CD28	CD28	CD137	CD3ζ

38A37331				31					
PLVX-hEF1a-38A37717	167	190	CD8α	38A37717	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37719	168	191	CD8α	38A37719	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37330	169	192	CD8α	38A37330	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37334	170	193	CD8α	38A37334	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37730	171	194	CD8α	38A37730	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37340	172	195	CD8α	38A37340	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37731	173	196	CD8α	38A37731	CD8α	CD28	CD28	CD137	CD3ζ
PLVX-hEF1a-38A37326	174	197	CD8α	38A37326	CD8α	CD28	CD28	CD137	CD3ζ
CD19 V_HH CAR	248	250	CD8α	CD19 V_HH	CD8α	CD28	CD28	NA	CD3ζ
CD20 V_HH CAR	249	251	CD8α	CD20 V_HH	CD8α	CD28	CD28	NA	CD3ζ
GSI5012 CAR	260	264	CD8α	38A37717	CD8α	CD8α	CD137	NA	CD3ζ

Multivalent chimeric antigen receptors

[0349] The present application also provides multivalent CARs that have two or more (such as about any one of 2, 3, 4, 5, 6, or more) antigen binding sites comprising single-domain antibodies. In some embodiments, the multivalent CAR targets a single antigen, and comprises two or more binding sites for the single antigen. In some embodiments, the multivalent CAR targets more than one antigen, and the multivalent CAR comprises two or more binding sites for at least one antigen. The binding sites specific for the same antigen may bind to the same epitope of the antigen or bind to different epitopes of the antigen. The binding sites specific for the same antigen may comprise the same or different single-domain antibodies.

[0350] In some embodiments, the present application provides a multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a plurality (such as about any one of 2, 3, 4, 5, 6, or more) of single-domain antibodies (sdAbs) specifically binding to an antigen (such as a tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the antigen is selected from the group consisting of CD19, CD20,

CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the plurality of sdAbs is camelid, chimeric, human, or humanized. In some embodiments, the plurality of single-domain antibodies is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CAR is monospecific. In some embodiments, the multivalent CAR is multispecific, such as bispecific.

[0351] In some embodiments, the present application provides a multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody specifically binding to a first epitope of an antigen (such as a tumor antigen), and a second single-domain antibody specifically binding to a second epitope of the antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope and the second epitope are different. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R,

CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first single-domain antibody and the second single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CAR is monospecific. In some embodiments, the multivalent CAR is multispecific, such as bispecific.

[0352] The multivalent CARs describe herein may be specially suitable for targeting multimeric antigens via synergistic binding by the different antigen binding sites, or for enhancing binding affinity or avidity to the antigen. Any of the single-domain antibodies described herein, such as the anti-CD19, anti-CD20, anti-BCMA, or anti-CD38 antibodies, may be used in the extracellular antigen binding domain of the multivalent CARs described herein. A list of exemplary monospecific multivalent chimeric antigen receptors, exemplary sequences, constructs and vectors thereof are shown in Table 5.

1. Multivalent BCMA CAR

[0353] In some embodiments, there is provided a multivalent CAR targeting BCMA (also referred herein as "multivalent BCMA CAR") comprising: (a) an extracellular antigen binding domain comprising a plurality (such as 2, 3, or more) of an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the plurality of the anti-BCMA sdAb is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent BCMA CAR is bivalent. In some embodiments, the multivalent BCMA CAR is trivalent. Any of the anti-BCMA sdAbs can be used to construct the multivalent BCMA CAR.

[0354] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a plurality (such as 2, 3, or more) of an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:7, a CDR2 comprising the amino acid sequence of SEQ ID NO:18, and a CDR3 comprising the

amino acid sequence of SEQ ID NO:29. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO:78. In some embodiments, the plurality of anti-BCMA sdAbs is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent BCMA CAR is bivalent. In some embodiments, the multivalent BCMA CAR is trivalent.

[0355] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb and the second anti-BCMA sdAb specifically binds to different epitopes on BCMA. In some embodiments, the first anti-BCMA sdAb is located at the N-terminus of the second anti-BCMA sdAb. In some embodiments, the first anti-BCMA sdAb is located at the C-terminus of the second anti-BCMA sdAb. In some embodiments, the first anti-BCMA sdAb and the second anti-BCMA sdAb is fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no

more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent BCMA CAR is bivalent. In some embodiments, the multivalent BCMA CAR is trivalent. In some embodiments, the extracellular antigen binding domain further comprises a third anti-BCMA sdAb that specifically binds to an epitope that is different from the first and the second anti-BCMA sdAb. Any of the anti-BCMA sdAbs can be used to construct the multivalent BCMA CAR.

[0356] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:16, a CDR2 comprising the amino acid sequence of SEQ ID NO:27, and a CDR3 comprising the amino acid sequence of SEQ ID NO:38; and wherein the second anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:9, a CDR2 comprising the amino acid sequence of SEQ ID NO:20, and a CDR3 comprising the amino acid sequence of SEQ ID NO:31. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 87. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 80. In some

embodiments, the first anti-BCMA sdAb is located at the N-terminus of the second anti-BCMA sdAb. In some embodiments, the first anti-BCMA sdAb is located at the C-terminus of the second anti-BCMA sdAb. In some embodiments, the first anti-BCMA sdAb and the second anti-BCMA sdAb is fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent BCMA CAR is bivalent.

[0357] In some embodiments, there is provided a multivalent BCMA CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 198-199 and 265-270. In some embodiments, there is provided a multivalent BCMA CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 198-199 and 265-270. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 198-199 and 265-270.

[0358] In some embodiments, there is provided an isolated nucleic acid encoding any of the multivalent BCMA CARs provided herein. In some embodiments, there is provided an isolated

nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 202-203 and 271-276. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 202-203 and 271-276. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the multivalent BCMA CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

2. Multivalent CD38 CAR

[0359] In some embodiments, there is provided a multivalent CAR targeting CD38 (also referred herein as "multivalent CD38 CAR") comprising: (a) an extracellular antigen binding domain comprising a plurality (such as 2, 3, or more) of an anti-CD38 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD38 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the plurality of the anti-CD38 sdAb is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling

domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CD38 CAR is bivalent. In some embodiments, the multivalent CD38 CAR is trivalent. Any of the anti-CD38 sdAbs can be used to construct the multivalent CD38 CAR.

[0360] In some embodiments, there is provided a multivalent CD38 CAR comprising: (a) an extracellular antigen binding domain comprising a plurality of anti-CD38 sdAbs; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein each of the plurality of anti-CD38 sdAbs comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:44, a CDR2 comprising the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:68. In some embodiments, the anti-CD38 sdAb is camelid, chimeric, human, or humanized. In some embodiments, each of the plurality of anti-CD38 sdAbs comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO:93. In some embodiments, the plurality of anti-CD38 sdAbs is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CD38 CAR is bivalent. In some embodiments, the multivalent CD38 CAR is trivalent.

[0361] In some embodiments, there is provided a multivalent CD38 CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-CD38 sdAb and a second anti-CD38 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-CD38 sdAb and the second anti-CD38 sdAb specifically binds to different epitopes on CD38. In some embodiments, the first anti-CD38 sdAb is located at the N-terminus of the second anti-CD38 sdAb. In some embodiments, the first anti-CD38 sdAb is located at the C-terminus of the second anti-CD38 sdAb. In some embodiments, the first anti-CD38 sdAb and the second anti-CD38 sdAb is fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CD38 CAR is bivalent. In some embodiments, the multivalent CD38 CAR is trivalent. In some embodiments, the extracellular antigen binding domain further comprises a third anti-CD38 sdAb that specifically binds to an epitope that is different from the first and the second anti-CD38 sdAb. Any of the anti-CD38 sdAbs can be used to construct the multivalent CD38 CAR.

[0362] In some embodiments, there is provided a multivalent CD38 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 200 or SEQ ID NO: 201. In some embodiments, there is provided a multivalent CD38 CAR comprising the amino acid sequence of SEQ ID NO: 200 or SEQ ID NO: 201. Also provided is a polypeptide comprising the amino acid sequence of SEQ ID NO: 200 or SEQ ID NO: 201.

[0363] In some embodiments, there is provided an isolated nucleic acid encoding any of the multivalent CD38 CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the nucleic acid sequence of SEQ ID NO: 204 or SEQ ID NO: 205. In some embodiments, there is provided an isolated nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 204 or SEQ ID NO: 205. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the multivalent CD38 CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

3. Other exemplary multivalent CARs

[0364] In some embodiments, there is provided a multivalent CAR targeting CD19 (also referred herein as "multivalent CD19 CAR") comprising: (a) an extracellular antigen binding domain comprising a plurality (such as 2, 3, or more) of an anti-CD19 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD19 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the plurality of the anti-CD19 sdAb is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations

thereof. In some embodiments, the multivalent CD19 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CD19 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CD19 CAR is bivalent. In some embodiments, the multivalent CD19 CAR is trivalent. Any of the anti-CD19 sdAbs can be used to construct the multivalent CD19 CAR.

[0365] In some embodiments, there is provided a multivalent CAR targeting CD20 (also referred herein as "multivalent CD20 CAR") comprising: (a) an extracellular antigen binding domain comprising a plurality (such as 2, 3, or more) of an anti-CD20 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD20 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the plurality of the anti-CD20 sdAb is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding

domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CD20 CAR is bivalent. In some embodiments, the multivalent CD20 CAR is trivalent. Any of the anti-CD20 sdAbs can be used to construct the multivalent CD20 CAR.

[0366] In some embodiments, there is provided a multivalent CAR targeting CD22 (also referred herein as "multivalent CD22 CAR") comprising: (a) an extracellular antigen binding domain comprising a plurality (such as 2, 3, or more) of an anti-CD22 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD22 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the plurality of the anti-CD22 sdAb is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CD22 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CD22 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CD22 CAR is bivalent. In some embodiments, the multivalent CD22 CAR is trivalent.

Table 5. Exemplary monospecific, multivalent CAR.

CAR	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extracellular Antigen binding domain					Hinge	TM	Intracellular signaling	
				sdAb #1	Lnk. #1 SEQ ID	sdAb #2	Lnk. #2 SEQ ID	sdAb #3			CO 1	Prim.
GSI5 014	198	202	CD 8α	269A 3734 6	144	269A 3734 6	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 015	199	203	CD 8α	269A 3734 6	144	269A 3734 6	144	269A 3734 6	CD8α	CD 8α	CD 137	CD3ζ
GSI5 016	200	204	CD 8α	38A3 7717	144	38A3 7717	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 017	201	205	CD 8α	38A3 7717	144	38A3 7717	144	38A3 7717	CD8α	CD 8α	CD 137	CD3ζ
GSI5 021	265	271	CD 8α	269A 3735 3	144	269A 3791 7	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 022	266	272	CD 8α	269A 3735 3	149	269A 3791 7	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 023	267	273	CD 8α	269A 3735 3	151	269A 3791 7	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 024	268	274	CD 8α	269A 3791 7	145	269A 3735 3	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 025	269	275	CD 8α	269A 3791 7	149	269A 3735 3	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GSI5 026	270	276	CD 8α	269A 3791 7	150	269A 3735 3	NA	NA	CD8α	CD 8α	CD 137	CD3ζ

Multispecific chimeric antigen receptor

[0367] The present application further provides multispecific chimeric antigen receptors targeting two or more (such as about any one of 2, 3, 4, 5, 6, or more) different antigens. In some embodiments, the multispecific CAR has one antigen binding site for each antigen. In some embodiments, the multispecific CAR has more than two binding sites for at least one antigen. Each antigen binding site may comprise a single-domain antibody. For example, in some

embodiments, the multispecific chimeric antigen receptor is a bispecific CAR comprising an extracellular antigen binding domain comprising two different sdAbs each specifically binding to an antigen. In some embodiments, the multispecific CAR is a trispecific CAR comprising an extracellular antigen binding domain comprising three different sdAbs each specifically binding to an antigen.

[0368] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody (sdAb) specifically binding to a first antigen (such as a first tumor antigen) and a second single-domain antibody (sdAb) specifically binding to a second antigen (such as a second tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first antigen is different from the second antigen. In some embodiments, the first antigen and/or the second antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first single-domain antibody and the second single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multispecific CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multispecific CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments,

the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ.

[0369] Depending on the desired antigen to be targeted, the CARs of the present application can be engineered to include the appropriate single-domain antibodies that are specific to the desired antigens. Any one or more of the anti-CD19, anti-CD20, anti-BCMA, or anti-CD38 antibodies described herein may be used in the extracellular antigen binding domain in the CARs of the present applications. The single-domain antibodies can be arranged in any suitable order. For example, the first single-domain antibody is fused at the N-terminus or the C-terminus of the second single-domain antibody. A suitable peptide linker may be placed between different single-domain antibodies to avoid steric hindrance between the single-domain antibodies. A list of exemplary bispecific chimeric antigen receptors, exemplary sequences, constructs and vectors thereof are shown in Table 6.

1. BCMA × CD38 CAR

[0370] In some embodiments, the CAR of the present application is a bispecific CAR simultaneously targeting BCMA and CD38. For example, the BCMA and CD38 can be used as candidates for targeting antigens expressed on multiple myeloma cells.

[0371] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting BCMA and CD38 (also referred herein as "BCMA × CD38 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody (sdAb) specifically binding to BCMA and a second single-domain antibody specifically binding to CD38; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first single-domain antibody and the second single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the first sdAb is fused at the N-terminus of the second sdAb. In some

embodiments, the first sdAb is fused at the C-terminus of the second sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA × CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA × CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ.

[0372] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting BCMA and CD38 (also referred herein as "BCMA × CD38 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA single-domain antibody and an anti-CD38 single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:7, a CDR2 comprising the amino acid sequence of SEQ ID NO:18, and a CDR3 comprising the amino acid sequence of SEQ ID NO:29; and wherein the anti-CD38 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:44, a CDR2 comprising the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:68. In some embodiments, the anti-BCMA sdAb and/or the anti-CD38 sdAb is camelid,

chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAbs comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO:78. In some embodiments, the anti-CD38 sdAbs comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO:93. In some embodiments, the anti-BCMA sdAb and the anti-CD38 sdAb are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-BCMA sdAb is fused at the N-terminus of the anti-CD38 sdAb. In some embodiments, the first anti-BCMA is fused at the C-terminus of the anti-CD38 sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA × CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA × CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide.

[0373] In some embodiments, there is provided a BCMA × CD38 CAR comprising a polypeptide comprising from the N-terminus to the C-terminus: a CD8α signal peptide, an anti-CD38 single-domain antibody, a peptide linker, an anti-BCMA single-domain antibody, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ; wherein the anti-BCMA single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:7, a CDR2 comprising the amino acid sequence of SEQ ID NO:18, and a CDR3 comprising the amino acid sequence of SEQ ID NO:29; and wherein the anti-CD38 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:44, a CDR2 comprising

the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:68. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the BCMA × CD38 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 207-211. In some embodiments, there is provided a BCMA × CD38 CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 207-211. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 207-211.

[0374] In some embodiments, there is provided a BCMA × CD38 CAR comprising a polypeptide comprising from the N-terminus to the C-terminus: a CD8α signal peptide, an anti-BCMA single-domain antibody, a peptide linker, an anti-CD38 single-domain antibody, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ; wherein the anti-BCMA single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:7, a CDR2 comprising the amino acid sequence of SEQ ID NO:18, and a CDR3 comprising the amino acid sequence of SEQ ID NO:29; and wherein the anti-CD38 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:44, a CDR2 comprising the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:68. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the BCMA × CD38 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 212-216. In some embodiments, there is provided a BCMA × CD38 CAR comprising t an amino acid sequence selected from the group consisting of SEQ ID NOs: 212-216. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 212-216.

[0375] In some embodiments, there is provided an isolated nucleic acid encoding any of the BCMA × CD38 CAR provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs:218-227. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs:218-227. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the BCMA × CD38 CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

2. CD19 × CD20 CAR

[0376] In some embodiments, B cell differentiation antigens such as CD19 and CD20 are candidates for target antigens in B cell lymphoma. Some of these antigens have been used as targets for passive immunotherapy with monoclonal antibodies with limited success. In some embodiments, the CAR of the present application is a bispecific CAR simultaneously targeting CD19 and CD20.

[0377] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting CD19 and CD20 (also referred herein as "CD19 × CD20 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody (sdAb) specifically binding to CD19 and a second single-domain antibody specifically binding to CD20; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first single-domain antibody and the second single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the first sdAb is fused at the N-terminus of the second sdAb. In some embodiments, the first sdAb is fused at the C-terminus of the second sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4,

CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ.

[0378] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting CD19 and CD20 (also referred herein as "CD19 × CD20 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 single-domain antibody and an anti-CD20 single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-CD19 single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3; and wherein the anti-CD20 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:4, a CDR2 comprising the amino acid sequence of SEQ ID NO:5, and a CDR3 comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, the anti-CD19 sdAb and/or the anti-CD20 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD19 sdAbs comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO:76. In some embodiments, the anti-CD20 sdAbs comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO:77. In some embodiments, the anti-CD19 sdAb and the anti-CD20 sdAb are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-CD19 sdAb is fused at the N-terminus

of the anti-CD20 sdAb. In some embodiments, the first anti-CD19 is fused at the C-terminus of the anti-CD20 sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises the amino acid sequence of SEQ ID NOs: 146. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide.

[0379] In some embodiments, there is provided a CD19 × CD20 CAR comprising a polypeptide comprising from the N-terminus to the C-terminus: a CD8α signal peptide, an anti-CD19 single-domain antibody, a peptide linker, an anti-CD20 single-domain antibody, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ; wherein the anti-CD19 single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3; and wherein the anti-CD20 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:4, a CDR2 comprising the amino acid sequence of SEQ ID NO:5, and a CDR3 comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises the amino acid sequence of SEQ ID NO: 146. In some embodiments, the CD19 × CD20 CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%,

89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 206. In some embodiments, there is provided a CD19 × CD20 CAR comprising the amino acid sequence of SEQ ID NO: 206. Also provided is a polypeptide comprising the amino acid sequence of SEQ ID NO: 206.

[0380] In some embodiments, there is provided an isolated nucleic acid encoding any of the CD19 × CD20 CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the nucleic acid sequence of SEQ ID NO:217. In some embodiments, there is provided an isolated nucleic acid comprising the nucleic acid sequence of SEQ ID NO:217. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the CD19 × CD20 CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

[0381] Currently, immunotherapies targeting CD19 have seen remarkable results in clinical trials. CD19 CAR-T cell-based clinical trials of short-term ALL treatment can achieve about 90% complete remission efficacy. However, approximately 10% of patients were found relapse after a few months' treatment. The main reason was that CD19 was lost during maturation of B cells to plasma cells and the residual tumor cells produced the CD19 antigen loss escape variants. The CD19 × CD20 CARs described herein may simultaneously target CD19 and CD20 tumor surface antigens, which may enhance systemic T cell antitumor activity, and reduce the target escape phenomena which caused at least 30% of leukemia relapse after CAR therapy.

3. Other exemplary multispecific CARs

[0382] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting CD19 and CD22 (also referred herein as "CD19 × CD22 CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 single-domain antibody and an anti-CD22 single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD19 sdAb and/or the anti-CD22 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD22 single-domain antibody and anti-CD22 single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments,

the anti-CD19 sdAb is fused at the N-terminus of the anti-CD22 sdAb. In some embodiments, the anti-CD19 sdAb is fused at the C-terminus of the anti-CD22 sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × CD22 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × CD22 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the anti-CD19 single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3.

[0383] In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting CD19 and BCMA (also referred herein as "CD19 × BCMA CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an

anti-CD19 single-domain antibody and an anti-BCMA single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD19 sdAb and/or the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA single-domain antibody and anti-BCMA single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-CD19 sdAb is fused at the N-terminus of the anti-BCMA sdAb. In some embodiments, the anti-CD19 sdAb is fused at the C-terminus of the anti-BCMA sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the anti-CD19 single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a

CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3.

Table 6. Exemplary bispecific CARs.

CAR	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extracellular Antigen binding domain			Hinge	TM	CO1	Intra.
				sdAb #1	Linker SEQ ID	sdAb# 2				
CD19 × CD20	206	217	CD8α	CD19 V_HH	146	CD20 V_HH	CD8α	CD28	CD28	CD3ζ
GSI5 001	207	218	CD8α	38A37 717	144	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GSI5 002	208	219	CD8α	38A37 717	145	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GSI5 003	209	220	CD8α	38A37 717	146	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GSI5 004	210	221	CD8α	38A37 717	147	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GSI5 005	211	222	CD8α	38A37 717	148	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GSI5 006	212	223	CD8α	269A3 7346	144	38A37 717	CD8α	CD8α	CD137	CD3ζ
GSI5 007	213	224	CD8α	269A3 7346	145	38A37 717	CD8α	CD8α	CD137	CD3ζ
GSI5 008	214	225	CD8α	269A3 7346	146	38A37 717	CD8α	CD8α	CD137	CD3ζ
GSI5 009	215	226	CD8α	269A3 7346	147	38A37 717	CD8α	CD8α	CD137	CD3ζ
GSI5 010	216	227	CD8α	269A3 7346	148	38A37 717	CD8α	CD8α	CD137	CD3ζ

Extracellular antigen binding domain

[0384] The extracellular antigen binding domain of the CARs described herein comprises one or more (such as any one of 1, 2, 3, 4, 5, 6 or more) single-domain antibodies. The single-domain antibodies can be fused to each other directly via peptide bonds, or via peptide linkers.

1. Single-domain antibodies

[0385] The CARs of the present application comprise an extracellular antigen binding domain comprising one or more single-domain antibodies. The sdAbs may be of the same of different

origins, and of the same or different sizes. Exemplary sdAbs include, but are not limited to, heavy chain variable domains from heavy-chain only antibodies (*e.g.*, V_HH or V_{NAR}), binding molecules naturally devoid of light chains, single domains (such as V_H or V_L) derived from conventional 4-chain antibodies, humanized heavy-chain only antibodies, human single-domain antibodies produced by transgenic mice or rats expressing human heavy chain segments, and engineered domains and single domain scaffolds other than those derived from antibodies. Any sdAbs known in the art or developed by the inventors, including the single-domain antibodies described in Section II of the present application, may be used to construct the CARs described herein. The sdAbs may be derived from any species including, but not limited to mouse, rat, human, camel, llama, lamprey, fish, shark, goat, rabbit, and bovine. Single-domain antibodies contemplated herein also include naturally occurring single-domain antibody molecules from species other than *Camelidae* and sharks.

[0386] In some embodiments, the sdAb is derived from a naturally occurring single-domain antigen binding molecule known as heavy chain antibody devoid of light chains (also referred herein as "heavy chain only antibodies"). Such single domain molecules are disclosed in WO 94/04678 and Hamers-Casterman, C. *et al.* (1993) *Nature* 363:446-448, for example. For clarity reasons, the variable domain derived from a heavy chain molecule naturally devoid of light chain is known herein as a V_HH to distinguish it from the conventional V_H of four chain immunoglobulins. Such a V_HH molecule can be derived from antibodies raised in *Camelidae* species, for example, camel, llama, vicuna, dromedary, alpaca and guanaco. Other species besides Camelidae may produce heavy chain molecules naturally devoid of light chain, and such V_HHs are within the scope of the present application.

[0387] V_HH molecules from Camelids are about 10 times smaller than IgG molecules. They are single polypeptides and can be very stable, resisting extreme pH and temperature conditions. Moreover, they can be resistant to the action of proteases which is not the case for conventional 4-chain antibodies. Furthermore, *in vitro* expression of V_HH s produces high yield, properly folded functional V_HHs. In addition, antibodies generated in Camelids can recognize epitopes other than those recognized by antibodies generated *in vitro* through the use of antibody libraries or via immunization of mammals other than Camelids (see, for example, WO9749805). As such, multispecific or multivalent CARs comprising one or more V_HH domains may interact more efficiently with targets than multispecific or multivalent CARs comprising antigen binding

fragments derived from conventional 4-chain antibodies. Since V_HHs are known to bind into 'unusual' epitopes such as cavities or grooves, the affinity of CARs comprising such V_HHs may be more suitable for therapeutic treatment than conventional multispecific polypeptides.

[0388] In some embodiments, the sdAb is derived from a variable region of the immunoglobulin found in cartilaginous fish. For example, the sdAb can be derived from the immunoglobulin isotype known as Novel Antigen Receptor (NAR) found in the serum of shark. Methods of producing single domain molecules derived from a variable region of NAR ("IgNARs") are described in WO 03/014161 and Streltsov (2005) *Protein Sci.* 14:2901-2909.

[0389] In some embodiments, the sdAb is recombinant, CDR-grafted, humanized, camelized, de-immunized and/or *in vitro* generated (*e.g.*, selected by phage display). In some embodiments, the amino acid sequence of the framework regions may be altered by "camelization" of specific amino acid residues in the framework regions. Camelization refers to the replacing or substitution of one or more amino acid residues in the amino acid sequence of a (naturally occurring) V_H domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_HH domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein. Such "camelizing" substitutions are preferably inserted at amino acid positions that form and/or are present at the V_H-V_L interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO 94/04678, Davies and Riechmann FEBS Letters 339: 285-290, 1994; Davies and Riechmann Protein Engineering 9 (6): 531-537, 1996; Riechmann J. Mol. Biol. 259: 957-969, 1996; and Riechmann and Muyldermans J. Immunol. Meth. 231: 25-38, 1999).

[0390] In some embodiments, the sdAb is a human single-domain antibody produced by transgenic mice or rats expressing human heavy chain segments. See, *e.g.*, US20090307787A1, U.S. Pat. No. 8,754,287, US20150289489A1, US20100122358A1, and WO2004049794. In some embodiments, the sdAb is affinity matured.

[0391] In some embodiments, naturally occurring V_HH domains against a particular antigen or target, can be obtained from (naïve or immune) libraries of Camelid V_HH sequences. Such methods may or may not involve screening such a library using said antigen or target, or at least one part, fragment, antigenic determinant or epitope thereof using one or more screening techniques known per se. Such libraries and techniques are for example described in WO

99/37681, WO 01/90190, WO 03/025020 and WO 03/035694. Alternatively, improved synthetic or semi-synthetic libraries derived from (naïve or immune) V_HH libraries may be used, such as V_HH libraries obtained from (naïve or immune) V_HH libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example described in WO 00/43507.

[0392] In some embodiments, the single-domain antibodies are generated from conventional four-chain antibodies. See, for example, EP 0 368 684, Ward et al. (Nature 1989 Oct. 12; 341 (6242): 544-6), Holt et al., Trends Biotechnol., 2003, 21(11):484-490; WO 06/030220; and WO 06/003388.

2. Antigens

[0393] The antigen(s) targeted by the CARs of the present application are cell surface molecules. The single-domain antibodies may be chosen to recognize an antigen that acts as a cell surface marker on target cells associated with a special disease state. In some embodiments, the antigen (such as the first antigen and/or the second antigen) is a tumor antigen. In some embodiments, the multispecific CARs target two or more tumor antigens. In some embodiments, the tumor antigen is associated with a B cell malignancy. Tumors express a number of proteins that can serve as a target antigen for an immune response, particularly T cell mediated immune responses. The antigens targeted by the CAR may be antigens on a single diseased cell or antigens that are expressed on different cells that each contribute to the disease. The antigens targeted by the CAR may be directly or indirectly involved in the diseases.

[0394] Tumor antigens are proteins that are produced by tumor cells that can elicit an immune response, particularly T-cell mediated immune responses. The selection of the targeted antigen of the invention will depend on the particular type of cancer to be treated. Exemplary tumor antigens include, for example, a glioma-associated antigen, carcinoembryonic antigen (CEA), β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CAIX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, M-CSF, prostase, prostate-specific antigen (PSA), PAP, NY-ESO-1, LAGE-la, p53, prostein, PSMA, HER2/neu, survivin and telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase, ephrinB2, CD22, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor and mesothelin.

[0395] In some embodiments, the tumor antigen comprises one or more antigenic cancer epitopes associated with a malignant tumor. Malignant tumors express a number of proteins that

can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase and gp100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD 19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma.

[0396] In some embodiments, the tumor antigen is a tumor-specific antigen (TSA) or a tumor-associated antigen (TAA). A TSA is unique to tumor cells and does not occur on other cells in the body. A TAA associated antigen is not unique to a tumor cell, and instead is also expressed on a normal cell under conditions that fail to induce a state of immunologic tolerance to the antigen. The expression of the antigen on the tumor may occur under conditions that enable the immune system to respond to the antigen. TAAs may be antigens that are expressed on normal cells during fetal development, when the immune system is immature, and unable to respond or they may be antigens that are normally present at extremely low levels on normal cells, but which are expressed at much higher levels on tumor cells.

[0397] Non-limiting examples of TSA or TAA antigens include the following: Differentiation antigens such as MART-1/MelanA (MART-I), gp 100 (Pmel 17), tyrosinase, TRP-1, TRP-2 and tumor-specific multilineage antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, pl5; overexpressed embryonic antigens such as CEA; overexpressed oncogenes and mutated tumor-suppressor genes such as p53, Ras, HER2/neu; unique tumor antigens resulting from chromosomal translocations; such as BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR; and viral antigens, such as the Epstein Barr virus antigens EBVA and the human papillomavirus (HPV) antigens E6 and E7. Other large, protein-based antigens include TSP-180, MAGE-4, MAGE-5, MAGE-6, RAGE, NY-ESO, pl85erbB2, pl80erbB-3, c-met, nm-23HI, PSA, TAG-72, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-Catenin, CDK4, Mum-1, p 15, p 16, 43-9F, 5T4, 791Tgp72, alpha-fetoprotein, beta-HCG, BCA225, BTAA, CA 125, CA 15-3\CA 27.29\BCAA, CA 195, CA 242, CA-50, CAM43, CD68\P1, CO-029, FGF-5, G250, Ga733\EpCAM, HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO- 1, RCAS 1,

SDCCAG16, TA-90\Mac-2 binding protein\cyclophilin C-associated protein, TAAL6, TAG72, TLP, and TPS.

[0398] In some embodiments, the antigen (such as the first antigen and/or the second antigen) are selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77.

3. Peptide linkers

[0399] The various single-domain antibodies in the multispecific or multivalent CARs described herein may be fused to each other via peptide linkers. In some embodiments, the single-domain antibodies are directly fused to each other without any peptide linkers. The peptide linkers connecting different single-domain antibodies may be the same or different. Different domains of the CARs may also be fused to each other via peptide linkers.

[0400] Each peptide linker in a CAR may have the same or different length and/or sequence depending on the structural and/or functional features of the single-domain antibodies and/or the various domains. Each peptide linker may be selected and optimized independently. The length, the degree of flexibility and/or other properties of the peptide linker(s) used in the CARs may have some influence on properties, including but not limited to the affinity, specificity or avidity for one or more particular antigens or epitopes. For example, longer peptide linkers may be selected to ensure that two adjacent domains do not sterically interfere with one another. For example, in a multivalent or multispecific CAR of the present application that comprise single-domain antibodies directed against a multimeric antigen, the length and flexibility of the peptide linkers are preferably such that it allows each single-domain antibody in the multivalent CAR to bind to the antigenic determinant on each of the subunits of the multimer. In some embodiments, a short peptide linker may be disposed between the transmembrane domain and the intracellular signaling domain of a CAR. In some embodiment, a peptide linker comprises flexible residues (such as glycine and serine) so that the adjacent domains are free to move relative to each other. For example, a glycine-serine doublet can be a suitable peptide linker.

[0401] The peptide linker can be of any suitable length. In some embodiments, the peptide linker is at least about any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100 or more amino acids long. In some embodiments, the peptide linker is no more than about any of 100, 75, 50, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8,

7, 6, 5 or fewer amino acids long. In some embodiments, the length of the peptide linker is any of about 1 amino acid to about 10 amino acids, about 1 amino acids to about 20 amino acids, about 1 amino acid to about 30 amino acids, about 5 amino acids to about 15 amino acids, about 10 amino acids to about 25 amino acids, about 5 amino acids to about 30 amino acids, about 10 amino acids to about 30 amino acids long, about 30 amino acids to about 50 amino acids, about 50 amino acids to about 100 amino acids, or about 1 amino acid to about 100 amino acids.

[0402] The peptide linker may have a naturally occurring sequence, or a non-naturally occurring sequence. For example, a sequence derived from the hinge region of heavy chain only antibodies may be used as the linker. *See,* for example, WO1996/34103. In some embodiments, the peptide linker is a flexible linker. Exemplary flexible linkers include glycine polymers $(G)_n$, glycine-serine polymers (including, for example, $(GS)_n$, $(GSGGS)_n$, $(GGGS)_n$, and $(GGGGS)_n$, where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers known in the art. In some embodiments, the peptide linker comprises the amino acid sequence GGGGS (SEQ ID NO: 144), $(GGGGS)_2$ (SEQ ID NO: 145), $(GGGS)_4$ (SEQ ID NO: 146), GGGGSGGGGSGGGGGGSGSGGGGS (SEQ ID NO: 147), GGGGSGGGGSGGGGGGSGSGGGGSGGGGSGGGGS (SEQ ID NO: 148), $(GGGGS)_3$ (SEQ ID NO: 149), $(GGGGS)_4$ (SEQ ID NO: 150), or $(GGGGS)_3$ (SEQ ID NO: 151).

Transmembrane domain

[0403] The CARs of the present application comprise a transmembrane domain that can be directly or indirectly fused to the extracellular antigen binding domain. The transmembrane domain may be derived either from a natural or from a synthetic source. As used herein, a "transmembrane domain" refers to any protein structure that is thermodynamically stable in a cell membrane, preferably a eukaryotic cell membrane. Transmembrane domains compatible for use in the CARs described herein may be obtained from a naturally occurring protein. Alternatively, it can be a synthetic, non-naturally occurring protein segment, *e.g.*, a hydrophobic protein segment that is thermodynamically stable in a cell membrane.

[0404] Transmembrane domains are classified based on the three dimensional structure of the transmembrane domain. For example, transmembrane domains may form an alpha helix, a complex of more than one alpha helix, a beta-barrel, or any other stable structure capable of spanning the phospholipid bilayer of a cell. Furthermore, transmembrane domains may also or alternatively be classified based on the transmembrane domain topology, including the number

of passes that the transmembrane domain makes across the membrane and the orientation of the protein. For example, single-pass membrane proteins cross the cell membrane once, and multi-pass membrane proteins cross the cell membrane at least twice (*e.g.*, 2, 3, 4, 5, 6, 7 or more times). Membrane proteins may be defined as Type I, Type II or Type III depending upon the topology of their termini and membrane-passing segment(s) relative to the inside and outside of the cell. Type I membrane proteins have a single membrane-spanning region and are oriented such that the N-terminus of the protein is present on the extracellular side of the lipid bilayer of the cell and the C-terminus of the protein is present on the cytoplasmic side. Type II membrane proteins also have a single membrane-spanning region but are oriented such that the C-terminus of the protein is present on the extracellular side of the lipid bilayer of the cell and the N-terminus of the protein is present on the cytoplasmic side. Type III membrane proteins have multiple membrane- spanning segments and may be further sub-classified based on the number of transmembrane segments and the location of N- and C-termini.

[0405] In some embodiments, the transmembrane domain of the CAR described herein is derived from a Type I single-pass membrane protein. In some embodiments, transmembrane domains from multi-pass membrane proteins may also be compatible for use in the CARs described herein. Multi-pass membrane proteins may comprise a complex (at least 2, 3, 4, 5, 6, 7 or more) alpha helices or a beta sheet structure. Preferably, the N-terminus and the C-terminus of a multi-pass membrane protein are present on opposing sides of the lipid bilayer, *e.g.*, the N-terminus of the protein is present on the cytoplasmic side of the lipid bilayer and the C-terminus of the protein is present on the extracellular side.

[0406] In some embodiments, the transmembrane domain of the CAR comprises a transmembrane domain chosen from the transmembrane domain of an alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, KIRDS2, OX40, CD2, CD27, LFA-1 (CDl la, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRFl), CD160, CD19, IL-2R beta, IL-2R gamma, IL-7R a, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDl ld, ITGAE, CD103, ITGAL, CDl la, LFA-1, ITGAM, CDl lb, ITGAX, CDl lc, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CDIOO (SEMA4D), SLAMF6

(NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, and/or NKG2C. In some embodiments, the transmembrane domain is derived from a molecule selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1.

[0407] In some embodiments, the transmembrane domain is derived from CD28. In some embodiments, the transmembrane domain is a transmembrane domain of CD28 comprising the amino acid sequence of SEQ ID NO: 133. In some embodiments, the transmembrane domain of CD28 is encoded by the nucleic acid sequence of SEQ ID NO: 135.

[0408] In some embodiments, the transmembrane domain is derived from CD8α. In some embodiments, the transmembrane domain is a transmembrane domain of CD8α comprising the amino acid sequence of SEQ ID NO: 132. In some embodiments, the transmembrane domain of CD8α is encoded by the nucleic acid sequence of SEQ ID NO: 134.

[0409] Transmembrane domains for use in the CARs described herein can also comprise at least a portion of a synthetic, non-naturally occurring protein segment. In some embodiments, the transmembrane domain is a synthetic, non-naturally occurring alpha helix or beta sheet. In some embodiments, the protein segment is at least approximately 20 amino acids, *e.g.*, at least 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more amino acids. Examples of synthetic transmembrane domains are known in the art, for example in U.S. Patent No.7,052,906 B1 and PCT Publication No. WO 2000/032776 A2.

[0410] The transmembrane domain may comprise a transmembrane region and a cytoplasmic region located at the C-terminal side of the transmembrane domain. The cytoplasmic region of the transmembrane domain may comprise three or more amino acids and, in some embodiments, helps to orient the transmembrane domain in the lipid bilayer. In some embodiments, one or more cysteine residues are present in the transmembrane region of the transmembrane domain. In some embodiments, one or more cysteine residues are present in the cytoplasmic region of the transmembrane domain. In some embodiments, the cytoplasmic region of the transmembrane domain comprises positively charged amino acids. In some embodiments, the cytoplasmic region of the transmembrane domain comprises the amino acids arginine, serine, and lysine.

[0411] In some embodiments, the transmembrane region of the transmembrane domain comprises hydrophobic amino acid residues. In some embodiments, the transmembrane domain

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of the CAR comprises an artificial hydrophobic sequence. For example, a triplet of phenylalanine, tryptophan and valine may be present at the C terminus of the transmembrane domain. In some embodiments, the transmembrane region comprises mostly hydrophobic amino acid residues, such as alanine, leucine, isoleucine, methionine, phenylalanine, tryptophan, or valine. In some embodiments, the transmembrane region is hydrophobic. In some embodiments, the transmembrane region comprises a poly-leucine-alanine sequence. The hydropathy, or hydrophobic or hydrophilic characteristics of a protein or protein segment, can be assessed by any method known in the art, for example the Kyte and Doolittle hydropathy analysis.

Intracellular signaling domain

[0412] The CARs of the present application comprise an intracellular signaling domain. The intracellular signaling domain is responsible for activation of at least one of the normal effector functions of the immune effector cell expressing the CARs. The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term "cytoplasmic signaling domain" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While usually the entire cytoplasmic signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the cytoplasmic signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term cytoplasmic signaling domain is thus meant to include any truncated portion of the cytoplasmic signaling domain sufficient to transduce the effector function signal.

[0413] In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell. In some embodiments, the CAR comprises an intracellular signaling domain consisting essentially of a primary intracellular signaling domain of an immune effector cell. "Primary intracellular signaling domain" refers to cytoplasmic signaling sequence that acts in a stimulatory manner to induce immune effector functions. In some embodiments, the primary intracellular signaling domain contains a signaling motif known as immunoreceptor tyrosine-based activation motif, or ITAM. An "ITAM," as used herein, is a conserved protein motif that is generally present in the tail portion of signaling molecules expressed in many immune cells. The motif may comprises two repeats of the amino acid sequence YxxL/I separated by 6-8 amino acids, wherein each x is independently any amino

acid, producing the conserved motif YxxL/Ix(6-8)YxxL/I. ITAMs within signaling molecules are important for signal transduction within the cell, which is mediated at least in part by phosphorylation of tyrosine residues in the ITAM following activation of the signaling molecule. ITAMs may also function as docking sites for other proteins involved in signaling pathways. Exemplary ITAM-containing primary cytoplasmic signaling sequences include those derived from CD3ζ, FcR gamma(FCER1G), FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

[0414] In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain consists of the cytoplasmic signaling domain of CD3ζ. In some embodiments, the primary intracellular signaling domain is a cytoplasmic signaling domain of wildtype CD3ζ. In some embodiments, the primary intracellular signaling domain of wildtype CD3ζ comprises the amino acid sequence of SEQ ID NO: 140. In some embodiments, the primary intracellular signaling domain of wildtype CD3ζ is encoded by the nucleic acid of SEQ ID NO: 142. In some embodiments, the primary intracellular signaling domain is a functional mutant of the cytoplasmic signaling domain of CD3ζ containing one or more mutations, such as Q65K. In some embodiments, the primary intracellular signaling domain of mutant CD3ζ comprises the amino acid sequence of SEQ ID NO: 141. In some embodiments, the primary intracellular signaling domain of mutant CD3ζis encoded by the nucleic acid of SEQ ID NO: 143.

Co-stimulatory signaling domain

[0415] Many immune effector cells require co-stimulation, in addition to stimulation of an antigen-specific signal, to promote cell proliferation, differentiation and survival, as well as to activate effector functions of the cell. In some embodiments, the CAR comprises at least one co-stimulatory signaling domain. The term "co-stimulatory signaling domain," as used herein, refers to at least a portion of a protein that mediates signal transduction within a cell to induce an immune response such as an effector function. The co-stimulatory signaling domain of the chimeric receptor described herein can be a cytoplasmic signaling domain from a co-stimulatory protein, which transduces a signal and modulates responses mediated by immune cells, such as T cells, NK cells, macrophages, neutrophils, or eosinophils. "Co-stimulatory signaling domain" can be the cytoplasmic portion of a co-stimulatory molecule. The term "co-stimulatory molecule"

refers to a cognate binding partner on an immune cell (such as T cell) that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the immune cell, such as, but not limited to, proliferation and survival.

[0416] In some embodiments, the intracellular signaling domain comprises a single co-stimulatory signaling domain. In some embodiments, the intracellular signaling domain comprises two or more (such as about any of 2, 3, 4, or more) co-stimulatory signaling domains. In some embodiments, the intracellular signaling domain comprises two or more of the same co-stimulatory signaling domains, for example, two copies of the co-stimulatory signaling domain of CD28. In some embodiments, the intracellular signaling domain comprises two or more co-stimulatory signaling domains from different co-stimulatory proteins, such as any two or more co-stimulatory proteins described herein. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3ζ) and one or more co-stimulatory signaling domains. In some embodiments, the one or more co-stimulatory signaling domains and the primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3ζ) are fused to each other via optional peptide linkers. The primary intracellular signaling domain, and the one or more co-stimulatory signaling domains may be arranged in any suitable order. In some embodiments, the one or more co-stimulatory signaling domains are located between the transmembrane domain and the primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3ζ). Multiple co-stimulatory signaling domains may provide additive or synergistic stimulatory effects.

[0417] Activation of a co-stimulatory signaling domain in a host cell (*e.g.*, an immune cell) may induce the cell to increase or decrease the production and secretion of cytokines, phagocytic properties, proliferation, differentiation, survival, and/or cytotoxicity. The co-stimulatory signaling domain of any co-stimulatory molecule may be compatible for use in the CARs described herein. The type(s) of co-stimulatory signaling domain is selected based on factors such as the type of the immune effector cells in which the effector molecules would be expressed (*e.g.*, T cells, NK cells, macrophages, neutrophils, or eosinophils) and the desired immune effector function (*e.g.*, ADCC effect). Examples of co-stimulatory signaling domains for use in the CARs can be the cytoplasmic signaling domain of co-stimulatory proteins, including, without limitation, members of the B7/CD28 family (*e.g.*, B7-1/CD80, B7-2/CD86, B7-H1/PD-L1, B7-H2, B7-H3, B7-H4, B7-H6, B7-H7, BTLA/CD272, CD28, CTLA-4, Gi24/VISTA/B7-H5,

ICOS/CD278, PD- 1, PD-L2/B7-DC, and PDCD6); members of the TNF superfamily (*e.g.*,4-1BB/TNFSF9/CD137, 4-1BB Ligand/TNFSF9, BAFF/BLyS/TNFSF13B, BAFF R/TNFRSF13C, CD27/TNFRSF7, CD27 Ligand/TNFSF7, CD30/TNFRSF8, CD30 Ligand/TNFSF8, CD40/TNFRSF5, CD40/TNFSF5, CD40 Ligand/TNFSF5, DR3/TNFRSF25, GITR/TNFRSF18, GITR Ligand/TNFSF18, HVEM/TNFRSF14, LIGHT/TNFSF14, Lymphotoxin-alpha/TNF-beta, OX40/TNFRSF4, OX40 Ligand/TNFSF4, RELT/TNFRSF19L, TACI/TNFRSF13B, TL1A/TNFSF15, TNF-alpha, and TNF RII/TNFRSF1B); members of the SLAM family (*e.g.*, 2B4/CD244/SLAMF4, BLAME/SLAMF8, CD2, CD2F-10/SLAMF9, CD48/SLAMF2, CD58/LFA-3, CD84/SLAMF5, CD229/SLAMF3, CRACC/SLAMF7, NTB-A/SLAMF6, and SLAM/CD150); and any other co-stimulatory molecules, such as CD2, CD7, CD53, CD82/Kai-1, CD90/Thy1, CD96, CD160, CD200, CD300a/LMIR1, HLA Class I, HLA- DR, Ikaros, Integrin alpha 4/CD49d, Integrin alpha 4 beta 1, Integrin alpha 4 beta 7/LPAM-1, LAG-3, TCL1A, TCL1B, CRTAM, DAP12, Dectin-1/CLEC7A, DPPIV/CD26, EphB6, TIM-1/KIM-1/HAVCR, TIM-4, TSLP, TSLP R, lymphocyte function associated antigen-1 (LFA-1), and NKG2C.

[0418] In some embodiments, the one or more co-stimulatory signaling domains are selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, CD3, lymphocyte function-associated antigen-1(LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and ligands that specially bind to CD83.

[0419] In some embodiments, the intracellular signaling domain in the CAR of the present application comprises a co-stimulatory signaling domain derived from CD28. In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3ζ and a co-stimulatory signaling domain of CD28. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain of CD28 comprising the amino acid sequence of SEQ ID NO: 136. In some embodiments, the co-stimulatory signaling domain of CD28 is encoded by the nucleic acid sequence of SEQ ID NO: 138. In some embodiments, the intracellular signaling domain is encoded by the nucleic acid sequence of SEQ ID NO: 228.

[0420] In some embodiments, the intracellular signaling domain in the CAR of the present application comprises a co-stimulatory signaling domain derived from CD137 (*i.e.*, 4-1BB). In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3ζ and a co-stimulatory signaling domain of CD137. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain of CD137

comprising the amino acid sequence of SEQ ID NO: 137. In some embodiments, the co-stimulatory signaling domain of CD137 is encoded by the nucleic acid sequence of SEQ ID NO: 139.

[0421] In some embodiments, the intracellular signaling domain in the CAR of the present application comprises a co-stimulatory signaling domain of CD28 and a co-stimulatory signaling domain of CD137. In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3ζ, a co-stimulatory signaling domain of CD28, and a co-stimulatory signaling domain of CD137. In some embodiments, the intracellular signaling domain comprises a polypeptide comprising from the N-terminus to the C-terminus: a co-stimulatory signaling domain of CD28, a co-stimulatory signaling domain of CD137, and a cytoplasmic signaling domain of CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain of CD28 comprising the amino acid sequence of SEQ ID NO: 136. In some embodiments, the co-stimulatory signaling domain of CD28 is encoded by the nucleic acid sequence of SEQ ID NO: 138. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain of CD137 comprising the amino acid sequence of SEQ ID NO: 137. In some embodiments, the co-stimulatory signaling domain of CD137 is encoded by the nucleic acid sequence of SEQ ID NO: 139.

[0422] Also within the scope of the present disclosure are variants of any of the co-stimulatory signaling domains described herein, such that the co-stimulatory signaling domain is capable of modulating the immune response of the immune cell. In some embodiments, the co-stimulatory signaling domains comprises up to 10 amino acid residue variations (*e.g.*, 1, 2, 3, 4, 5, or 8) as compared to a wild-type counterpart. Such co-stimulatory signaling domains comprising one or more amino acid variations may be referred to as variants. Mutation of amino acid residues of the co-stimulatory signaling domain may result in an increase in signaling transduction and enhanced stimulation of immune responses relative to co-stimulatory signaling domains that do not comprise the mutation. Mutation of amino acid residues of the co-stimulatory signaling domain may result in a decrease in signaling transduction and reduced stimulation of immune responses relative to co-stimulatory signaling domains that do not comprise the mutation.

Hinge region

[0423] The CARs of the present application may comprise a hinge domain that is located between the extracellular antigen binding domain and the transmembrane domain. A hinge domain is an amino acid segment that is generally found between two domains of a protein and may allow for flexibility of the protein and movement of one or both of the domains relative to one another. Any amino acid sequence that provides such flexibility and movement of the extracellular antigen binding domain relative to the transmembrane domain of the effector molecule can be used.

[0424] The hinge domain may contain about 10-100 amino acids, *e.g.*, about any one of 15-75 amino acids, 20-50 amino acids, or 30-60 amino acids. In some embodiments, the hinge domain may be at least about any one of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, or 75 amino acids in length.

[0425] In some embodiments, the hinge domain is a hinge domain of a naturally occurring protein. Hinge domains of any protein known in the art to comprise a hinge domain are compatible for use in the chimeric receptors described herein. In some embodiments, the hinge domain is at least a portion of a hinge domain of a naturally occurring protein and confers flexibility to the chimeric receptor. In some embodiments, the hinge domain is derived from CD8α. In some embodiments, the hinge domain is a portion of the hinge domain of CD8α, *e.g.*, a fragment containing at least 15 (*e.g.*, 20, 25, 30, 35, or 40) consecutive amino acids of the hinge domain of CD8α. In some embodiments, the hinge domain of CD8α comprises the amino acid sequence of SEQ ID NO: 130. In some embodiments, the hinge domain of CD8α is encoded by the nucleic acid sequence of SEQ ID NO: 131.

[0426] Hinge domains of antibodies, such as an IgG, IgA, IgM, IgE, or IgD antibodies, are also compatible for use in the pH-dependent chimeric receptor systems described herein. In some embodiments, the hinge domain is the hinge domain that joins the constant domains CH1 and CH2 of an antibody. In some embodiments, the hinge domain is of an antibody and comprises the hinge domain of the antibody and one or more constant regions of the antibody. In some embodiments, the hinge domain comprises the hinge domain of an antibody and the CH3 constant region of the antibody. In some embodiments, the hinge domain comprises the hinge domain of an antibody and the CH2 and CH3 constant regions of the antibody. In some embodiments, the antibody is an IgG, IgA, IgM, IgE, or IgD antibody. In some embodiments, the

antibody is an IgG antibody. In some embodiments, the antibody is an IgG1, IgG2, IgG3, or IgG4 antibody. In some embodiments, the hinge region comprises the hinge region and the CH2 and CH3 constant regions of an IgG1 antibody. In some embodiments, the hinge region comprises the hinge region and the CH3 constant region of an IgG1 antibody.

[0427] Non-naturally occurring peptides may also be used as hinge domains for the chimeric receptors described herein. In some embodiments, the hinge domain between the C-terminus of the extracellular ligand-binding domain of an Fc receptor and the N- terminus of the transmembrane domain is a peptide linker, such as a (GxS)n linker, wherein x and n, independently can be an integer between 3 and 12, including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more.

Signal peptide

[0428] The CARs of the present application may comprise a signal peptide (also known as a signal sequence) at the N-terminus of the polypeptide. In general, signal peptides are peptide sequences that target a polypeptide to the desired site in a cell. In some embodiments, the signal peptide targets the effector molecule to the secretory pathway of the cell and will allow for integration and anchoring of the effector molecule into the lipid bilayer. Signal peptides including signal sequences of naturally occurring proteins or synthetic, non-naturally occurring signal sequences, which are compatible for use in the CARs described herein will be evident to one of skill in the art. In some embodiments, the signal peptide is derived from a molecule selected from the group consisting of CD8α, GM-CSF receptor α, and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8α. In some embodiments, the signal peptide of CD8α comprises the amino acid sequence of SEQ ID NO: 127. In some embodiments, the signal peptide of CD8α is encoded by the nucleic acid sequence of SEQ ID NO: 128 or SEQ ID NO: 129.

IV. Engineered immune effector cells

[0429] Further provided in the present application are host cells (such as immune effector cells) comprising any one of the CARs described herein.

[0430] Thus, in some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a multispecific (such as bispecific) chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a

first single-domain antibody (sdAb) specifically binding to a first antigen (such as a first tumor antigen) and a second single-domain antibody (sdAb) specifically binding to a second antigen (such as a second tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first antigen is different from the second antigen. In some embodiments, the first antigen and/or the second antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first single-domain antibody and the second single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multispecific CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multispecific CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling

domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0431] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a BCMA × CD38 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA single-domain antibody and an anti-CD38 single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-BCMA sdAb and/or the anti-CD38 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA single-domain antibody and the anti-CD38 single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-BCMA sdAb is fused at the N-terminus of the anti-CD38 sdAb. In some embodiments, the anti-BCMA sdAb is fused at the C-terminus of the anti-CD38 sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA × CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane

domain. In some embodiments, the BCMA × CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the anti-BCMA single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:7, a CDR2 comprising the amino acid sequence of SEQ ID NO:18, and a CDR3 comprising the amino acid sequence of SEQ ID NO:29. In some embodiments, the anti-CD38 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:44, a CDR2 comprising the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:68. In some embodiments, the BCMA × CD38 CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 207-216. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0432] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a CD19 × CD20 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 single-domain antibody and an anti-CD20 single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD19 sdAb and/or the anti-CD20 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD20 single-domain antibody and anti-CD20 single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-CD19 sdAb is fused at the N-terminus of the anti-CD20 sdAb. In some embodiments, the anti-CD19 sdAb is fused at the C-terminus of the anti-CD20 sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular

signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the anti-CD19 single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the anti-CD20 antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:4, a CDR2 comprising the amino acid sequence of SEQ ID NO:5, and a CDR3 comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, the CD19 × CD20 CAR comprises the amino acid sequence of SEQ ID NO: 206. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0433] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a CD19 × CD22 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 single-domain antibody and an anti-CD22 single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD19 sdAb and/or the anti-CD22 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD22 single-domain antibody and anti-

CD22 single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-CD19 sdAb is fused at the N-terminus of the anti-CD22 sdAb. In some embodiments, the anti-CD19 sdAb is fused at the C-terminus of the anti-CD22 sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × CD22 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × CD22 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the anti-CD19 single-domain antibody comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0434] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a CD19 × BCMA CAR comprising a polypeptide comprising: (a) an

extracellular antigen binding domain comprising an anti-CD19 single-domain antibody and an anti-BCMA single-domain antibody; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-CD19 sdAb and/or the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA single-domain antibody and anti-BCMA single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the anti-CD19 sdAb is fused at the N-terminus of the anti-BCMA sdAb. In some embodiments, the anti-CD19 sdAb is fused at the C-terminus of the anti-BCMA sdAb. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 144-151. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 × BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 × BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the anti-CD19 single-domain antibody comprises a

CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0435] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a multivalent chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a plurality of single-domain antibodies (sdAbs) specifically binding to an antigen (such as a tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the plurality of sdAbs is camelid, chimeric, human, or humanized. In some embodiments, the plurality of single-domain antibodies is fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a

CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CAR is monospecific. In some embodiments, the multivalent CAR is multispecific, such as bispecific. In some embodiments, the multivalent CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 198-201.

[0436] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a multivalent chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first single-domain antibody specifically binding to a first epitope of an antigen (such as a tumor antigen), and a second single-domain antibody specifically binding to a second epitope of the antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope and the second epitope are different. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first single-domain antibody and the second single-domain antibody are fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the

polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0437] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a CD19 chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD19 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-CD19 sdAb comprises the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:2, and a CDR3 comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the anti-CD19 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD19 sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 76. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD19 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD19 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the CD19 CAR comprises the amino acid

sequence of SEQ ID NO: 248. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0438] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a CD20 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD20 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-CD20 sdAb comprises the amino acid sequence of SEQ ID NO:4, a CDR2 comprising the amino acid sequence of SEQ ID NO:5, and a CDR3 comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, the anti-CD20 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD20 sdAb further comprises an FR1 comprising the amino acid sequence of SEQ ID NO: 244, an FR2 comprising the amino acid sequence of SEQ ID NO: 245, an FR3 comprising the amino acid sequence of SEQ ID NO: 246, and/or an FR4 comprising the amino acid sequence of SEQ ID NO: 247. In some embodiments, the anti-CD20 sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 77. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD20 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD20 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3ζ. In some

embodiments, the CD20 CAR comprises the amino acid sequence of SEQ ID NO: 249. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0439] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a BCMA CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises any one of the following:

(12) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:18; and a CDR3 comprising the amino acid sequence of SEQ ID NO:29;

(13) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:19; and a CDR3 comprising the amino acid sequence of SEQ ID NO:30;

(14) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:20; and a CDR3 comprising the amino acid sequence of SEQ ID NO:31;

(15) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:21; and a CDR3 comprising the amino acid sequence of SEQ ID NO:32;

(16) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:22; and a CDR3 comprising the amino acid sequence of SEQ ID NO:33;

(17) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:23; and a CDR3 comprising the amino acid sequence of SEQ ID NO:34;

(18) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:24; and a CDR3 comprising the amino acid sequence of SEQ ID NO:35;

(19) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:25; and a CDR3 comprising the amino acid sequence of SEQ ID NO:36;

(20) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:26; and a CDR3 comprising the amino acid sequence of SEQ ID NO:37;

(21) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:27; and a CDR3 comprising the amino acid sequence of SEQ ID NO:38; or

(22) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:28; and a CDR3 comprising the amino acid sequence of SEQ ID NO:39.

In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence from the group consisting of SEQ ID NO:78-88. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal

peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ.In some embodiments, the BCMA CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 152-162. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0440] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a CD38 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-CD38 sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises any one of the following:

(13) a CDR1 comprising the amino acid sequence of SEQ ID NO:40; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:64;

(14) a CDR1 comprising the amino acid sequence of SEQ ID NO:41; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:65;

(15) a CDR1 comprising the amino acid sequence of SEQ ID NO:42; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:66;

(16) a CDR1 comprising the amino acid sequence of SEQ ID NO:43; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:67;

(17) a CDR1 comprising the amino acid sequence of SEQ ID NO:44; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:68;

(18) a CDR1 comprising the amino acid sequence of SEQ ID NO:45; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:69;

(19) a CDR1 comprising the amino acid sequence of SEQ ID NO:46; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:70;

(20) a CDR1 comprising the amino acid sequence of SEQ ID NO:47; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:71;

(21) a CDR1 comprising the amino acid sequence of SEQ ID NO:48; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:72;

(22) a CDR1 comprising the amino acid sequence of SEQ ID NO:49; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:73;

(23) a CDR1 comprising the amino acid sequence of SEQ ID NO:50; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:74; or

(24) a CDR1 comprising the amino acid sequence of SEQ ID NO:51; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:75.

In some embodiments, the anti-CD38 sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-CD38 sdAb comprises a V_HH domain comprising an amino acid sequence from the group consisting of SEQ ID NO:89-100. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the CD38 CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the CD38 CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-

terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the anti-CD38 sdAb, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, there is provided a CD38 CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 163-174. In some embodiments, the engineered immune effector cell is a T cell, an NK cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic.

[0441] Also provided are engineered immune effector cells comprising (or expressing) two or more different CARs. Any two or more of the CARs described herein may be expressed in combination. The CARs may target different antigens, thereby providing synergistic or additive effects. As the single-domain antibodies in the extracellular antigen binding domains of the CARs have only single antigen variable chains (such as heavy chains), such CAR-expresing cells do not have variable chain mispairing problems, as seen in engineered immune effector cells co-expressing two or more scFv-based CARs. Exemplary engineered immune effector cells co-expressing two V_HH-based CARs are illustrated in FIG. 1E. One of skill in the art would recognize that CARs based on other sdAbs or having other structures as described herein may be co-expressed in the engineered immune effector cells as well. The two or more CARs may be encoded on the same vector or different vectors.

[0442] The engineered immune effector cell may further express one or more therapeutic proteins and/or immunomodulators, such as immune checkpoint inhibitors. See, for example, International Patent Application NOs. PCT/CN2016/073489 and PCT/CN2016/087855.

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Vectors

[0443] The present application provides vectors for cloning and expressing any one of the CARs described herein. In some embodiments, the vector is suitable for replication and integration in eukaryotic cells, such as mammalian cells. In some embodiments, the vector is a viral vector. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, lentiviral vector, retroviral vectors, vaccinia vector, herpes simplex viral vector, and derivatives thereof. Viral vector technology is well known in the art and is described, for example, in Sambrook *et al.* (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals.

[0444] A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. The heterologous nucleic acid can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to the engineered mammalian cell *in vitro* or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In some embodiments, lentivirus vectors are used. In some embodiments, self-inactivating lentiviral vectors are used. For example, self-inactivating lentiviral vectors carrying the immunomodulator (such as immune checkpoint inhibitor) coding sequence and/or self-inactivating lentiviral vectors carrying chimeric antigen receptors can be packaged with protocols known in the art. The resulting lentiviral vectors can be used to transduce a mammalian cell (such as primary human T cells) using methods known in the art. Vectors derived from retroviruses such as lentivirus are suitable tools to achieve long-term gene transfer, because they allow long-term, stable integration of a transgene and its propagation in progeny cells. Lentiviral vectors also have low immunogenicity, and can transduce non-proliferating cells.

[0445] In some embodiments, the vector comprises any one of the nucleic acids encoding a CAR described herein. The nucleic acid can be cloned into the vector using any known molecular cloning methods in the art, including, for example, using restriction endonuclease sites and one or more selectable markers. In some embodiments, the nucleic acid is operably linked to a promoter. Varieties of promoters have been explored for gene expression in mammalian cells, and any of the promoters known in the art may be used in the present invention. Promoters may

be roughly categorized as constitutive promoters or regulated promoters, such as inducible promoters.

[0446] In some embodiments, the nucleic acid encoding the CAR is operably linked to a constitutive promoter. Constitutive promoters allow heterologous genes (also referred to as transgenes) to be expressed constitutively in the host cells. Exemplary constitutive promoters contemplated herein include, but are not limited to, Cytomegalovirus (CMV) promoters, human elongation factors-1alpha (hEF1α), ubiquitin C promoter (UbiC), phosphoglycerokinase promoter (PGK), simian virus 40 early promoter (SV40), and chicken β-Actin promoter coupled with CMV early enhancer (CAGG). The efficiencies of such constitutive promoters on driving transgene expression have been widely compared in a huge number of studies. For example, Michael C. Milone *et al* compared the efficiencies of CMV, hEF1α, UbiC and PGK to drive chimeric antigen receptor expression in primary human T cells, and concluded that hEF1α promoter not only induced the highest level of transgene expression, but was also optimally maintained in the CD4 and CD8 human T cells (Molecular Therapy, 17(8): 1453-1464 (2009)). In some embodiments, the nucleic acid encoding the CAR is operably linked to a hEF1α promoter.

[0447] In some embodiments, the nucleic acid encoding the CAR is operably linked to an inducible promoter. Inducible promoters belong to the category of regulated promoters. The inducible promoter can be induced by one or more conditions, such as a physical condition, microenvironment of the engineered immune effector cell, or the physiological state of the engineered immune effector cell, an inducer (*i.e.*, an inducing agent), or a combination thereof. In some embodiments, the inducing condition does not induce the expression of endogenous genes in the engineered mammalian cell, and/or in the subject that receives the pharmaceutical composition. In some embodiments, the inducing condition is selected from the group consisting of: inducer, irradiation (such as ionizing radiation, light), temperature (such as heat), redox state, tumor environment, and the activation state of the engineered mammalian cell.

[0448] In some embodiments, the vector also contains a selectable marker gene or a reporter gene to select cells expressing the CAR from the population of host cells transfected through lentiviral vectors. Both selectable markers and reporter genes may be flanked by appropriate regulatory sequences to enable expression in the host cells. For example, the vector may contain

transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the nucleic acid sequences.

[0449] In some embodiments, the vector comprises more than one nucleic acid encoding CARs. In some embodiments, the vector comprises a nucleic acid comprising a first nucleic acid sequence encoding a first CAR and a second nucleic acid sequence encoding a second CAR, wherein the first nucleic acid is operably linked to the second nucleic acid via a third nucleic acid sequence encoding a self-cleaving peptide. In some embodiments, the self-cleaving peptide is selected from the group consisting of T2A, P2A and F2A. In some embodiments, the T2A peptide has an amino acid sequence of SEQ ID NO: 254. In some embodiments, the T2A peptide is encoded by the nucleic acid sequence of SEQ ID NO: 255. In some embodiments, there is provided an isolated nucleic acid encoding a BCMA CAR and a CD38 CAR, comprising the nucleic acid sequence of SEQ ID NO: 239.

Immune effector cells

[0450] "Immune effector cells" are immune cells that can perform immune effector functions. In some embodiments, the immune effector cells express at least FcγRIII and perform ADCC effector function. Examples of immune effector cells which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells, neutrophils, and eosinophils.

[0451] In some embodiments, the immune effector cells are T cells. In some embodiments, the T cells are CD4+/CD8-, CD4-/CD8+, CD4+/CD8+, CD4-/CD8-, or combinations thereof. In some embodiments, the T cells produce IL-2, TFN, and/or TNF upon expressing the CAR and binding to the target cells, such as CD20+ or CD19+ tumor cells. In some embodiments, the CD8+ T cells lyse antigen-specific target cells upon expressing the CAR and binding to the target cells.

[0452] In some embodiments, the immune effector cells are NK cells. In other embodiments, the immune effector cells can be established cell lines, for example, NK-92 cells.

[0453] In some embodiments, the immune effector cells are differentiated from a stem cell, such as a hematopoietic stem cell, a pluripotent stem cell, an iPS, or an embryonic stem cell.

[0454] The engineered immune effector cells are prepared by introducing the CARs into the immune effector cells, such as T cells. In some embodiments, the CAR is introduced to the immune effector cells by transfecting any one of the isolated nucleic acids or any one of the

vectors described in Section III. In some embodiments, the CAR is introduced to the immune effector cells by inserting proteins into the cell membrane while passing cells through a microfluidic system, such as CELL SQUEEZE® (*see*, for example, U.S. Patent Application Publication No. 20140287509).

[0455] Methods of introducing vectors or isolated nucleic acids into a mammalian cell are known in the art. The vectors described can be transferred into an immune effector cell by physical, chemical, or biological methods.

[0456] Physical methods for introducing the vector into an immune effector cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. *See*, for example, Sambrook *et al.* (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. In some embodiments, the vector is introduced into the cell by electroporation.

[0457] Biological methods for introducing the vector into an immune effector cell include the use of DNA and RNA vectors. Viral vectors have become the most widely used method for inserting genes into mammalian, *e.g.*, human cells.

[0458] Chemical means for introducing the vector into an immune effector cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle *in vitro* is a liposome (*e.g.*, an artificial membrane vesicle).

[0459] In some embodiments, RNA molecules encoding any of the CARs described herein may be prepared by a conventional method (*e.g.*, *in vitro* transcription) and then introduced into the immune effector cells via known methods such as mRNA electroporation. See, *e.g.*, Rabinovich *et al.*, Human Gene Therapy 17:1027-1035.

[0460] In some embodiments, the transduced or transfected immune effector cell is propagated ex vivo after introduction of the vector or isolated nucleic acid. In some embodiments, the transduced or transfected immune effector cell is cultured to propagate for at least about any of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, or 14 days. In some embodiments, the transduced or transfected immune effector cell is further evaluated or screened to select the engineered mammalian cell.

[0461] Reporter genes may be used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, *e.g.*, enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (*e.g.*, Ui-Tei *et al.* FEBS Letters 479: 79-82 (2000)). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially.

[0462] Other methods to confirm the presence of the nucleic acid encoding the CARs in the engineered immune effector cell, include, for example, molecular biological assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; biochemical assays, such as detecting the presence or absence of a particular peptide, *e.g.*, by immunological methods (such as ELISAs and Western blots).

1. Sources of T Cells

[0463] Prior to expansion and genetic modification of the T cells, a source of T cells is obtained from an individual. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In some embodiments, any number of T cell lines available in the art, may be used. In some embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. In some embodiments, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In some embodiments, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In some embodiments, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Again, surprisingly, initial activation steps in the absence of calcium lead to magnified activation. As

those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics Cell Saver 5) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca^{2+}-free, Mg^{2+}-free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

[0464] In some embodiments, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient or by counterflow centrifugal elutriation. A specific subpopulation of T cells, such as CD3+, CD28+, CD4+, CD8+, CD45RA+, and CD45RO+T cells, can be further isolated by positive or negative selection techniques. For example, in some embodiments, T cells are isolated by incubation with anti-CD3/anti-CD28 (i.e., 3×28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, for a time period sufficient for positive selection of the desired T cells. In some embodiments, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In some embodiments, the time period is 10 to 24 hours. In some embodiments, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immune-compromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells. Thus, by simply shortening or lengthening the time T cells are allowed to bind to the CD3/CD28 beads and/or by increasing or decreasing the ratio of beads to T cells (as described further herein), subpopulations of T cells can be preferentially selected for or against at culture initiation or at other time points during the process. Additionally, by increasing or decreasing the ratio of anti-CD3 and/or anti-CD28 antibodies on the beads or other surface, subpopulations of T cells can be preferentially selected for or against at culture initiation or at other desired time points. The skilled artisan would recognize that multiple rounds of selection

can also be used. In some embodiments, it may be desirable to perform the selection procedure and use the "unselected" cells in the activation and expansion process. "Unselected" cells can also be subjected to further rounds of selection.

[0465] Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In certain embodiments, it may be desirable to enrich for or positively select for regulatory T cells which typically express CD4+, CD25+, CD62Lhi, GITR+, and FoxP3+. Alternatively, in certain embodiments, T regulatory cells are depleted by anti-C25 conjugated beads or other similar method of selection.

[0466] For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (*e.g.*, particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (i.e., leukemic blood, tumor tissue, etc.). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[0467] In some embodiments, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (*e.g.*, particles such as beads),

interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In some embodiments, the concentration of cells used is 5×10^6/ml. In some embodiments, the concentration used can be from about 1×10^5/ml to 1×10^6/ml, and any integer value in between.

[0468] In some embodiments, the cells may be incubated on a rotator for varying lengths of time at varying speeds at either 2-10°C, or at room temperature.

[0469] T cells for stimulation can also be frozen after a washing step. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or culture media containing 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin and 7.5% DMSO, or 31.25% Plasmalyte-A, 31.25% Dextrose 5%, 0.45% NaCl, 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin, and 7.5% DMSO or other suitable cell freezing media containing for example, Hespan and PlasmaLyte A, the cells then are frozen to −80°C. at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at −20°C. or in liquid nitrogen.

[0470] In some embodiments, cryopreserved cells are thawed and washed as described herein and allowed to rest for one hour at room temperature prior to activation.

[0471] Also contemplated in the present application is the collection of blood samples or apheresis product from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as T cells, isolated and frozen for later use in T cell therapy for any number of diseases or conditions that would benefit from T cell therapy, such as those described herein. In one embodiment a blood sample or an apheresis is taken from a generally healthy subject. In certain embodiments, a blood sample or an apheresis is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet developed a

disease, and the cells of interest are isolated and frozen for later use. In certain embodiments, the T cells may be expanded, frozen, and used at a later time. In certain embodiments, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but prior to any treatments. In a further embodiment, the cells are isolated from a blood sample or an apheresis from a subject prior to any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, cytoxan, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun 73:316-321, 1991; Bierer et al., Curr. Opin. Immun. 5:763-773, 1993). In a further embodiment, the cells are isolated for a patient and frozen for later use in conjunction with (*e.g.*, before, simultaneously or following) bone marrow or stem cell transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cells are isolated prior to and can be frozen for later use for treatment following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan.

[0472] In some embodiments, T cells are obtained from a patient directly following treatment. In this regard, it has been observed that following certain cancer treatments, in particular treatments with drugs that damage the immune system, shortly after treatment during the period when patients would normally be recovering from the treatment, the quality of T cells obtained may be optimal or improved for their ability to expand ex vivo. Likewise, following ex vivo manipulation using the methods described herein, these cells may be in a preferred state for enhanced engraftment and in vivo expansion. Thus, it is contemplated within the context of the present invention to collect blood cells, including T cells, dendritic cells, or other cells of the hematopoietic lineage, during this recovery phase. Further, in certain embodiments, mobilization (for example, mobilization with GM-CSF) and conditioning regimens can be used to create a condition in a subject wherein repopulation, recirculation, regeneration, and/or expansion of particular cell types is favored, especially during a defined window of time following therapy.

Illustrative cell types include T cells, B cells, dendritic cells, and other cells of the immune system.

2. Activation and Expansion of T Cells

[0473] Whether prior to or after genetic modification of the T cells with the CARs described herein, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Pat. Nos. 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005.

[0474] Generally, T cells can be expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the T cells. In particular, T cell populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (*e.g.*, bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4+ T cells or CD8+ T cells, an anti-CD3 antibody and an anti-CD28 antibody. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, Besancon, France) can be used as can other methods commonly known in the art (Berg et al., Transplant Proc. 30(8):3975-3977, 1998; Haanen et al., J. Exp. Med. 190(9):13191328, 1999; Garland et al., J. Immunol Meth. 227(1-2):53-63, 1999).

[0475] In some embodiments, the primary stimulatory signal and the co-stimulatory signal for the T cell may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When coupled to a surface, the agents may be coupled to the same surface (i.e., in "cis" formation) or to separate surfaces (i.e., in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In one embodiment, the agent providing the co-stimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In certain embodiments, both agents can be in solution. In another embodiment, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing Fc receptors or an

antibody or other binding agent which will bind to the agents. In this regard, see for example, U.S. Patent Application Publication Nos. 20040101519 and 20060034810 for artificial antigen presenting cells (aAPCs) that are contemplated for use in activating and expanding T cells in the present invention.

[0476] In some embodiments, the T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In an alternative embodiment, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In a further embodiment, the beads and cells are first concentrated by application of a force, such as a magnetic force, resulting in increased ligation of cell surface markers, thereby inducing cell stimulation.

[0477] By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3×28 beads) to contact the T cells. In one embodiment the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, preferably PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (i.e., 100%) may comprise the target cell of interest. Accordingly, any cell number is within the context of the present invention. In certain embodiments, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in one embodiment, a concentration of about 2 billion cells/ml is used. In another embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells. Such populations of cells may have therapeutic value and would be desirable to obtain in certain embodiments. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[0478] In some embodiments, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may be cultured for 21 days. In one embodiment of the invention the beads and the T cells are cultured together for about eight days. In another embodiment, the beads and T cells are cultured together for 2-3 days. Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell culture include an appropriate media (*e.g.*, Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGFβ, and TNF-α or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, α-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37 °C) and atmosphere (*e.g.*, air plus 5% CO_2). T cells that have been exposed to varied stimulation times may exhibit different characteristics. For example, typical blood or apheresed peripheral blood mononuclear cell products have a helper T cell population (TH, CD4+) that is greater than the cytotoxic or suppressor T cell population (TC, CD8). Ex vivo expansion of T cells by stimulating CD3 and CD28 receptors produces a population of T cells that prior to about days 8-9 consists predominately of TH cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of TC cells. Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of TH cells may be advantageous. Similarly, if an antigen-specific subset of TC cells has been isolated it may be beneficial to expand this subset to a greater degree.

[0479] Further, in addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated T cell product for specific purposes.

V. Pharmaceutical compositions

[0480] Further provided by the present application are pharmaceutical compositions comprising any one of the single-domain antibodies (such as anti-CD19, anti-CD20, anti-BCMA, or anti-CD38 sdAbs), or any one of the engineered immune effector cells comprising any one of the CARs as described herein, and a pharmaceutically acceptable carrier. Pharmaceutical compositions can be prepared by mixing a single-domain antibody, or a plurality of engineered immune effector cells having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions.

[0481] Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers, antioxidants including ascorbic acid, methionine, Vitamin E, sodium metabisulfite; preservatives, isotonicifiers, stabilizers, metal complexes (*e.g.* Zn-protein complexes); chelating agents such as EDTA and/or non-ionic surfactants.

[0482] Buffers are used to control the pH in a range which optimizes the therapeutic effectiveness, especially if stability is pH dependent. Buffers are preferably present at concentrations ranging from about 50 mM to about 250 mM. Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof. For example, citrate, phosphate, succinate, tartrate, fumarate, gluconate, oxalate, lactate, acetate. Additionally, buffers may comprise histidine and trimethylamine salts such as Tris.

[0483] Preservatives are added to retard microbial growth, and are typically present in a range from 0.2%-1.0% (w/v). Suitable preservatives for use with the present invention include octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium halides (*e.g.*, chloride, bromide, iodide), benzethonium chloride; thimerosal, phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol, 3-pentanol, and m-cresol.

[0484] Tonicity agents, sometimes known as "stabilizers" are present to adjust or maintain the tonicity of liquid in a composition. When used with large, charged biomolecules such as proteins and antibodies, they are often termed "stabilizers" because they can interact with the charged

groups of the amino acid side chains, thereby lessening the potential for inter and intra-molecular interactions. Tonicity agents can be present in any amount between 0.1% to 25% by weight, preferably 1 to 5%, taking into account the relative amounts of the other ingredients. Preferred tonicity agents include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol.

[0485] Additional excipients include agents which can serve as one or more of the following: (1) bulking agents, (2) solubility enhancers, (3) stabilizers and (4) and agents preventing denaturation or adherence to the container wall. Such excipients include: polyhydric sugar alcohols (enumerated above); amino acids such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, threonine, etc.; organic sugars or sugar alcohols such as sucrose, lactose, lactitol, trehalose, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (*e.g.*, inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thio sulfate; low molecular weight proteins such as human serum albumin, bovine serum albumin, gelatin or other immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides (*e.g.*, xylose, mannose, fructose, glucose; disaccharides (*e.g.*, lactose, maltose, sucrose); trisaccharides such as raffinose; and polysaccharides such as dextrin or dextran.

[0486] Non-ionic surfactants or detergents (also known as "wetting agents") are present to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the active therapeutic protein or antibody. Non-ionic surfactants are present in a range of about 0.05 mg/ml to about 1.0 mg/ml, preferably about 0.07 mg/ml to about 0.2 mg/ml.

[0487] Suitable non-ionic surfactants include polysorbates (20, 40, 60, 65, 80, etc.), polyoxamers (184, 188, etc.), PLURONIC® polyols, TRITON®, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.), lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, sucrose fatty acid ester, methyl celluose and carboxymethyl cellulose. Anionic detergents that can be used include sodium lauryl sulfate, dioctyle sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents include benzalkonium chloride or benzethonium chloride.

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CLAIMS

1. A chimeric antigen receptor (CAR) comprising a polypeptide comprising:
   (a) an extracellular antigen binding domain comprising a first single-domain antibody (sdAb) specifically binding to a first antigen and a second sdAb specifically binding to a second antigen, wherein each of the first and second sdAbs is a V_HH domain;
   (b) a transmembrane domain; and
   (c) an intracellular signaling domain.

2. The CAR of claim 1, wherein the first sdAb is positioned N-terminal of the second sdAb.

3. The CAR of claim 1, wherein the first sdAb is positioned C-terminal of the second sdAb.

4. The CAR of any one of claims 1-3, wherein the first sdAb and/or the second sdAb are camelid, chimeric, human, or humanized.

5. The CAR of any one of claims 1-4, wherein the first sdAb and the second sdAb are directly fused to each other via a peptide bond.

6. The CAR of any one of claims 1-4, wherein the first sdAb and the second sdAb are fused to each other via a peptide linker.

7. The CAR of claim 6, wherein the peptide linker is 1 to 50 amino acids long.

8. The CAR of any one of claims 1-7, wherein the first antigen and the second antigen are selected from the group consisting of CD19, CD20, CD22, CD33, CD38, B-cell maturation antigen (BCMA), CS1, receptor tyrosine kinase like orphan receptor 1 (ROR1), Glypican 3 (GPC3), CD123, interleukin-13 receptor (IL-13R), CD138, c-Met, epidermal growth factor receptor variant III (EGFRvIII), GD-2, New York esophageal squamous cell carcinoma 1 (NY-ESO-1), Melanoma-associated antigen 3 (MAGE A3), and glycolipid F77.

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9. The CAR of any one of claims 1-8, wherein the first sdAb is an anti-BCMA sdAb.

10. The CAR of any one of claims 1-8, wherein the first sdAb is an anti-CD38 sdAb.

11. The CAR of any one of claims 1-8, wherein the first sdAb is an anti-CD19 sdAb.

12. The CAR of any one of claims 1-8, wherein the first sdAb is an anti-CD20 sdAb.

13. The CAR of any one of claims 1-8, wherein the first sdAb is an anti-CD22 sdAb.

14. The CAR of any one of claims 1-13, wherein the first and second sdAbs are different.

15. The CAR of any one of claims 1-14, wherein the first antigen is different from the second antigen.

16. The CAR of any one of claims 1-13, which is a multispecific CAR.

17. The CAR of claim 16, wherein the multispecific CAR is a bispecific CAR.

18. The CAR of any one of claims 14-17, wherein the first sdAb is an anti-BCMA sdAb, and the second sdAb is an anti-CD38 sdAb.

19. The CAR of any one of claims 14-17, wherein the first sdAb is an anti-BCMA sdAb, and the second sdAb is an anti-CD19 sdAb.

20. The CAR of any one of claims 14-17, wherein the first sdAb is an anti-CD19 sdAb, and the second sdAb is an anti-CD20 sdAb.

21. The CAR of any one of claims 14-17, wherein the first sdAb is an anti-CD19 sdAb, and the second sdAb is an anti-CD22 sdAb.

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22. The CAR of any one of claims 1-13, wherein the first antigen is the same as the second antigen.

23. The CAR of any one of claims 1-7, wherein each of the first sdAb and the second sdAb is an anti-BCMA sdAb.

24. The CAR of claim 22 or 23, wherein the first sdAb and the second sdAb specifically bind to a same epitope.

25. The CAR of claim 22 or 23, wherein the first sdAb and the second sdAb specifically bind to different epitopes.

26. The CAR of any one of claims 1-7, wherein the first sdAb and/or the second sdAb is an anti-CD19 sdAb comprising a complementarity determining region 1 (CDR1) comprising the amino acid sequence of SEQ ID NO: 1, a CDR2 comprising the amino acid sequence of SEQ ID NO: 2, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 3.

27. The CAR of any one of claims 1-7, wherein the first sdAb and/or the second sdAb is an anti-CD20 sdAb comprising a complementarity determining region 1 (CDR1) comprising the amino acid sequence of SEQ ID NO: 4, a CDR2 comprising the amino acid sequence of SEQ ID NO: 5, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 6.

28. The CAR of any one of claims 1-7, wherein the first sdAb and/or the second sdAb is an anti-CD38 sdAb comprising a complementarity determining region (CDR) comprising any one of the following:
    (1) a CDR1 comprising the amino acid sequence of SEQ ID NO: 40; a CDR2 comprising the amino acid sequence of SEQ ID NO: 52; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 64;
    (2) a CDR1 comprising the amino acid sequence of SEQ ID NO: 41; a CDR2 comprising the amino acid sequence of SEQ ID NO: 53; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 65;

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(3) a CDR1 comprising the amino acid sequence of SEQ ID NO: 42; a CDR2 comprising the amino acid sequence of SEQ ID NO: 54; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 66;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO: 43; a CDR2 comprising the amino acid sequence of SEQ ID NO: 55; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 67;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO: 44; a CDR2 comprising the amino acid sequence of SEQ ID NO: 56; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 68;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO: 45; a CDR2 comprising the amino acid sequence of SEQ ID NO: 57; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 69;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO: 46; a CDR2 comprising the amino acid sequence of SEQ ID NO: 58; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 70;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO: 47; a CDR2 comprising the amino acid sequence of SEQ ID NO: 59; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 71;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO: 48; a CDR2 comprising the amino acid sequence of SEQ ID NO: 60; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 72;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO: 49; a CDR2 comprising the amino acid sequence of SEQ ID NO: 61; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 73;

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO: 50; a CDR2 comprising the amino acid sequence of SEQ ID NO: 62; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 74; or

(12) a CDR1 comprising the amino acid sequence of SEQ ID NO: 51; a CDR2 comprising the amino acid sequence of SEQ ID NO: 63; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 75.

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29. The CAR of any one of claims 1-28, wherein the transmembrane domain is from CD8α, CD4, CD28, CD137, CD80, CD86, CD152, or programmed cell death protein 1 (PD1).

30. The CAR of any one of claims 1-29, wherein the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell.

31. The CAR of claim 30, wherein the primary intracellular signaling domain is from CD3ζ.

32. The CAR of any one of claims 1-31, wherein the intracellular signaling domain comprises a co-stimulatory signaling domain.

33. The CAR of claim 32, wherein the co-stimulatory signaling domain is from a co-stimulatory molecule which is: CD27, CD28, CD137, OX40, CD30, CD40, CD3, lymphocyte function-associated antigen 1 (LFA-1), CD2, CD7, LIGHT, natural killer group 2C (NKG2C), B7-H3, CD83, or any combination thereof.

34. The CAR of any one of claims 1-33, further comprising a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain.

35. The CAR of any one of claims 1-34, further comprising a signal peptide located at the N-terminus of the polypeptide.

36. The CAR of claim 1, comprising the amino acid sequence of any one of SEQ ID NOs: 200, 201, and 206 to 216.

37. An isolated nucleic acid comprising a nucleic acid sequence encoding the CAR of any one of SEQ ID NOs: 200, 201, and 206 to 216.

38. A vector comprising the isolated nucleic acid of claim 37.

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39. An engineered immune effector cell comprising the CAR of any one of claims 1-35, the isolated nucleic acid of claim 37, or the vector of claim 38.

40. The engineered immune effector cell of claim 38, wherein the immune effector cell is a T cell.

41. A pharmaceutical composition comprising the engineered immune effector cell of claim 39 or 40, and a pharmaceutically acceptable carrier.

42. The pharmaceutical composition of claim 41, for use in the treatment of cancer.

43. Use of the pharmaceutical composition of claim 41 for the treatment of cancer.

44. Use of the pharmaceutical composition of claim 41 for the manufacture of a medicament for the treatment of cancer.

45. The CAR of any one of claims 1-36, for use in the treatment of cancer.

46. Use of the CAR of any one of claims 1-36 for the treatment of cancer.

47. Use of the CAR of any one of claims 1-36 for the manufacture of a medicament for the treatment of cancer.

48. The engineered immune effector cell of claim 39 or 40, for use in the treatment of cancer.

49. Use of the engineered immune effector cell of claim 39 or 40, for the treatment of cancer.

50. Use of the engineered immune effector cell of claim 39 or 40, for the manufacture of a medicament for the treatment of cancer.

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51. A polypeptide comprising a first single-domain antibody (sdAb) specifically binding to a first antigen and a second sdAb specifically binding to a second antigen, wherein each of the first and second sdAbs is a V_HH domain, for use in the production of an extracellular antigen binding domain of a candidate chimeric antigen receptor (CAR).

52. The polypeptide for use of claim 51, wherein the CAR is as defined in any one of claims 1-36.

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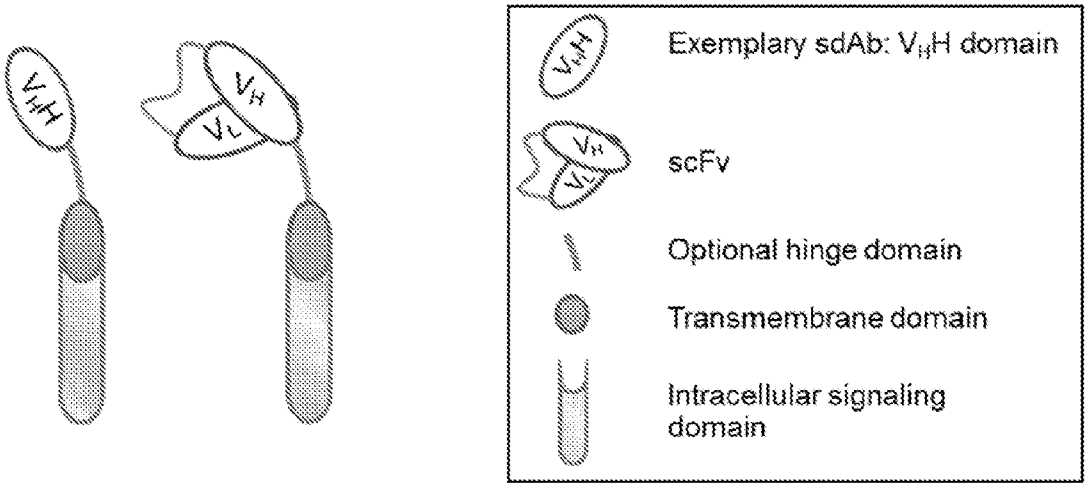

Exemplary sdAb: VHH domain
scFv
Optional hinge domain
Transmembrane domain
Intracellular signaling domain

FIG. 1A

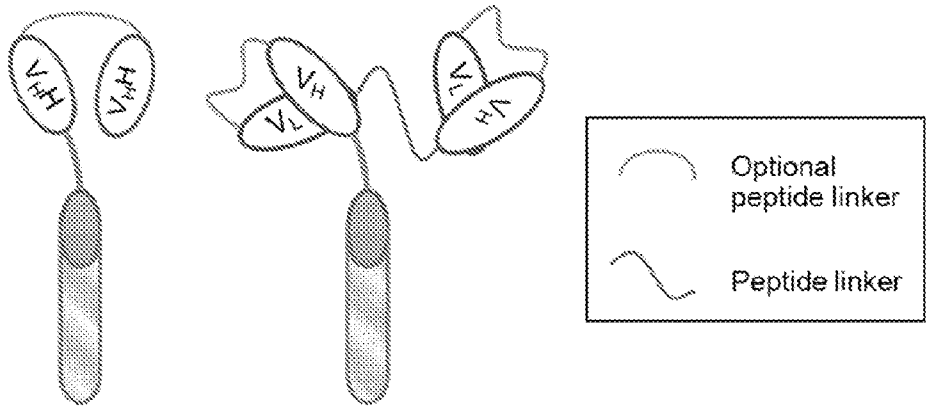

Optional peptide linker
Peptide linker

FIG. 1B

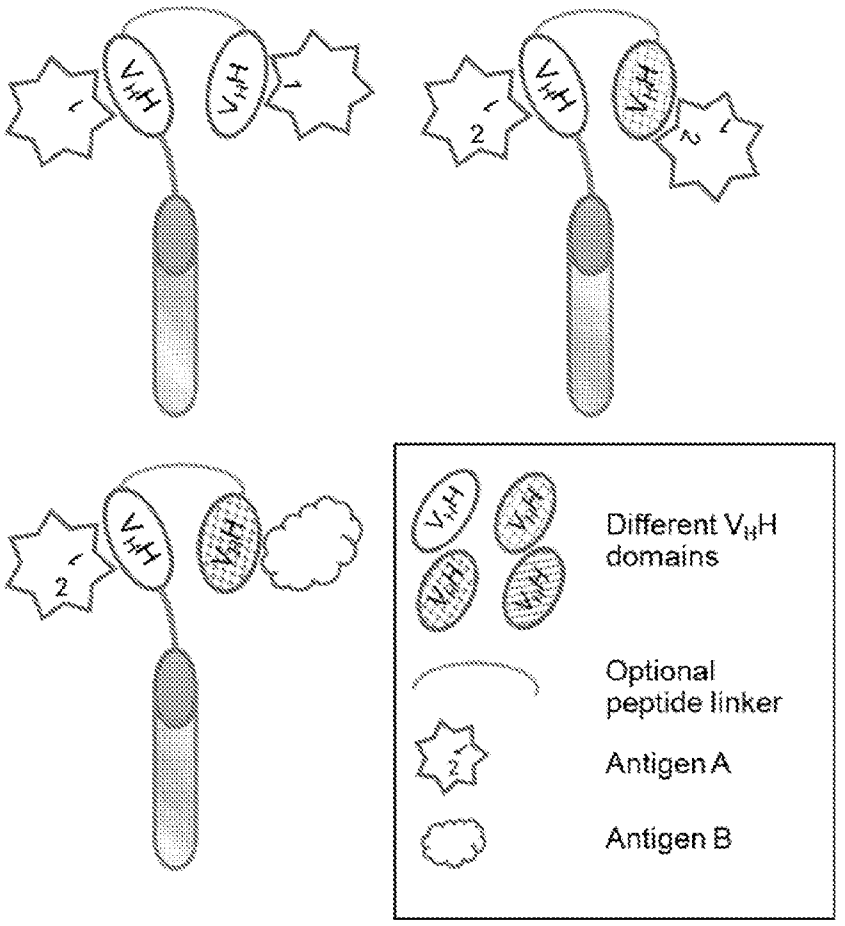

Different V_HH domains
Optional peptide linker
Antigen A
Antigen B

FIG. 1C

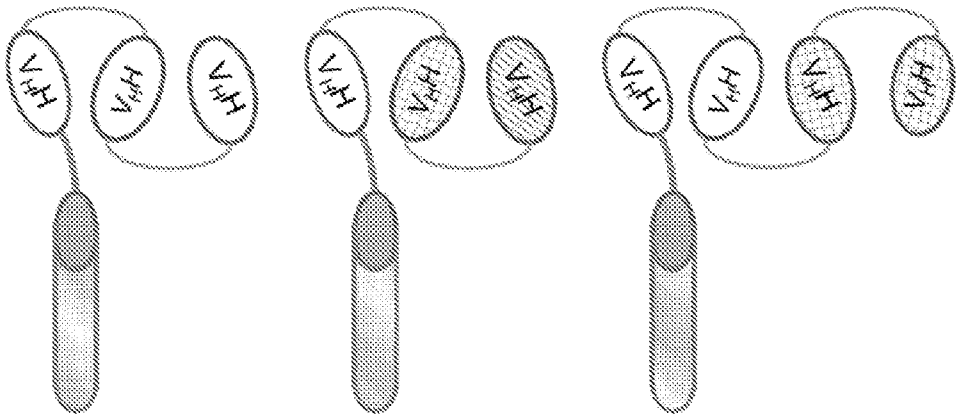

VHH
VHH
VHH
VHH
VHH
VHH
VHH
VHH
VHH
VHH

FIG. 1D

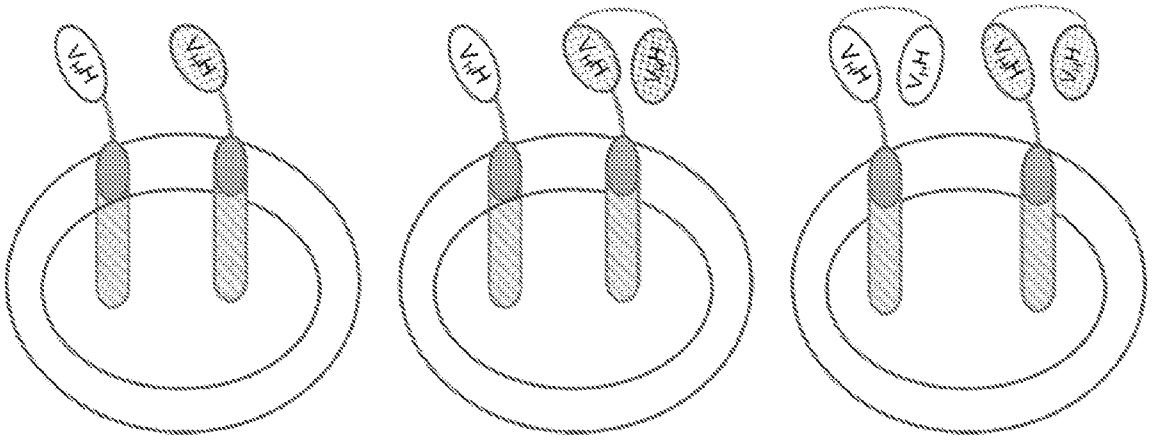

VHH
VHH
VHH
VHH
VHH
VHH
VHH
VHH
VHH

FIG. 1E

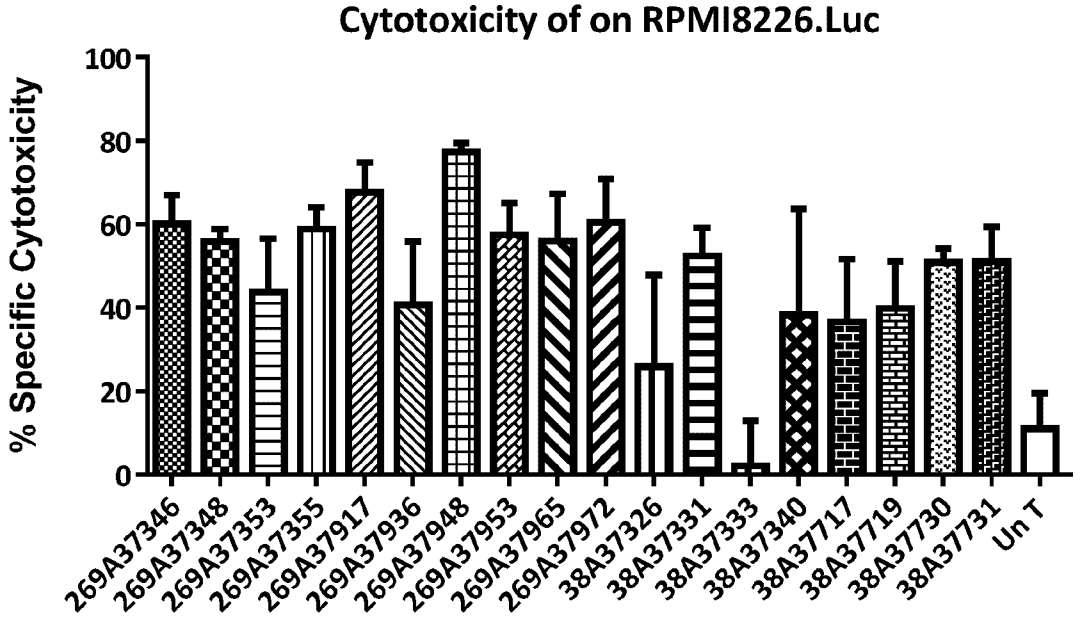
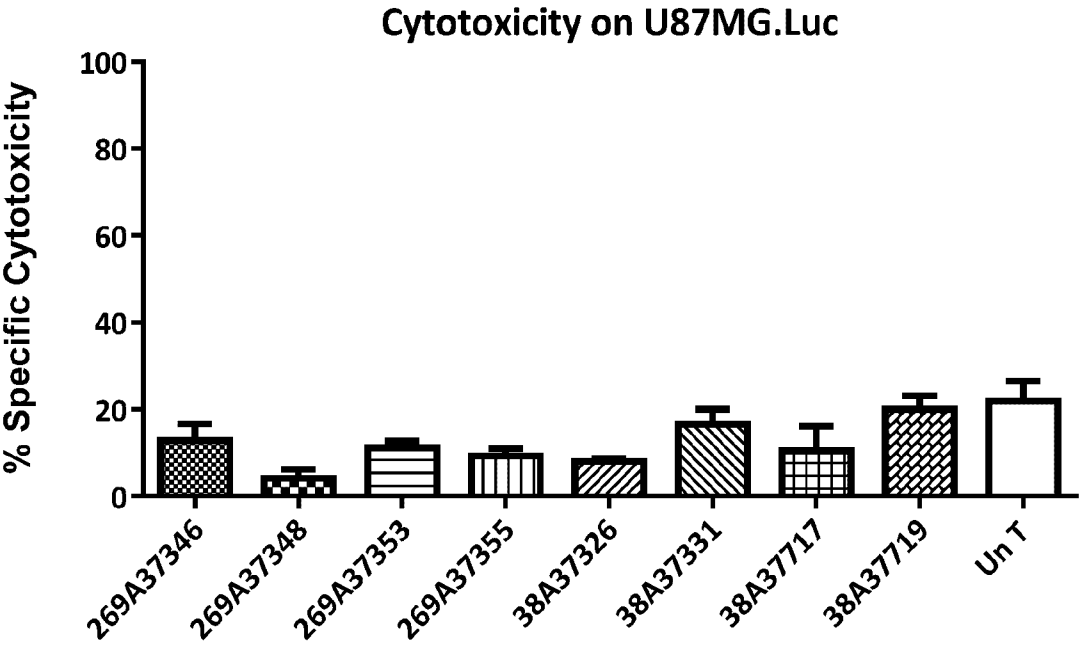

Cytotoxicity of on RPMI8226.Luc
% Specific Cytotoxicity
100
80
60
40
20
0
269A37346
269A37348
269A37353
269A37355
269A37917
269A37936
269A37948
269A37953
269A37965
269A37972
38A37326
38A37331
38A37333
38A37340
38A37717
38A37719
38A37730
38A37731
Un T

FIG. 2A


Cytotoxicity on U87MG.Luc
% Specific Cytotoxicity
100
80
60
40
20
0
269A37346
269A37348
269A37353
269A37355
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38A37331
38A37717
38A37719
Un T

FIG. 2B

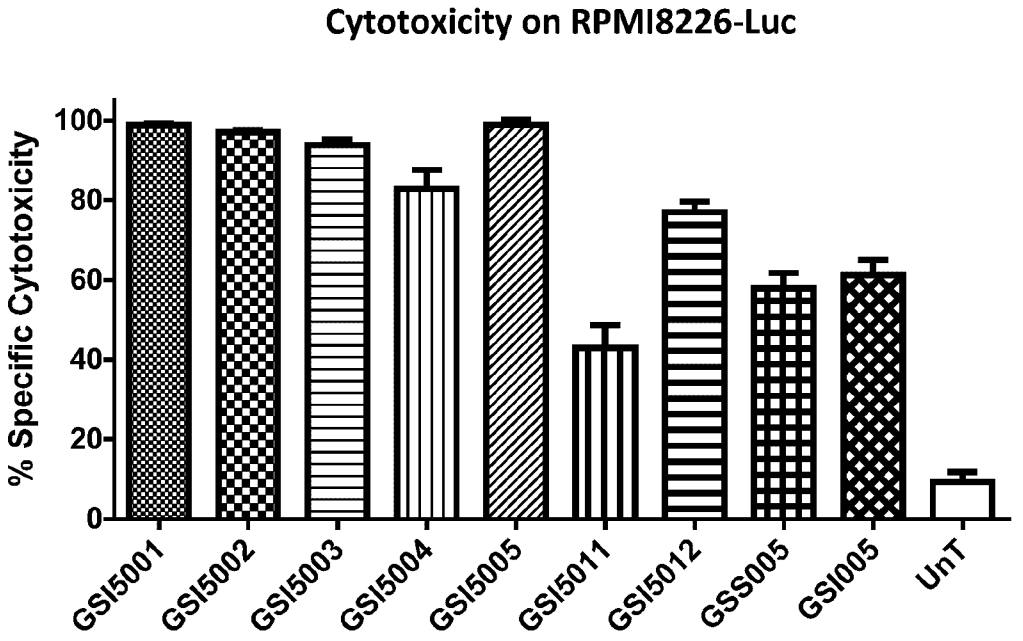

Cytotoxicity on RPMI8226-Luc
% Specific Cytotoxicity
100
80
60
40
20
0
GSI5001
GSI5002
GSI5003
GSI5004
GSI5005
GSI5011
GSI5012
GSS005
GSI005
UnT

FIG. 3A

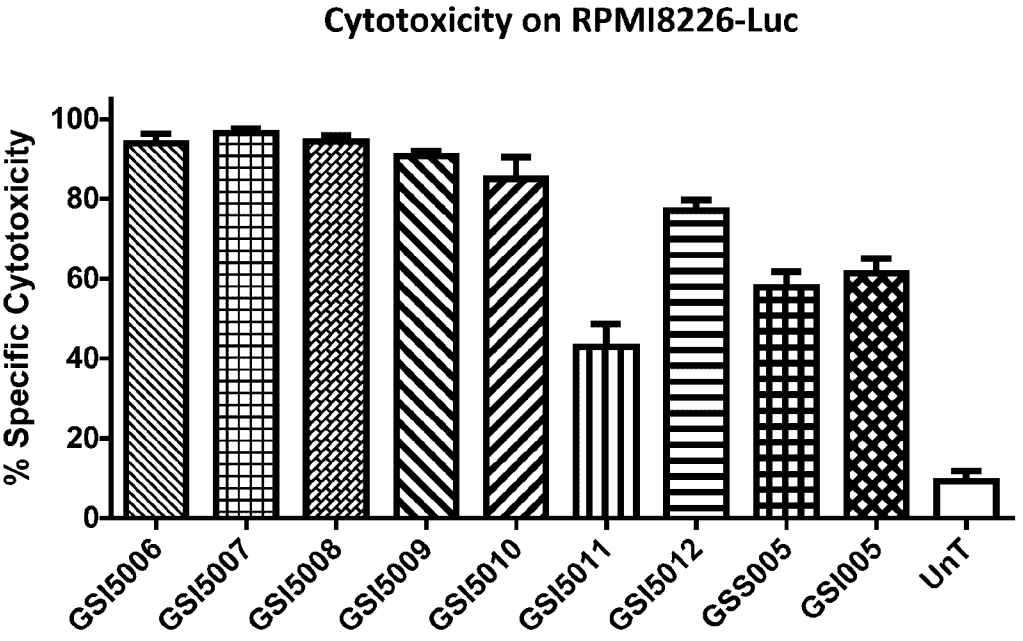

Cytotoxicity on RPMI8226-Luc
% Specific Cytotoxicity
100
80
60
40
20
0
GSI5006
GSI5007
GSI5008
GSI5009
GSI5010
GSI5011
GSI5012
GSS005
GSI005
UnT

FIG. 3B

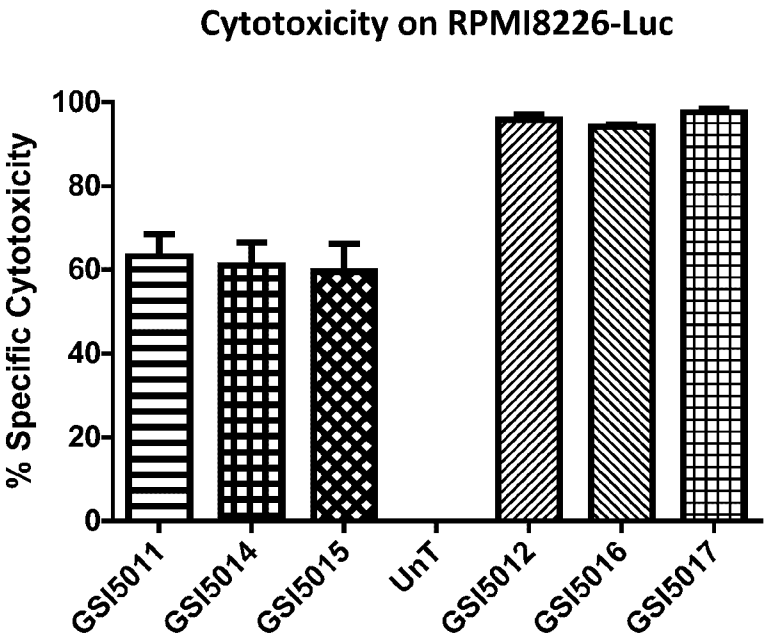
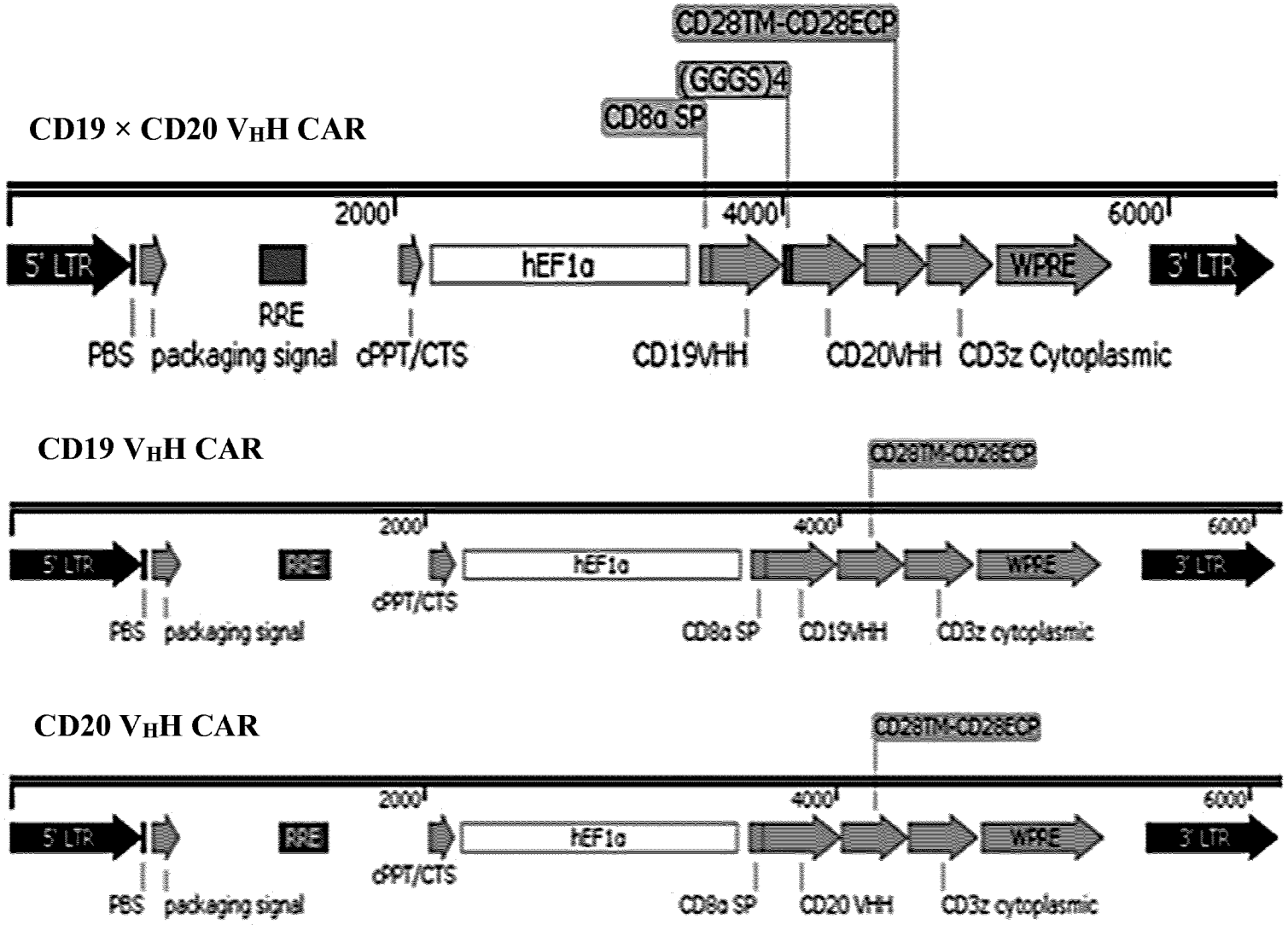

Cytotoxicity on RPMI8226-Luc
% Specific Cytotoxicity
100
80
60
40
20
0
GSI5011
GSI5014
GSI5015
UnT
GSI5012
GSI5016
GSI5017

FIG. 4

FIG. 5



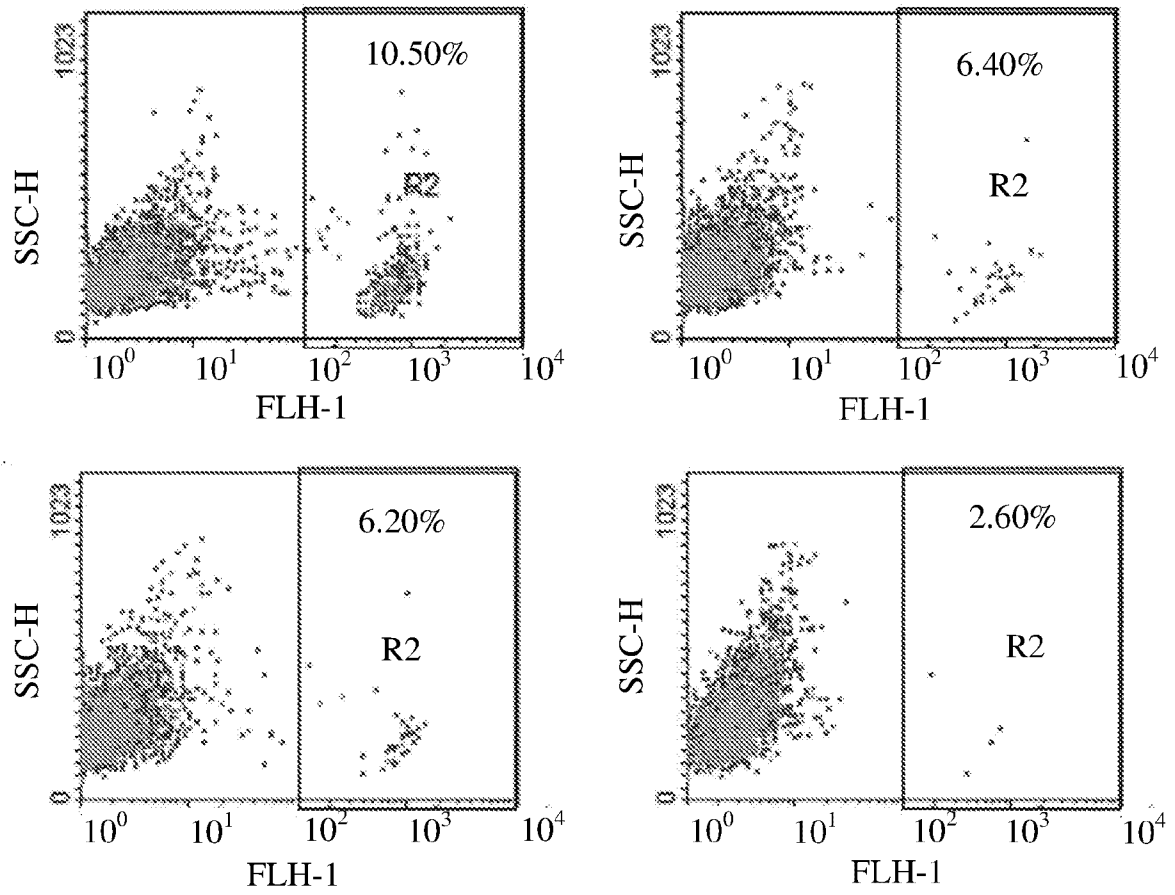


FIG. 6

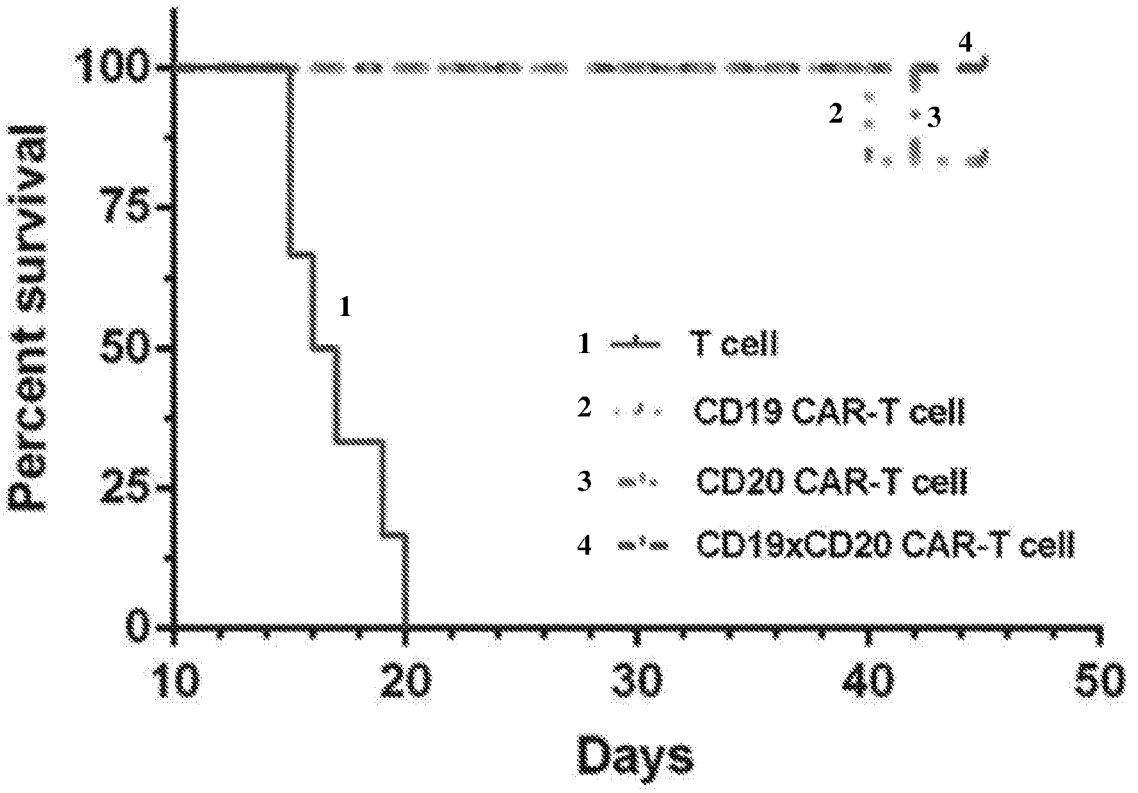

Percent survival
100
75
50
25
0
10
20
30
40
50
Days
1
2
3
4
1 T cell
2 CD19 CAR-T cell
3 CD20 CAR-T cell
4 CD19xCD20 CAR-T cell

FIG. 7

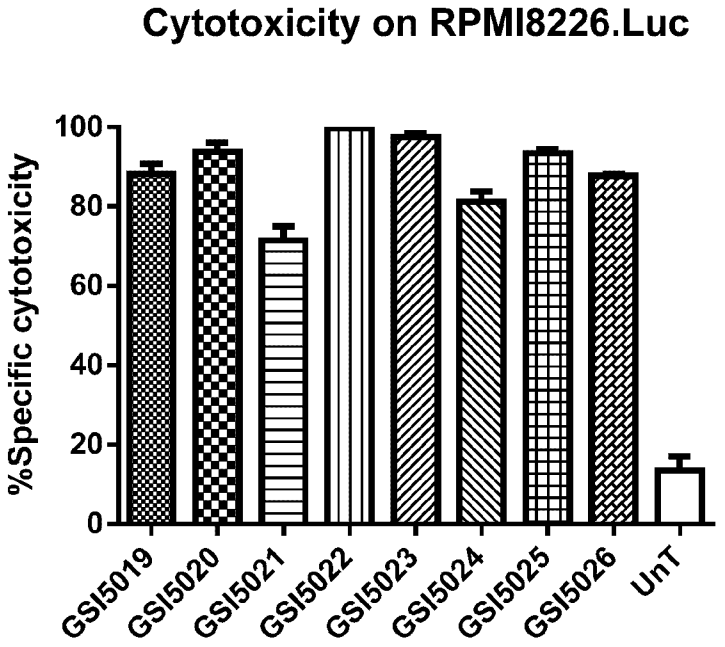

Cytotoxicity on RPMI8226.Luc
%Specific cytotoxicity
100
80
60
40
20
0
GSI5019
GSI5020
GSI5021
GSI5022
GSI5023
GSI5024
GSI5025
GSI5026
UnT

FIG. 8A

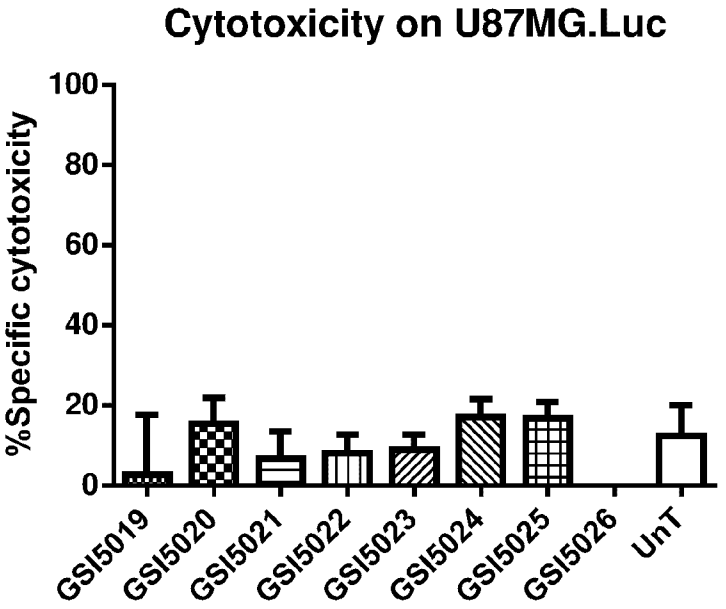

Cytotoxicity on U87MG.Luc
%Specific cytotoxicity
100
80
60
40
20
0
GSI5019
GSI5020
GSI5021
GSI5022
GSI5023
GSI5024
GSI5025
GSI5026
UnT

FIG.8B