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Fortsættes ...

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# DESCRIPTION

Description

## FIELD OF THE PRESENT INVENTION

**[0001]** The present invention relates to chimeric antigen receptor comprising single-domain antibodies, engineered immune effector cells, and uses thereof. The present disclosure further relates to activation and expansion of cells for therapeutic uses, especially for chimeric antigen receptor-based T cell immunotherapy.

## BACKGROUND OF THE PRESENT INVENTION

**[0002]** 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.

**[0003]** WO 2013/123061 relates to scFv-based bispecific CARs.

**[0004]** Grada et al., Molecular Therapy-Nucleic Acids, 2013, Vol. 2, e105, studied a TanCAR that mediated bispecific activation and targeting of T cells.

**[0005]** Jamnani et al., Biochimica et Biophysica Acta (BBA), 2014, Vol. 1840, No. 1, pages 378-386, reported oligoclonal anti-HER2 CARs comprising a single V<sub>H</sub>H domain.

**[0006]** Single-domain antibodies (sdAbs) are different from conventional 4-chain antibodies by having a single monomeric antibody variable domain. For example, camels 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<sub>H</sub>H), which can have high affinity to an antigen without the aid of a light chain. Camelid V<sub>H</sub>H is known as the smallest functional

antigen-binding fragment with a molecular weight of approximately 15 kD.

#### **BRIEF SUMMARY OF THE PRESENT INVENTION**

**[0007]** The present invention provides chimeric antigen receptors (CARs) based on single-domain antibodies ( $V_{HH}$ ), engineered immune effector cells, and uses thereof in cancer immunotherapy as defined in the claims.

**[0008]** The present invention provides a chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first sdAb specifically binding to a first antigen and a second sdAb specifically binding to a second antigen, wherein each of the first and second sdAb is a  $V_{HH}$ ; (b) a transmembrane domain; and (c) an intracellular signaling domain; wherein the first sdAb is an anti-BCMA sdAb comprising 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 sdAb is an anti-BCMA sdAb comprising 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.

**[0009]** In some embodiments, the first sdAb is located at the N-terminus or C-terminus of the second sdAb.

**[0010]** In some embodiments, the first sdAb and/or the second sdAb are camelid, chimeric, or humanized.

**[0011]** In some embodiments, the first sdAb and the second sdAb are directly fused to each other via a peptide bond.

**[0012]** In some embodiments, the first sdAb and the second sdAb are fused to each other via a peptide linker. In one embodiment, the peptide linker is no more than 50 amino acids long.

**[0013]** In some embodiments, the transmembrane domain is the transmembrane domain of a molecule selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1.

**[0014]** In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell. In one embodiment, the primary intracellular signaling domain is the primary intracellular signaling domain of CD3 $\zeta$ .

**[0015]** In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is the co-



stimulatory signaling domain of 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 and combinations thereof, preferably CD137.

**[0016]** The present invention also provides the CAR of the invention further comprising a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. The present invention also provides the CAR of the invention further comprising a signal peptide located at the N-terminus of the polypeptide.

**[0017]** The present invention also provides an isolated nucleic acid comprising a nucleic acid sequence encoding the CAR of the invention.

**[0018]** The present invention also provides a vector comprising the isolated nucleic acid of the invention. In one embodiment, the vector is a lentiviral vector. In one embodiment, the lentiviral vector is a self-inactivating lentiviral vector.

**[0019]** The present invention also provides an engineered immune effector cell comprising the CAR of the invention, the isolated nucleic acid of the invention, or the vector of the invention. In one embodiment, the immune effector cell is a T cell.

**[0020]** The present invention also provides a pharmaceutical composition comprising the engineered immune effector cell of the invention, and a pharmaceutically acceptable carrier.

**[0021]** The present invention also provides a pharmaceutical composition of the invention for use in a method of treating cancer in an individual. In one embodiment, the engineered immune effector cell is autologous.

**[0022]** In some embodiments, the cancer is multiple myeloma, acute lymphoblastic leukemia, or chronic lymphocytic leukemia. In one embodiment, the cancer is multiple myeloma.

**[0023]** Disclosed as reference is an anti-BCMA sdAb comprising the CDR regions of any one of SEQ ID NOs:78, 79, 81 to 86 and 88. Disclosed but not claimed, 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;

(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;

(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. Disclosed as reference, the anti-BCMA sdAb comprises a V<sub>H</sub>H domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO:78, 79, 81 to 86 and 88. A first anti-BCMA sdAb comprised in the CAR of the invention 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 a second anti-BCMA sdAb comprised in the CAR of the invention 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. The anti-BCMA sdAbs comprised in the CAR of the invention may comprise a VHH domain comprising an amino acid sequence of SEQ ID NO: 87 and 80, respectively.

**[0024]** Disclosed as reference, there is an anti-BCMA heavy-chain only antibody (HCAB) or an antigen binding protein comprising any one of the anti-BCMA sdAbs described above.

**[0025]** The CAR of the invention is a BCMA chimeric antigen receptor comprising: (a) an extracellular antigen binding domain comprising a first and a second anti-BCMA sdAb as defined in the claims; (b) a transmembrane domain; and (c) an intracellular signaling domain. The CAR may be described as monospecific. The CAR may be described as multivalent (such as bivalent or trivalent).

**[0026]** In some embodiments, the first sdAb and the second sdAb specifically bind to the same epitope. In some embodiments, the first sdAb and the second sdAb specifically bind to different

epitopes.

**[0027]** In some embodiments according to any one of the CARs of the invention, the first sdAb and/or the second sdAb are camelid, chimeric, human, or humanized.

**[0028]** In some embodiments according to any one of the CARs of the invention 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.

**[0029]** In some embodiments according to any one of the CARs of the invention, the transmembrane domain is that of a molecule selected from the group consisting of CD8 $\alpha$ , 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.

**[0030]** In some embodiments according to any one of the CARs of the invention, 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 that of CD3 $\zeta$ . In some embodiments, the primary intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 140 or SEQ ID NO: 141.

**[0031]** In some embodiments according to any one of the CARs of the invention, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of 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 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.

**[0032]** In some embodiments according to any one of the CARs of the invention, 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 CD8 $\alpha$ . In some embodiments, the hinge domain comprises the amino acid sequence of SEQ ID NO: 130.

**[0033]** 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 CD8 $\alpha$ , GM-CSF receptor  $\alpha$ , and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8 $\alpha$ . In some embodiments, the signal peptide comprises the amino acid sequence of SEQ ID NO: 127.

**[0034]** CARs of the invention are listed in Table 5. Disclosed as reference in Tables 4, 5 and 6, the CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 152-174, 198-201, 206-216, 248-249 and 257-260. The present invention provides a CAR comprising an amino acid sequence selected from SEQ ID NOs: 265-270.

**[0035]** Disclosed as reference is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 76 to 79, 81 to 86, 88 to 100, 152-174, 198-201, 206-216, 248-249 and 257-260. The present invention provides a CAR comprising a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 80, 87 and 265-270.

**[0036]** One aspect of the present invention provides an isolated nucleic acid comprising a nucleic acid sequence encoding any one of the CARs of the invention. The present invention provides an isolated nucleic acid comprising a nucleic acid sequence selected from SEQ ID NO: 105, 112 and 271-276. Disclosed as reference, the nucleic acid sequence is selected from the group consisting of SEQ ID NOs: 175-197, 202-205, 217-227, 250-251 and 261-264. 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.

**[0037]** One aspect of the present invention provides a vector comprising the isolated nucleic acids of the invention. 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.

**[0038]** One aspect of the present invention provides an engineered immune effector cell, comprising any one of the CARs of the invention or any one of the isolated nucleic acids of the invention, or any one of the vectors of the invention. In some embodiments, the engineered immune effector cell comprises or expresses two or more CARs of the invention 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.

**[0039]** One aspect of the present invention provides a pharmaceutical composition comprising any one of the engineered immune effector cells of the invention and a pharmaceutically acceptable carrier. Further provided is the pharmaceutical composition of the invention for use in a method of treating cancer in an individual. 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.

**[0040]** Disclosed as reference is 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 examples, there is a method of treating a disease (such as cancer) in an individual, comprising administering to the individual an effective amount of the pharmaceutical composition.

**[0041]** Also provided are uses kits, and articles of manufacture comprising any one of the CARs, engineered immune effector cells, isolated nucleic acids, or vectors of the invention. The invention is set out in the appended set of claims. The embodiments and/or examples of the following description which are not covered by the appended claims are not considered to be part of the present invention. Any references in the description to methods of treatment refer to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treatment of the human (or animal) body by therapy (or for diagnosis).

## BRIEF DESCRIPTION OF THE DRAWINGS

### **[0042]**

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.

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.

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<sub>H</sub>H domain specifically binding to antigen A, and a second V<sub>H</sub>H domain specifically binding to antigen B. Antigen A and antigen B are different antigens.

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

FIG. 1E shows exemplary engineered immune effector cells co-expressing two different V<sub>H</sub>H-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.

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.

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.

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

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

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

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.

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.

FIG. 7 shows results of an *in vivo* antitumor assay of T cells expressing an exemplary bispecific CAR targeting CD 19 and CD20.

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.

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 INVENTION

**[0043]** The present invention provides a multivalent CAR as defined in the claims. 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.

**[0044]** 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 invention, 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.

**[0045]** Accordingly, disclosed as reference is 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 examples, 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 examples, the first antigen is CD19, and the second antigen is CD20. In some examples, the first antigen is CD19, and the second antigen is CD22.

**[0046]** The present invention provides a multivalent chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first and a second single-domain antibody (sdAb) specifically binding to an antigen as defined in the claims; (b) a transmembrane domain; and (c) an intracellular signaling domain.

**[0047]** In another aspect, there is provided a multivalent chimeric antigen receptor (CAR) of the invention 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.

**[0048]** Further disclosed as reference are novel anti-CD19, anti-CD20, anti-BCMA, and anti-CD38 single-domain antibodies and chimeric antigen receptors comprising any one of the sdAbs.

**[0049]** Engineered immune effector cells (such as T cells) comprising the of CARs of the invention, pharmaceutical composition of the invention, and the pharmaceutical composition of the invention for use in methods of treating cancer are also provided herein.

## **I. Definitions**

**[0050]** 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.



**[0051]** 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')<sub>2</sub>, 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.

**[0052]** 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<sub>H</sub>) followed by three constant domains (C<sub>H</sub>) for each of the α and γ chains and four C<sub>H</sub> domains for μ and ε isotypes. Each L chain has at the N-terminus, a variable domain (V<sub>L</sub>) followed by a constant domain at its other end. The V<sub>L</sub> is aligned with the V<sub>H</sub> and the C<sub>L</sub> is aligned with the first constant domain of the heavy chain (C<sub>H1</sub>). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a V<sub>H</sub> and V<sub>L</sub> 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. Parslow (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<sub>H</sub>), 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<sub>H</sub> sequence and function, e.g., humans express the following subclasses: IgG1, IgG2A, IgG2B, IgG3, IgG4, IgA1 and IgA2.

**[0053]** 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 HCAs.

**[0054]** 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 HCAs,

and their heavy chain variable domains are referred herein as "V<sub>H</sub>Hs". Some V<sub>H</sub>Hs 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<sub>H</sub>H 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.

**[0055]** 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.

**[0056]** 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<sub>H</sub>" and "V<sub>L</sub>", 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<sub>H</sub>H". V<sub>H</sub>H is thus a special type of V<sub>H</sub>.

**[0057]** 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.

**[0058]** 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 invention 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).

**[0059]** The term "naked antibody" refers to an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

**[0060]** 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.

**[0061]** 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.

**[0062]** 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.

**[0063]** "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.

**[0064]** "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, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

**[0065]** "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.

**[0066]** 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).

**[0067]** 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<sup>®</sup> 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."

**[0068]** "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.

**[0069]** 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.

**[0070]** 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).

**[0071]** 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)

**[0072]** 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
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

**[0073]** 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.

**[0074]** 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).

**[0075]** 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.

**[0076]** 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.

**[0077]** "Framework" or "FR" residues are those variable-domain residues other than the HVR residues as herein defined.

**[0078]** 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<sub>L</sub> or V<sub>H</sub> framework sequences. Generally, the selection of human immunoglobulin V<sub>L</sub> or V<sub>H</sub> 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<sub>L</sub>, the subgroup may be subgroup kappa I, kappa II, kappa III or kappa IV as in Kabat *et al.*, supra. Additionally, for the V<sub>H</sub>, 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.

**[0079]** 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.

**[0080]** 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 examples, 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).

**[0081]** 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  $\leq 1 \mu\text{M}$ ,  $\leq 100 \text{ nM}$ ,  $\leq 10 \text{ nM}$ ,  $\leq 1 \text{ nM}$ , or  $\leq 0.1 \text{ nM}$ . 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.

**[0082]** 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.

**[0083]** 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).

**[0084]** "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 example, the antibody effector functions of complement binding, complement dependent cytotoxicity and antibody dependent cytotoxicity are affected. In some examples, 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<sub>H</sub>2 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)).

**[0085]** "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).

**[0086]** 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.

**[0087]** "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 ( $K_d$ ). 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. Specific illustrative examples for measuring binding affinity are described in the following.

**[0088]** A "blocking" antibody or an "antagonist" antibody is one that inhibits or reduces a biological activity of the antigen it binds. In some examples, blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

**[0089]** 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.

**[0090]** "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<sup>™</sup> (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.

**[0091]** "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." 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.

**[0092]** 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.

**[0093]** 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.

**[0094]** 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.

**[0095]** 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."

**[0096]** 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.

**[0097]** "Allogeneic" refers to a graft derived from a different individual of the same species.

**[0098]** 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.

**[0099]** 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.

**[0100]** 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.

**[0101]** 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 uses of the present invention contemplate any one or more of these aspects of treatment.

**[0102]** 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.

**[0103]** 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.

**[0104]** "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.

**[0105]** "Neoadjuvant setting" refers to a clinical setting in which the method is carried out before the primary/definitive therapy.

**[0106]** 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.

**[0107]** 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.

**[0108]** "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 dextrans; 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<sup>™</sup>, polyethylene glycol (PEG), and PLURONICS<sup>™</sup> or polyethylene glycol (PEG).

**[0109]** 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.

**[0110]** 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.

**[0111]** 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.

**[0112]** 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 invention, may be one which is suitable for parenteral or intravenous administration.

**[0113]** 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.

**[0114]** It is understood that embodiments of the present invention provided herein include "consisting" and/or "consisting essentially of" embodiments.

**[0115]** 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".

**[0116]** 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.

**[0117]** The term "about X-Y" used herein has the same meaning as "about X to about Y."

**[0118]** 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**

**[0119]** Disclosed herein as reference are 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.**

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Ab	Ex. AA SEQ ID	Ex. NA SEQ ID	CDR1	CDR2	CDR3
Exemplary Anti-CD19 single-domain antibodies					
CD19 V <sub>H</sub> H	76	101	INRMG (SEQ ID NO: 1)	SITVRGITNYADSVK G (SEQ ID NO: 2)	VSSNRDPDY (SEQ ID NO: 3)
Exemplary Anti-CD20 single-domain antibodies					
CD20 V <sub>H</sub> H	77	102	IGTMG (SEQ ID NO: 4)	AIRWSTGGTRYADS VKG (SEQ ID NO: 5)	DRLSLDLSGRYHYN PAVYDY (SEQ ID NO: 6)
Exemplary Anti-BCMA single-domain antibodies					
269A3 7346	78	103	SGFTLDYYAIG (SEQ ID NO: 7)	CISRSDGSTYYADSV KG (SEQ ID NO: 18)	AGADCSGYLRDYEF (SEQ ID NO: 29)
269A3 7348	79	104	SGRTFSTYGMA (SEQ ID NO: 8)	SKASMNYSGRITYY ADSVKG (SEQ ID NO: 19)	AGTGCSTYGCFDAQ IIDY (SEQ ID NO: 30)
269A3 7917	80	105	SGRTFTMG (SEQ ID NO: 9)	AISLSPTLAYYAESV KG (SEQ ID NO: 20)	ADRKSVMSIRPDY (SEQ ID NO: 31)
269A3 7355	81	106	SGGIFVINAMG (SEQ ID NO: 10)	SIRGLGRITNYDDSV KG (SEQ ID NO: 21)	VYVTLLGGVNRDY (SEQ ID NO: 32)
269A3 7915	82	107	SGRTFSSIVMG (SEQ ID NO: 11)	AIMWNDGITYLQDS VKG (SEQ ID NO: 22)	ASKGRYSEYEF (SEQ ID NO: 33)
269A3 7936	83	108	SGFTFDRAVIV (SEQ ID NO: 12)	FIKPSDGTIYYIDSLK G (SEQ ID NO: 23)	ASPEDWYTDWIDW SIYR (SEQ ID NO: 34)
269A3 7953	84	109	STYTVNSDVM G (SEQ ID NO: 13)	AIMWNDGITYLQDS VKG (SEQ ID NO: 24)	ASKGRYSEYEF (SEQ ID NO: 35)

Exemplary Anti-BCMA single-domain antibodies					
269A3 7965	85	110	SGATLTNDHM A (SEQ ID NO: 14)	AIDWSGRRTNYADP VEG (SEQ ID NO: 25)	VLRAWISYDNDY (SEQ ID NO: 36)
269A3 7972	86	111	SGGTLSKNTVA (SEQ ID NO: 15)	SITWDGRRTYYADS VKG (SEQ ID NO: 26)	DLGKWPAGPADY (SEQ ID NO: 37)
269A3 7353	87	112	SEHTFSSHVMG (SEQ ID NO: 16)	VIGWRDISTSYADS VKG (SEQ ID NO: 27)	ARRIDAADFDS (SEQ ID NO: 38)
269A3 7948	88	113	SGRAFSTYFMA (SEQ ID NO: 17)	GIAWSGGSTAYADS	SRGIEVEEFGA (SEQ ID NO: 39)
				VKG (SEQ ID NO: 28)	
Exemplary Anti-CD38 single-domain antibodies					
38A37 333	89	114	SGLTFSSYPMM (SEQ ID NO: 40)	RISDSGGYTNYDDS VKG (SEQ ID NO: 52)	ILGLPT (SEQ ID NO: 64)
38A37 336	90	115	SGFTFSSNWM Y (SEQ ID NO: 41)	TISTDGRGTYYKDS VKG (SEQ ID NO: 53)	KEPRVLMAYLRNLG DFGS (SEQ ID NO: 65)
38A37 699	91	116	SGRIFSINAMG (SEQ ID NO: 42)	AISTAGSTNYGDSV KG (SEQ ID NO: 54)	LNFPYPVY (SEQ ID NO: 66)
38A37 331	92	117	SGSIFKVERVF AMS (SEQ ID NO: 43)	SISSGETTTYADSVK G (SEQ ID NO: 55)	ADHTFTGDF (SEQ ID NO: 67)
38A37 717	93	118	TGKVFSIYDMG (SEQ ID NO: 44)	EITSSGTTHYDDFVS G (SEQ ID NO: 56)	NHVFGGSY (SEQ ID NO: 68)

Exemplary Anti-CD38 single-domain antibodies					
38A37 719	94	119	SASIFTRLPMG (SEQ ID NO: 45)	GIVPSGRINYADSVK G (SEQ ID NO: 57)	ADTFPLPT (SEQ ID NO: 69)
38A37 330	95	120	SGRAYATMA (SEQ ID NO: 46)	HLRVSGDTTYTDS VKG (SEQ ID NO: 58)	GPYGILAAARVSNP GNYDY (SEQ ID NO: 70)
38A37 334	96	121	SGLTFSSYIMG (SEQ ID NO: 47)	EISSGGMTSYADSV KG (SEQ ID NO: 59)	APERGSIWYSRYEY KY (SEQ ID NO: 71)
38A37 730	97	122	SQGIFTINAMG (SEQ ID NO: 48)	EVSSGGRTDYADSV KG (SEQ ID NO: 60)	VSGWHVFGDRIV (SEQ ID NO: 72)
38A37 340	98	123	SGRTFSSYAMA (SEQ ID NO: 49)	SISTSGGITDYADSV KG (SEQ ID NO: 61)	ARTWYLRTSLQYD Y (SEQ ID NO: 73)
38A37 731	99	124	SGTIVSISTMG (SEQ ID NO: 50)	TITRRGRNTYTDSV KG (SEQ ID NO: 62)	AEVQLDIWASAYDY (SEQ ID NO: 74)
38A37 326	100	125	SGRTYAMG (SEQ ID NO: 51)	TISGAGNTKYADSV KG (SEQ ID NO: 63)	AGKWFPAANEY (SEQ ID NO: 75)
(*= sdAb comprised in the CAR of the invention. Other sdAbs are disclosed as reference.)					

### Anti-BCMA single-domain antibodies

**[0120]** Disclosed herein as reference are 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.

**[0121]** 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 myeloma 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 inducing 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 myeloma. 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.

**[0122]** In the CAR of the invention, there is an anti-BCMA single-domain antibody comprising all three CDRs of the amino acid sequence of SEQ ID NO: 80. In the CAR of the invention, there is also an anti-BCMA single-domain antibody comprising all three CDRs of the amino acid sequence of SEQ ID NO: 87. 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.

**[0123]** Disclosed as reference, there is 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, 8, 10-15 and 17; (b) a CDR2 comprising an amino acid sequence selected from SEQ ID NO:18, 19, 21-26 and 28; and (c) a CDR3 comprising an amino acid sequence selected from SEQ ID NO:29, 30, 32-37 and 39. In some examples, the anti-BCMA single-domain antibody is camelid. In some examples, the anti-BCMA single-domain antibody is humanized. In some examples, the anti-BCMA single-domain antibody comprises an acceptor human framework, e.g., a human immunoglobulin framework or a human consensus framework. The anti-BCMA sdAbs included in the CAR of the invention comprise a CDR1 comprising the amino acid sequence of SEQ ID NO: 9 or 16, a CDR2 comprising the amino acid sequence of SEQ ID NO:20 or 27 and a CDR3 comprising the amino acid sequence of SEQ ID NO: 31 or 38, respectively. The anti-BCMA sdAb may be camelid. The anti-BCMA sdAb may be humanized. The anti-BCMA sdAb may comprise a human framework, e.g. a human immunoglobulin framework or a human consensus framework.

**[0124]** Disclosed as reference, there is 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 examples, 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 examples, the anti-BCMA single-domain antibody is affinity matured. In some examples, the anti-BCMA single-domain antibody is camelid. In some examples, the anti-BCMA single-domain antibody is humanized. In some examples, the anti-BCMA single-domain antibody comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

**[0125]** The CAR of the invention comprises a second 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.

**[0126]** The CAR of the invention comprises a first 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.

**[0127]** The anti-BCMA sdAb included in the CAR of the invention may comprise a V<sub>H</sub>H 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: 80 and 87. In some embodiments, a V<sub>H</sub>H 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: 80 and 87. 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: 80 and 87, including post-

translational modifications of that sequence.

**[0128]** In some embodiments, a second anti-BCMA single-domain antibody included in the CAR of the invention comprises a V<sub>H</sub>H domain having the amino acid sequence of SEQ ID NO: 80. In some embodiments, a second polypeptide included in the CAR of the invention comprises the amino acid sequence of SEQ ID NO: 80. In some embodiments, a first anti-BCMA single-domain antibody included in the CAR of the invention comprises a V<sub>H</sub>H domain having the amino acid sequence of SEQ ID NO: 87. In some embodiments, a first polypeptide included in the CAR of the invention comprises the amino acid sequence of SEQ ID NO: 87.

**[0129]** In some examples, 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 examples, 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 examples, there is an antibody that specifically binds to the same epitope as any of the anti-BCMA single-domain antibodies provided herein. For example, in some examples, there is an antibody that binds to the same epitope as an anti-BCMA single-domain antibody comprising any one of the amino acid sequences of SEQ ID NOs:78-88.

**[0130]** Disclosed as reference is 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 examples competitive binding may be determined using an ELISA assay. For example, in some examples there is an antibody that specifically binds to BCMA competitively with an anti-BCMA single-domain antibody comprising any one of the amino acid sequences of SEQ ID NOs:78-88.

**[0131]** Disclosed as reference, there is an anti-BCMA antibody or antigen binding protein comprising any one of the anti-BCMA single-domain antibodies described above. In some examples the anti-BCMA antibody is a monoclonal antibody, including a camelid, chimeric, humanized or human antibody. In some examples the anti-BCMA antibody is an antibody fragment, e.g., a V<sub>H</sub>H fragment. In some examples 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 examples the Fc region has reduced or minimized effector function.

**[0132]** An anti-BCMA antibody (such as anti-BCMA single-domain antibody) or antigen binding protein disclosed herein as reference incorporate any of the features, singly or in combination, as described in Sections 1-7 of "Features of antibodies" below.

**[0133]** In some embodiments, there is provided an isolated nucleic acid encoding the anti-BCMA single-domain antibodies comprised in the CAR of the invention. 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: 105 and 112. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 105 and 112. 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. A method of making an anti-BCMA antibody is disclosed wherein the method comprises culturing a host cell comprising a nucleic acid encoding the anti-BCMA antibody, 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). Nucleic acid sequences selected from the group consisting of SEQ ID NOs: 103, 104, 106-111 and 113 are disclosed herein as reference.

## Features of antibodies

### 1. Antibody Affinity

**[0134]** In some embodiments, the antibody has a dissociation constant ( $K_d$ ) of  $\leq 1\mu\text{M}$ ,  $\leq 100\text{ nM}$ ,  $\leq 10\text{ nM}$ ,  $\leq 1\text{ nM}$ ,  $\leq 0.1\text{ nM}$ ,  $\leq 0.01\text{ nM}$ , or  $\leq 0.001\text{ nM}$  (e.g.  $10^{-8}\text{ M}$  or less, e.g. from  $10^{-8}\text{ M}$  to  $10^{-13}\text{ M}$ , e.g., from  $10^{-9}\text{ M}$  to  $10^{-13}\text{ M}$ ).

**[0135]**  $K_d$  can be measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version or  $V_{\text{H}}\text{H}$  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 ( $^{125}\text{I}$ )-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  $5\mu\text{g/ml}$  of a capturing anti-Fab antibody (Cappel Labs) in  $50\text{ mM}$  sodium carbonate ( $\text{pH } 9.6$ ), and subsequently blocked with  $2\%$  (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately  $23^\circ\text{C}$ ). In a non-adsorbent plate (Nunc #269620),  $100\text{ pM}$  or  $26\text{ pM}$  [ $^{125}\text{I}$ ]-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,  $150\mu\text{l/well}$  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.

**[0136]** K<sub>d</sub> can be 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<sub>H</sub>H 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<sub>on</sub>) and dissociation rates (k<sub>off</sub>) 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 (K<sub>d</sub>) is calculated as the ratio k<sub>off</sub>/k<sub>on</sub>. See, e.g., Chen et al., J. Mol. Biol. 293:865-881 (1999). If the on-rate exceeds 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> 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 spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

## 2. Antibody Fragments

**[0137]** The CAR of the invention comprises sdAbs that each comprises a VHH.

**[0138]** In some examples an antibody disclosed herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>, Fv, and scFv fragments, V<sub>H</sub>H, 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, Rosenberg 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')<sub>2</sub> fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

**[0139]** 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).

**[0140]** 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

**[0141]** In some embodiments, the antibody 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.

**[0142]** 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.

**[0143]** 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).

**[0144]** 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)).

**[0145]** 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')<sub>2</sub> 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.

**[0146]** 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  $V_HH$ s 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.

**[0147]** 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**

**[0148]** In some embodiments, the antibody 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.

**[0149]** 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.

**[0150]** 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 humanhuman 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).

**[0151]** 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.

**[0152]** One technique for obtaining V<sub>H</sub>H 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<sub>H</sub>H sequences (such as a blood sample, serum sample or sample of B-cells), and then generating V<sub>H</sub>H 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

**[0153]** Antibodies 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.

**[0154]** In certain phage display methods, repertoires of V<sub>H</sub> and V<sub>L</sub> 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.

**[0155]** Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

## **6. Multispecific Antibodies**

**[0156]** An antibody may be a multispecific antibody, *e.g.* a bispecific antibody. Multispecific antibodies are antibodies that have binding specificities for at least two different sites. One of the binding specificities may be for an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38, and the other is for any other antigen. 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.

**[0157]** Bispecific antibodies can be prepared as full length antibodies or antibody fragments. Techniques for making multispecific antibodies include, but are not limited to, recombinant coexpression 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**

**[0158]** In some embodiments, amino acid sequence variants of the antibodies 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**

**[0159]** In some embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the 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
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

Original Residue	Exemplary Substitutions	Preferred Substitutions
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

**[0160]** Amino acids may be grouped according to common side-chain properties:

1. (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
2. (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
3. (3) acidic: Asp, Glu;
4. (4) basic: His, Lys, Arg;
5. (5) residues that influence chain orientation: Gly, Pro;
6. (6) aromatic: Trp, Tyr, Phe.

**[0161]** Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

**[0162]** 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 can be mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

**[0163]** 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 examples 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.

**[0164]** In some examples, 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 examples of the variant V<sub>H</sub>H sequences, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

**[0165]** 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.

**[0166]** 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**

**[0167]** In some embodiments, the antibody 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.

**[0168]** 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 may be made in order to create antibody variants with certain improved properties.

**[0169]** Antibody variants may be 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  $\pm 3$  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).

**[0170]** 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**

**[0171]** One or more amino acid modifications may be introduced into the Fc region of an antibody 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.

**[0172]** An antibody variant that may possess 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(γ)RIII only, whereas monocytes express Fc(γ)RI, Fc(γ)RII and Fc(γ)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, ACT1™ 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)).

**[0173]** 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).

**[0174]** 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).)

**[0175]** An antibody variant may comprise 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).

**[0176]** Alterations may be 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).

**[0177]** 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).

**[0178]** 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**

**[0179]** 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**

**[0180]** In some embodiments, the antibody 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, propylene glycol homopolymers, polypropylene 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.*

**[0181]** In some embodiments, there are conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation. 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**

**[0182]** The antibodies (such as single-domain antibodies) described herein may be prepared using any methods known in the art or as described herein.

**[0183]** 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<sub>H</sub>H<sub>5</sub>) 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.

**[0184]** 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**

**[0185]** 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,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N}=\text{C}=\text{NR}$ , where R and  $\text{R}^1$  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.

**[0186]** The animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100  $\mu\text{g}$  or 5  $\mu\text{g}$  of 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

**[0187]** 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.

**[0188]** 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).

**[0189]** 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).

**[0190]** 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.

**[0191]** 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.

**[0192]** Preferred immortalized myeloma cells are those that fuse efficiently, support stable highlevel 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)).

**[0193]** 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).

**[0194]** 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 art. For example, binding affinity may be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

**[0195]** 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.

**[0196]** 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.

**[0197]** 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 Plickthun, *ImmunoL Revs.* 130:151-188 (1992).

**[0198]** 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.

**[0199]** 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.

**[0200]** The monoclonal antibodies described herein may be 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.

**[0201]** 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**

**[0202]** Polynucleic acid sequences encoding the antibodies used in the invention 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.

**[0203]** 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.



**[0204]** 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<sup>TM</sup>-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as *E. coli* LE392.

**[0205]** The expression vector 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.

**[0206]** 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. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. Heterologous promoters may be utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

**[0207]** 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.

**[0208]** 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, lpp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. The signal sequences used in both cistrons of the expression system may be STII signal sequences or variants thereof.

**[0209]** The production of antibodies can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. 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, may be 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).

**[0210]** Disclosed is 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. 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.

**[0211]** 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.

#### **b) Prokaryotic Host Cells.**

**[0212]** Prokaryotic host cells suitable for expressing the antibodies include Archaeobacteria 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 example *E. coli* cells are used as hosts.

**[0213]** 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 lq lacL8 AompT A(nmpc-fepE) degP41 kan<sup>R</sup> (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.

**[0214]** 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**

**[0215]** 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.

**[0216]** Prokaryotic cells used to produce the antibodies 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 example, 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.

**[0217]** 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.

**[0218]** 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.

**[0219]** If an inducible promoter is used in the expression vector, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect, 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.

**[0220]** The expressed antibodies 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.

**[0221]** 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.

**[0222]** 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<sub>550</sub> 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.

**[0223]** To improve the production yield and quality of the antibodies, 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.

**[0224]** 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).

**[0225]** *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.

#### **d) Protein Purification**

**[0226]** 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.

**[0227]** In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the antibodies comprising an Fc region. Protein A is a 41kD cell wall protein from *Staphylococcus aureus* 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 nonspecifically bound to the solid phase. Finally the antibodies of interest is recovered from the solid phase by elution.

#### 4. Recombinant Production in Eukaryotic Cells

**[0228]** 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

**[0229]** 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.

**[0230]** The DNA for such precursor region is ligated in reading frame to DNA encoding the antibodies.

##### b) Origin of Replication

**[0231]** 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

**[0232]** 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.

**[0233]** 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.

**[0234]** 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,

such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

**[0235]** 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).

**[0236]** 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**

**[0237]** 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 bases 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. At 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.

**[0238]** 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.

**[0239]** 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.

**[0240]** 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**

**[0241]** Transcription of a DNA encoding the antibodies 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,  $\alpha$ -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**

**[0242]** 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**

**[0243]** 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).

**[0244]** 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**

**[0245]** The host cells used to produce the antibodies 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<sup>™</sup> 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**

**[0246]** 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.

**[0247]** 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. 5:1567-1575 (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<sub>H</sub>3 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.

**[0248]** 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).

**[0249]** (The section "Immunoconjugates" is disclosed as reference. The embodiments disclosed in **Immunoconjugates** this section are not embodiments of the invention.)

**[0250]** In some embodiments, there are also 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.

**[0251]** 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.

**[0252]** 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, curcumin, croton, *saponaire officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

**[0253]** 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  $\text{At}^{211}$ ,  $\text{I}^{131}$ ,  $\text{I}^{125}$ ,  $\text{Y}^{90}$ ,  $\text{Re}^{186}$ ,  $\text{Re}^{188}$ ,  $\text{Sm}^{153}$ ,  $\text{Bi}^{212}$ ,  $\text{P}^{32}$ ,  $\text{Pb}^{212}$  and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example  $\text{Tc}^{99\text{m}}$  or  $\text{I}^{123}$ , 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.

**[0254]** 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.

[0255] The immunoconjugates 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**

[0256] The section "methods and Compositions for Diagnostics and Detection" is disclosed as reference. The embodiments disclosed in this section are not embodiments of the invention.

[0257] 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.

[0258] 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 CD 19, and detecting whether a complex is formed between the anti-CD19 antibody and CD 19. 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.

[0259] 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.

**[0260]** 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.

**[0261]** 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.

**[0262]** 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  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ , fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase,  $\beta$ -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

**[0263]** The present invention provides a CAR as defined in the claims. A chimeric antigen receptor (CAR) may comprise an extracellular antigen binding domain comprising one or more single-domain antibodies (such as V<sub>H</sub>Hs). 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<sub>H</sub>H domains (*i.e.*, V<sub>H</sub>H-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<sub>H</sub>H domains in the exemplary CARs of FIGs. 1A-1D may be substituted with other sdAbs.

**[0264]** A chimeric antigen receptor (CAR) comprising a polypeptide may comprise: (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. The antigen can be 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. The sdAb can be camelid, chimeric, human, or humanized. In some embodiments, the transmembrane domain is selected from the group consisting of CD8 $\alpha$ , 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 that of CD3 $\zeta$ . In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of 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 and combinations thereof. In some embodiments, the CAR further comprises a hinge domain (such as a CD8 $\alpha$  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 CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain of CD28, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain of CD28, a second co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain of CD137, and intracellular signaling domain of CD3 $\zeta$ . The CAR of the invention may be described as monospecific. The CAR of the invention may be described as multivalent, such as bivalent or trivalent.

#### **Chimeric antigen receptors of specific targets**

**[0265]** Cars can comprise 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 as reference.

**[0266]** 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.

### BCMA CAR

**[0267]** A CAR targeting BCMA (also referred herein as "BCMA CAR") may comprise 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 that of CD3 $\zeta$ . In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of that of 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, and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8 $\alpha$  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 CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen-binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain of CD28, a second co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen-binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . The BCMA CAR can be described as monospecific.

**[0268]** A BCMA CAR may comprise 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:

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; or

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.

**[0269]** The anti-BCMA sdAb may be camelid, chimeric, human, or humanized. The anti-BCMA sdAb may comprise a V<sub>H</sub>H domain comprising an amino acid sequence from the group consisting of SEQ ID NO:80 and 87. 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 that of CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of 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 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 costimulatory signaling domain of CD28, a second co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of D3ζ. 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 of CD137, and a primary intracellular signaling domain of D3ζ. The BCMA CAR can be described as monospecific.

**[0270]** Disclosed as reference there is 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. Disclosed as reference, there is a BCMA CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 152-162, and 257-259. Also disclosed as reference is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 152-162, and 257-259.

**[0271]** Disclosed as reference, there is an isolated nucleic acid encoding any of the BCMA CARs disclosed herein. Disclosed as reference there is 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. Disclosed as reference, there is an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 175-185, and 261-263. The isolated nucleic acid can be a DNA. The isolated nucleic acid can be an RNA. A vector can comprise any one of the nucleic acids encoding the BCMA CARs described above. The vector can be an expression vector. The vector can be a viral vector, such as a lentiviral vector.

**Table 4. Exemplary monospecific, monovalent CARs.** (disclosed as reference)

Ex. Vector or CAR name	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extracellular. sdAb	Hinge	TM	Intracellular signaling		
							CO1	CO2	Prim.
BCMA CAR									
PLVX- hEF1a- 269A37346	152	175	CD8α	269A37 346	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37348	153	176	CD8α	269A37 348	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37917	154	177	CD8α	269A37 917	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37355	155	178	CD8α	269A37 355	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37915	156	179	CD8α	269A37 915	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37936	157	180	CD8α	269A37 936	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37953	158	181	CD8α	269A37 953	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37965	159	182	CD8α	269A37 965	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37972	160	183	CD8α	269A37 972	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 269A37353	161	184	CD8α	269A37 353	CD8α	CD28	CD28	CD137	CD3ζ

Ex. Vector or CAR name	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extracellular. sdAb	Hinge	TM	Intracellular signaling		
							CO1	CO2	Prim.
BCMA CAR									
PLVX- hEF1a- 269A37948	162	185	CD8α	269A37 948	CD8α	CD28	CD28	CD137	CD3ζ
GSI5011 CAR	257	261	CD8α	269A37 346	CD8α	CD8α	CD137	NA	CD3ζ
GSI5019 CAR	258	262	CD8α	269A37 353	CD8α	CD8α	CD137	NA	CD3ζ
GSI5020 CAR	259	263	CD8α	269A37 917	CD8α	CD8α	CD137	NA	CD3ζ
CD38 CAR									
PLVX- hEF1a- 38A37333	163	186	CD8α	38A373 33	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37336	164	187	CD8α	38A373 36	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37699	165	188	CD8α	38A376 99	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37331	166	189	CD8α	38A373 31	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37717	167	190	CD8α	38A377 17	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37719	168	191	CD8α	38A377 19	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37330	169	192	CD8α	38A373 30	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37334	170	193	CD8α	38A373 34	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a- 38A37730	171	194	CD8α	38A377 30	CD8α	CD28	CD28	CD137	CD3ζ
PLVX- hEF1a-	172	195	CD8α	38A373 40	CD8α	CD28	CD28	CD137	CD3ζ

CD38 CAR									
38A37340									
PLVX-hEF1a-38A37731	173	196	CD8 $\alpha$	38A37731	CD8 $\alpha$	CD28	CD28	CD137	CD3 $\zeta$
PLVX-hEF1a-38A37326	174	197	CD8 $\alpha$	38A37326	CD8 $\alpha$	CD28	CD28	CD137	CD3 $\zeta$
CD19 V <sub>H</sub> H CAR	248	250	CD8 $\alpha$	CD19 V <sub>H</sub> H	CD8 $\alpha$	CD28	CD28	NA	CD3 $\zeta$
CD20 V <sub>H</sub> H CAR	249	251	CD8 $\alpha$	CD20 V <sub>H</sub> H	CD8 $\alpha$	CD28	CD28	NA	CD3 $\zeta$
GSI5012 CAR	260	264	CD8 $\alpha$	38A37717	CD8 $\alpha$	CD8 $\alpha$	CD137	NA	CD3 $\zeta$

### Multivalent chimeric antigen receptors

**[0272]** The present invention provides multivalent anti-BCMA CARs as defined in the claims. 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.

**[0273]** A multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor may comprise 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. The antigen may be selected be 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. The plurality of sdAbs may be camelid, chimeric, human, or humanized. The plurality of singledomain antibodies may be fused to each other via peptide bonds or peptide linkers. Each peptide linker may be no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. The transmembrane domain may be selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1. The intracellular signaling domain may comprise a primary intracellular signaling domain of an immune effector cell (such as T cell). The primary intracellular signaling domain may be derived from D3 $\zeta$ . The intracellular signaling domain may comprise a co-stimulatory signaling domain. The costimulatory signaling domain may be 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. The multivalent CAR may further comprise a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the

transmembrane domain. The multivalent CAR may further comprise a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. The polypeptide may comprise from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . The multivalent CAR may be monospecific. The multivalent CAR may be multispecific, such as bispecific.

**[0274]** A multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor may comprise 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. The antigen may be 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. The first sdAb and/or the second sdAb may be camelid, chimeric, human, or humanized. The first single-domain antibody and the second single-domain antibody may be fused to each other via a peptide bond or a peptide linker. The peptide linker may be no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. The transmembrane domain may be selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1. The intracellular signaling domain may comprise a primary intracellular signaling domain of an immune effector cell (such as T cell). The primary intracellular signaling domain may be derived from D3 $\zeta$ . The intracellular signaling domain may comprise a co-stimulatory signaling domain. The co-stimulatory signaling domain may be 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. The multivalent CAR further may comprise a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. The multivalent CAR may further comprise a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. The polypeptide may comprise from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . The multivalent CAR may be monospecific. The multivalent CAR may be multispecific, such as bispecific.

**[0275]** 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

**[0276]** A multivalent CAR targeting BCMA (also referred herein as "multivalent BCMA CAR") may comprise (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. The anti-BCMA sdAb may be camelid, chimeric, human, or humanized. The plurality of the anti-BCMA sdAb may be fused to each other via peptide bonds or peptide linkers. The peptide linker may be no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. The intracellular signaling domain may comprise a primary intracellular signaling domain of an immune effector cell (such as T cell). The primary intracellular signaling domain may be derived from D3 $\zeta$ . The intracellular signaling domain may comprise a co-stimulatory signaling domain. The co-stimulatory signaling domain may be 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. The multivalent BCMA CAR may further comprise a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. The multivalent BCMA CAR may further comprise a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. The polypeptide may comprise from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . The multivalent BCMA CAR may be bivalent, The multivalent BCMA CAR may be trivalent. Any of the anti-BCMA sdAbs can be used to construct the multivalent BCMA CAR.

**[0277]** A multivalent BCMA CAR may comprise: (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. The first anti-BCMA sdAb may be located at the N-terminus of the second anti-BCMA sdAb. The first anti-BCMA sdAb may be located at the C-terminus of the second anti-BCMA sdAb. The first anti-BCMA sdAb and the second anti-BCMA sdAb may be fused to each other via a peptide bond or a peptide linker. The peptide linker may be no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. The intracellular signaling domain may comprise a primary intracellular signaling domain of an immune effector cell (such as T cell). The primary intracellular signaling domain may be derived from D3 $\zeta$ . The intracellular signaling domain may comprise a costimulatory signaling domain. The co-stimulatory signaling domain may be 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. The multivalent BCMA CAR may further comprise a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. The multivalent

BCMA CAR may further comprise a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. The polypeptide may comprise from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . The multivalent BCMA CAR may be bivalent, The multivalent BCMA CAR may be trivalent. The extracellular antigen binding domain may further comprise 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.

**[0278]** The present invention provides 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<sub>H</sub>H domain comprising the amino acid sequence of SEQ ID NO: 87. In some embodiments, the first anti-BCMA sdAb comprises a V<sub>H</sub>H 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 that of D3 $\zeta$ . In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of 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 and combinations thereof. In some embodiments, the multivalent BCMA CAR further comprises a hinge domain (such as a CD8 $\alpha$  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 CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . The multivalent BCMA CAR can be described as bivalent.

**[0279]** 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: 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: 265-270. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 265-270.

**[0280]** In some embodiments, there is provided an isolated nucleic acid encoding any of the multivalent BCMA CARs of the invention. 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: 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: 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 of the invention. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector.

**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.
GS15 014	198	202	CD 8 $\alpha$	269A 3734 6	144	269A 3734 6	NA	NA	CD8 $\alpha$	CD 8 $\alpha$	CD 137	CD3 $\zeta$
GS15 015	199	203	CD 8 $\alpha$	269A 3734 6	144	269A 3734 6	144	269A 3734 6	CD8 $\alpha$	CD 8 $\alpha$	CD 137	CD3 $\zeta$
GS15 016	200	204	CD 8 $\alpha$	3 8 A3 7717	144	38A3 7717	NA	NA	CD8 $\alpha$	CD 8 $\alpha$	CD 137	CD3 $\zeta$
GS15 017	201	205	CD 8 $\alpha$	3 8 A3 7717	144	38A3 7717	144	38A3 7717	CD8 $\alpha$	CD 8 $\alpha$	CD 137	CD3 $\zeta$
GS15 021	265	271	CD 8 $\alpha$	269A 3735 3	144	269A 3791 7	NA	NA	CD8 $\alpha$	CD 8 $\alpha$	CD 137	CD3 $\zeta$
GS15 022	266	272	CD 8 $\alpha$	269A 3735 3	149	269A 3791 7	NA	NA	CD8 $\alpha$	CD 8 $\alpha$	CD 137	CD3 $\zeta$
GS15 267	273		CD	269A	151	269A	NA	NA	CD8 $\alpha$	CD	CD	CD3 $\zeta$

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.
023			8α	3735 3		3791 7				8α	137	
GS15 024	268	274	CD 8α	269A 3791 7	145	269A 3735 3	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GS15 025	269	275	CD 8α	269A 3791 7	149	269A 3735 3	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
GS15 026	270	276	CD 8α	269A 3791 7	150	269A 3735 3	NA	NA	CD8α	CD 8α	CD 137	CD3ζ
(*= multivalent BCMA CARs of the invention. Other CARs are disclosed as reference.)												

**Multispecific chimeric antigen receptor (The section "Multispecific chimeric antigen receptor" is disclosed as reference. The embodiments disclosed in this section are not embodiments of the invention.)**

**[0281]** The present disclosure 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.

**[0282]** 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 CD8 $\alpha$ , 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 D3 $\zeta$ . 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 CD8 $\alpha$  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 CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from D3 $\zeta$ .

**[0283]** Depending on the desired antigen to be targeted, the CARs 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 disclosure. 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 as reference.

## **1. BCMA $\times$ CD38 CAR**

**[0284]** In some embodiments, the CAR 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.

**[0285]** 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 D3ζ. 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 D3ζ.

**[0286]** 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<sub>H</sub>H domain comprising the amino acid sequence of SEQ ID NO:78. In some embodiments, the anti-CD38 sdAbs comprises a V<sub>H</sub>H 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 CD8 $\alpha$ , 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 D3 $\zeta$ . 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  $\times$  CD38 CAR further comprises a hinge domain (such as a CD8 $\alpha$  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  $\times$  CD38 CAR further comprises a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide.

**[0287]** In some embodiments, there is provided a BCMA  $\times$  CD38 CAR comprising a polypeptide comprising from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, an anti-CD38 single-domain antibody, a peptide linker, an anti-BCMA single-domain antibody, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3 $\zeta$ ; 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  $\times$  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  $\times$  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.

**[0288]** 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 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.

**[0289]** 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**

**[0290]** 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 is a bispecific CAR simultaneously targeting CD19 and CD20.

**[0291]** 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 CD8 $\alpha$ , 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 D3 $\zeta$ . 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  $\times$  CD20 CAR further comprises a hinge domain (such as a CD8 $\alpha$  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  $\times$  CD20 CAR further comprises a signal peptide (such as a CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from D3 $\zeta$ .

**[0292]** In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting CD19 and CD20 (also referred herein as "CD19  $\times$  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<sub>H</sub>H domain comprising the amino acid sequence of SEQ ID NO:76. In some embodiments, the anti-CD20 sdAbs comprises a V<sub>H</sub>H 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 CD8 $\alpha$ , 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 D3 $\zeta$ . 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  $\times$  CD20 CAR further comprises a hinge domain (such as a CD8 $\alpha$  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  $\times$  CD20 CAR further comprises a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide.

**[0293]** In some embodiments, there is provided a CD19  $\times$  CD20 CAR comprising a polypeptide comprising from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, an anti-CD19 single-domain antibody, a peptide linker, an anti-CD20 single-domain antibody, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from CD3 $\zeta$ ; 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  $\times$  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  $\times$  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.

**[0294]** In some embodiments, there is provided an isolated nucleic acid encoding any of the CD19  $\times$  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.

**[0295]** 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

**[0296]** 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 D3ζ. 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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from D3 $\zeta$ . 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.

**[0297]** In some embodiments, there is provided a multispecific (such as bispecific) chimeric antigen receptor targeting CD19 and BCMA (also referred herein as "CD19  $\times$  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 CD8 $\alpha$ , 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 D3 $\zeta$ . 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  $\times$  BCMA CAR further comprises a hinge domain (such as a CD8 $\alpha$  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  $\times$  BCMA CAR further comprises a signal peptide (such as a CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from D3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a co-stimulatory signaling domain derived from CD28, and a primary intracellular signaling domain derived from D3 $\zeta$ . 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. (disclosed as reference)**

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 <sub>H</sub> H	146	CD20 V <sub>H</sub> H	CD8α	CD28	CD28	CD3ζ
GS15 001	207	218	CD8α	38A37 717	144	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GS15 002	208	219	CD8α	38A37 717	145	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GS15 003	209	220	CD8α	38A37 717	146	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GS15 004	210	221	CD8α	38A37 717	147	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GS15 005	211	222	CD8α	38A37 717	148	269A3 7346	CD8α	CD8α	CD137	CD3ζ
GS15 006	212	223	CD8α	269A3 7346	144	38A37 717	CD8α	CD8α	CD137	CD3ζ
GS15 007	213	224	CD8α	269A3 7346	145	38A37 717	CD8α	CD8α	CD137	CD3ζ
GS15 008	214	225	CD8α	269A3 7346	146	38A37 717	CD8α	CD8α	CD137	CD3ζ
GS15 009	215	226	CD8α	269A3 7346	147	38A37 717	CD8α	CD8α	CD137	CD3ζ
GS15 010	216	227	CD8α	269A3 7346	148	38A37 717	CD8α	CD8α	CD137	CD3ζ

#### Extracellular antigen binding domain

**[0298]** The extracellular antigen binding domain of the CARs of the invention comprises a first and a second anti-BCMA sdAb as defined in the claims.

**[0299]** The single-domain antibodies can be fused to each other directly via peptide bonds, or via peptide linkers.

## 1. Single-domain antibodies

**[0300]** The CARs of the present invention comprise an extracellular antigen binding domain comprising a first and a second sdAb, each of which is a VHH. The sdAbs may be of the same or 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.

**[0301]** 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  $V_{HH}$ s are within the scope of the present invention.

**[0302]**  $V_{HH}$  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  $V_{HH}$ s. 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  $V_{HH}$ s are known to bind into 'unusual' epitopes such as cavities or grooves, the affinity of CARs comprising such  $V_{HH}$ s may be more suitable for therapeutic treatment than conventional multispecific polypeptides.

**[0303]** 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.

**[0304]** 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).

**[0305]** The sdAb can be 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. The sdAb can be affinity matured.

**[0306]** Naturally occurring  $V_HH$  domains against a particular antigen or target, can be obtained from (naive 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 (naive or immune)  $V_HH$  libraries may be used, such as  $V_HH$  libraries obtained from (naive or immune)  $V_HH$  libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example described in WO 00/43507.

**[0307]** The single-domain antibodies can be 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

**[0308]** The antigen targeted by the CARs of the present invention is BCMA. Other antigen are disclosed as reference. 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 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.

**[0309]** 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 will depend on the particular type of cancer to be treated. Exemplary tumor antigens include, for example, a glioma-associated antigen, carcinoembryonic antigen (CEA),  $\beta$ -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-Ia, 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.

**[0310]** The tumor antigen can comprise 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.

**[0311]** The tumor antigen may be 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.

**[0312]** 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, p15; 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, p185erbB2, p180erbB-3, c-met, nm-23H1, 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.

**[0313]** The antigen (such as the first antigen and/or the second antigen) may be selected from the group consisting of CD19, CD20, CD22, CD33, CD38, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77.

### **3. Peptide linkers**

**[0314]** 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.

**[0315]** 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 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.

**[0316]** 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 acid 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.

**[0317]** 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)<sub>n</sub>, glycine-serine polymers (including, for example, (GS)<sub>n</sub>, (GSGGS)<sub>n</sub>, (GGGS)<sub>n</sub>, and (GGGGS)<sub>n</sub>, 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)<sub>2</sub> (SEQ ID NO: 145), (GGGS)<sub>4</sub> (SEQ ID NO: 146), GGGSGGGSGGGGGSGSGGGGS (SEQ ID NO: 147), GGGSGGGSGGGGGSGSGGGSGGGSGGGGS (SEQ ID NO: 148), (GGGGS)<sub>3</sub> (SEQ ID NO: 149), (GGGGS)<sub>4</sub> (SEQ ID NO: 150), or (GGGGS)<sub>3</sub> (SEQ ID NO: 151).

### **Transmembrane domain**

**[0318]** The CARs of the present invention 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.

**[0319]** 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.

**[0320]** 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.

**[0321]** 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 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRFI), CD160, CD19, IL-2R beta, IL-2R gamma, IL-7R alpha, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (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 that of a molecule selected from the group consisting of CD8alpha, CD4, CD28, CD137, CD80, CD86, CD152 and PD1.

**[0322]** In some embodiments, the transmembrane domain is that of 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.

**[0323]** In some embodiments, the transmembrane domain is that of CD8alpha. In some

embodiments, the transmembrane domain is a transmembrane domain of CD8 $\alpha$  comprising the amino acid sequence of SEQ ID NO: 132. In some embodiments, the transmembrane domain of CD8 $\alpha$  is encoded by the nucleic acid sequence of SEQ ID NO: 134.

**[0324]** 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.

**[0325]** 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.

**[0326]** In some embodiments, the transmembrane region of the transmembrane domain comprises hydrophobic amino acid residues. In some embodiments, the transmembrane domain 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**

**[0327]** The CARs of the present invention 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.

**[0328]** 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 CD3 $\zeta$ , FcR gamma(FCER1G), FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

**[0329]** In some embodiments, the primary intracellular signaling domain is that of CD3 $\zeta$ . In some embodiments, the intracellular signaling domain consists of the cytoplasmic signaling domain of CD3 $\zeta$ . In some embodiments, the primary intracellular signaling domain is a cytoplasmic signaling domain of wildtype CD3 $\zeta$ . In some embodiments, the primary intracellular signaling domain of wildtype CD3 $\zeta$  comprises the amino acid sequence of SEQ ID NO: 140. In some embodiments, the primary intracellular signaling domain of wildtype CD3 $\zeta$  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 CD3 $\zeta$  containing one or more mutations, such as Q65K. In some embodiments, the primary intracellular signaling domain of mutant CD3 $\zeta$  comprises the amino acid sequence of SEQ ID NO: 141. In some embodiments, the primary intracellular signaling domain of mutant CD3 $\zeta$  is encoded by the nucleic acid of SEQ ID NO: 143.

#### **Co-stimulatory signaling domain**

**[0330]** 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.

**[0331]** 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 CD3 $\zeta$ ) 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 CD3 $\zeta$ ) 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 CD3 $\zeta$ ). Multiple co-stimulatory signaling domains may provide additive or synergistic stimulatory effects.

**[0332]** 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, GITRLigand/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.

**[0333]** 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, and B7-H3.

**[0334]** In some embodiments, the intracellular signaling domain in the CAR of the present invention comprises a co-stimulatory signaling domain of CD28. In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3 $\zeta$  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.

**[0335]** In some embodiments, the intracellular signaling domain in the CAR of the present invention comprises a co-stimulatory signaling domain of CD137 (*i.e.*, 4-1BB). In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3 $\zeta$  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.

**[0336]** In some embodiments, the intracellular signaling domain in the CAR of the present invention 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 CD3 $\zeta$ , 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 CD3 $\zeta$ . 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.

**[0337]** 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**

**[0338]** The CARs of the present invention 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.

**[0339]** 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.

**[0340]** 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 CD8 $\alpha$ . In some embodiments, the hinge domain is a portion of the hinge domain of CD8 $\alpha$ , e.g., a fragment containing at least 15 (e.g., 20, 25, 30, 35, or 40) consecutive amino acids of the hinge domain of CD8 $\alpha$ . In some embodiments, the hinge domain of CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 130. In some embodiments, the hinge domain of CD8 $\alpha$  is

encoded by the nucleic acid sequence of SEQ ID NO: 131.

**[0341]** 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.

**[0342]** 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)<sub>n</sub> 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**

**[0343]** The CARs of the present invention 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 CD8 $\alpha$ , GM-CSF receptor  $\alpha$ , and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8 $\alpha$ . In some embodiments, the signal peptide of CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 127. In some embodiments, the signal peptide of CD8 $\alpha$  is encoded by the nucleic acid sequence of SEQ ID NO: 128 or SEQ ID NO: 129.

#### **IV. Engineered immune effector cells**

**[0344]** Further provided are immune effector cells comprising any one of the CARs of the invention.

**[0345]** An engineered immune effector cell (such as T cell) may comprise 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. The antigen may be 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. The plurality of sdAbs may be camelid, chimeric, human, or humanized. The plurality of single-domain antibodies may be to each other via peptide bonds or peptide linkers. Each peptide linker may be no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. The transmembrane domain may be selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1. The intracellular signaling domain may comprise a primary intracellular signaling domain of an immune effector cell (such as T cell). The primary intracellular signaling domain may be derived from CD3 $\zeta$ . The intracellular signaling domain may comprise a co-stimulatory signaling domain. The co-stimulatory signaling domain may be 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. The multivalent CAR may further comprise a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. The multivalent CAR may further comprise a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. The polypeptide may comprise from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3 $\zeta$ . In some embodiments, the multivalent CAR is monospecific. The multivalent CAR may be multispecific, such as bispecific. The multivalent CAR may comprise an amino acid sequence selected from the group consisting of SEQ ID NOs: 198-201.

**[0346]** An engineered immune effector cell (such as T cell) may comprise 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. The antigen may be 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. The first sdAb and/or the second sdAb may be camelid, chimeric, human, or humanized. The first single-domain antibody and the second single-domain antibody may be fused to each other via a peptide bond or a peptide linker. The peptide linker may be no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. The transmembrane domain may be selected from the group consisting

of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1. The intracellular signaling domain may comprise a primary intracellular signaling domain of an immune effector cell (such as T cell). The primary intracellular signaling domain may be derived from CD3 $\zeta$ . The intracellular signaling domain comprises may be co-stimulatory signaling domain. The co-stimulatory signaling domain may be 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. The multivalent CAR may further comprise a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. The multivalent CAR may further comprise a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. The polypeptide may comprise from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3 $\zeta$ . The engineered immune effector cell may be 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. The engineered immune effector cell may be autologous. The engineered immune effector cell may be allogenic.

**[0347]** In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a BCMA CAR of the invention comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first 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.

**[0348]** In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V<sub>H</sub>H domain comprising an amino acid sequence from the group consisting of SEQ ID NO:80 and 87. 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 that of CD3 $\zeta$ . In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of 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, and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8 $\alpha$  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 CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen-binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a first co-

stimulatory signaling domain of CD28, a second co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the BCMA CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 265-270. 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.

**[0349]** 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-expressing 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<sub>H</sub>H-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.

**[0350]** 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.

## Vectors

**[0351]** The present invention provides vectors for cloning and expressing any one of the CARs of the invention. 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.

**[0352]** 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.

**[0353]** In some embodiments, the vector comprises any one of the nucleic acids encoding a CAR of the invention. 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.

**[0354]** 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 (hEF1 $\alpha$ ), ubiquitin C promoter (UbiC), phosphoglycerokinase promoter (PGK), simian virus 40 early promoter (SV40), and chicken  $\beta$ -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, hEF1 $\alpha$ , UbiC and PGK to drive chimeric antigen receptor expression in primary human T cells, and concluded that hEF1 $\alpha$  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 hEF1 $\alpha$  promoter.

**[0355]** 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. The inducing condition may not induce the expression of endogenous genes in the engineered mammalian cell, and/or in the subject that receives the pharmaceutical composition. The

inducing condition can be 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.

**[0356]** 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.

**[0357]** 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.

### **Immune effector cells**

**[0358]** "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.

**[0359]** In some embodiments, the immune effector cells are T cells. In some embodiments, the T cells are CD4<sup>+</sup>/CD8<sup>-</sup>, CD4<sup>-</sup>/CD8<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>+</sup>, CD4<sup>-</sup>/CD8<sup>-</sup>, 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<sup>+</sup> or CD19<sup>+</sup> tumor cells. In some embodiments, the CD8<sup>+</sup> T cells lyse antigen-specific target cells upon expressing the CAR and binding to the target cells.

**[0360]** 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.

**[0361]** 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.

**[0362]** The engineered immune effector cells are prepared by introducing the CARs into the immune effector cells, such as T cells. The CAR can be 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. The CAR can be 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).

**[0363]** 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.

**[0364]** 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. The vector can be introduced into the cell by electroporation.

**[0365]** 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.

**[0366]** 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).

**[0367]** 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.

**[0368]** The transduced or transfected immune effector cell can be propagated *ex vivo* after introduction of the vector or isolated nucleic acid. The transduced or transfected immune effector cell can be 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. The transduced or transfected immune effector cell can be further evaluated or screened to select the engineered mammalian cell.

**[0369]** 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.

**[0370]** 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 (The section "Sources of T cells" is disclosed as reference. The embodiments in this section are not embodiments of the invention.)**

**[0371]** 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<sup>2+</sup>-free, Mg<sup>2+</sup>-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.

**[0372]** 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.

**[0373]** 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-CD25 conjugated beads or other similar method of selection.

**[0374]** 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.

**[0375]** 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  $5 \times 10^6$ /ml. In some embodiments, the concentration used can be from about  $1 \times 10^5$ /ml to  $1 \times 10^6$ /ml, and any integer value in between.

**[0376]** 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.

**[0377]** 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.

**[0378]** 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.

**[0379]** Also disclosed as reference 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.

**[0380]** 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 (The section "Activation and Expansion of T Cells" is disclosed as reference. The embodiments in this section are not embodiments of the invention.)**

**[0381]** 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.

**[0382]** 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<sup>+</sup> T cells or CD8<sup>+</sup> 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): 1319-1328, 1999; Garland et al., J. Immunol Meth. 227(1-2): 53-63, 1999).

**[0383]** 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.

**[0384]** 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.

**[0385]** By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3 $\times$ 28 beads) to contact the T cells. In one embodiment the cells (for example, 10<sup>4</sup> to 10<sup>9</sup> 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<sup>+</sup> T cells that normally have weaker CD28 expression.

**[0386]** 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- $\gamma$ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF $\beta$ , and TNF- $\alpha$  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,  $\alpha$ -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<sub>2</sub>). 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<sup>+</sup>) 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.

**[0387]** 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**

**[0388]** Further provided by the present invention are pharmaceutical compositions comprising any one of the engineered immune effector cells comprising any one of the CARs of the invention, and a pharmaceutically acceptable carrier. Pharmaceutical compositions can be prepared by mixing 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.

**[0389]** 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.

**[0390]** 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.

**[0391]** 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.

**[0392]** 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.

**[0393]** Additional excipients include agents which can serve as one or more of the following: (1) bulking agents, (2) solubility enhancers, (3) stabilizers and (4) 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, myoinositol, myoinositol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol,  $\alpha$ -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.

**[0394]** 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.

**[0395]** Suitable non-ionic surfactants include polysorbates (20, 40, 60, 65, 80, etc.), polyoxamers (184, 188, etc.), PLURONIC<sup>®</sup> polyols, TRITON<sup>®</sup>, polyoxyethylene sorbitan monoethers (TWEEN<sup>®</sup>-20, TWEEN<sup>®</sup>-80, etc.), laurmacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, sucrose fatty acid ester, methyl cellulose 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.

**[0396]** In order for the pharmaceutical compositions to be used for in vivo administration, they must be sterile. The pharmaceutical composition may be rendered sterile by filtration through sterile filtration membranes. The pharmaceutical compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

**[0397]** The route of administration is in accordance with known and accepted methods, such

as by single or multiple bolus or infusion over a long period of time in a suitable manner, e.g., injection or infusion by subcutaneous, intravenous, intraperitoneal, intramuscular, intraarterial, intralesional or intraarticular routes, topical administration, inhalation or by sustained release or extended-release means.

**[0398]** Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

**[0399]** The pharmaceutical compositions described herein may also contain more than one active compound or agent as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition may comprise a cytotoxic agent, chemotherapeutic agent, cytokine, immunosuppressive agent, or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

**[0400]** The active ingredients may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 18th edition.

## **VI. Uses in methods of treating cancer**

**[0401]** The present invention further provides a CAR or a pharmaceutical composition of the invention for use in methods of cell immunotherapy. In some embodiments, the cell immunotherapy is for treating cancer, including but not limited to hematological malignancies and solid tumors. Any of the single-domain antibodies, chimeric antigen receptors, and engineered immune effector cells described herein may be used in the method of treating cancer. The CARs described herein may be useful for treating tumors having antigen loss escape mutations, and for reducing resistance to existing therapies. In some embodiments, the compositions of the invention may be used in methods for treating other diseases that are associated with the antigens specifically recognized by the single-domain antibodies or CARs, including, for example, autoimmune diseases.

**[0402]** Disclosed as reference, there is a method of treating a cancer in an individual (such as a human individual), comprising administering to the individual an effective amount of a pharmaceutical composition comprising: (1) 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; and (2) a pharmaceutically acceptable carrier. In some examples, 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 examples, the plurality of sdAbs is camelid, chimeric, human, or humanized. In some examples, the plurality of single-domain antibodies is fused to each other via peptide bonds or peptide linkers. In some examples, 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 examples, the transmembrane domain is selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some examples, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some examples, the primary intracellular signaling domain is derived from CD3 $\zeta$ . In some examples, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some examples, 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 examples, the multivalent CAR further comprises a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some examples, the multivalent CAR further comprises a signal peptide (such as a CD8 $\alpha$  signal peptide) located at the N-terminus of the polypeptide. In some examples, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3 $\zeta$ . In some examples, the multivalent CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 198-201, and 265-270. In some examples, the engineered immune effector cell is autologous. In some examples, the engineered immune effector cell is allogenic. In some examples, the cancer is a liquid cancer, such as multiple myeloma, acute lymphoblastic leukemia, or chronic lymphocytic leukemia. In some examples, the cancer is a solid cancer.

**[0403]** Disclosed as reference, there is a method of treating a cancer in an individual (such as a human individual), comprising administering to the individual an effective amount of a pharmaceutical composition comprising: (1) 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; and (2) a pharmaceutically acceptable carrier. In some examples, 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 examples, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some examples, 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 examples, 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 examples, the transmembrane domain is selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some examples, embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some examples, the primary intracellular signaling domain is derived from CD3 $\zeta$ . In some examples, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some examples, 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 examples, the multivalent CAR further comprises a hinge domain (such as a CD8 $\alpha$  hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some examples, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3 $\zeta$ . In some examples, the engineered immune effector cell is autologous. In some examples, the engineered immune effector cell is allogenic. In some examples, the cancer is a liquid cancer, such as multiple myeloma, acute lymphoblastic leukemia, or chronic lymphocytic leukemia. In some examples, the cancer is a solid cancer.

**[0404]** The present invention provides a pharmaceutical composition of the invention for use in a method of treating a cancer in an individual (such as a human individual) wherein the method comprises administering to the individual an effective amount of a pharmaceutical composition comprising: (1) an engineered immune effector cell (such as T cell) comprising a BCMA CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain; and (2) a pharmaceutically acceptable carrier, wherein the first 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; and wherein the second 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.

**[0405]** In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V<sub>H</sub>H domain comprising an amino acid sequence from the group consisting of SEQ ID NO:80 and 87. 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 that of CD3 $\zeta$ . In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is that of 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 and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8 $\alpha$  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 CD8 $\alpha$  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 CD8 $\alpha$  signal peptide, the extracellular antigen binding domain, a CD8 $\alpha$  hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain of CD28, a second co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8 $\alpha$  signal peptide, the extracellular antigen-binding domain, a CD8 $\alpha$  hinge domain, a CD8 $\alpha$  transmembrane domain, a co-stimulatory signaling domain of CD137, and a primary intracellular signaling domain of CD3 $\zeta$ . In some embodiments, the BCMA CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 265-270. 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, such as multiple myeloma, acute lymphoblastic leukemia, or chronic lymphocytic leukemia. In some embodiments, the cancer is a solid cancer.

**[0406]** Disclosed as reference, there is a method of treating a disease (such as cancer) in an individual (such as a human individual), comprising administering to the individual an effective amount of a pharmaceutical composition comprising an anti-BCMA single-domain antibody and a pharmaceutically acceptable carrier, wherein the anti-BCMA sdAb comprises any one of the following:

1. (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. (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. (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. (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. (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. (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. (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. (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. (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. (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. (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.

**[0407]** In some examples, the anti-BCMA sdAb comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 78-88.

**[0408]** The methods described herein are suitable for treating various cancers, including both solid cancer and liquid cancer. The methods are applicable to cancers of all stages, including early stage, advanced stage and metastatic cancer. The methods described herein may be used as a first therapy, second therapy, third therapy, or combination therapy with other types of cancer therapies known in the art, such as chemotherapy, surgery, radiation, gene therapy, immunotherapy, bone marrow transplantation, stem cell transplantation, targeted therapy, cryotherapy, ultrasound therapy, photodynamic therapy, radio-frequency ablation or the like, in an adjuvant setting or a neoadjuvant setting.

**[0409]** Administration of the pharmaceutical compositions may be carried out in any convenient manner, including by injection, ingestion, transfusion, implantation or transplantation. The compositions may be administered to a patient transarterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, intravenously, or intraperitoneally. In some embodiments, the pharmaceutical composition is administered systemically. In some embodiments, the pharmaceutical composition is



administered to an individual by infusion, such as intravenous infusion. Infusion techniques for immunotherapy are known in the art (see, e.g., Rosenberg et al., *New Eng. J. of Med.* 319: 1676 (1988)). In some embodiments, the pharmaceutical composition is administered to an individual by intradermal or subcutaneous injection. In some embodiments, the compositions are administered by intravenous injection. In some embodiments, the compositions are injected directly into a tumor, or a lymph node. In some embodiments, the pharmaceutical composition is administered locally to a site of tumor, such as directly into tumor cells, or to a tissue having tumor cells.

**[0410]** Multiple myeloma (MM) is an incurable aggressive plasma malignancy, which is categorized as a B-cell neoplasia and proliferates in uncontrollable method in the bone marrow, interfering with the normal metabolic production of blood cells and causing painful bone lesions (Garfall, A.L. et al., *Discovery Med.* 2014, 17, 37). Multiple myeloma can present clinically with hypercalcemia, renal insufficiency, anemia, bony lesions, bacterial infections, hyperviscosity, and amyloidosis (Robert Z. Orlowski, *Cancer Cell.* 2013, 24(3)). According to investigation and statistics, nearly 86,000 patients will be diagnosed each year with myeloma, and while about 63,000 patients die every year from the disease-related complications (Becker, 2011). Because of an aging populace, it is predicted that the number of cases of myeloma will increase year by year. Like many cancers, there is no known cause of multiple myeloma, and no cure. Some treatments for multiple myeloma are similar to treatments for other cancers, such as chemotherapy or radiation therapy, stem cell transplant or bone marrow transplant, targeted therapy or biological therapy (George, 2014). Antibody-based cell immunotherapies have demonstrated substantial clinical benefit for patients with hematological malignancies, particular in B cell Non-Hodgkin's lymphoma. There is a need for effective immunotherapeutic agent for treating multiple myeloma.

**[0411]** Dosages and desired drug concentration of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary artisan. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. "The Use of Interspecies Scaling in Toxicokinetics," In *Toxicokinetics and New Drug Development*, Yacobi et al., Eds, Pergamon Press, New York 1989, pp. 42-46. It is within the scope of the present invention that different formulations will be effective for different treatments and different disorders, and that administration intended to treat a specific organ or tissue may necessitate delivery in a manner different from that to another organ or tissue.

**[0412]** Disclosed as reference, wherein the pharmaceutical composition comprises any one of the single-domain antibodies described herein, the pharmaceutical composition is administered at a dosage of about 10 ng/kg up to about 100 mg/kg of body weight of the individual or more per day, for example, at about 1 mg/kg/day to 10 mg/kg/day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Pat. No. 4,657,760; 5,206,344; or 5,225,212.

**[0413]** In some embodiments, wherein the pharmaceutical composition comprises any one of the engineered immune cells of the invention, the pharmaceutical composition is administered at a dosage of at least about any of  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$ ,  $10^8$ , or  $10^9$  cells/kg of body weight of the individual. In some embodiments, the pharmaceutical composition is administered at a dosage of any of about  $10^4$  to about  $10^5$ , about  $10^5$  to about  $10^6$ , about  $10^6$  to about  $10^7$ , about  $10^7$  to about  $10^8$ , about  $10^8$  to about  $10^9$ , about  $10^4$  to about  $10^9$ , about  $10^4$  to about  $10^6$ , about  $10^6$  to about  $10^8$ , or about  $10^5$  to about  $10^7$  cells/kg of body weight of the individual.

**[0414]** In some embodiments, the pharmaceutical composition is administered for a single time. In some embodiments, the pharmaceutical composition is administered for multiple times (such as any of 2, 3, 4, 5, 6, or more times). In some embodiments, the pharmaceutical composition is administered once per week, once 2 weeks, once 3 weeks, once 4 weeks, once per month, once per 2 months, once per 3 months, once per 4 months, once per 5 months, once per 6 months, once per 7 months, once per 8 months, once per 9 months, or once per year. In some embodiments, the interval between administrations is about any one of 1 week to 2 weeks, 2 weeks to 1 month, 2 weeks to 2 months, 1 month to 2 months, 1 month to 3 months, 3 months to 6 months, or 6 months to a year. The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

**[0415]** Moreover, dosages may be administered by one or more separate administrations, or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

## **VII. Kits and articles of manufacture**

**[0416]** Further provided are kits, unit dosages, and articles of manufacture comprising any of the chimeric antigen receptors, or the engineered immune effector cells of the invention. In some embodiments, a kit is provided which contains any one of the pharmaceutical compositions of the invention and preferably provides instructions for its use.

**[0417]** The kits of the present invention are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. Kits may optionally provide additional components such as buffers and interpretative information. The present invention thus also provides articles of manufacture, which include vials (such as sealed vials), bottles, jars, flexible packaging, and the like.

**[0418]** The article of manufacture can comprise a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials,

syringes, *etc.* The containers may be formed from a variety of materials such as glass or plastic. Generally, the container holds a composition which is effective for treating a disease or disorder (such as cancer) described herein, and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The label or package insert indicates that the composition is used for treating the particular condition in an individual. The label or package insert will further comprise instructions for administering the composition to the individual. The label may indicate directions for reconstitution and/or use. The container holding the pharmaceutical composition may be a multi-use vial, which allows for repeat administrations (e.g. from 2-6 administrations) of the reconstituted formulation. Package insert refers to instructions customarily included in commercial packages of therapeutic products that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products. Additionally, the article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

**[0419]** The kits or article of manufacture may include multiple unit doses of the pharmaceutical composition and instructions for use, packaged in quantities sufficient for storage and use in pharmacies, for example, hospital pharmacies and compounding pharmacies.

**[0420]** The examples below are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way. The following examples and detailed description are offered by way of illustration and not by way of limitation.

## EXAMPLES

**[0421]** The examples described herein are not intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (for example, amounts, temperature, *etc.*), but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

### Example 1. Preparation of single-domain antibodies

**[0422]** To develop single-domain antibodies with high binding affinity to specified antigens, llamas were immunized and a phage-display library was constructed to identify V<sub>H</sub>H leads. Distinct clones were picked at random and were classified according to the heavy chain complementarity determining region 3 (CDR3), a region that can play a major role in antigen

binding.

**[0423]** In order to obtain the single-domain antibodies, llamas were immunized regularly with respective immunogens, which can include recombinant human BCMA protein having a C-terminal Fc tag (ACRO Biosystems, Cat No.:BC7-H5254), recombinant human CD38 protein having a C-terminal His tag (ACRO Biosystems, Cat No.: CD8-H5224), recombinant human CD19 protein having a C-terminal Fc tag (ACRO Biosystems, Cat No.:CD9-H5229) and recombinant human CD20 protein having a C-terminal Fc tag (ACRO Biosystems, Cat No.: CD0-H526a).

**[0424]** A process of generating anti-BCMA single-domain antibodies is described below as an example for generating single-domain antibodies against various antigens. Generation of anti-human CD38, anti-human-CD19 and anti-CD20 single-domain antibodies were performed with similar processes as described below. Other protocols for preparing single-domain antibodies have been described. See, for example, Els Pardon et al, Nature Protocol, 2014; 9(3): 674.

## **1. Animal immunization and immune response assay**

### **1.1 Animal immunization**

**[0425]** Each immunogen comprising a recombinant antigen protein was mixed with adjuvant or PBS and injected to a llama. The animals were immunized by service vendor (Cedarline) for seven times, typically with 200 µg immunogen and CFA (Complete Freund's Adjuvant) each time at about 1-week to 2-week intervals. Peripheral blood samples were collected at the pre-immunization stage and after the 5th and 7th immunization. After multiple rounds of immunization, immune reactions of the llamas against the target antigen were evaluated to confirm the titer of antigen-specific single-domain antibodies. Lymphocytes were isolated by gradient centrifugation from about 100 ml of peripheral blood. The cells were supplemented with RNALATER™ and stored at -80°C. Sera were obtained by centrifugation of anti-coagulated blood samples and stored at -80°C.

### **1.2 IgG fractionation**

**[0426]** IgG-subclass fractionation was carried out according to GenScript's Standard Operating Procedure. The IgG subclasses were fractionated from terminal bleed serum using Protein G and Protein A resins. The 1 ml serum sample was loaded onto a 1 ml HITRAP® Protein G HP column, and the column was washed with 10 ml phosphate buffer (20 mM, pH 7.0). The IgG3 (MW 100,000 Da) fraction was eluted with 0.15 M NaCl, 0.58% acetic acid (pH 3.5), and the eluate was neutralized with 1 M Tris-HCl (pH 9.0) to pH 7.4. Subsequently, the IgG1 (MW 170,000 Da) fraction was eluted with 0.1 M glycine-HCl (pH 2.7), and the eluate was

neutralized with 1 M Tris-HCl (pH 8.5) to pH 7.4. The flow-through of HITRAP<sup>®</sup> Protein G HP column was then loaded onto a 1 ml HITRAP<sup>®</sup> Protein A HP column, and the column was washed with 20 ml phosphate buffer (20 mM, pH 7.0). The IgG2 (MW 100,000 Da) fraction was eluted with 0.15 M NaCl, 0.58% acetic acid (pH 4.5), and the eluate was neutralized with 1M Tris-HCl (pH 9.0) to pH 7.4. The concentrations of the purified IgG1, IgG2 and IgG3 antibodies were determined by OD280, and the purity of each was assessed by both reducing and non-reducing SDS-PAGE analysis.

### 1.3 Immune response assay

**[0427]** Immune response of the llamas was evaluated by ELISA, in which the serum samples and purified IgGs were assayed for binding to immobilized immunogens. Sera collected pre-immunization, after 5th immunization and at terminal bleed were evaluated. The antigen (*i.e.*, recombinant human antigen protein) was diluted in coating buffer at 4 µg/ml. The microtiter plate was coated with diluted antigen at 4°C overnight. The plate was then washed 3 times with washing buffer followed by blocking at room temperature for 2 hours. The plate was subsequently washed 4 times with washing buffer. A series of diluted sera or IgGs were added to the plate and incubated at room temperature for 1.5 hours. The plate was then washed 4 times with washing buffer. HRP-conjugated anti-llama IgG secondary antibody was added to the plate and incubated at room temperature for 1 hour. After washing, the TMB substrate was added to each well and incubated for 10 minutes before stopping with 1 M HCl. To quantify binding, absorbance at 450 nm was measured for each well using a MK3 spectrometer.

## 2. V<sub>H</sub>H phage display library construction

### 2.1 RNA extraction

**[0428]** Total RNA was extracted from the isolated lymphocytes (from 1.1.1) using TRIZOL<sup>®</sup> Reagent according to the manufacturer's protocol. Quantity and quality of the total RNA were assessed by gel electrophoresis and quantified by measuring absorbance at OD260/280.

### 2.2 RT-PCR and V<sub>H</sub>H amplification

**[0429]** Total RNA was reverse transcribed into cDNA with an oligo(dT)<sub>20</sub> primer using PRIMESCRIPT<sup>™</sup> 1st Strand cDNA Synthesis Kit according to the manufacturer's protocol. Six forward and two reverse specific degenerate primers were designed to amplify the V<sub>H</sub>H fragments, which had two BglI restriction sites introduced. The V<sub>H</sub>H fragments were amplified

according to GenScript's standard operating procedure (SOP) as described below.

**[0430]** The variable regions of the heavy-chain immunoglobulins (*i.e.*, V<sub>H</sub>Hs) were amplified using a two-step polymerase chain reaction (PCR). In the first PCR, 100 ng of cDNA template was mixed with primers CALL001 (SEQ ID NO: 229) and CALL002 (SEQ ID NO: 230). The DNA products from the first PCR reaction were analyzed by agarose gel electrophoresis. After gel purification, the DNA products of the first PCR were used as templates in the second PCR. The second PCR was performed with the primers BACK-1 (SEQ ID NO: 231), BACK-2 (SEQ ID NO: 232) and PMCF (SEQ ID NO: 233). The amplified second PCR products containing V<sub>H</sub>H PCR fragments were gel purified and enzyme digested, and then inserted into phagemid plasmids. The recombinant plasmids with V<sub>H</sub>H gene fragments were electro-transferred into *E.coli* cells in order to generate the phage display V<sub>H</sub>H immune library.

**[0431]** The procedure of the PCR reaction has an initial denaturation step at 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and followed by a final extension step at 72°C for 7 min.

### 2.3 Phage library construction

**[0432]** The V<sub>H</sub>H PCR products were obtained by amplification using different primer pairs. The PCR products were then digested with BglI and gel purified. The gel purified fragments were inserted into GenScript's in-house phagemid vector. A pilot library was constructed to optimize the ligation and transformation conditions. The optimized ligation and transformation conditions were employed to develop the phagemid library. A small portion of the transformed cells was diluted and streaked on 2×YT plates supplemented with 100 µg/ml ampicillin. The colonies were counted to calculate the library size. Positive clones were randomly picked and sequenced to assess the quality of the library. The rest of the transformed cells were streaked onto YT plates supplemented with 100 µg/ml ampicillin and 2% glucose. Lawns of colonies were scraped off the plates. A small aliquot of the cells was used for library plasmid isolation. The rest was supplemented with glycerol and stored at -80°C as stock.

## 3. Phage display panning

### 3.1 Bio-panning

**[0433]** The constructed V<sub>H</sub>H phage library was panned against recombinant human BCMA protein and CHO cells expressing human BCMA (*i.e.*, CHO-BCMA cells, prepared in house by Legend Biotec) respectively using a standard procedure developed by GenScript. The library stock was grown to log phase, and then the library was rescued with M13KO7 helper phage

and was amplified overnight at 25°C in a shaker. The phage was then precipitated with PEG/NaCl, re-suspended in PBS and stored at -80°C. For solid phase panning, microplate wells were coated with recombinant human BCMA protein in PBS at 4°C overnight. For liquid phase panning, CHO-BCMA cells were blocked with blocking buffer at room temperature for 1 hour. During the coating or blocking step, phage particles were pre-incubated with the blocking buffer and Fc control protein in microplate wells. After pre-incubation, phage particles were added to the wells coated with BCMA proteins or CHO-BCMA solution respectively and incubated for 1 hour. After incubation, unbound and nonspecifically bound phages were washed away by rinsing the wells or the CHO-BCMA cells with PBST for six times supplemented with two additional PBS washes. The bound phage particles were eluted by 100 mM triethylamine (TEA), and the eluate was neutralized by 1 M Tris-HCl (pH 7.4). Half of the eluate was then used to infect exponentially growing *E. coli* TG1 cells ( $OD_{600} = 0.4 \sim 0.6$ ) for output titration.

### 3.2 Phage ELISA

**[0434]** Phage ELISA was performed to identify clones specific to the target antigens. Individual output phage clones were grown in 96-deep-well plate and rescued by M13KO7 helper phage overnight. To identify clones that bind to antigen proteins, 96-well ELISA microtiter plates were coated with recombinant human BCMA protein and Fc control protein respectively in coating buffer overnight at 4°C, and the plates were then blocked with blocking buffer. After blocking, approximately 50 µl per well of phage supernatant from the overnight cell culture was added to the plates for 1.5-hour incubation at 4°C. The plates were washed four times, and the HRP-conjugated anti-M13 monoclonal antibody was added to the plates for 45-minute incubation at 4°C. The plates were again washed five times and substrate solution was added to the wells for developing. Absorption at 450 nm was measured for each well.

**[0435]** To identify clones that bind CHO-BCMA cells, the CHO-BCMA cells were blocked with blocking buffer at room temperature for 1 hour. After blocking, approximately 20 µl per well of phage supernatant from the overnight cell culture was added to the cell solutions for 1-hour incubation at room temperature. After the cells were washed 4 times, the HRP-conjugated anti-M13 monoclonal antibody was added for 30-minute incubation at room temperature. The cells were washed five times and substrate solution was then added for developing. The absorption was measured at 450 nm.

**[0436]** After panning, phage ELISA positive single clones were randomly selected and DNA was prepared from output phage using plasmid extraction kits. The inserts in the plasmids were sequenced. One or more  $V_HH$  sequences were obtained for each target antigen, see, for example,

**[0437]** Table 2.

### Example 2. Preparation of Monospecific $V_HH$ Chimeric Antigen Receptors

**[0438]** A CAR backbone sequence encoding a CAR backbone polypeptide comprising from the N-terminus to the C-terminus: a CD8a hinge domain, a CD28 transmembrane domain, a CD28 cytoplasmic domain, a CD137 cytoplasmic domain, and a CD3 $\zeta$  cytoplasmic domain were chemically synthesized and cloned into a pre-modified lentiviral vector downstream and operably linked to a constitutive hEF1 $\alpha$  promoter. The resulting CAR backbone vector was named "PLLV-hEF1 $\alpha$ -8281373." Multi-cloning sites (MCS) in the vector allowed insertion of a nucleic acid sequence comprising a Kozak sequence (SEQ ID NO: 126) operably linked to a nucleic acid sequence encoding a CD8 $\alpha$  signal peptide fused to the N-terminus of a V<sub>H</sub>H fragment into the PLLV-hEF1 $\alpha$ -8281373 vector, upstream and operably linked to the CAR backbone sequence.

**[0439]** To construct a monospecific CAR having a single V<sub>H</sub>H domain using the PLLV-hEF1 $\alpha$ -8281373 backbone, the nucleic acid sequence encoding the V<sub>H</sub>H domain was operably linked to the 3' of the nucleic acid sequence encoding the CD8 $\alpha$  signal peptide. The fusion nucleic acid sequence was chemically synthesized and cloned into the PLLV-hEF1 $\alpha$ -8281373 CAR backbone via the EcoRI (SEQ ID NO: 234: 5'-GAATTC-3') and SpeI (SEQ ID NO: 235: 5'-ACTAGT-3') restriction sites by molecular cloning techniques known in the art. Table 4 lists the vectors that were constructed to express the exemplary monospecific, monovalent anti-BCMA and anti-CD38 CARs.

**[0440]** For ease of further inserting additional sequences, such as a nucleotide encoding a second V<sub>H</sub>H, when designing a monospecific CAR construct (e.g., anti-BCMA or anti-CD38), restriction sites including HpaI (SEQ ID NO: 236: 5'-GTTAAC-3'), MluI (SEQ ID NO: 237: 5'-ACGCGT-3'), NsiI (SEQ ID NO: 238: 5'-ATGCAT-3') sites were included between the CD8 $\alpha$  signal peptide nucleic acid sequence and the V<sub>H</sub>H nucleic acid sequence.

**[0441]** The lentivirus packaging plasmid mixture including pCMV- $\Delta$ R-8.74 and pMD2.G (Addgene#12259) was pre-mixed with the vectors PLLV-hEF1 $\alpha$ -8281373 having V<sub>H</sub>H fragments (Table 4) at a pre-optimized ratio with polyetherimide (PEI), then mixed properly and incubated at room temperature for 5 minutes. The transfection mix was then added dropwise to the HEK293 cells and mixed gently. Afterwards, cells were incubated overnight in a 37°C and 5% CO<sub>2</sub> cell incubator. The supernatants were collected after centrifugation at 4°C, 500 g for 10 min.

**[0442]** The virus-containing supernatants were filtered through a 0.45 $\mu$ m PES filter, followed by ultra-centrifugation for lentivirus concentration. After ultra-centrifugation, the supernatants were carefully discarded and the virus pellets were rinsed cautiously with pre-chilled DPBS. The virus was aliquoted properly, then stored at -80°C immediately. The virus titer was determined by p24 based on HTRF kit developed by GenScript.

#### **PBMC preparation**



**[0443]** Leukocytes were collected from healthy donors by apheresis, and cell concentration was adjusted to  $5 \times 10^6$  cells /ml in R10 medium. Leukocytes were then mixed with 0.9% NaCl solution at 1:1 (v/v) ratio. 3 ml lymphoprep medium was added to a 15 ml centrifuge tube, and 6 ml of diluted lymphocyte mix was slowly layered on top of the lymphoprep medium. The lymphocyte mix was centrifuged at 800 g for 30 minutes without brakes at 20°C. Lymphocyte buffy coat was then collected with a 200 µl pipette. The harvested fraction was diluted at least 6 folds with 0.9% NaCl or R10 to reduce density of the solution. The harvested fraction was then centrifuged at 250g for 10 minutes at 20°C. The supernatant was aspirated completely, and 10 ml of R10 was added to the cell pellet to resuspend the cell pellet. The mixture was further centrifuged at 250 g for 10 minutes at 20°C. The supernatant was again aspirated. 2 ml of 37°C pre-warmed R10 with 100IU/ml IL-2 was added to the cell pellet, and the cell pellet was re-suspended softly. The cell number was determined following Trypan Blue staining, and this PBMC sample was ready for later experiments.

#### **T cell purification**

**[0444]** Human T cells were purified from PBMCs using Miltenyi Pan T cell isolation kit (Cat#130-096-535), following manufacturer's protocol as described below. Cell number was first determined and the cell suspension was centrifuged at 300 g for 10 minutes. The supernatant was then aspirated completely, and the cell pellets were re-suspended in 40 µl buffer per  $10^7$  total cells. 10 µl of Pan T Cell Biotin-Antibody Cocktail was added per  $10^7$  total cells, mixed thoroughly and incubated for about 5 minutes in the refrigerator (2~8°C). 30 µl of buffer was then added per  $10^7$  cells. 20 µl of Pan T Cell MicroBead Cocktail was added per  $10^7$  cells. The cell suspension mixture was mixed well and incubated for an additional 10 minutes in the refrigerator (2~8°C). A minimum of 500 µl is required for magnetic separation. For magnetic separation, an LS column was placed in the magnetic field of a suitable MACS Separator. The column was prepared by rinsing with 3 ml of buffer. The cell suspension was then applied onto the column, and flow-through containing the unlabeled cells was collected, which represented the enriched T cell fractions. Additional T cells were collected by washing the column with 3 ml of buffer and collecting unlabeled cells that pass through. These unlabeled cells again represented the enriched T cells, and were combined with the flow-through from previous step. The pooled enriched T cells were then centrifuged and re-suspended in R10+100IU/ml IL-2.

**[0445]** The prepared T cells were subsequently pre-activated for 48-96 hours with human T cell activation/expansion kit (Miltenyi#130-091-441) according to manufacturer's protocol in which anti-CD3/CD28 MACSiBead particles were added at a bead-to-cell ratio of 1:2.

**[0446]** The pre-activated T cells were transduced with lentivirus stock in the presence of 7

µg/ml polybrene with centrifugation at 1200 g, 32°C for 1.5 h. The transduced cells were then transferred to the cell culture incubator for transgene expression under suitable conditions.

**[0447]** On day 3 or day 7 post-transduction, transduced T cells were harvested and co-incubated with tumor cells at an effector (CAR-T) to target cell ratio of 20:1 for 20 hours. Target cells were either human multiple myeloma cell line RPMI8226.Luc or human glioblastoma cell line U87MG.Luc cells, both cell lines engineered in house to express firefly luciferase. To assay the cytotoxicity of CAR-T on tumor cells, One-glo luminescent luciferase assay reagents (Promega#E6110) were prepared according to manufacturer's protocol and added to the co-cultured cells to detect the remaining luciferase activity in the well. Since luciferase is expressed only in RPMI8226.Luc or U87MG.Luc cells, the remaining luciferase activity in the well correlates directly to the number of viable target cells in the well. The maximum luciferase activity was obtained by adding culture media to target cells in absence of effector cells. The minimum luciferase activity was determined by adding Triton X-100 at a final concentration of 1% at the time when the cytotoxicity assays were initiated. The specific cytotoxicity was calculated by the formula: Specific Cytotoxicity%= 100% \* (1-(RLUsample-RLUmin)/(RLUmax-RLUmin)).

**[0448]** Monospecific CAR clones targeting BCMA (CD269) were coded starting with the digits "269" while monospecific CAR clones targeting CD38 were similarly coded starting with the digits "38." As shown in FIG. 2A, the selected clones exhibited different levels of cytotoxicity on multiple myeloma cell line RPMI8226.Luc cells, with over 60% monospecific V<sub>H</sub>H-based CAR-Ts showing >50% cytotoxicity against RPMI8226.Luc cells. Clones 269A37346, 269A37348, 269A37353, 269A37355, 38A37326, 38A37331, 38A37717, and 38A37719 based CAR-T were selected for further testing. In particular, clones 269A37346, 269A37348, 269A37353, 269A37355, 38A37326, 38A37331, 38A37717 based CAR-T exhibited potent cytotoxicity on multiple myeloma cell line RPMI8226.Luc cells with more than 20%-30% increase in RPMI8226.Luc cell killing by CAR-T treatment as compared with untransduced control T cells (UnT). Nevertheless, such cytotoxicity increase did not occur on human glioblastoma cell line U87MG.Luc cells (see FIG. 2B). No significant cytotoxicity effects were detected on U87MG.Luc by these monospecific V<sub>H</sub>H-based CAR-T cells as compared to UnT controls. The observation above indicated that some of these clones might be target specific and potent on BCMA or CD38 positive cells.

### **Example 3. Preparation of exemplary bispecific or multivalent chimeric antigen receptors**

**[0449]** Potentially potent clones as described in Example 2 could also be favorable candidates for generating bispecific or multivalent V<sub>H</sub>H-based CARs. Two representative V<sub>H</sub>H clones (anti-BCMA V<sub>H</sub>H clone 269A37346 and anti-CD38 V<sub>H</sub>H clone 38A37717) were selected to construct various exemplary CAR constructs.

**[0450]** BCMA × CD38 V<sub>H</sub>H-based CARs can be generated by combining BCMA specific V<sub>H</sub>H and CD38 specific V<sub>H</sub>H via a suitable peptide linker (e.g. Gly-Ser polymer) followed by a CAR signal domain backbone vector. Exemplary bispecific BCMA×CD38 CAR constructs (GSI5001 to GSI5010) are listed in Table 6. First, the amino acid sequence of anti-BCMA V<sub>H</sub>H and anti-CD38 V<sub>H</sub>H were connected together via a Gly-Ser linker that could be of different lengths. Then the linked amino acid sequence was placed after the CD8α signal peptide sequence. This combined sequence including Kozak-CD8α signal peptide-bispecific V<sub>H</sub>H was directly synthesized and cloned into a PLLV-hEF1α-81373 CAR backbone via EcoRI and SpeI restriction sites, by regular molecular cloning protocols known in the art. The CAR backbone sequence encodes a CAR backbone polypeptide comprising from the N-terminus to the C-terminus: a CD8a hinge domain, a CD8a transmembrane domain, a CD137 cytoplasmic domain, and a CD3ζ cytoplasmic domain were chemically synthesized and cloned into a pre-modified lentiviral vector downstream and operably linked to a constitutive hEF1α promoter. The resulting CAR backbone vector was named "PLLV-hEF1α-81373."

**[0451]** Additionally, an exemplary co-expression vector encoding BCMA CAR and CD38 CAR were constructed by combining BCMA specific V<sub>H</sub>H-based CAR and CD38 specific V<sub>H</sub>H-based CAR in a single CAR vector via a suitable splice based linker (T2A, P2A, or F2A). For example, the GSI5013 construct has a nucleic acid sequence of SEQ ID NO: 239. The nucleic acid sequence in GSI5013 encodes from the 5' to the 3': CD8a signal peptide, 38A37717 V<sub>H</sub>H, CD8 α hinge domain, CD8a transmembrane domain, CD137 co-stimulatory domain, CD3ζ cytoplasmic signaling domain, T2A, CD8a signal peptide, 269A37346 V<sub>H</sub>H, CD8 α hinge domain, CD8a transmembrane domain, CD137 co-stimulatory domain, and CD3ζ cytoplasmic signaling domain.

**[0452]** Multivalent V<sub>H</sub>H CARs can be constructed by introducing a nucleic acid sequencing encoding multiple copies of a single V<sub>H</sub>H fused to each other by peptide linkers into a CAR signal domain backbone vector. Exemplary monovalent multivalent CAR constructs (GSI5014, GSI5015, GSI5016, GSI5017) are listed in Table 5. These constructs were prepared by linking 2-3 copies of V<sub>H</sub>H by Glycine-serine linker followed by directly synthesizing this linked sequence in combination with Kozak-CD8α signal peptide nucleic acid sequence, and cloning into the PLLV-hEF1α-81373 CAR backbone via EcoRI and SpeI restriction sites. As controls, single copy V<sub>H</sub>H were also cloned into the same PLLV-hEF1α-81373 CAR backbone by identical methods (GSI5011 and GSI5012, listed in Table 4).

**[0453]** Lentiviral vectors carrying CAR genes of GSI5001 to GSI5017 were packaged and titrated with protocols as described in Example 2. Using protocols described in Example 2, human PBMCs were prepared from peripheral bloods of volunteers for further isolation of primary human T cells using Miltenyi human PanT cell isolation kits. The purified T cells were pre-activated and expanded using Miltenyi anti-CD3/CD28 micro-beads as described in Example 2. The pre-activated T cells were then transduced with lentivirus stock in the presence of 7 µg/ml polybrene by centrifugation at 1200 g, 32°C for 1.5 h. The transduced

cells were then transferred to the cell culture incubator for transgene expression under suitable conditions.

**[0454]** On day 3 post transduction, transduced T cells were harvested and co-incubated with tumor cells at an effector (CAR-T) to target cell ratio of 20: 1 for 20 hours. To assay the cytotoxicity of CAR-T on tumor cells, One-glo luminescent luciferase assay reagents (Promega#E6110) were added to the co-cultured cells and the specific cytotoxicity for each CAR-T was measured as described in Example 2.

**[0455]** The copy numbers of integrated CAR genes for each group was determined by a semiquantitative PCR (q-PCR) assay. Briefly, genomic DNA from each group of CAR-T was prepared with Gentra Puregene Cell Kit (Qiagen). The concentration of genomic DNA was determined by Nanodrop, and 10ng genomic DNA sample was processed for a standardized q-PCR assay with SYBR Green Realtime PCR Master mix plus (Toyobo) on ABI#7300 q-PCR instrument using CAR specific primers (forward primer 137P2F (SEQ ID NO: 252): 5'-GTCCTTCTCCTGTCACTGGTTAT-3'; and reverse primer 137P2R (SEQ ID NO: 253): 5'-TCTTCTTCTTCTGGAAATCGGCA-3'). The relative copy number of each integrated CAR gene was calculated based on a standard curve established using plasmid containing target sequences.

**[0456]** As shown in Table 7 below, the copy numbers for each CAR-T preparation were determined and the data suggested high target gene integration into the genome of the T cells.

TABLE 7. Genome integration copy numbers.

CAR-T cells with constructs	Copies/ng gDNA
GSI5001	18257060
GSI5002	15105810
GSI5003	17307510
GSI5004	2735165
GSI5005	Not processed
GSI5006	6692277
GSI5007	6929693
GSI5008	15549250
GSI5009	10602720
GSI5010	7353348
GSI5011	3089537
GSI5012	650551.3
GSS005	1070972
GSI005	321521
UnT	72.77
water	117

**[0457]** As illustrated in FIGs. 3A-3B, mono-specific V<sub>H</sub>H CAR against BCMA (GSI5011) and mono-specific V<sub>H</sub>H CAR against CD38 (GSI5012) showed potent cytotoxicity on multiple myeloma cell line RPMI8226.Luc. With GSI5011 CAR, 42.98±2.86% RPMI8226.Luc cells were lysed, and with GSI5012, 61.25±1.92% RPMI8226.Luc cells were lysed, as compared with the non-specific lysis by untransduced control T cells (UnT, 9.25±1.11%).

**[0458]** The bispecific CARs GSI5001-GSI5010 elicited potent specific lysis of multiple myeloma cell line RPMI8226.Luc as compared to untransduced control T cells (UnT). As shown in FIGs. 3A and 3B, the specific percentage of lysis of RPMI8226.Luc cells was 98.91±0.17% for GSI5001-expressing CAR-T cells, 97.10±0.26% for GSI5002-expressing CAR-T cells, 93.85±0.69% for GSI5003-expressing CAR-T cells, 82.81±2.40% for GSI5004-expressing CAR-T cells, 98.95±0.66% for GSI5005-expressing CAR-T cells, 93.91±1.25% for GSI5006-expressing CAR-T cells, 96.49±1.05% for GSI5007-expressing CAR-T cells, 94.41±0.75% for GSI5008-expressing CAR-T cells, 90.72±0.62% for GSI5009-expressing CAR-T cells, and 85.05±2.69% for GSI5010-expressing CAR-T cells, as compared to the non-specific lysis by untransduced control T cells (UnT, 9.25±1.11%). These results also implicated that the shorter Gly-Ser linker seemed to be showing slightly better cytotoxicity performance on target tumor cells. Further studies could be performed under sub-optimal assay conditions to study such effects. Moreover, the order of anti-BCMA V<sub>H</sub>H and anti-CD38 V<sub>H</sub>H in the vector did not show significant influence on the final cytotoxicity performance on RPMI8226.Luc cells. Two scFv-based CAR-T cells were also prepared in the assay, in which GSS005 was an anti-BCMA scFv-based CAR while GSI005 was an anti-CD38 scFv-based CAR. Both GSS005 and GSI005 showed potent specific lysis against RPMI8226.Luc cells as well (57.94±1.91% for GSS5005, 61.25±1.92% for GSI005).

**[0459]** Engineered T cells with BCMA-specific monovalent CAR (GSI5011), BCMA specific bivalent CAR (GSI5012) or BCMA specific trivalent CAR (GSI5013) engineered T cells, and engineered T cells with CD38-specific monovalent CAR (GSI5012), CD38 specific bivalent CAR (GSI5016) or CD38 specific trivalent CAR (GSI5017) were prepared and cytotoxicity assays were performed on RPMI8226.Luc cells as described above. As shown in FIG. 4, the specific percentage lysis of RPMI8226.Luc cells were 63.25±2.64% by GSI5011-expressing CAR-T cells, 61.04±2.75% by GSI5014-expressing CAR-T cells, and 59.57±2.64% by GSI5015-expressing CAR-T cells, as compared to 0.05%±2.33% by untransduced control T cells (UnT). Also, as depicted in FIG. 4, the specific lysis of RPMI8226.Luc cells by anti-CD38 V<sub>H</sub>H CAR-T were 95.79±0.62% by GSI5012-expressing CAR-T cells, 94.16±0.31% by GSI5016-expressing CAR-T cells, and 97.61±0.77% by GSI5015-expressing CAR-T cells, as compared to 57.92% ±2.88% by untransduced control T cells (UnT). The data suggested that these CARs with different antigen binding modalities had potent antitumor activity against BCMA positive cells.

Reference

**Example 4. Preparation, *in vitro* and *in vivo* assays of an exemplary CD19×CD20 CAR**

**[0460]** Exemplary anti-CD19 sdAb and anti-CD20 sdAb were obtained by similar procedures as in Example 1. These single-domain antibodies are listed in Table 2. Monospecific CD19 CAR based on the CD19 V<sub>H</sub>H and monospecific CD20 CAR based on CD20 V<sub>H</sub>H were prepared as described in Example 2, and listed in Table 4. An exemplary bispecific CD19×CD20 CAR based on the CD19 V<sub>H</sub>H and CD20 V<sub>H</sub>H was constructed as described in Example 3, and listed in Table 6. The CAR backbone vector used for constructing the exemplary CD19 CAR, CD20 CAR and the CD19×CD20 CAR encodes from the N-terminus to the C-terminus: a CD8a signal peptide, CD8α hinge domain, a CD28 transmembrane domain, cytoplasmic domain of CD28, and cytoplasmic domain of CD3ζ. FIG. 5 shows the constructs of the CD19 CAR, the CD20 CAR and the bispecific CD19×CD20 CAR. Engineered CAR-T cells expressing the CD19 CAR, the CD20 CAR or the bispecific CD19×CD20 CAR were prepared as described in Example 2.

**[0461]** Cytotoxicity of the CAR-T cells were determined in a 4h co-culture assay. In the experiments, the prepared CAR-T cells were centrifugally collected, then diluted to desired concentrations by DPBS with 10% human AB serum, and cultured on 96 well-plates. Raji tumor cells, known to exhibit strong expression of CD19 and CD20, were labeled with Calcein-AM (BD Biosciences). The labeled CAR-T cells and Raji cells were cultured at an effector to target ratio of 10:1 at 37°C for 4 h. Subsequently, the cytotoxicity of CAR-T cells was detected by FACS.

**[0462]** As shown in FIG. 6 top left panel, there was no significant Raji cell lysis by untransduced T cells. The cytotoxicity on Raji cells was around 40% either by CD19 (39%, top right panel) or CD20 (41%, bottom left panel) monospecific V<sub>H</sub>H CAR-T cells. When the same CD19 V<sub>H</sub>H and CD20 V<sub>H</sub>H were fused to serve as the extracellular antigen binding domain in the same construct, *i.e.* in the CD19×CD20 bispecific CAR-T cells, the cytotoxicity of the Raji tumor cells (75.24%) were enhanced as compared to mono-specific CD19 V<sub>H</sub>H CAR-T or CD20 V<sub>H</sub>H CAR-T cells (FIG. 6 bottom right panel).

### Mouse Tumor Model Studies

**[0463]** NOG mice were infused with 4×10<sup>6</sup> Raji cells per mouse through tail vein injection. After 10 days, the mice were divided evenly into four groups at random, where each group was injected with equivalent doses of untransduced T cells, CD19 CAR-T cells, CD20 CAR-T cells, or CD19×CD20 bispecific CAR-T cells respectively, and observed continuously for 5 weeks.

**[0464]** As shown in FIG. 7, the *in vivo* survival data suggested that the overall survival rate for mice treated with CD19×CD20 bispecific CARs was higher than that with CD 19 or CD20 monospecific CAR-T cells.

### Example 5. Preparation of exemplary monospecific, bivalent CARs having two different BCMA binding domains

**[0465]** Potential V<sub>H</sub>H clones as described in Example 2 could also be used to generate monospecific, multivalent CARs having two or more different target binding domains in the extracellular antigen binding domain. Two representative anti-BCMA sdAb clones (clone 269A37353 and clone 269A37917) were selected to construct various exemplary monospecific, bivalent CAR constructs in the present example.

**[0466]** BCMA targeting bivalent CARs (*i.e.*, bivalent BCMA CARs) can be generated by fusing two different BCMA-specific V<sub>H</sub>H domains via a suitable peptide linker (*e.g.* Gly-Ser polymer), and subsequently inserting the fusion construct into a CAR signal domain backbone vector. Exemplary bivalent BCMA CAR constructs (GSI5021-GSI5026) having two different BCMA binding domains 269A37353 and 269A37917 are listed in Table 5. Peptide linkers of various lengths were used in different constructs. Constructs GSI5021-GSI5023 each encoded from the N-terminus to the C-terminus: CD8 $\alpha$  signal peptide, 269A37353, peptide linker, 269A37917, CD8 $\alpha$  hinge, CD8 $\alpha$  transmembrane domain, cytoplasmic domain of CD137, and cytoplasmic domain of human CD3 $\zeta$ . Constructs GSI5024-GSI5026 each encoded from the N-terminus to the C-terminus: CD8 $\alpha$  signal peptide, 269A37917, peptide linker, 269A37353, CD8 $\alpha$  hinge, CD8 $\alpha$  transmembrane domain, cytoplasmic domain of CD137, and cytoplasmic domain of human CD3 $\zeta$ . Each construct was further fused to a Kozak sequence at the 5' to provide the full coding sequence. The full coding sequence was directly synthesized and cloned into a PLLV-hEF1 $\alpha$ -81373 CAR backbone via EcoRI and SpeI restriction sites using common molecular cloning protocols known in the art. By contrast, monospecific, monovalent CARs (GSI5019 and GSI5020, listed in Table 4) having the same CAR signal domain backbone were constructed using similar methods.

**[0467]** Lentiviral vectors carrying each of the CAR constructs GSI5019-GSI5026 were packaged and titrated with protocols as described in Example 2. Using protocols described in Example 2, human PBMCs were prepared from peripheral blood samples from volunteers for further isolation of primary human T cells using Miltenyi human PanT cell isolation kits. The purified T cells were pre-activated and expanded using Miltenyi anti-CD3/CD28 micro-beads as described in Example 2.

**[0468]** The pre-activated T cells were then transduced with lentivirus stock in the presence of 7  $\mu$ g/ml polybrene by centrifugation at 1200 g, 32°C for 1.5 h. The transduced cells were then transferred to the cell culture incubator for transgene expression under suitable conditions.

**[0469]** On day 3 post transduction, transduced T cells were harvested and co-incubated with tumor cells (RPMI8226.Luc cells or U87MG.Luc cells) at an effector (CAR-T) to target cell ratio of 20:1 for 20 hours. RPMI8226.Luc cells are multiple myeloma cells expressing luciferase, and are BCMA positive. U87MG.Luc cells are glioblastoma cells expressing luciferase, and are

BCMA negative. To assay the cytotoxicity of the CAR-Ts on tumor cells, One-glo luminescent luciferase assay reagents (Promega#E6110) were added to the co-cultured cells and the specific cytotoxicity for each CAR-T was measured as described in Example 2.

**[0470]** As shown in FIG. 8A, the specific percentage of lysis of RPMI8226.Luc cells were  $88.21 \pm 1.29\%$  by GSI5019-expressing CAR-T cells,  $93.84 \pm 1.13\%$  by GSI5020-expressing CAR-T cells,  $71.45 \pm 1.79\%$  by GSI5021-expressing CAR-T cells,  $99.80 \pm 0.45\%$  by GSI5022-expressing CAR-T cells,  $97.46 \pm 0.50\%$  by GSI5023-expressing CAR-T cells,  $81.29 \pm 1.27\%$  by GSI5024-expressing CAR-T cells,  $93.50 \pm 0.47\%$  by GSI5025-expressing CAR-T cells,  $87.83 \pm 0.23\%$  by GSI5026-expressing CAR-T cells, respectively, as compared to  $13.49\% \pm 1.75\%$  by untransduced control T cells (UnT). Also, as depicted in FIG. 8B, the specific percentage of lysis of BCMA-negative cell line U87MG.Luc was  $2.84 \pm 7.41\%$  by GSI5019-expressing CAR-T cells,  $15.50 \pm 2.24\%$  by GSI5020-expressing CAR-T cells,  $6.74 \pm 3.37\%$  by GSI5021-expressing CAR-T cells,  $8.03 \pm 2.36\%$  by GSI5022-expressing CAR-T cells,  $9.00 \pm 1.88\%$  by GSI5023-expressing CAR-T cells,  $17.03 \pm 2.27\%$  by GSI5024-expressing CAR-T cells,  $16.81 \pm 1.98\%$  by GSI5025-expressing CAR-T cells,  $-11.55 \pm 5.43\%$  by GSI5026-expressing CAR-T cells, as compared to  $12.49\% \pm 3.79\%$  by untransduced control T cells (UnT). The data suggests that the multivalent CARs with different antigen-binding modalities had potent antitumor activity against BCMA positive cells, but not against BCMA negative cells.

## REFERENCES CITED IN THE DESCRIPTION

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**Patentkrav**

1. Kimær antigenreceptor (CAR) omfattende et polypeptid omfattende:
  - (a) et ekstracellulært antigenbindende domæne omfatter et første enkelt-domæneantistof (sdAb), der specifikt binder til et første antigen, og et  
5 andet sdAb, der specifikt binder til et andet antigen, hvor hver af det første og andet sdAb er et V<sub>H</sub>H;  
(b) et transmembrandomæne; og  
(c) et intracellulær signaleringsdomæne;  
hvor det første sdAb er et anti-BCMA sdAb omfattende en CDR1  
10 omfattende aminosyresekvensen af SEQ ID NO: 16, en CDR2 omfattende aminosyresekvensen af SEQ ID NO:27 og en CDR3 omfattende aminosyresekvensen af SEQ ID NO:38; og  
hvor det andet sdAb er et anti-BCMA sdAb omfattende en CDR1 omfattende aminosyresekvensen af SEQ ID NO:9, en CDR2 omfattende  
15 aminosyresekvensen af SEQ ID NO:20 og en CDR3 omfattende aminosyresekvensen af SEQ ID NO:31.
2. CAR'en ifølge krav 1, hvor det første sdAb er lokaliseret ved N-terminus eller C-terminus af det andet sdAb.  
20
3. CAR'en ifølge krav 1 eller 2, hvor det første sdAb og/eller det andet sdAb er camelid, kimært eller humaniseret.
4. CAR'en ifølge et hvilket som helst af kravene 1-3, hvor det første sdAb og det  
25 andet sdAb er direkte fusioneret til hinanden via en peptidbinding.
5. CAR'en ifølge et hvilket som helst af kravene 1-3, hvor det første sdAb og det andet sdAb er fusioneret til hinanden via en peptidlinker.
- 30 6. CAR'en ifølge krav 5, hvor peptidlinkeren ikke er mere end 50 aminosyrer lang.
7. CAR'en ifølge et hvilket som helst af kravene 1-6, hvor transmembrandomænet er transmembrandomænet af et molekyle valgt fra gruppen bestående af CD8α,

CD4, CD28, CD137, CD80, CD86, CD152 og PD1.

**8.** CAR'en ifølge et hvilket som helst af kravene 1-7, hvor det intracellulære signaleringsdomæne omfatter et primært intracellulært signaleringsdomæne af en  
5 immuneffektorcelle.

**9.** CAR'en ifølge krav 8, hvor det primære intracellulære signaleringsdomæne er det primære intracellulære signaleringsdomæne af CD3ζ.

10 **10.** CAR'en ifølge et hvilket som helst af kravene 1-9, hvor det intracellulære signaleringsdomæne omfatter et co-stimulatorisk signaleringsdomæne.

**11.** CAR'en ifølge krav 10, hvor det co-stimulatoriske signaleringsdomæne er det co-stimulatoriske signaleringsdomæne af et co-stimulatisk molekyle valgt fra  
15 gruppen bestående af CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3 og kombinationer deraf, fortrinsvis CD137.

**12.** CAR'en ifølge et hvilket som helst af kravene 1-11, yderligere omfattende et hængselsdomæne lokaliseret mellem C-terminus af det ekstracellulære antigen-  
20 bindende domæne og N-terminus af transmembrandomænet.

**13.** CAR'en ifølge et hvilket som helst af kravene 1-12, yderligere omfattende et signalpeptid lokaliseret ved N-terminus af polypeptidet.

25 **14.** Isoleret nukleinsyre omfattende en nukleinsyresekvens, der koder for CAR'en ifølge et hvilket som helst af kravene 1-13.

**15.** Vektor omfattende den isolerede nukleinsyre ifølge krav 14.

30 **16.** Vektoren ifølge krav 15, hvor vektoren er en lentiviral vektor.

**17.** Vektoren ifølge krav 16, hvor den lentivirale vektor er en selv-inaktiverende lentiviral vektor.

**18.** Modificeret immuncellule omfattende CAR'en ifølge et hvilket som helst af kravene 1-13, den isolerede nukleinsyre ifølge krav 14 eller vektoren ifølge et hvilket som helst af kravene 15-17.

5 **19.** Den modificerede immuncellule ifølge krav 18, hvor immuncellule-  
cellen er en T-celle.

**20.** Farmaceutisk sammensætning omfattende den modificerede immuncellule-  
celle ifølge krav 18 eller 19, og en farmaceutisk acceptabel bærer.

10

**21.** Farmaceutisk sammensætning som defineret i krav 20 til anvendelse i en  
fremgangsmåde til behandling af kræft hos et individ.

**22.** Den farmaceutiske sammensætning til anvendelse ifølge krav 21, hvor den  
15 modificerede immuncellule er autolog.

**23.** Den farmaceutiske sammensætning til anvendelse ifølge krav 21 eller 22,  
hvor kræftformen er multipelt myelom, akut lymfoblastisk leukæmi eller kronisk  
lymfatisk leukæmi.

20

**24.** Den farmaceutiske sammensætning til anvendelse ifølge krav 23, hvor  
kræftformen er multipelt myelom.

25

# DRAWINGS

Drawing

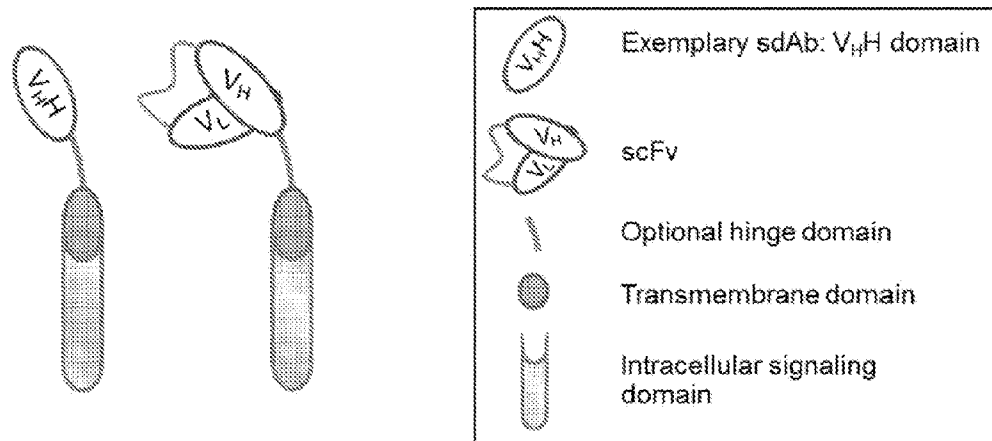


FIG. 1A

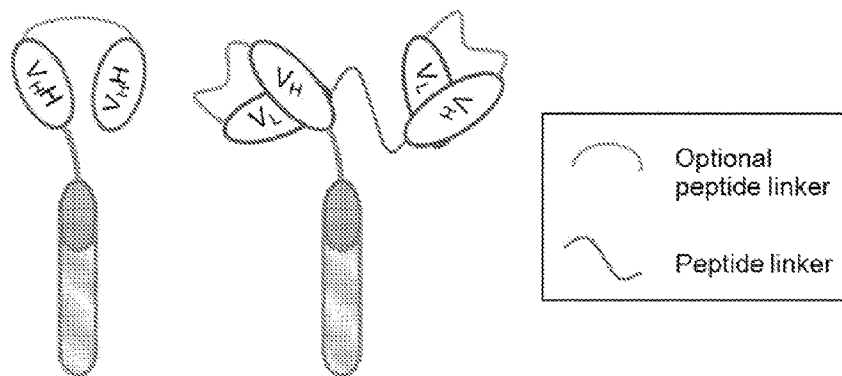


FIG. 1B

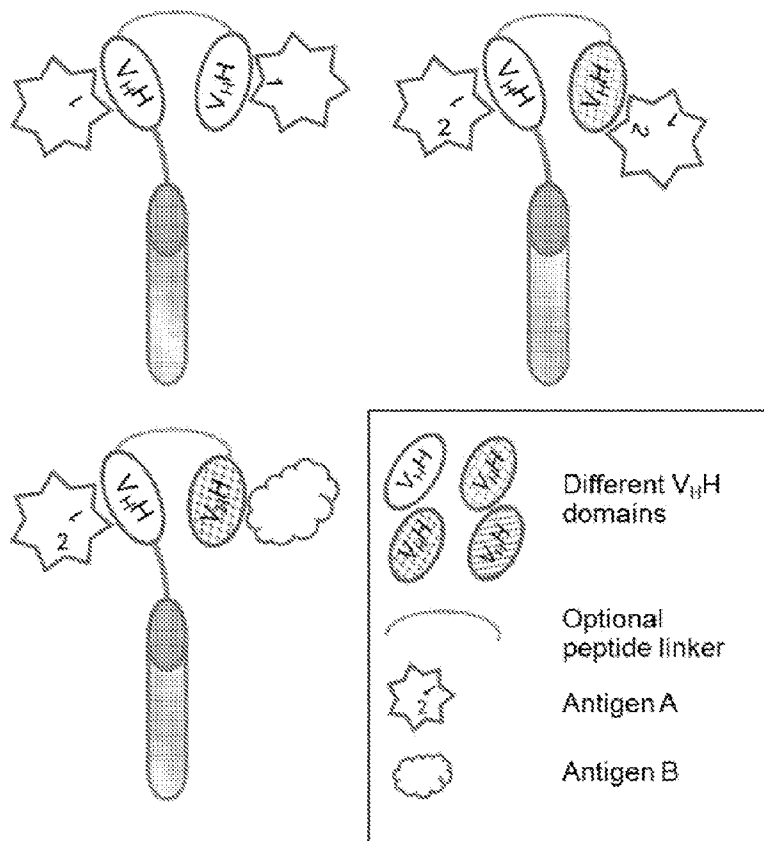


FIG. 1C

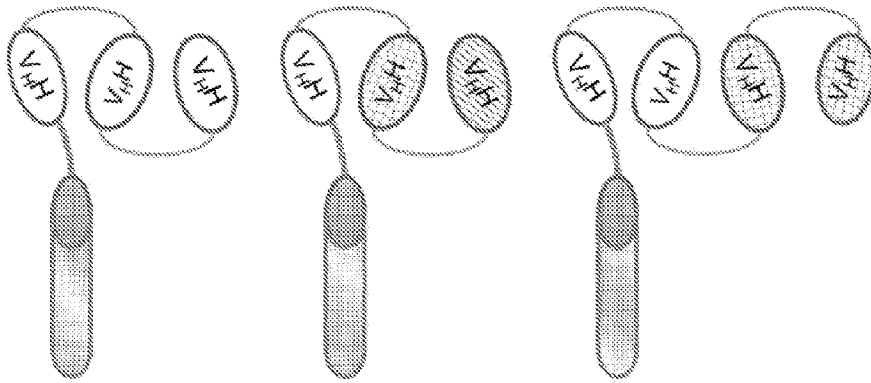


FIG. 1D

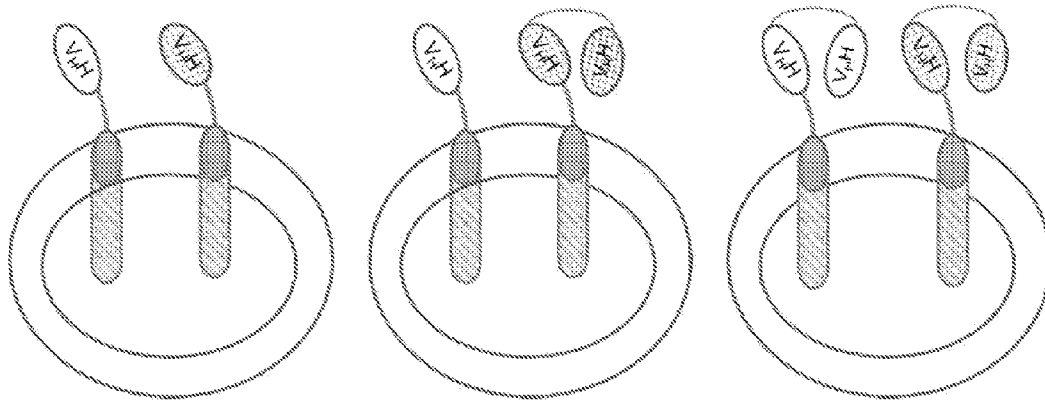


FIG. 1E

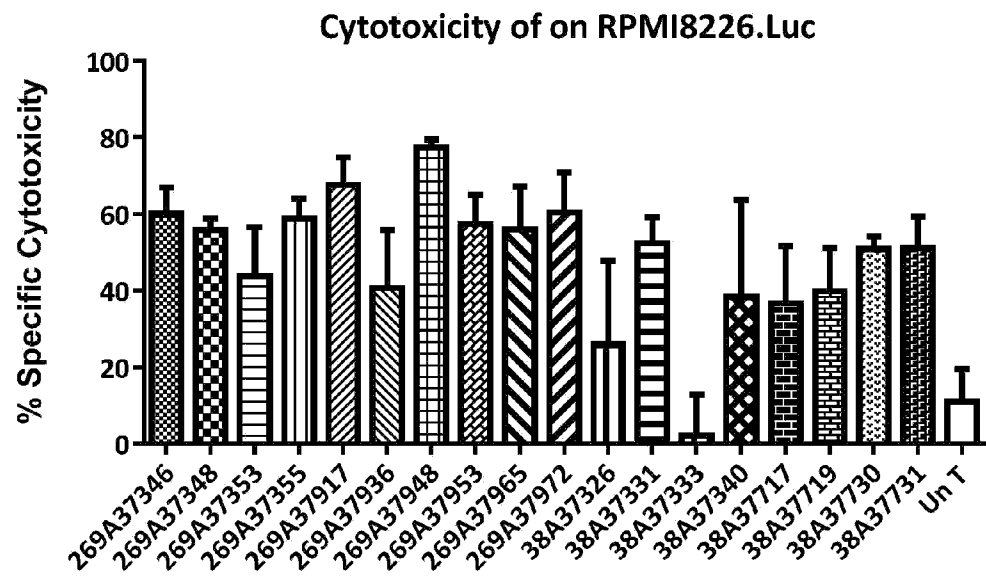


FIG. 2A

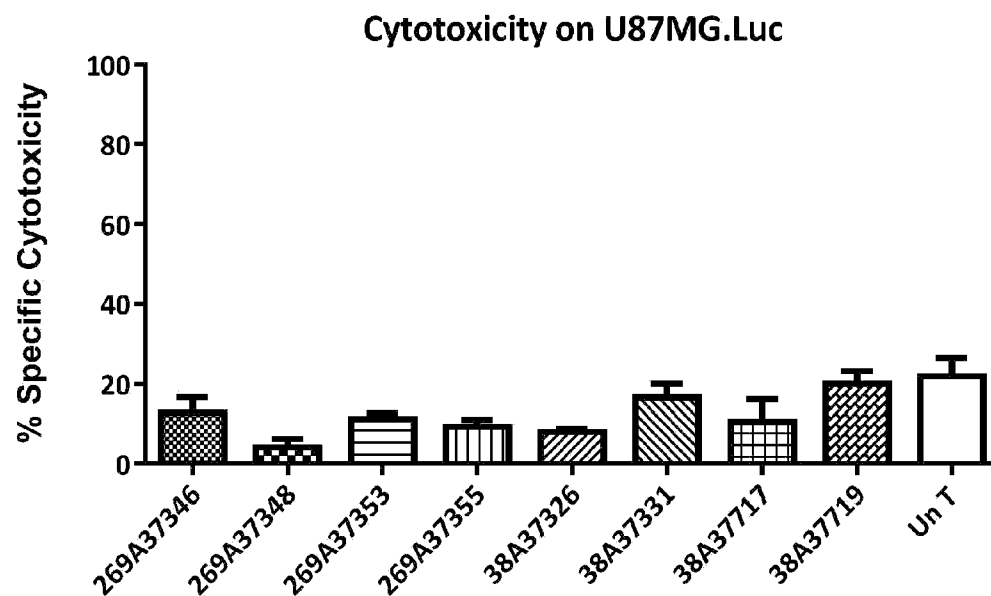


FIG. 2B



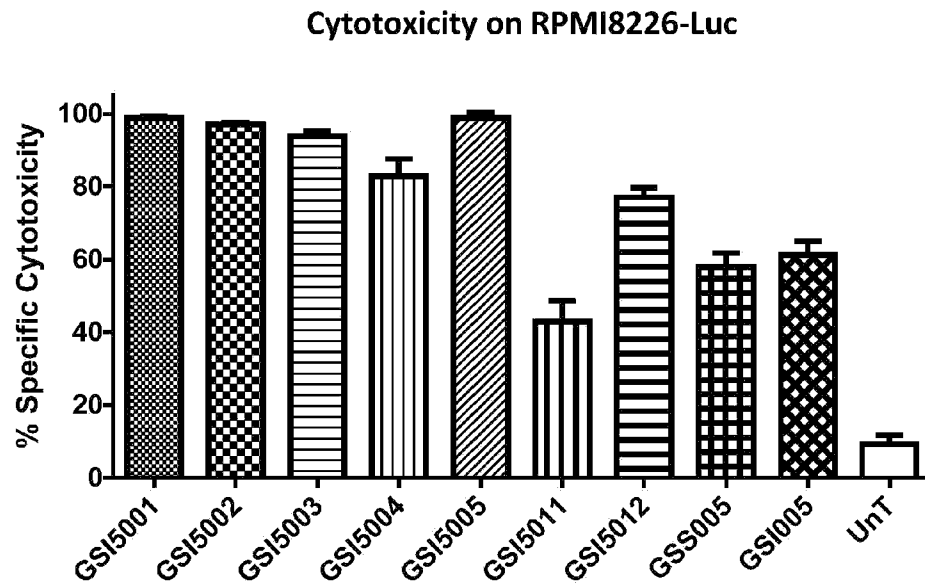


FIG. 3A

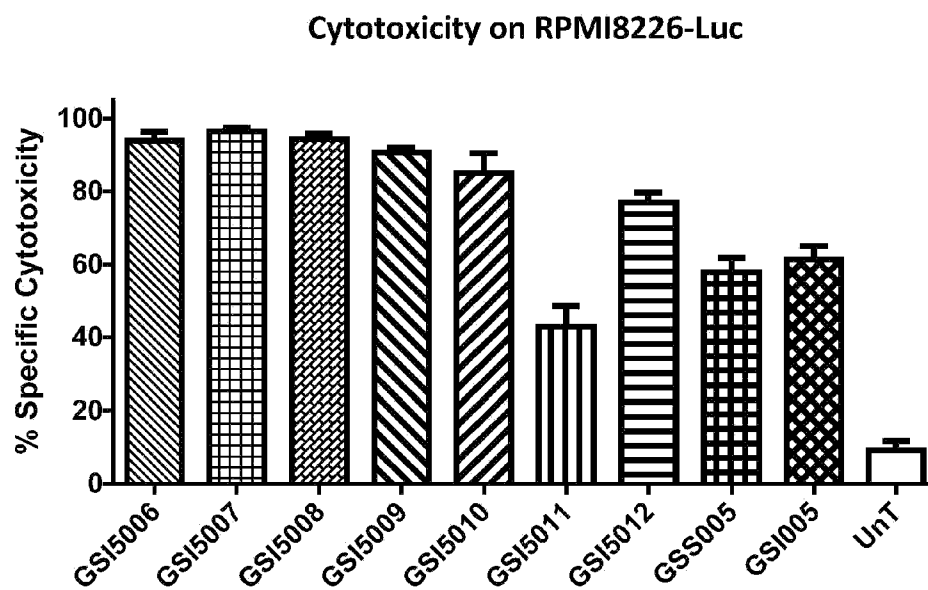


FIG. 3B

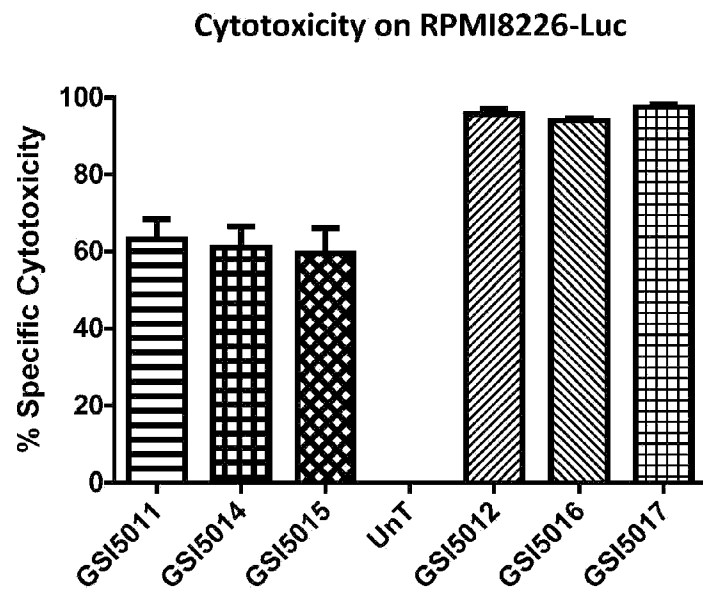
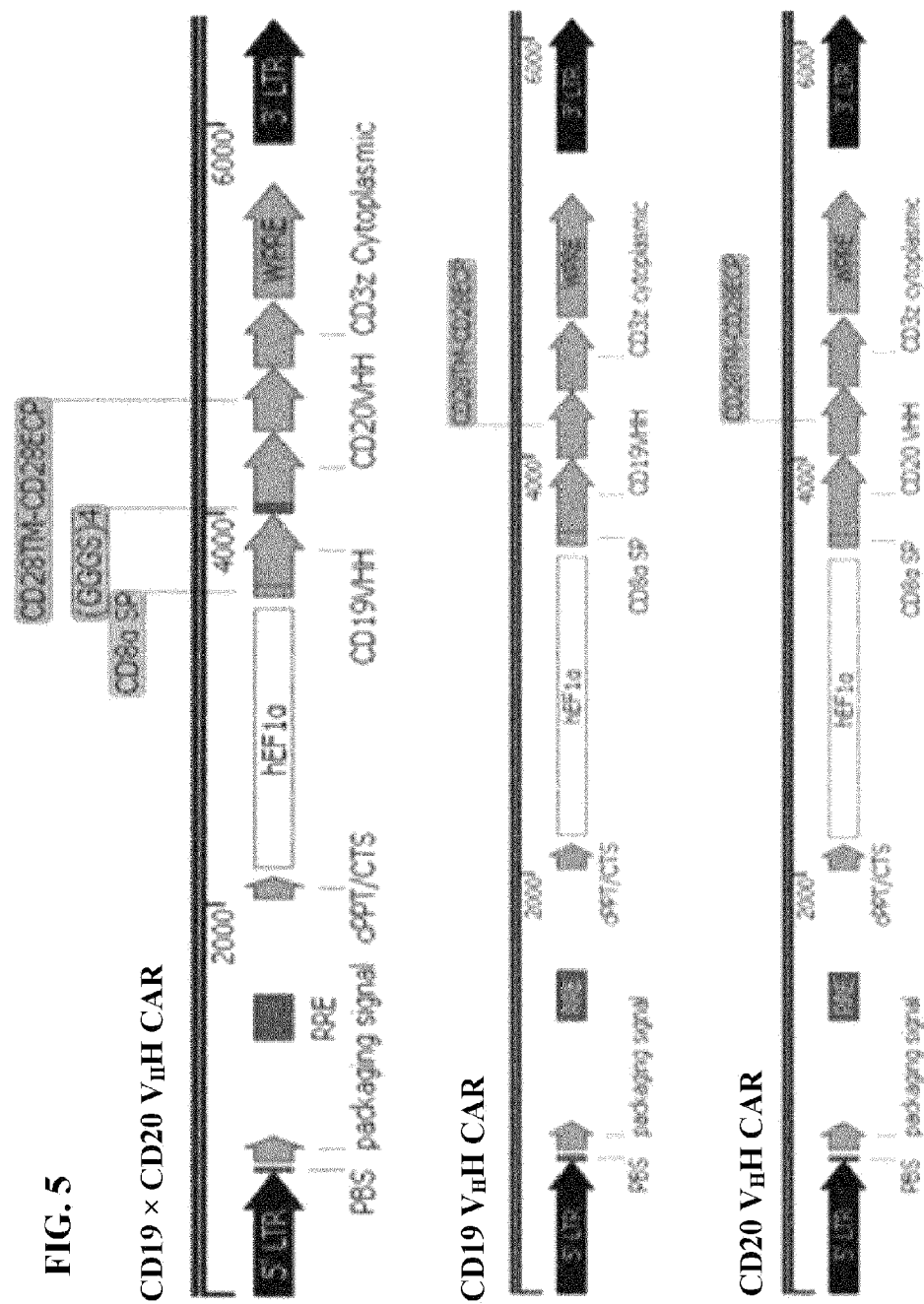
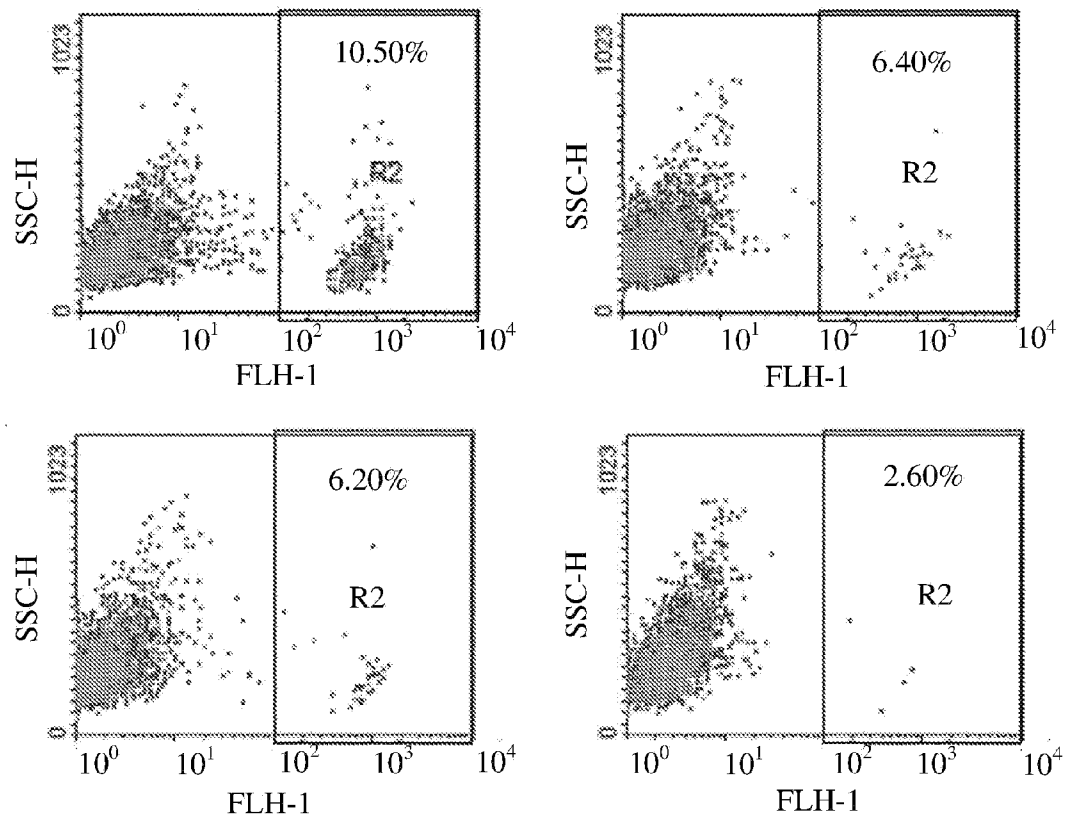


FIG. 4

FIG. 5



**FIG. 6**

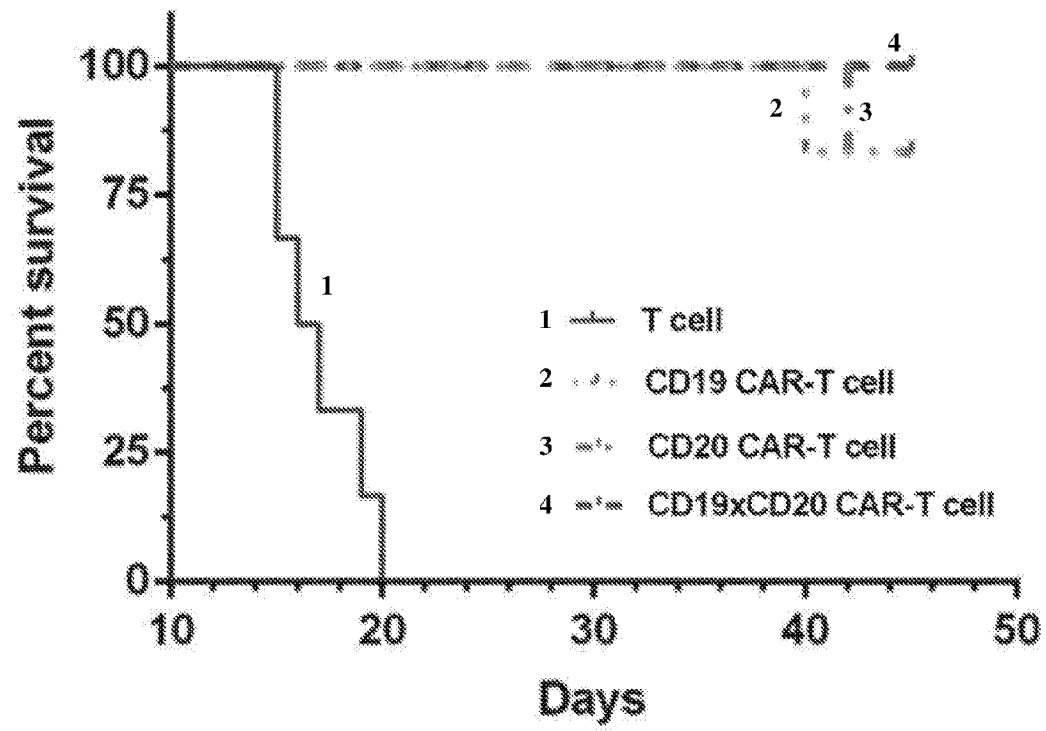
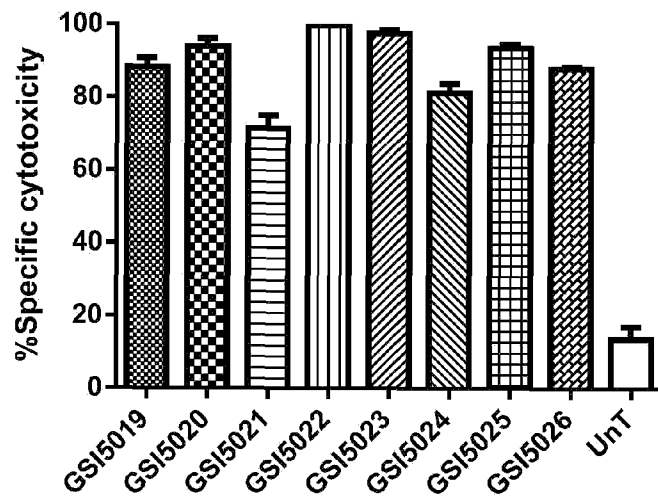
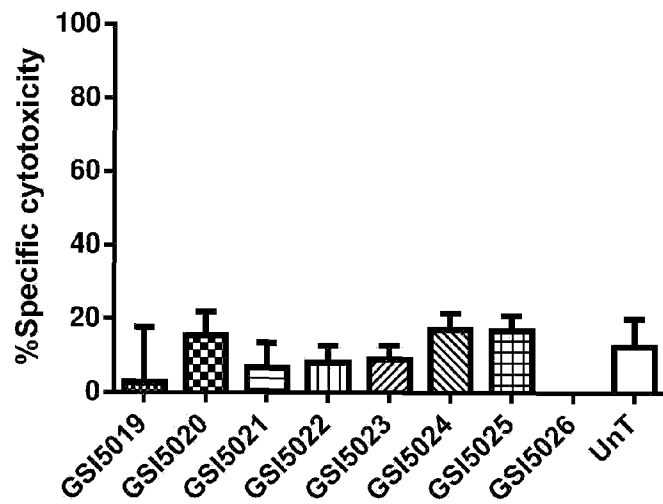


FIG. 7

**Cytotoxicity on RPMI8226.Luc****FIG. 8A****Cytotoxicity on U87MG.Luc****FIG.8B**

## SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.

