CHIMERIC ANTIGEN RECEPTORS TARGETING BCMA AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefits of International Patent Application No. PCT/CN2016/094408 filed August 10, 2016, the contents of which are incorporated herein by reference in their entirety.

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

[0002] The contents of the following submission on ASCII text file are incorporated herein by reference in their entirety: a computer readable form (CRF) of the Sequence Listing (file name: 761422000640SEQLISTING.txt, date recorded: August 10, 2017, size: 520 KB).

FIELD OF THE PRESENT APPLICATION

[0003] The present invention relates to single-domain antibodies, chimeric antigen receptors and engineered immune effector cells that target BCMA, and methods of use thereof.

BACKGROUND OF THE PRESENT APPLICATION

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

[0005] Single-domain antibodies (sdAbs) are different from conventional 4-chain antibodies by having a single monomeric antibody variable domain. For example, camelids and sharks produce sdAbs named heavy chain-only antibodies (HcAbs), which naturally lack light chains. The antigen-binding fragment in each arm of the camelid heavy-chain only antibodies has a

single heavy chain variable domain (V_HH), which can have high affinity to an antigen without the aid of a light chain. Camelid V_HH is known as the smallest functional antigen-binding fragment with a molecular weight of approximately 15 kD.

[0006] Multiple myeloma (MM) is an incurable aggressive plasma malignancy, which is categorized as a B-cell neoplasia and proliferates in uncontrollably 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. Although current therapies for multiple myeloma often lead to remissions, nearly all patients eventually relapse. There is a need for effective immunotherapeutic agent for treating multiple myeloma.

[0007] The LCAR-B38M disclosed in the current invention is a bivalent BCMA targeting CAR-T which have already shown clinical advantages in terms of both safety and efficacy in treating refractory or relapsed multiple myeloma patients in a clinical trial. In an early clinical trial, 33 out of 35 (94%) patients had clinical remission of multiple myeloma upon receiving LCAR-B38M CAR-T cells. Most patients had only mild side effects. The study was presented by the major inventor at both the 2017 ASCO Annual Meeting (Abstract LBA3001) and the press briefing which recruited extensive media coverage (http://www.ascopost.com/News/55713).

[0008] Overall, the objective response rate was 100%, and 33 patients (94%) had an evident clinical remission of myeloma (complete response, very good partial response, or partial response) within 2 months of receiving CAR T cells. After following the group for a period of more than 4 months, of the 19 patients, 14 have reached stringent complete response criteria, 1

patient has reached partial response, and 4 patients have achieved very good partial remission criteria in efficacy.

[0009] Since the outstanding efficacy and safety profile obtained from LCAR-B38M clinical trial are significantly superior than a few other BCMA CAR-T trials reported at the same time in the ASCO, the works had been widely recognized as an "revolutionized breakthrough"in the immunotherapy field. It is notable that all these BCMA CAR design are conventional CAR in which BCMA antigen binding domain is composed of a monovalent ScFv antibody.

[0010] The disclosures of all publications, patents, patent applications and published patent applications referred to herein are hereby incorporated herein by reference in their entirety.

BRIEF SUMMARY OF THE PRESENT APPLICATION

[0011] The present application provides anti-BCMA single-domain antibodies (sdAb), chimeric antigen receptors (CARs) comprising one or more anti-BCMA sdAbs (such as V_HH fragments), engineered immune effector cells, and methods of use thereof in cancer immunotherapy.

[0012] One aspect of the present application provides an anti-BCMA sdAb comprising the CDR regions of any one of SEQ ID NOs: 115-152. In some embodiments, the anti-BCMA sdAb comprises any one of the following: (1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77; (2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78; (3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; (4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80; (5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81; (6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82; (7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid

sequence of SEQ ID NO:83; (8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84; (9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85; (10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; (11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87; (12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88; (13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89; (14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90; (15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; (16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92; (17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93; (18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; (19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95; (20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; (21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97; (22) a

CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98; (23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99; (24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100; (25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101; (26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102; (27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103; (28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104; (29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105; (30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106; (31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107; (32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108; (33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109; (34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110; (35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid

sequence of SEQ ID NO:111; (36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112; (37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or (38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 115-152.

[0013] In some embodiments, there is provided an anti-BCMA heavy-chain only antibody (HCAB) or an antigen binding protein comprising any one of the anti-BCMA sdAbs described above. Also provided are BCMA epitopes that any one of the anti-BCMA sdAbs described above specifically bind to, and anti-BCMA antibodies (such as anti-BCMA sdAbs) that compete with any one of the anti-BCMA sdAbs described above.

[0014] In some embodiments according to any one of the anti-BCMA sdAbs described above, the anti-BCMA sdAb is a camelid antibody. In some embodiments, the anti-BCMA sdAb is a chimeric antibody. In some embodiments, the anti-BCMA sdAb is humanized. In some embodiments, the anti-BCMA sdAb is a V_HH fragment.

[0015] One aspect of the present application provides a BCMA chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb (such as any one of the anti-BCMA sdAbs described above); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the CAR is monospecific. In some embodiments, the CAR is monovalent. In some embodiments, the CAR is multivalent (such as bivalent or trivalent). In some embodiments, the CAR is multispecific (such as bispecific). In some embodiments, the extracellular antigen binding domain comprises at least two anti-BCMA sdAbs (such as any one or more of the anti-BCMA sdAbs described above).

[0016] One aspect of the present application provides a multivalent chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety and a second BCMA binding moiety; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, one or

more of the first BCMA binding moiety and the second BCMA binding moiety is an anti-BCMA sdAb. In some embodiments, the first BCMA binding moiety is a first anti-BCMA sdAb and the second BCMA binding moiety is a second anti-BCMA sdAb. In some embodiments, the first BCMA binding moiety is an anti-BCMA sdAb and the second BCMA binding moiety is derived from a human antibody. In some embodiments, the first BCMA binding moiety is an anti-BCMA sdAb and the second BCMA binding moiety is a polypeptide ligand of BCMA. In some embodiments, the first BCMA binding moiety and the second BCMA binding moiety specifically bind to the same epitope on BCMA. In some embodiments, the first BCMA binding moiety and the second BCMA binding moiety specifically bind to the different epitopes on BCMA. In some embodiments, the first BCMA binding moiety and/or the second BCMA binding moiety specifically binds to an epitope on BCMA derived from an amino acid sequence selected from SEQ ID NOs: 388-394. In some embodiments, the first BCMA binding moiety specifically binds to an epitope derived from SEQ ID NO: 389 and/or 390. In some embodiments, the second BCMA binding moiety specifically binds to an epitope derived from SEQ ID NO: 391 and/or 392.

[0017] One aspect of the present application provides a multivalent (such as bivalent or trivalent) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb (such as any one of the anti-BCMA sdAbs described above) and a second anti-BCMA sdAb (such as any one of the anti-BCMA sdAbs described above); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the first anti-BCMA sdAb and the second anti-BCMA sdAb specifically bind to the same epitope on BCMA. In some embodiments, the first anti-BCMA sdAb and the second anti-BCMA sdAb specifically bind to different epitopes on BCMA. In some embodiments, the first anti-BCMA sdAb and/or the second anti-BCMA sdAb specifically binds to an epitope on BCMA derived from an amino acid sequence selected from SEQ ID NOs: 388-394. In some embodiments, the first anti-BCMA sdAb specifically binds to an epitope derived from SEQ ID NO: 389 and/or 390. In some embodiments, the second anti-BCMA sdAb specifically binds to an epitope derived from SEQ ID NO: 391 and/or 392.

[0018] In some embodiments according to any one of the multivalent CARs provided above, the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) is located at the N-terminus of the second BCMA binding moiety (*e.g.*, the second anti-BCMA sdAb). In some embodiments,

the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) is located at the C-terminus of the second BCMA binding moiety (*e.g.*, the second anti-BCMA sdAb). In some embodiments, the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) and the second BCMA binding moiety (*e.g.*, the second anti-BCMA sdAb) are directly fused to each other via a peptide bond. In some embodiments, the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) and the second BCMA binding moiety (*e.g.*, the second anti-BCMA 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: 208-215.

[0019] In some embodiments according to any one of the CARs (including multivalent CARs) described above, the transmembrane domain is derived from a molecule selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the transmembrane domain is derived from CD8α or CD28. In some embodiments, the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 193 or 194.

[0020] In some embodiments according to any one of the CARs (including multivalent CARs) described above, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as a T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the primary intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 197 or 198.

[0021] In some embodiments according to any one of the CARs (including multivalent CARs) described above, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the co-stimulatory signaling domain comprises a cytoplasmic domain of CD28 and/or a cytoplasmic domain of CD137. In some embodiments, the co-stimulatory signaling domain comprises the amino acid sequence of SEQ ID NO: 195 and/or SEQ ID NO: 196.

[0022] In some embodiments according to any one of the CARs (including multivalent CARs) described above, the CAR further comprises a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In

some embodiments, the hinge domain is derived from CD8α. In some embodiments, the hinge domain comprises the amino acid sequence of SEQ ID NO: 192.

[0023] In some embodiments according to any one of the CARs (including multivalent CARs) described above, the CAR further comprises a signal peptide located at the N-terminus of the polypeptide. In some embodiments, the signal peptide is derived from a molecule selected from the group consisting of CD8α, GM-CSF receptor α, and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8α. In some embodiments, the signal peptide comprises the amino acid sequence of SEQ ID NO: 191.

[0024] One aspect of the present application provides a CAR as listed in Tables 4 and 5. In some embodiments, the CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 216-256 and 298-335.

[0025] One aspect of the present application provides a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152, 216-256 and 298-335.

[0026] One aspect of the present application provides an isolated nucleic acid comprising a nucleic acid sequence encoding any one of the anti-BCMA sdAbs or CARs (including multivalent CARs) described above. In some embodiments, the nucleic acid sequence is selected from the group consisting of SEQ ID NOs: 153-190, 257-297 and 336-373. In some embodiments, the isolated nucleic acid further comprises a first nucleic acid sequence encoding a first CAR, wherein the second nucleic acid sequence encoding second CAR is operably linked to the first nucleic acid sequence via a third nucleic acid sequence encoding a self-cleaving peptide, such as a T2A, P2A, or F2A peptide. Wherein the third nucleic acid sequence is SEQ ID NO: 385. In some embodiments, the isolated nucleic acid is a DNA molecule. In some embodiments, the isolated nucleic acid is an RNA molecule.

[0027] One aspect of the present application provides a vector comprising any one of the isolated nucleic acids described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector. In some embodiments, the vector is a lentiviral vector. In some embodiments, the vector is a non-viral vector.

[0028] One aspect of the present application provides an engineered immune effector cell, comprising any one of the CARs (including multivalent CARs) provided above, or any one of the isolated nucleic acids described above, or any one of the vectors described above. In some embodiments, the 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.

[0029] One aspect of the present application provides a pharmaceutical composition comprising any one of the engineered immune effector cells described above and a pharmaceutically acceptable carrier. Further provided is a method of treating cancer in an individual, comprising administering to the individual an effective amount of any one of the pharmaceutical compositions described above. In some embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic. In some embodiments, the cancer is a liquid cancer. In some embodiments, the cancer is multiple myeloma, acute lymphoblastic leukemia, or chronic lymphocytic leukemia. In some embodiments, the cancer is a solid cancer, such as glioblastoma. In some embodiments, the cancer is refractory or relapsed multiple myeloma.

[0030] One aspect of the present application provides a pharmaceutical composition comprising any one of the anti-BCMA sdAbs described above and a pharmaceutically acceptable carrier. In some embodiments, there is provided a method of treating a disease (such as cancer) in an individual, comprising administering to the individual an effective amount of the pharmaceutical composition.

[0031] Also provided are methods of use, kits, and articles of manufacture comprising any one of the anti-BCMA sdAbs, CARs (including multivalent CARs), engineered immune effector cells, isolated nucleic acids, or vectors described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] FIGs. 1A-1B show results of an *in vitro* cytotoxicity assay of T cells expressing exemplary monospecific CARs comprising various anti-BCMA sdAbs against RPMI8226.Luc cells (FIG. 1A), or U87MG.Luc cells (FIG. 1B).

[0033] FIGs. 2A-2C show results of an *in vitro* cytotoxicity assay of T cells expressing exemplary monospecific CARs comprising various anti-BCMA sdAbs against RPMI8226.Luc cells (FIG. 2A), K562.BCMA.Luc cells (FIG. 2B), or K562.CD19.Luc cells (FIG. 2C).

[0034] FIG. 3 shows results of an *in vitro* IFNγ release assay of T cells expressing exemplary monospecific CARs comprising various anti-BCMA sdAbs against K562.BCMA.Luc cells.

[0035] FIGs. 4A-4C show results of an *in vitro* cytotoxicity assay of T cells expressing exemplary multivalent BCMA CARs against RPMI8226.Luc cells (FIGs. 4A-4B) or U87MG.Luc cells (FIG. 4C).

[0036] FIGs. 5A-5E show results of an *in vitro* cytotoxicity assay of T cells expressing exemplary bivalent BCMA CARs against RPMI8226.Luc cells (FIG. 5A), K562.CD19.Luc cells (FIG. 5B), A549.Luc cells (FIG. 5C), U87MG.Luc cells (FIG. 5D), or Raji.Luc cells (FIG. 5E).

[0037] FIG. 5F shows results of an *in vitro* cytotoxicity assay of T cells expressing exemplary bivalent BCMA CARs against K562.BCMA.Luc cells and K562.CD38.Luc cells.

[0038] FIG. 6A shows results of an *in vitro* IFNγ release assay of T cells expressing exemplary bivalent BCMA CARs against K562.BCMA.Luc cells at two different effector cell to target cell ratios.

[0039] FIG. 6B shows results of an *in vitro* IFNγ release assay of T cells expressing exemplary bivalent BCMA CARs against RPMI8226.Luc, A549.Luc, K562.CD38.Luc and Raji.Luc cells.

[0040] FIGs.7A-7C show binding of three exemplary VHH fragments to K562.BCMA.Luc cells and K562.CD38.Luc cells (negative control).

[0041] FIG.8A shows a crystal structure of the extracellular domain of BCMA. FIG. 8B shows BCMA epitope peptides.

[0042] FIGs.9A-9B show results of epitope mapping assays of VHH1 and VHH2.

[0043] FIG.10 shows results of a competitive binding assay using CHO-BCMA cells.

[0044] FIG. 11 shows *in vitro* cytotoxicity of donor-derived T cells expressing LCAR-B38M against RPMI8226.Luc cells.

[0045] FIG. 12A shows *in vitro* cytotoxicity of LCAR-B38M CAR-T cells prepared from a selected donor against RPMI8226.Luc cells. FIGs. 12B-12E show *in vivo* anti-tumor activity of LCAR-B38M CAR-T cells in tumor xenograft mice model. FIG. 12B shows bioluminescence imaging data in LCAR-B38M CAR-T treated mice and untransduced T cell (UnT) treated mice. FIG. 12C shows the study design and bioluminescence images of mice in CAR-T group and UnT group. FIG. 12D shows images of livers from UnT-treated mice. FIG. 12E shows an *ex vivo* luciferase assay validating tumors in the livers of UnT-treated mice.

[0046] FIGs. 13A-13F show clinical parameters of two monkeys treated with LCAR-B38M CAR-T cells. The clinical parameters monitored in the study include body temperature (FIG.

13A), body weight (FIG. 13B), Complete Blood Count (CBC, FIGs. 13C and 13D), as well as serum chemistry and cytokine levels (FIGs. 13E and 13F).

[0047] FIG. 14A-C show the in vitro cytotoxicity assays about LCAR-B38M CAR-T cells and LCAR-B27S CAR-T cells prepared from the same three multiple myeloma patients respectively. FIG.14A shows the in vitro cytotoxicity results of LCAR-B38M CAR-T cells and LCAR-B27S CAR-T cells prepared from multiple myeloma patient A. FIG.14B shows the in vitro cytotoxicity results of LCAR-B38M CAR-T cells and LCAR-B27S CAR-T cells prepared from multiple myeloma patient B. FIG.14C shows the in vitro cytotoxicity results of LCAR-B38M CAR-T cells and LCAR-B27S CAR-T cells prepared from multiple myeloma patient C.

[0048] FIG. 15A 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 VHH domain. The schematic structure on the right shows an exemplary monospecific monovalent CAR having an extracellular antigen binding domain comprising a scFv domain.

[0049] FIG. 15B 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.

[0050] FIG. 15C shows schematic structures of exemplary bivalent and bispecific V_HH-based CARs. The schematic structure in the top left panel shows an exemplary mono-epitope, 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 bi-epitope, bivalent CAR having an extracellular antigen binding domain comprising a first V_HH domain specifically binding to epitope 1 of antigen A, and a second V_HH domain specifically binding to epitope 2 of antigen A. Epitope 1 and epitope 2 of antigen A may be different in their structures and/or sequences. The schematic structure in the bottom left panel shows an exemplary bispecific CAR having an extracellular antigen binding

domain comprising a first V_HH domain specifically binding to antigen A, and a second V_HH domain specifically binding to antigen B. Antigen A and antigen B are different antigens.

[0051] FIG. 15D shows schematic structures of exemplary V_HH-based CARs having three or more V_HH domains. The CARs may have a plurality of V_HH domains fused to each other directly or via peptide linkers. The V_HH domains may be the same or different. Different V_HH domains may specifically bind to different epitopes on the same antigen or different antigens.

[0052] FIG. 15E shows exemplary engineered immune effector cells co-expressing two different V_HH-based CARs. The exemplary engineered immune effector cell in the left panel co-expresses two different monospecific, monovalent CARs. The exemplary engineered immune effector cell in the middle panel co-expresses a monospecific, monovalent CAR and a bispecific or bivalent CAR. The exemplary engineered immune effector cell in the right panel co-expresses two different bispecific or bivalent CARs. The CARs may recognize different antigens.

DETAILED DESCRIPTION OF THE PRESENT APPLICATION

[0053] The present application provides anti-BCMA single-domain antibodies (sdAb) and chimeric antigen receptors (CARs) comprising an extracellular antigen binding domain comprising one or more BCMA binding moieties (such as anti-BCMA sdAbs). Multivalent CARs comprising at least two binding moieties (such as sdAbs) that specifically bind to a single antigen are also provided. In some embodiments, the present application provides multivalent (such as bivalent or trivalent) CARs comprising at least two anti-BCMA sdAbs. In some embodiments, the at least two anti-BCMA sdAbs are different anti-BCMA sdAbs that specifically bind to different epitopes on BCMA. The anti-BCMA sdAbs, CARs and engineered immune cells expressing CARs described in the present application are useful agents for cancer treatment.

[0054] Notably, the present application has demonstrated superior efficacy of bivalent bi-epitope CARs comprising two anti-BCMA sdAbs targeting different BCMA epitopes (*e.g.*, LCAR-B38M), in treating multiple myeloma among human patients. At an interim analysis of a Phase I/II clinical trial, 100% of patients with relapsed or refractory multiple myeloma responded to the LCAR-B38M CAR-T treatment. 94% of the patients had evident clinical remission of myeloma within two months of receiving the CAR-T treatment. Patients who reached Stringent Complete Response (sCR) criteria remained free of minimal residual disease after more than a year of receiving the CAR-T treatment. Additionally, the LCAR-B38M CAR-T treatment was

well tolerated by the patients as most patients only experienced mild and manageable cytokine release syndrome, a common side effect of CAR-T cell-based therapy. No patients experienced neurological side effects. In comparison, a pilot clinical study of a monovalent CAR comprising a single anti-BCMA sdAb showed lower objective response rate and complete remission rate, and higher relapse rate among treated patients. Prior to this application, all BCMA CARs under clinical studies had only one BCMA binding moiety in the extracellular antigen binding domain. The improved clinical efficacy and safety of the multivalent BCMA CARs of the present application are unexpected.

[0055] Accordingly, one aspect of the present application provides a multivalent CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a plurality of a single-domain antibody (sdAb) specifically binding to BCMA; (b) a transmembrane domain; and (c) an intracellular signaling domain.

[0056] In another aspect, there is provided a multivalent CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope is different from the second epitope.

[0057] Further provided are novel anti-BCMA sdAbs and CARs comprising any one or more of the anti-BCMA sdAbs described herein.

[0058] Engineered immune effector cells (such as T cells) comprising the CARs, pharmaceutical compositions, kits, articles of manufacture and methods of treating cancer using the engineered immune effectors cells or the sdAbs are also described herein.

I. Definitions

[0059] The term "antibody" includes monoclonal antibodies (including full length 4-chain antibodies or full length heavy-chain only antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, multispecific antibodies (*e.g.*, bispecific antibodies, diabodies, and single-chain molecules), as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv). The term "immunoglobulin" (Ig) is used interchangeably with "antibody"

herein. Antibodies contemplated herein include single-domain antibodies, such as heavy chain only antibodies.

[0060] The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains. An IgM antibody consists of 5 of the basic heterotetramer units along with an additional polypeptide called a J chain, and contains 10 antigen binding sites, while IgA antibodies comprise from 2-5 of the basic 4-chain units which can polymerize to form polyvalent assemblages in combination with the J chain. In the case of IgGs, the 4-chain unit is generally about 150,000 Daltons. Each L chain is linked to an H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (V_H) followed by three constant domains (C_H) for each of the α and γ chains and four C_H domains for μ and ε isotypes. Each L chain has at the N-terminus, a variable domain (V_L) followed by a constant domain at its other end. The V_L is aligned with the V_H and the C_L is aligned with the first constant domain of the heavy chain (C_H1). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a V_H and V_L together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see *e.g.*, *Basic and Clinical Immunology,* 8th Edition, Daniel P. Sties, Abba I. Terr and Tristram G. Parsolw (eds), Appleton & Lange, Norwalk, Conn., 1994, page 71 and Chapter 6. The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains (C_H), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, having heavy chains designated α, δ, ε, γ and μ, respectively. The γ and α classes are further divided into subclasses on the basis of relatively minor differences in the C_H sequence and function, *e.g.*, humans express the following subclasses: IgG1, IgG2A, IgG2B, IgG3, IgG4, IgA1 and IgA2.

[0061] The term "heavy chain-only antibody" or "HCAb" refers to a functional antibody, which comprises heavy chains, but lacks the light chains usually found in 4-chain antibodies. Camelid animals (such as camels, llamas, or alpacas) are known to produce HCAbs.

[0062] The term "single-domain antibody" or "sdAb" refers to a single antigen-binding polypeptide having three complementary determining regions (CDRs). The sdAb alone is capable of binding to the antigen without pairing with a corresponding CDR-containing polypeptide. In some cases, single-domain antibodies are engineered from camelid HCAbs, and their heavy chain variable domains are referred herein as "V_HHs". Some V_HHs may also be known as Nanobodies. Camelid sdAb is one of the smallest known antigen-binding antibody fragments (see, *e.g.*, Hamers-Casterman *et al.*, Nature 363:446-8 (1993); Greenberg *et al.*, Nature 374:168-73 (1995); Hassanzadeh-Ghassabeh *et al.*, Nanomedicine (Lond), 8:1013-26 (2013)). A basic V_HH has the following structure from the N-terminus to the C-terminus: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3.

[0063] 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.

[0064] The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domains of the heavy chain and light chain may be referred to as "V_H" and "V_L", respectively. These domains are generally the most variable parts of the antibody (relative to other antibodies of the same class) and contain the

antigen binding sites. Heavy-chain only antibodies from the Camelid species have a single heavy chain variable region, which is referred to as "V_HH". V_HH is thus a special type of V_H.

[0065] 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.

[0066] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (*e.g.*, isomerizations, amidations) that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture or recombinantly, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present application may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler

and Milstein., *Nature,* 256:495-97 (1975); Hongo *et al.*, *Hybridoma,* 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567), phage-display technologies (see, *e.g.*, Clackson *et al.*, *Nature,* 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222: 581-597 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.*, WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits *et al.*, *Nature* 362: 255-258 (1993); Bruggemann *et al.*, *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al.*, *Bio/Technology* 10: 779-783 (1992); Lonberg *et al.*, *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

[0067] The term "naked antibody" refers to an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

[0068] 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.

[0069] 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.

[0070] 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.

[0071] "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.

[0072] "Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the V_H and V_L antibody domains connected into a single polypeptide chain. Preferably, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of the sFv, see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0073] "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.

[0074] 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).

[0075] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is(are) identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include PRIMATTZFD® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, *e.g.*, immunizing macaque monkeys with an antigen of interest. As used herein, "humanized antibody" is used a subset of "chimeric antibodies."

[0076] "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.

[0077] 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.

[0078] 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, sdAbs 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).

[0079] 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)

[0080] 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

Loop	Kabat	AbM	Chothia	Contact
H3	H95-H102	H95-H102	H96-H101	H93-H101

[0081] 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.

[0082] The amino acid residues of a sdAb (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).

[0083] 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.

[0084] 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.

[0085] "Framework" or "FR" residues are those variable-domain residues other than the HVR residues as herein defined.

[0086] A "human consensus framework" or "acceptor human framework" is a framework that represents the most commonly occurring amino acid residues in a selection of human immunoglobulin V_L or V_H framework sequences. Generally, the selection of human immunoglobulin V_L or V_H sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat *et al.*, *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Examples include for the V_L, the subgroup may be subgroup kappa I, kappa II, kappa III or kappa IV as in Kabat *et al.*, supra. Additionally, for the VH, the subgroup may be subgroup I, subgroup II, or subgroup III as in Kabat *et al.* Alternatively, a human consensus framework can be derived from the above in which particular residues, such as when a human framework residue is selected based on its homology to the donor framework by aligning the donor framework sequence with a collection of various human framework sequences. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain pre-existing amino acid sequence changes. In some embodiments, the number of pre-existing amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less.

[0087] 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.

[0088] An "affinity-matured" antibody is one with one or more alterations in one or more HVRs thereof that result in an improvement in the affinity of the antibody for antigen, compared

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

[0089] 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 ≦1 μM, ≦100 nM, ≦10 nM, ≦1 nM, or ≦0.1 nM. In some embodiments, an antigen binding protein (such as a CAR or an sdAb) specifically binds an epitope on a protein that is conserved among the protein from different species. In some embodiments, specific binding can include, but does not require exclusive binding.

[0090] 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 different antigens. "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 antigen.

[0091] 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).

[0092] "Antibody effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody—dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.*, B cell receptors); and B cell activation. "Reduced or minimized" antibody effector function means that which is reduced by at least 50% (alternatively 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) from the wild type or unmodified antibody. The determination of antibody effector function is readily determinable and measurable by one of ordinary skill in the art. In a preferred embodiment, the antibody effector functions of complement binding, complement dependent cytotoxicity and antibody dependent cytotoxicity are affected. In some embodiments, effector function is eliminated through a mutation in the constant region that eliminated glycosylation, *e.g.*, "effector-less mutation." In one aspect, the effector-less mutation is an N297A or DANA mutation (D265A+N297A) in the C_H2 region. Shields *et al.*, *J. Biol. Chem.* 276 (9): 6591-6604 (2001). Alternatively, additional mutations resulting in reduced or eliminated effector function include: K322A and L234A/L235A (LALA). Alternatively, effector function can be reduced or eliminated through production techniques, such as expression in host cells that do not glycosylate (*e.g.*, *E. coli.*) or in which result in an altered glycosylation pattern that is ineffective or less effective at promoting effector function (*e.g.*, Shinkawa *et al.*, *J. Biol. Chem.* 278(5): 3466-3473 (2003).

[0093] "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).

[0094] 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.

[0095] "Binding affinity" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an antibody or a CAR) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity that reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen, or CAR and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of

measuring binding affinity are known in the art, any of which can be used for purposes of the present application. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

[0096] A "blocking" antibody or an "antagonist" antibody is one that inhibits or reduces a biological activity of the antigen it binds. In some embodiments, blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

[0097] "Percent (%) amino acid sequence identity" and "homology" with respect to a peptide, polypeptide or antibody sequence are defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific peptide or polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or MEGALIGN™ (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0098] "Chimeric antigen receptor" or "CAR" as used herein refers to genetically engineered receptors, which can be used to graft one or more antigen specificity onto immune effector cells, such as T cells. Some CARs are also known as "artificial T-cell receptors," "chimeric T cell receptors," or "chimeric immune receptors." In some embodiments, the CAR comprises an extracellular antigen binding domain specific for one or more antigens (such as tumor antigens), a transmembrane domain, and an intracellular signaling domain of a T cell and/or other receptors. "CAR-T" refers to a T cell that expresses a CAR. "BCMA CAR" refers to a CAR having an extracellular binding domain specific for BCMA. "Bi-epitope CAR" refers to a CAR having an extracellular binding domain specific for two different epitopes on BCMA.

[0099] 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.

[0100] 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.

[0101] 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.

[0102] 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."

[0103] 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.

[0104] "Allogeneic" refers to a graft derived from a different individual of the same species.

[0105] 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.

[0106] 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.

[0107] 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.

[0108] As used herein, "treatment" or "treating" is an approach for obtaining beneficial or desired results including clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, one or more of the following: alleviating one or more symptoms resulting from the disease, diminishing the extent of the disease, stabilizing the disease (*e.g.*, preventing or delaying the worsening of the disease), preventing or delaying the spread (*e.g.*, metastasis) of the disease, preventing or delaying the recurrence of the disease, delay or slowing the progression of the disease, ameliorating the disease state, providing a remission (partial or total) of the disease, decreasing the dose of one or more other medications required to treat the disease, delaying the progression of the disease, increasing the quality of life, and/or prolonging survival. Also encompassed by "treatment" is a reduction of pathological consequence of cancer. The methods of the present application contemplate any one or more of these aspects of treatment.

[0109] 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.

[0110] The term "effective amount" used herein refers to an amount of an agent, such as a sdAb, 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.

[0111] "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.

[0112] "Neoadjuvant setting" refers to a clinical setting in which the method is carried out before the primary/definitive therapy.

[0113] 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.

[0114] 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.

[0115] "Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counterions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™ or polyethylene glycol (PEG).

[0116] 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.

[0117] 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.

[0118] 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.

[0119] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein or antibody formulation in a diluent such that the protein is dispersed throughout. The reconstituted formulation is suitable for administration (*e.g.* subcutaneous administration) to a patient to be treated with the protein of interest and, In some embodiments of the present application, may be one which is suitable for parenteral or intravenous administration.

[0120] 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.

[0121] It is understood that embodiments of the present application described herein include “consisting” and/or “consisting essentially of” embodiments.

[0122] 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".

[0123] 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.

[0124] The term “about X-Y” used herein has the same meaning as “about X to about Y.”

[0125] 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. Anti-BCMA Single-Domain Antibodies

[0126] One aspect of the present application provides isolated single-domain antibodies (referred herein as “anti-BCMA sdAbs”) that specifically bind to BCMA, such as human BCMA. In some embodiments, the anti-BCMA sdAb modulates BCMA activity. In some embodiments, the anti-BCMA sdAb is an antagonist antibody. Further provided are antigen-binding fragments derived from any one of the anti-BCMA sdAbs described herein, and antigen binding proteins comprising any one of the anti-BCMA sdAbs described herein. Exemplary anti-BCMA sdAbs are listed in Table 2 below.

Table 2. Exemplary anti-BCMA sdAbs.

SdAb	Ex. AA SEQ ID	Ex. NA SEQ ID	CDR1	CDR2	CDR3
269A37346	115	153	DYYAIG (SEQ ID NO: 1)	CISRSDGSTYYADSVKG (SEQ ID NO: 39)	AGADCSGYLRDYEF (SEQ ID NO: 77)
269A37348	116	154	TYGMA (SEQ ID NO: 2)	SKASMNYSGRTYYADSVKG (SEQ ID NO: 40)	AGTGCSTYGCFDAQIIDY (SEQ ID NO: 78)

269A37917	117	155	TFTMG (SEQ ID NO: 3)	AISLSPTLAYYAESVKG (SEQ ID NO: 41)	ADRKSVMSIRPDY (SEQ ID NO: 79)
269A37355	118	156	INAMG (SEQ ID NO: 4)	SIRGLGRTNYDDSVKG (SEQ ID NO: 42)	VYVTLLGGVNRDY (SEQ ID NO: 80)
269A37915	119	157	SIVMG (SEQ ID NO: 5)	AIMWNDGITYLQDSVKG (SEQ ID NO: 43)	ASKGRYSEYEY (SEQ ID NO: 81)
269A37936	120	158	RAVIV (SEQ ID NO: 6)	FIKPSDGTIYYIDSLKG (SEQ ID NO: 44)	ASPEDWYTDWIDWSIYR (SEQ ID NO: 82)
269A37953	121	159	SDVMG (SEQ ID NO: 7)	AIMWNDGITYLQDSVKG (SEQ ID NO: 45)	ASKGRYSEYEY (SEQ ID NO: 83)
269A37965	122	160	NDHMA (SEQ ID NO: 8)	AIDWSGRTTNYADPVEG (SEQ ID NO: 46)	VLRAWISYDNDY (SEQ ID NO: 84)
269A37972	123	161	KNTVA (SEQ ID NO: 9)	SITWDGRTTYYADSVKG (SEQ ID NO: 47)	DLGKWPAGPADY (SEQ ID NO: 85)
269A37353	124	162	SHVMG (SEQ ID NO: 10)	VIGWRDISTSYADSVKG (SEQ ID NO: 48)	ARRIDAADFDS (SEQ ID NO: 86)
269A37948	125	163	TYFMA (SEQ ID NO: 11)	GIAWSGGSTAYADSVKG (SEQ ID NO: 49)	SRGIEVEEFGA (SEQ ID NO: 87)
269B005	126	164	INVMA (SEQ ID NO: 12)	AVTRDGRKSCGDSVKG (SEQ ID NO: 50)	DGWGATTLDYTYGMDY (SEQ ID NO: 88)
269B023	127	165	TFTMG (SEQ ID NO: 13)	SITWDGRSAYYAESVKG (SEQ ID NO: 51)	DRKSVMSIRPDY (SEQ ID NO: 89)
269B024	128	166	INAMG (SEQ ID NO: 14)	TITRGGSTNYGPSVKG (SEQ ID NO: 52)	ERLDGSGYGYEYDY (SEQ ID NO: 90)
269B028	129	167	KNTVA (SEQ ID NO: 15)	SITCDGRTTYYANSVNG (SEQ ID NO: 53)	YRKSIMSIQPDY (SEQ ID NO: 91)
269B030	130	168	SIVMG (SEQ ID NO: 16)	AIMWNDGLTYLQGSVKG (SEQ ID NO: 54)	DRKSVMSIRPDY (SEQ ID NO: 92)
269B038	131	169	TFTMG (SEQ ID NO: 17)	AISLSPTLAYYAESVKG (SEQ ID NO: 55)	RRIDAADFDS (SEQ ID NO: 93)
269B054	132	170	KNTVA (SEQ ID NO: 18)	SITWDGRTTYYADSVKG (SEQ ID NO: 56)	LGKWPAGPADY (SEQ ID NO: 94)
269B059	133	171	INTMD (SEQ ID NO: 19)	AISLSPTLAYYAESVKG (SEQ ID NO: 57)	DRKSVMSIRPDY (SEQ ID NO: 95)
269B060	134	172	KNTVA (SEQ ID NO: 20)	SITCDGRTTYYANSVKG (SEQ ID NO: 58)	LGKWPAGSADY (SEQ ID NO: 96)
269B069	135	173	DYWMH (SEQ ID NO: 21)	SIDTSGQTTYYADSLKG (SEQ ID NO: 59)	RYRGGTWYGMAN (SEQ ID NO: 97)
269B074	136	174	SNTMA (SEQ ID NO: 22)	STTWNGRSTYYADSVKG (SEQ ID NO: 60)	LGKWPAGPADY (SEQ ID NO: 98)
269B076	137	175	TFTMG (SEQ ID NO: 23)	DISGGRTNYADSVKG (SEQ ID NO: 61)	DRKSVMSIRPDY (SEQ ID NO: 99)
269B079	138	176	VAAISL (SEQ ID NO: 24)	FTISRDNAKNTVVLQMNSLKP (SEQ ID NO: 62)	DRKSVMSIRPDY (SEQ ID NO: 100)
269B083	139	177	KNTVA (SEQ ID NO: 25)	SITWDGRTTYYADSVKG (SEQ ID NO: 63)	TASCHLFGLGSGAFVS (SEQ ID NO: 101)

269B085	140	178	TFTMG (SEQ ID NO: 26)	AISLSPTLAYYAESVKG (SEQ ID NO: 64)	SKDRYSEYEY (SEQ ID NO: 102)
269B093	141	179	TFTMG (SEQ ID NO: 27)	AISLSPTLAYYAESVKG KG (SEQ ID NO: 65)	KNGGPVDY (SEQ ID NO: 103)
269B094	142	180	SIVMG (SEQ ID NO: 28)	AIMWNDGITYLQDSVK G (SEQ ID NO: 66)	SKGRYSEYEY (SEQ ID NO: 104)
269B104	143	181	TFTMG (SEQ ID NO: 29)	AINLSPTLTYYAESVKG (SEQ ID NO: 67)	ERKSVMAIPPDY (SEQ ID NO: 105)
269B109	144	182	TFTMG (SEQ ID NO: 30)	SITLIPTFPYYAYSVKG (SEQ ID NO: 68)	YRKYLMSILPDY (SEQ ID NO: 106)
269B110	145	183	TFTMG (SEQ ID NO: 31)	AISLSPTLAYYAESVKG (SEQ ID NO: 69)	NRNSQRVIAALSWIGM NY (SEQ ID NO: 107)
269B113	146	184	TFTMG (SEQ ID NO: 32)	AISLSPTLAYYAESVKG (SEQ ID NO: 70)	RRIDAADFDS (SEQ ID NO: 108)
269B126	147	185	TFTMG (SEQ ID NO: 33)	VIGWRDINASYADSVK G (SEQ ID NO: 71)	RRIDATDFDS (SEQ ID NO: 109)
269B129	148	186	NHVMG (SEQ ID NO: 34)	VIGWRDISTSYADSVKG (SEQ ID NO: 72)	RRIDAADFDS (SEQ ID NO: 110)
269B131	149	187	NYILA (SEQ ID NO: 35)	HISRSGGKSGYGDSVK G (SEQ ID NO: 73)	PLWYGSPTLIDY (SEQ ID NO: 111)
269B135	150	188	TFTMG (SEQ ID NO: 36)	AISLSPTLAYYAESVK G (SEQ ID NO: 74)	DRKSVMSIRPDY (SEQ ID NO: 112)
269B136	151	189	TFTMG (SEQ ID NO: 37)	AISLSPTLAYYAEPVK G (SEQ ID NO: 75)	DRKSVMSIRPDY (SEQ ID NO: 113)
269B139	152	190	NNFVMG (SEQ ID NO: 38)	AISLSPTLAYYVESVKG (SEQ ID NO: 76)	DRKSVMSIRPDY (SEQ ID NO: 114)

[0127] 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 including ligand (APRIL) (*e.g.* Mackay et al., 2003 and Kalled et al., Immunological Review, 204: 43-54, 2005). BCMA can be a suitable tumor antigen target for immunotherapeutic agents against multiple myeloma. Antibodies of high affinity can block the binding between BCMA and its native ligands BAFF and APRIL. The anti-BCMA sdAbs can be used in combination with cell immunotherapy using CAR-T cells, for example, to enhance cytotoxic effects against tumor cells.

[0128] In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 115. In some embodiments, there is

provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 116. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 117. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 118. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 119. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 120. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 121. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 122. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 123. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 124. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 125. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 126. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 127. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 128. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 129. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 130. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 131. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 132. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 133. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 134. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all

three CDRs of the amino acid sequence of SEQ ID NO: 135. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 136. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 137. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 138. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 139. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 140. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 141. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 142. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 143. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 144. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 145. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 146. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 147. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 148. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 149. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 150. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 151. In some embodiments, there is provided an anti-BCMA sdAb comprising one, two, or all three CDRs of the amino acid sequence of SEQ ID NO: 152. In some embodiments, the anti-BCMA sdAb is camelid. In some embodiments, the anti-BCMA sdAb is humanized. In some embodiments, the anti-BCMA sdAb comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0129] In some embodiments, there is provided an anti-BCMA sdAb 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: 1-38; (b) a CDR2 comprising an amino acid sequence selected from SEQ ID NO: 39-76; and (c) a CDR3 comprising an amino acid sequence selected from SEQ ID NO: 77-114. In some embodiments, the anti-BCMA sdAb is camelid. In some embodiments, the anti-BCMA sdAb is humanized. In some embodiments, the anti-BCMA sdAb comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0130] In some embodiments, there is provided an anti-BCMA sdAb 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:1-38; (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:39-76; 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: 77-114. In some embodiments, a CDR having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-BCMA sdAb comprising that sequence retains the ability to bind to BCMA. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 having about any one of 1, 2, 3, or 4 amino acid substitutions (*e.g.*, conservative substitutions), insertions, or deletions to an amino acid sequence selected from SEQ ID NO:1-38; (b) a CDR2 having about any one of 1, 2, 3, or 4 amino acid substitutions (*e.g.*, conservative substitutions), insertions, or deletions to an amino acid sequence selected from SEQ ID NO:39-76; and (c) a CDR3 having about any one of 1, 2, 3, or 4 amino acid substitutions (*e.g.*, conservative substitutions), insertions, or deletions to an amino acid sequence selected from SEQ ID NO: 77-114. In some embodiments, the anti-BCMA sdAb is affinity matured. In some embodiments, the anti-BCMA sdAb is camelid. In some embodiments, the anti-BCMA sdAb is humanized. In some embodiments, the anti-BCMA sdAb comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0131] In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 1; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 39; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 77. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 2; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 40; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 78. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 3; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 41; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 79. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 4; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 42; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 80. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 5; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 43; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 81. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 6; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 44; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 82. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 7; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 45; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 83. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 8; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 46; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 84. In some embodiments, there is provided an anti-BCMA sdAb 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: 47; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 85. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID

NO: 10; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 48; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 86. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 11; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 49; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 87. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 12; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 50; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 88. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 13; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 51; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 89. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 14; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 52; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 90. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 15; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 53; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 91. In some embodiments, there is provided an anti-BCMA sdAb 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: 54; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 92. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 17; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 55; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 93. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 18; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 56; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 94. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 19; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 57; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 95. In

some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 20; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 58; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 96. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 21; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 59; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 97. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 22; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 60; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 98. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 23; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 61; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 99. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 24; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 62; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 100. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 25; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 63; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 101. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 26; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 64; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 102. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 27; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 65; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 103. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 28; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 66; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 104. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid

sequence of SEQ ID NO: 29; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 67; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 105. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 30; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 68; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 106. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 31; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 69; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 107. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 32; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 70; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 108. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 33; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 71; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 109. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 34; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 72; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 110. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 35; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 73; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 111. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 36; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 74; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 112. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 37; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 75; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 113. In some embodiments, there is provided an anti-BCMA sdAb comprising three CDRs comprising: (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 38; (b) a CDR2 comprising the amino acid sequence of SEQ ID NO:

76; and (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 114. In some embodiments, the anti-BCMA sdAb is camelid. In some embodiments, the anti-BCMA sdAb is humanized. In some embodiments, the anti-BCMA sdAb comprises an acceptor human framework, *e.g.*, a human immunoglobulin framework or a human consensus framework.

[0132] In some embodiments, the anti-BCMA sdAb, including any of the embodiments described above (*i.e.*, anti-BCMA sdAb comprising specific CDR1, CDR2, and/or CDR3) comprises a V_HH domain having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NO: 115-152. In some embodiments, a V_HH sequence having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the reference sequence, but the anti-BCMA sdAb 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: 115-152. In some embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (i.e., in the FRs). Optionally, the anti-BCMA sdAb comprises an amino acid sequence selected from SEQ ID NO: 115-152, including post-translational modifications of that sequence.

[0133] In some embodiments, there is provided an isolated anti-BCMA sdAb comprising a V_HH domain having an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152. In some embodiments, there is provided a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152.

[0134] In some embodiments, functional epitopes can be mapped by combinatorial alanine scanning. In this process, a combinatorial alanine-scanning strategy can be used to identify amino acids in the BCMA protein that are necessary for interaction with anti-BCMA sdAbs. In some embodiments, the epitope is conformational and crystal structure of anti-BCMA sdAb bound to BCMA may be employed to identify the epitopes. In some embodiments, the present application provides an epitope of BCMA derived from an amino acid sequence selected from the group consisting of SEQ ID NOs: 388-394. In some embodiments, the present application provides an epitope of BCMA comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 388-394.

[0135] In some embodiments, the present application provides antibodies which compete with any one of the anti-BCMA sdAbs described herein for binding to BCMA. In some embodiments, the invention provides antibodies which compete with the anti-BCMA sdAbs provided herein for binding to an epitope on the BCMA. In some embodiments, an antibody is provided that binds to the same epitope as an anti-BCMA sdAb comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152. In some embodiments, an antibody is provided that specifically binds to BCMA competitively with an anti-BCMA sdAb comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152.

[0136] In some embodiments, competition assays may be used to identify a monoclonal antibody that competes with an anti-BCMA sdAb described herein for binding to BCMA. Competition assays can be used to determine whether two antibodies bind the same epitope by recognizing identical or sterically overlapping epitopes or one antibody competitively inhibits binding of another antibody to the antigen. In certain embodiments, such a competing antibody binds to the same epitope (*e.g.*, a BCMA epitope derived from an amino acid sequence selected from the group consisting of SEQ ID NOs: 388-394) that is bound by an antibody described herein. Exemplary competition assays include, but are not limited to, routine assays such as those provided in Harlow and Lane (1988) Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in Methods in Molecular Biology vol. 66 (Humana Press, Totowa, N.J.). In some embodiments, two antibodies are said to bind to the same epitope if each blocks binding of the other by 50% or more. In some embodiments, the antibody that competes with an anti-BCMA sdAb described herein is a camelid, chimeric, humanized or human antibody. In some embodiments, the present application provides an antibody that competes with a camelid, chimeric, humanized, or human anti-BCMA sdAb as described herein.

[0137] In some embodiments, there is provided an anti-BCMA antibody or antigen binding protein comprising any one of the anti-BCMA sdAbs described above. In some embodiments, the anti-BCMA antibody is a monoclonal antibody, including a camelid, chimeric, humanized or human antibody. In some embodiments, the anti-BCMA antibody is an antibody fragment, *e.g.*, a V_HH fragment. In some embodiments, the anti-BCMA antibody is a full-length heavy-chain only

antibody comprising an Fc region of any antibody class or isotype, such as IgG1 or IgG4. In some embodiments, the Fc region has reduced or minimized effector function.

[0138] In some embodiments, the anti-BCMA antibody (such as anti-BCMA sdAb) or antigen binding protein according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 of "Features of antibodies" below.

[0139] In some embodiments, there is provided an isolated nucleic acid encoding any one of the anti-BCMA antibodies (such as anti-BCMA sdAbs) described above. In some embodiments, an isolated nucleic acid encoding an anti-BCMA sdAb 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: 153-190. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 153-190. In some embodiments, a vector (*e.g.*, expression vector) comprising such nucleic acid are provided. In some embodiments, a host cell comprising such nucleic acid is provided. In some embodiments, a method of making an anti-BCMA antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the anti-BCMA antibody, as provided above, under conditions suitable for expression of the anti-BCMA antibody, and optionally recovering the anti-BCMA antibody from the host cell (or host cell culture medium).

Features of antibodies

1. Antibody Affinity

[0140] In some embodiments, an anti-BCMA antibody provided herein has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (*e.g.* 10^{-8} M or less, *e.g.* from 10^{-8} M to 10^{-13} M, *e.g.*, from 10^{-9} M to 10^{-13} M).

[0141] In some embodiments, Kd is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version or V_HH fragment of an antibody of interest and its antigen as described by the following assay. For example, solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, *e.g.*, Chen et al., *J. Mol. Biol.* 293:865-881(1999)).

[0142] In some embodiments, Kd is measured using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE ®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab or V_HH of the antibody of interest (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE ® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, *e.g.*, Chen et al., *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-AMINCO ™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

2. Antibody Fragments

[0143] In some embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, $F(ab')_2$, Fv, and scFv fragments, V_HH, and other fragments described below. For a review of certain antibody fragments, see Hudson et al. *Nat. Med.* 9:129-134 (2003). For a review of scFv fragments, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

[0144] 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).

[0145] 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

[0146] In some embodiments, the antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, *e.g.*, in U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (*e.g.*, a variable region derived from a camelid species, such as llama) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

[0147] 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.

[0148] 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).

[0149] 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)).

[0150] In some embodiments, the sdAbs 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 sdAbs requires the introduction and mutagenesis of a limited amount of amino acids in a single polypeptide chain. This is in contrast to humanization of scFv, Fab', (Fab')2 and IgG, which requires the introduction of amino acid changes in two chains, the light and the heavy chain and the preservation of the assembly of both chains.

[0151] Single-domain antibodies comprising a V_HH domain can be humanized to have human-like sequences. In some embodiments, the FR regions of the V_HH domain used herein comprise at least about any one of 50%, 60%, 70%, 80%, 90%, 95% or more of amino acid sequence homology to human V_H framework regions. One exemplary class of humanized V_HH domains is characterized in that the V_HHs carry an amino acid from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, or glutamine at position 45, such as, for example, L45 and a tryptophan at position 103, according to the Kabat numbering. As such, polypeptides belonging to this class show a high amino acid sequence homology to human V_H framework

regions and said polypeptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanization.

[0152] Another exemplary class of humanized Camelid sdAbs 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

[0153] In some embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr. Opin. Pharmacol.* 5: 368-74 (2001) and Lonberg, *Curr. Opin. Immunol.* 20:450-459 (2008). Transgenic mice or rats capable of producing fully human sdAbs are known in the art. See, *e.g.*, US20090307787A1, U.S. Pat. No. 8,754,287, US20150289489A1, US20100122358A1, and WO2004049794.

[0154] 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.

[0155] Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (*See, e.g.*, Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human antibodies generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

[0156] 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.

[0157] One technique for obtaining V_HH sequences directed against a particular antigen or target involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (i.e. so as to raise an immune response and/or heavy chain antibodies directed against said antigen or target), obtaining a suitable biological sample from said transgenic mammal that contains (nucleic acid sequences encoding) said V_HH sequences (such as a blood sample, serum sample or sample of B-cells), and then generating V_HH sequences directed against said antigen or target, starting from said sample, using any suitable technique known per se (such as any of the methods described herein or a hybridoma technique). For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO 02/085945, WO 04/049794 and WO 06/008548 and Janssens et al., Proc. Natl. Acad. Sci. USA. 2006 Oct. 10; 103(41):15130-5 can be used. For example, such heavy chain antibody expressing mice can express heavy chain antibodies with any suitable (single) variable domain, such as (single) variable domains from natural sources (*e.g.* human (single) variable domains,

Camelid (single) variable domains or shark (single) variable domains), as well as for example synthetic or semi-synthetic (single) variable domains.

5. Library-Derived Antibodies

[0158] Antibodies of the present application may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, *e.g.*, in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, *e.g.*, in the McCafferty et al., *Nature* 348:552-554; Clackson et al., *Nature* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Marks and Bradbury, in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132(2004). Methods for constructing sdAb libraries have been described, for example, see U.S. Pat. NO. 7371849.

[0159] In certain phage display methods, repertoires of V_H and V_L genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (*e.g.*, from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J,* 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

[0160] Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

6. Multispecific Antibodies

[0161] In some embodiments, an antibody provided herein is a multispecific antibody, *e.g.* a bispecific antibody. Multispecific antibodies are antibodies that have binding specificities for at least two different sites. In some embodiments, one of the binding specificities is for an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38, and the other is for any other antigen. In some embodiments, bispecific antibodies may bind to two different epitopes of an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express an antigen selected from the group consisting of CD19, CD20, BCMA, and CD38.

[0162] Bispecific antibodies can be prepared as full length antibodies or antibody fragments. Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)), WO 93/08829, and Traunecker et al., *EMBO J.* 10: 3655 (1991)), and "knob-in-hole" engineering (*see, e.g.*, U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (*see, e.g.*, US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (*see, e.g.*, Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992)); using "diabody" technology for making bispecific antibody fragments (see, *e.g.*, Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (*see, e.g.*, Gruber et al., *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, *e.g.*, in Tutt et al. *J. Immunol.* 147: 60 (1991); and creating polypeptides comprising tandem single-domain antibodies (*see,* e.g, U.S. Patent Application No. 20110028695; and Conrath et al. J. Biol. Chem., 2001; 276(10):7346-50). Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (*see, e.g.,* US 2006/0025576A1).

7. Antibody Variants

[0163] In some embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other

biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleic acid sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, *e.g.*, antigen-binding.

a) Substitution, Insertion, and Deletion Variants

[0164] In some embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in Table 3 under the heading of "Preferred substitutions." More substantial changes are provided in Table 3 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, *e.g.*, retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE 3. Amino Acid Substitutions

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

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

[0165] Amino acids may be grouped according to common side-chain properties:

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

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

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

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

(6) aromatic: Trp, Tyr, Phe.

[0166] Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0167] One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (*e.g.*, improvements) in certain biological properties (*e.g.*, increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, *e.g.*, using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (*e.g.* binding affinity).

[0168] Alterations (*e.g.*, substitutions) may be made in HVRs, *e.g.*, to improve antibody affinity. Such alterations may be made in HVR "hotspots," *i.e.*, residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (*see, e.g.*, Chowdhury,

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

[0169] In some embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (*e.g.*, conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or CDRs. In some embodiments of the variant V_HH sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

[0170] 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.

[0171] 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

[0172] In some embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

[0173] Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. *See, e.g.*, Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, *e.g.*, mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the present application may be made in order to create antibody variants with certain improved properties.

[0174] In some embodiments, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (*e.g.*, complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (EU numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, *i.e.*, between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. *See, e.g.*, US Patent Publication Nos. US

2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); US Patent Application No. US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8,* knockout CHO cells (*see, e.g.*, Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

[0175] 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

[0176] In some embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions.

[0177] In some embodiments, the present application contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half-life of the antibody *in vivo* is important yet certain effector

functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express Fc(RI, Fc(RII and Fc(RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (*see, e.g.* Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (*see* Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (*see*, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes et al. *Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. *See, e.g.*, C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (*see*, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. et al., *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half-life determinations can also be performed using methods known in the art (*see, e.g.*, Petkova, S.B. et al., *Int'l. Immunol.* 18(12):1759-1769 (2006)).

[0178] 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).

[0179] 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).)

[0180] In some embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, *e.g.*, substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

[0181] In some embodiments, alterations are made in the Fc region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), *e.g.*, as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

[0182] 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).

[0183] 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

[0184] 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

[0185] In some embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, *etc.*

[0186] In some embodiments, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In some embodiments, the nonproteinaceous moiety is a carbon nanotube (Kam et al., *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

Methods of preparation

[0187] The antibodies (such as sdAbs) described herein may be prepared using any methods known in the art or as described herein.

[0188] Methods of preparing sdAbs have been described. See, for example, Els Pardon et al, *Nature Protocol*, 2014; 9(3): 674. Single-domain antibodies (such as V_HHs) may be obtained using methods known in the art such as by immunizing a *Camelid* species (such as camel or llama) and obtaining hybridomas therefrom, or by cloning a library of sdAbs using molecular

biology techniques known in the art and subsequent selection by ELISA with individual clones of unselected libraries or by using phage display.

[0189] For recombinant production of the sdAbs, the nucleic acids encoding the sdAbs are isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the sdAb 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

[0190] Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, *e.g.*, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are independently lower alkyl groups. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

[0191] The animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg or the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to fourteen days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitable to enhance the immune response.

2. Monoclonal Antibodies

[0192] 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.

[0193] 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).

[0194] 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).

[0195] 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.

[0196] 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.

[0197] Preferred immortalized myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 cells (and derivatives thereof, *e.g.*, X63-Ag8-653) available from the American Type Culture Collection, Manassas, Va. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0198] 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).

[0199] The culture medium in which the hybridoma cells are cultured can be assayed for the presence of monoclonal antibodies directed against the desired antigen. Preferably, the binding affinity and specificity of the monoclonal antibody can be determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked assay (ELISA). Such techniques and assays are known in the in art. For example, binding affinity may be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

[0200] 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.

[0201] 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.

[0202] Monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567, and as described above. DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, in order to synthesize monoclonal antibodies in such recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Pliickthun, *Immunol. Revs.* 130:151-188 (1992).

[0203] In a further embodiment, antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nucl. Acids Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[0204] 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.

[0205] The monoclonal antibodies described herein may by monovalent, the preparation of which is well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and a modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues may be substituted with another amino acid residue or are deleted so as to prevent crosslinking. In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly Fab fragments, can be accomplished using routine techniques known in the art.

[0206] 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

[0207] Polynucleotide sequences encoding the antibodies of the present application can be obtained using standard recombinant techniques. Desired polynucleotide 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.

[0208] 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.

[0209] In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as GEM™-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as *E. coli* LE392.

[0210] The expression vector of the present application may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5′) to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, *e.g.* the presence or absence of a nutrient or a change in temperature.

[0211] A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the present application. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

[0212] 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.

[0213] In one aspect, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (*i.e.* cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In some embodiments of the present application, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

[0214] In some embodiments, the production of the antibodies according to the present application can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In some embodiments, polypeptide components, such as the polypeptide encoding the V_H domain of the first antigen binding portion optionally fused to the second antigen binding portion, and the polypeptide encoding the V_L domain of the first antigen binding portion optionally fused to the second antigen binding portion, are expressed, folded and assembled to form functional antibodies within the cytoplasm. Certain host strains (*e.g.*, the *E. coli* trxB$^-$ strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun *Gene,* 159:203 (1995).

[0215] The present invention provides an expression system in which the quantitative ratio of expressed polypeptide components can be modulated in order to maximize the yield of secreted and properly assembled the antibodies of the present application. Such modulation is accomplished at least in part by simultaneously modulating translational strengths for the polypeptide components. One technique for modulating translational strength is disclosed in Simmons *et al.*, U.S. Pat. No. 5,840,523. It utilizes variants of the translational initiation region (TIR) within a cistron. For a given TIR, a series of amino acid or nucleic acid sequence variants

can be created with a range of translational strengths, thereby providing a convenient means by which to adjust this factor for the desired expression level of the specific chain. TIR variants can be generated by conventional mutagenesis techniques that result in codon changes which can alter the amino acid sequence, although silent changes in the nucleic acid sequence are preferred. Alterations in the TIR can include, for example, alterations in the number or spacing of Shine-Dalgarno sequences, along with alterations in the signal sequence. One method for generating mutant signal sequences is the generation of a "codon bank" at the beginning of a coding sequence that does not change the amino acid sequence of the signal sequence (*i.e.*, the changes are silent). This can be accomplished by changing the third nucleotide position of each codon; additionally, some amino acids, such as leucine, serine, and arginine, have multiple first and second positions that can add complexity in making the bank. This method of mutagenesis is described in detail in Yansura *et al.* (1992) *METHODS: A Companion to Methods in Enzymol.* 4:151-158.

[0216] Preferably, a set of vectors is generated with a range of TIR strengths for each cistron therein. This limited set provides a comparison of expression levels of each chain as well as the yield of the desired protein products under various TIR strength combinations. TIR strengths can be determined by quantifying the expression level of a reporter gene as described in detail in Simmons *et al.* U.S. Pat. No. 5,840,523. Based on the translational strength comparison, the desired individual TIRs are selected to be combined in the expression vector constructs of the present application.

b) Prokaryotic Host Cells.

[0217] Prokaryotic host cells suitable for expressing the antibodies of the present application include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (*e.g.*, *E. coli*), *Bacilli* (*e.g.*, *B. subtilis*), Enterobacteria, *Pseudomonas* species (*e.g.*, *P. aeruginosa*), *Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla*, or *Paracoccus*. In some embodiments, gram-negative cells are used. In some embodiments, *E. coli* cells are used as hosts for the invention. Examples of *E. coli* strains include strain W3110 (Bachmann, *Cellular and Molecular Biology*, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 AfhuA (AtonA) ptr3 lac Iq lacL8 AompT A(nmpc-fepE) degP41 kan^R (U.S.

Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308(ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass *et al.*, *Proteins,* 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli, Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon.

[0218] 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

[0219] 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.

[0220] Prokaryotic cells used to produce the antibodies of the present application are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

[0221] 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.

[0222] 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.

[0223] If an inducible promoter is used in the expression vector of the present application, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the present application, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium (see, *e.g.*, Simmons *et al.*, *J. Immunol. Methods* (2002), 263:133-147). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

[0224] The expressed antibodies of the present application are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

[0225] 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.

[0226] During the fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, *e.g.*, an OD_{550} of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

[0227] To improve the production yield and quality of the antibodies of the present application, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen *et al.* (1999) *J Bio Chem* 274:19601-19605; Georgiou *et al.*, U.S. Pat. No. 6,083,715; Georgiou *et al.*, U.S. Pat. No. 6,027,888; Bothmann and Pluckthun (2000) J. Biol. Chem. 275:17100-17105; Ramm and Pluckthun (2000) J. Biol. Chem. 275:17106-17113; Arie *et al.* (2001) *Mol. Microbiol.* 39:199-210.

[0228] 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).

[0229] *E. coli* strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins may be used as host cells in the expression system encoding the antibodies of the present application.

d) Protein Purification

[0230] 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.

[0231] In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the antibodies comprising an Fc region of the present application. Protein A is a 41kD cell wall protein from *Staphylococcus aureas* which binds with a high affinity to the Fc region of antibodies. Lindmark *et al* (1983) *J. Immunol. Meth.* 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants. The solid phase is then washed to remove contaminants non-specifically bound to the solid phase. Finally the antibodies of interest is recovered from the solid phase by elution.

4. Recombinant Production in Eukaryotic Cells

[0232] 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

[0233] 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.

[0234] The DNA for such precursor region is ligated in reading frame to DNA encoding the antibodies of the present application.

b) Origin of Replication

[0235] 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

[0236] 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.

[0237] 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.

[0238] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up nucleic acid encoding the antibodies of the present application, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

[0239] 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).

[0240] 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

[0241] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the desired polypeptide sequences. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 based upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of the transcription of many genes is a CNCAAT region where N may be any nucleotide. A the 3′ end of most eukaryotic is an AATAAA sequence that may be

the signal for addition of the poly A tail to the 3′ end of the coding sequence. All of these sequences may be inserted into eukaryotic expression vectors.

[0242] 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.

[0243] 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.

[0244] 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

[0245] Transcription of a DNA encoding the antibodies of the present application by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on

enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5′ or 3′ to the polypeptide encoding sequence, but is preferably located at a site 5′ from the promoter.

f) Transcription Termination Component

[0246] 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

[0247] 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).

[0248] 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

[0249] The host cells used to produce the antibodies of the present application may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.* 58:44 (1979), Barnes *et al.*, *Anal. Biochem.* 102:255 (1980), U.S. Pat. No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

i) Protein Purification

[0250] 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.

[0251] The protein composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify the antibodies that are based on human immunoglobulins containing 1, 2, or 4 heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human 3 (Guss *et al.*, *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABXTMresin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[0252] Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*, from about 0-0.25M salt).

Immunoconjugates

[0253] In some embodiments, the present application also provides immunoconjugates comprising any of the antibodies (such as sdAbs) 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.

[0254] 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.

[0255] In some embodiments, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[0256] In some embodiments, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

[0257] 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.

[0258] The immunuoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (*e.g.*, from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

Methods and Compositions for Diagnostics and Detection

[0259] In some embodiments, any of the antibodies (such as sdAbs) provided herein is useful for detecting the presence of BCMA 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.

[0260] In some embodiments, an anti-BCMA antibody (such as any one of the anti-BCMA sdAbs 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 certain embodiments, labeled anti-BCMA sdAbs are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, *e.g.*, through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, *e.g.*, firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, *e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

III. Chimeric antigen receptors

[0262] One aspect of the present application provides a chimeric antigen receptor (CAR) comprising an extracellular antigen binding domain comprising one or more single-domain antibodies (such as V_HHs). Any one of the anti-BCMA sdAbs described in Section II can be used in the CARs described herein. Exemplary structures of CARs are shown in FIGs. 15A-15D.

[0263] In some embodiments, there is provided a CAR targeting BCMA (also referred herein as "BCMA CAR") comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1,

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

[0264] In some embodiments, there is provided a BCMA CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises any one of the following: (1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77; (2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78; (3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; (4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80; (5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81; (6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of

SEQ ID NO:82; (7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83; (8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84; (9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85; (10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; (11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87; (12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88; (13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89; (14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90; (15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; (16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92; (17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93; (18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; (19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95; (20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; (21) a CDR1 comprising

the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97; (22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98; (23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99; (24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100; (25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101; (26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102; (27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103; (28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104; (29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105; (30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106; (31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107; (32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108; (33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109; (34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID

NO:110; (35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111; (36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112; (37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or (38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 115-152. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some

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

[0265] In some embodiments, there is provided a BCMA CAR comprising a polypeptide having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 216-256 and 298-335. In some embodiments, there is provided a BCMA CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 216-256 and 298-335. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 216-256 and 298-335.

[0266] In some embodiments, there is provided an isolated nucleic acid encoding any of the BCMA CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 257-297 and 336-373. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 257-297 and 336-373. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the BCMA CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector. In some embodiments, the vector is a non-viral vector. Exemplary monovalent BCMA CARs are shown in Table 4 below.

Table 4. Exemplary monovalent BCMA CARs.

Ex. Vector or CAR name	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extra-cellular. sdAb	Hinge	TM	Intracellular signaling		
							CO1	CO2	Prim.
PLLV-hEF1a-269A37346	216	257	CD8α	269A37346	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37348	217	258	CD8α	269A37348	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37917	218	259	CD8α	269A37917	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37355	219	260	CD8α	269A37355	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-	220	261	CD8α	269A379	CD8α	CD28	CD28	CD137	CD3ζ

269A37915				15					
PLLV-hEF1a-269A37936	221	262	CD8α	269A37936	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37953	222	263	CD8α	269A37953	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37965	223	264	CD8α	269A37965	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37972	224	265	CD8α	269A37972	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37353	225	266	CD8α	269A37353	CD8α	CD28	CD28	CD137	CD3ζ
PLLV-hEF1a-269A37948	226	267	CD8α	269A37948	CD8α	CD28	CD28	CD137	CD3ζ
GSI5011 CAR	227	268	CD8α	269A37346	CD8α	CD8α	CD137	NA	CD3ζ
GSI5019 CAR	228	269	CD8α	269A37353	CD8α	CD8α	CD137	NA	CD3ζ
GSI5020 CAR	229	270	CD8α	269A37917	CD8α	CD8α	CD137	NA	CD3ζ
269B005S	230	271	CD8α	269B005	CD8α	CD8α	CD137	NA	CD3ζ
269B023S	231	272	CD8α	269B023	CD8α	CD8α	CD137	NA	CD3ζ
269B024S	232	273	CD8α	269B024	CD8α	CD8α	CD137	NA	CD3ζ
269B028S	233	274	CD8α	269B028	CD8α	CD8α	CD137	NA	CD3ζ
269B030S	234	275	CD8α	269B030	CD8α	CD8α	CD137	NA	CD3ζ
269B038S	235	276	CD8α	269B038	CD8α	CD8α	CD137	NA	CD3ζ
269B054S	236	277	CD8α	269B054	CD8α	CD8α	CD137	NA	CD3ζ
269B059S	237	278	CD8α	269B059	CD8α	CD8α	CD137	NA	CD3ζ
269B060S	238	279	CD8α	269B060	CD8α	CD8α	CD137	NA	CD3ζ
269B069S	239	280	CD8α	269B069	CD8α	CD8α	CD137	NA	CD3ζ
269B074S	240	281	CD8α	269B074	CD8α	CD8α	CD137	NA	CD3ζ
269B076S	241	282	CD8α	269B076	CD8α	CD8α	CD137	NA	CD3ζ
269B079S	242	283	CD8α	269B083	CD8α	CD8α	CD137	NA	CD3ζ
269B083S	243	284	CD8α	269B085	CD8α	CD8α	CD137	NA	CD3ζ
269B085S	244	285	CD8α	269B093	CD8α	CD8α	CD137	NA	CD3ζ
269B093S	245	286	CD8α	269B094	CD8α	CD8α	CD137	NA	CD3ζ
269B094S	246	287	CD8α	269B104	CD8α	CD8α	CD137	NA	CD3ζ
269B104S	247	288	CD8α	269B109	CD8α	CD8α	CD137	NA	CD3ζ
269B109S	248	289	CD8α	269B110	CD8α	CD8α	CD137	NA	CD3ζ
269B110S	249	290	CD8α	269B113	CD8α	CD8α	CD137	NA	CD3ζ
269B113S	250	291	CD8α	269B126	CD8α	CD8α	CD137	NA	CD3ζ
269B126S	251	292	CD8α	269B129	CD8α	CD8α	CD137	NA	CD3ζ
269B129S	252	293	CD8α	269B131	CD8α	CD8α	CD137	NA	CD3ζ
269B131S	253	294	CD8α	269B135	CD8α	CD8α	CD137	NA	CD3ζ
269B135S	254	295	CD8α	269B136	CD8α	CD8α	CD137	NA	CD3ζ
269B136S	255	296	CD8α	269B139	CD8α	CD8α	CD137	NA	CD3ζ

269B139S	256	297	CD8α	269B024	CD8α	CD8α	CD137	NA	CD3ζ

Multivalent chimeric antigen receptors

[0267] The present application also provides multivalent CARs that have two or more (such as about any one of 2, 3, 4, 5, 6, or more) binding moieties that specifically bind to an antigen, such as BCMA. In some embodiments, one or more of the binding moieties are antigen binding fragments. In some embodiments, one or more of the binding moieties comprise single-domain antibodies. In some embodiments, one or more of the binding moieties are derived from camelid antibodies. In some embodiments, one or more of the binding moieties are derived from a four-chain antibody. In some embodiments, one or more of the binding moieties are scFvs. In some embodiments, one or more of the binding moieties are derived from human antibodies. In some embodiments, one or more of the binding moieties are polypeptide ligands or other non-antibody polypeptides that specifically bind to the antigen. In some embodiments, the multivalent CAR is monospecific, *i.e.*, the multivalent CAR targets a single antigen, and comprises two or more binding sites for the single antigen. In some embodiments, the multivalent CAR is multispecific, *i.e.*, the multivalent CAR targets more than one antigen, and the multivalent CAR comprises two or more binding sites for at least one antigen. The binding moieties specific for the same antigen may bind to the same epitope of the antigen (*i.e.*, "mono-epitope CAR") or bind to different epitopes (*i.e.*, "multi-epitope CAR" such as bi-epitope CAR or tri-epitope CAR) of the antigen. The binding sites specific for the same antigen may comprise the same or different sdAbs.

[0268] In some embodiments, the present application provides a multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a plurality (such as at least about any one of 2, 3, 4, 5, 6, or more) of binding moieties specifically binding to an antigen (such as a tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77.

[0269] In some embodiments, the present application provides a multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a plurality (such as at least about any one of 2, 3, 4, 5, 6, or more) of single-domain antibodies (sdAbs) specifically binding

to an antigen (such as a tumor antigen); (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77.

[0270] In some embodiments, the present application provides a multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first binding moiety specifically binding to a first epitope of an antigen (such as a tumor antigen), and a second binding moiety specifically binding to a second epitope of the antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope and the second epitope are different. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77. In some embodiments, the first binding moiety is an sdAb and the second binding moiety is derived from a human antibody (*e.g.*, an scFv). In some embodiments, the first binding moiety is an sdAb and the second binding moiety is a polypeptide ligand. In some embodiments, the first epitope is the same as the second epitope. In some embodiments, the first epitope is different from the second epitope. In some embodiments, the multivalent CAR specifically binds to two different epitopes on an antigen. In some embodiments, the multivalent CAR specifically binds to three or more different epitopes on an antigen.

[0271] In some embodiments, the present application provides a multivalent (such as bivalent, trivalent, or of higher number of valencies) chimeric antigen receptor comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first sdAb specifically binding to a first epitope of an antigen (such as a tumor antigen), and a second sdAb specifically binding to a second epitope of the antigen; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope and the second epitope are different. In some embodiments, the antigen is selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77.

[0272] In some embodiments, the binding moieties, such as sdAbs (including the plurality of sdAbs, or the first sdAb and/or the second sdAb) are camelid, chimeric, human, or humanized.

In some embodiments, the binding moieties or sdAbs are fused to each other via peptide bonds or peptide linkers. In some embodiments, each peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent CAR is monospecific. In some embodiments, the multivalent CAR is multispecific, such as bispecific.

[0273] 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 anti-BCMA sdAbs described herein may be used in the extracellular antigen binding domain of the multivalent CARs described herein. A list of exemplary multivalent BCMA CARs, exemplary sequences, constructs and vectors thereof are shown in Table 5.

[0274] In some embodiments, there is provided a multivalent CAR targeting BCMA comprising: (a) an extracellular antigen binding domain comprising a plurality (such as at least about any one of 2, 3, 4 or more) of a BCMA binding moiety (*e.g.*, an anti-BCMA sdAb); (b) a transmembrane domain; and (c) an intracellular signaling domain. Any of the anti-BCMA sdAbs

can be used to construct the multivalent BCMA CAR. In some embodiments, the extracellular antigen binding domain specifically binds to a single epitope of BCMA, and these CARs are referred herein as mono-epitope multivalent BCMA CARs.

[0275] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a plurality (such as at least about any one of 2, 3, 4 or more) of an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:1, a CDR2 comprising the amino acid sequence of SEQ ID NO:39, and a CDR3 comprising the amino acid sequence of SEQ ID NO:77.

[0276] In some embodiments, there is provided a multivalent BCMA CAR (also referred herein as "multi-epitope multivalent CAR") comprising: (a) an extracellular antigen binding domain comprising at least two (such as any one of 2, 3, 4, or more) BCMA binding moieties; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the at least two BCMA binding moieties specifically bind to at least two different epitopes on BCMA. In some embodiments, the extracellular antigen binding domain comprises a first BCMA binding moiety and a second BCMA binding moiety. In some embodiments, the first BCMA binding moiety is an anti-BCMA sdAb and the second BCMA binding moiety is derived from a human antibody (*e.g.*, an scFv). In some embodiments, the first BCMA binding moiety is an sdAb and the second BCMA binding moiety is a BCMA polypeptide ligand. In some embodiments, the first anti-BCMA binding moiety and/or the second BCMA binding moiety specifically binds to an epitope on BCMA derived from an amino acid sequence selected from SEQ ID NOs: 388-394. In some embodiments, the first BCMA binding moiety specifically binds to an epitope derived from SEQ ID NO: 389 and/or 390. In some embodiments, the second BCMA binding moiety specifically binds to an epitope derived from SEQ ID NO: 391 and/or 392.

[0277] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb and the second anti-BCMA sdAb specifically binds to different epitopes on BCMA. Any of the anti-BCMA sdAbs can be used to construct the multivalent BCMA CAR. In some embodiments, the first anti-BCMA sdAb and/or the second anti-BCMA sdAb specifically binds to an epitope on BCMA derived from an amino acid sequence selected

from SEQ ID NOs: 388-394. In some embodiments, the first anti-BCMA sdAb specifically binds to an epitope derived from SEQ ID NO: 389 and/or 390. In some embodiments, the second anti-BCMA sdAb specifically binds to an epitope derived from SEQ ID NO: 391 and/or 392.

[0278] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:3, a CDR2 comprising the amino acid sequence of SEQ ID NO:41, and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; and wherein the second anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:10, a CDR2 comprising the amino acid sequence of SEQ ID NO:48, and a CDR3 comprising the amino acid sequence of SEQ ID NO:86. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 117. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 124. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 124. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 117.

[0279] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:10, a CDR2 comprising the amino acid sequence of SEQ ID NO:48, and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; and wherein anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:11, a CDR2 comprising the amino acid sequence of SEQ ID NO:49, and a CDR3 comprising the amino acid sequence of SEQ ID NO:87. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 124. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 125.

[0280] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein

the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:7, a CDR2 comprising the amino acid sequence of SEQ ID NO:45, and a CDR3 comprising the amino acid sequence of SEQ ID NO:83; and wherein anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:11, a CDR2 comprising the amino acid sequence of SEQ ID NO:49, and a CDR3 comprising the amino acid sequence of SEQ ID NO:87. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 121. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 125.

[0281] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:15, a CDR2 comprising the amino acid sequence of SEQ ID NO:53, and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; and wherein anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:18, a CDR2 comprising the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:94. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 129. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 132.

[0282] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:18, a CDR2 comprising the amino acid sequence of SEQ ID NO:56, and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; and wherein anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:20, a CDR2 comprising the amino acid sequence of SEQ ID NO:58, and a CDR3 comprising the amino acid sequence of SEQ ID NO:96. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 132. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 134.

[0283] In some embodiments, there is provided a multivalent BCMA CAR comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb and a second anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:20, a CDR2 comprising the amino acid sequence of SEQ ID NO:58, and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; and wherein anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO:28, a CDR2 comprising the amino acid sequence of SEQ ID NO:66, and a CDR3 comprising the amino acid sequence of SEQ ID NO:104. In some embodiments, the first anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 134. In some embodiments, the second anti-BCMA sdAb comprises a V_HH domain comprising the amino acid sequence of SEQ ID NO: 142.

[0284] In some embodiments, the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) is located at the N-terminus of the second BCMA binding moiety (*e.g.*, the second anti-BCMA sdAb). In some embodiments, the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) is located at the C-terminus of the second BCMA binding moiety (*e.g.*, the second anti-BCMA sdAb). In some embodiments, the first BCMA binding moiety (*e.g.*, the first anti-BCMA sdAb) and the second BCMA binding moiety (*e.g.*, the second anti-BCMA sdAb) 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 intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multivalent BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-

terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the multivalent BCMA CAR is bivalent. In some embodiments, the multivalent BCMA CAR is trivalent. In some embodiments, the multivalent BCMA CAR specifically binds to two different epitopes on BCMA. In some embodiments, the multivalent BCMA CAR specifically binds to three or more different epitopes on BCMA.

[0285] 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: 298-335. In some embodiments, there is provided a multivalent BCMA CAR comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 298-335. Also provided is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 298-335.

[0286] In some embodiments, there is provided an isolated nucleic acid encoding any of the multivalent BCMA CARs provided herein. In some embodiments, there is provided an isolated nucleic acid having at least about any one of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 336-373. In some embodiments, there is provided an isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 336-373. In some embodiments, the isolated nucleic acid is a DNA. In some embodiments, the isolated nucleic acid is an RNA. In some embodiments, there is provided a vector comprising any one of the nucleic acids encoding the multivalent BCMA CARs described above. In some embodiments, the vector is an expression vector. In some embodiments, the vector is a viral vector, such as a lentiviral vector. In some embodiments, the vector is a non-viral vector. Exemplary multivalent BCMA CARs are shown in Table 5 below.

Table 5. Exemplary multivalent BCMA CARs.

CAR	Ex. AA SEQ ID	Ex. NA SEQ ID	SP	Extracellular Antigen binding domain					Hinge	TM	Intracellular signaling	
				sdAb #1	Lnk. #1 SEQ	sdAb #2	Lnk. #2 SEQ	sdAb #3			CO 1	Prim.

					ID		ID					
GSI50 14	298	336	CD8α	269A 37346	208	269A 37346	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 15	299	337	CD8α	269A 37346	208	269A 37346	208	269A 37346	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 21	300	338	CD8α	269A 37353	208	269A 37917	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 22	301	339	CD8α	269A 37353	213	269A 37917	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 23	302	340	CD8α	269A 37353	215	269A 37917	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 24	303	341	CD8α	269A 37917	209	269A 37353	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 25	304	342	CD8α	269A 37917	213	269A 37353	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
GSI50 26	305	343	CD8α	269A 37917	214	269A 37353	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 001	306	344	CD8α	269A 37353	208	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 002	307	345	CD8α	269A 37353	213	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 003	308	346	CD8α	269A 37353	215	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 004	309	347	CD8α	269A 37948	209	269A 37353	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 005	310	348	CD8α	269A 37948	213	269A 37353	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 006	311	349	CD8α	269A 37948	214	269A 37353	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 007	312	350	CD8α	269A 37953	208	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 008	313	351	CD8α	269A 37953	213	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 009	314	352	CD8α	269A 37953	215	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 010	315	353	CD8α	269A 37953	209	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 011	316	354	CD8α	269A 37953	213	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 012	317	355	CD8α	269A 37953	214	269A 37948	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 013	318	356	CD8α	269B 028	208	269B 054	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR	319	357	CD8α	269B	213	269B	NA	NA	CD	CD	CD	CD3ζ

014				028		054			8α	8α	137	
BCAR 015	320	358	CD8α	269B 028	215	269B 054	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 016	321	359	CD8α	269B 028	209	269B 054	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 017	322	360	CD8α	269B 028	213	269B 054	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 018	323	361	CD8α	269B 028	214	269B 054	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 019	324	362	CD8α	269B 054	208	269B 060	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 020	325	363	CD8α	269B 054	213	269B 060	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 021	326	364	CD8α	269B 054	215	269B 060	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 022	327	365	CD8α	269B 054	209	269B 060	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 023	328	366	CD8α	269B 054	213	269B 060	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 024	329	367	CD8α	269B 054	214	269B 060	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 025	330	368	CD8α	269B 060	208	269B 094	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 026	331	369	CD8α	269B 060	213	269B 094	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 027	332	370	CD8α	269B 060S	215	269B 094	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 028	333	371	CD8α	269B 060	209	269B 094	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 029	334	372	CD8α	269B 060	213	269B 094	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ
BCAR 030	335	373	CD8α	269B 060	214	269B 094	NA	NA	CD 8α	CD 8α	CD 137	CD3ζ

Multispecific chimeric antigen receptor

[0287] The present application further provides multispecific chimeric antigen receptors targeting two or more (such as about any one of 2, 3, 4, 5, 6, or more) different antigens. In some embodiments, the multispecific CAR has one antigen binding site for each antigen. In some embodiments, the multispecific CAR has more than two binding sites for at least one antigen. Each antigen binding site may comprise a sdAb. For example, in some embodiments, the multispecific CAR 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.

[0288] 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 BCMA and a second single-domain antibody (sdAb) specifically binding to a second antigen (such as a 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 second antigen is 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. In some embodiments, the first sdAb and/or the second sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first sdAb and the second sdAb are fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multispecific CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the multispecific CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain

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

Extracellular antigen binding domain

[0289] The extracellular antigen binding domain of the CARs described herein comprises one or more (such as any one of 1, 2, 3, 4, 5, 6 or more) binding moieties, such as sdAbs. In some embodiments, the one or more binding moieties are antibodies or antigen-binding fragments thereof. In some embodiments, the one or more binding moieties are derived from four-chain antibodies. In some embodiments, the one or more binding moieties are derived from camelid antibodies. In some embodiments, the one or more binding moieties are derived from human antibodies. In some embodiments, the one or more binding moieties are non-antibody binding proteins, such as polypeptide ligands or engineered proteins that bind to an antigen. The binding moieties can be fused to each other directly via peptide bonds, or via peptide linkers.

1. Single-domain antibodies

[0290] In some embodiments, the CAR comprises an extracellular antigen binding domain comprising one or more sdAbs. The sdAbs may be of the same of different origins, and of the same or different sizes. Exemplary sdAbs include, but are not limited to, heavy chain variable domains from heavy-chain only antibodies (*e.g.*, V_HH or V_{NAR}), binding molecules naturally devoid of light chains, single domains (such as V_H or V_L) derived from conventional 4-chain antibodies, humanized heavy-chain only antibodies, human sdAbs 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 sdAbs 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 sdAb molecules from species other than *Camelidae* and sharks.

[0291] In some embodiments, the sdAb is derived from a naturally occurring single-domain antigen binding molecule known as heavy chain antibody devoid of light chains (also referred herein as "heavy chain only antibodies"). Such single domain molecules are disclosed in WO

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

[0292] V_HH molecules from Camelids are about 10 times smaller than IgG molecules. They are single polypeptides and can be very stable, resisting extreme pH and temperature conditions. Moreover, they can be resistant to the action of proteases which is not the case for conventional 4-chain antibodies. Furthermore, *in vitro* expression of V_HH s produces high yield, properly folded functional V_HHs. In addition, antibodies generated in Camelids can recognize epitopes other than those recognized by antibodies generated *in vitro* through the use of antibody libraries or via immunization of mammals other than Camelids (see, for example, WO9749805). As such, multispecific or multivalent CARs comprising one or more V_HH domains may interact more efficiently with targets than multispecific or multivalent CARs comprising antigen binding fragments derived from conventional 4-chain antibodies. Since V_HHs are known to bind into 'unusual' epitopes such as cavities or grooves, the affinity of CARs comprising such V_HHs may be more suitable for therapeutic treatment than conventional multispecific polypeptides.

[0293] 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.

[0294] 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).

[0295] In some embodiments, the sdAb is a human sdAb produced by transgenic mice or rats expressing human heavy chain segments. See, *e.g.*, US20090307787A1, U.S. Pat. No. 8,754,287, US20150289489A1, US20100122358A1, and WO2004049794. In some embodiments, the sdAb is affinity matured.

[0296] In some embodiments, naturally occurring V_HH domains against a particular antigen or target, can be obtained from (naïve or immune) libraries of Camelid V_HH sequences. Such methods may or may not involve screening such a library using said antigen or target, or at least one part, fragment, antigenic determinant or epitope thereof using one or more screening techniques known per se. Such libraries and techniques are for example described in WO 99/37681, WO 01/90190, WO 03/025020 and WO 03/035694. Alternatively, improved synthetic or semi-synthetic libraries derived from (naïve or immune) V_HH libraries may be used, such as V_HH libraries obtained from (naïve or immune) V_HH libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example described in WO 00/43507.

[0297] In some embodiments, the sdAbs are generated from conventional four-chain antibodies. See, for example, EP 0 368 684, Ward et al. (Nature 1989 Oct. 12; 341 (6242): 544-6), Holt et al., Trends Biotechnol., 2003, 21(11):484-490; WO 06/030220; and WO 06/003388.

2. Antigens

[0298] The antigen(s) targeted by the CARs of the present application are cell surface molecules. The binding moieties (such as sdAbs) may be chosen to recognize an antigen that acts as a cell surface marker on target cells associated with a special disease state. In some embodiments, the antigen (such as the first antigen and/or the second antigen) is a tumor antigen. In some embodiments, the multispecific CARs target two or more tumor antigens. In some embodiments, the tumor antigen is associated with a B cell malignancy. Tumors express a number of proteins that can serve as a target antigen for an immune response, particularly T cell

mediated immune responses. The antigens targeted by the CAR may be antigens on a single diseased cell or antigens that are expressed on different cells that each contribute to the disease. The antigens targeted by the CAR may be directly or indirectly involved in the diseases.

[0299] Tumor antigens are proteins that are produced by tumor cells that can elicit an immune response, particularly T-cell mediated immune responses. The selection of the targeted antigen of the invention will depend on the particular type of cancer to be treated. Exemplary tumor antigens include, for example, a glioma-associated antigen, carcinoembryonic antigen (CEA), β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CAIX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, M-CSF, prostase, prostate-specific antigen (PSA), PAP, NY-ESO-1, LAGE-la, p53, prostein, PSMA, HER2/neu, survivin and telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase, ephrinB2, CD22, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor and mesothelin.

[0300] In some embodiments, the tumor antigen comprises one or more antigenic cancer epitopes associated with a malignant tumor. Malignant tumors express a number of proteins that can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase and gp100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD 19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma.

[0301] In some embodiments, the tumor antigen is a tumor-specific antigen (TSA) or a tumor-associated antigen (TAA). A TSA is unique to tumor cells and does not occur on other cells in the body. A TAA associated antigen is not unique to a tumor cell, and instead is also expressed on a normal cell under conditions that fail to induce a state of immunologic tolerance to the antigen. The expression of the antigen on the tumor may occur under conditions that enable the immune system to respond to the antigen. TAAs may be antigens that are expressed on normal cells during fetal development, when the immune system is immature, and unable to respond or

they may be antigens that are normally present at extremely low levels on normal cells, but which are expressed at much higher levels on tumor cells.

[0302] Non-limiting examples of TSA or TAA antigens include the following: Differentiation antigens such as MART-1/MelanA (MART-I), gp 100 (Pmel 17), tyrosinase, TRP-1, TRP-2 and tumor-specific multilineage antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, pl5; overexpressed embryonic antigens such as CEA; overexpressed oncogenes and mutated tumor-suppressor genes such as p53, Ras, HER2/neu; unique tumor antigens resulting from chromosomal translocations; such as BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR; and viral antigens, such as the Epstein Barr virus antigens EBVA and the human papillomavirus (HPV) antigens E6 and E7. Other large, protein-based antigens include TSP-180, MAGE-4, MAGE-5, MAGE-6, RAGE, NY-ESO, pl85erbB2, pl80erbB-3, c-met, nm-23HI, PSA, TAG-72, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-Catenin, CDK4, Mum-1, p 15, p 16, 43-9F, 5T4, 791Tgp72, alpha-fetoprotein, beta-HCG, BCA225, BTAA, CA 125, CA 15-3\CA 27.29\BCAA, CA 195, CA 242, CA-50, CAM43, CD68\P1, CO-029, FGF-5, G250, Ga733\EpCAM, HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO- 1, RCAS 1, SDCCAG16, TA-90\Mac-2 binding protein\cyclophilin C-associated protein, TAAL6, TAG72, TLP, and TPS.

[0303] In some embodiments, the antigen (such as the first antigen and/or the second antigen) are selected from the group consisting of CD19, CD20, CD22, CD33, CD38, BCMA, CS1, ROR1, GPC3, CD123, IL-13R, CD138, c-Met, EGFRvIII, GD-2, NY-ESO-1, MAGE A3, and glycolipid F77.

3. Peptide linkers

[0304] The various binding moieties (such as sdAbs) in the multispecific or multivalent CARs described herein may be fused to each other via peptide linkers. In some embodiments, the binding moieties (such as sdAbs) are directly fused to each other without any peptide linkers. The peptide linkers connecting different binding moieties (such as sdAbs) may be the same or different. Different domains of the CARs may also be fused to each other via peptide linkers.

[0305] 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 sdAbs and/or the various domains. Each peptide linker may be selected and optimized independently. The length, the degree of flexibility and/or other properties of the peptide linker(s) used in the CARs may have some

influence on properties, including but not limited to the affinity, specificity or avidity for one or more particular antigens or epitopes. For example, longer peptide linkers may be selected to ensure that two adjacent domains do not sterically interfere with one another. For example, in a multivalent or multispecific CAR of the present application that comprise sdAbs directed against a multimeric antigen, the length and flexibility of the peptide linkers are preferably such that it allows each sdAb 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.

[0306] The peptide linker can be of any suitable length. In some embodiments, the peptide linker is at least about any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100 or more amino acids long. In some embodiments, the peptide linker is no more than about any of 100, 75, 50, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5 or fewer amino acids long. In some embodiments, the length of the peptide linker is any of about 1 amino acid to about 10 amino acids, about 1 amino acids to about 20 amino acids, about 1 amino acid to about 30 amino acids, about 5 amino acids to about 15 amino acids, about 10 amino acids to about 25 amino acids, about 5 amino acids to about 30 amino acids, about 10 amino acids to about 30 amino acids long, about 30 amino acids to about 50 amino acids, about 50 amino acids to about 100 amino acids, or about 1 amino acid to about 100 amino acids.

[0307] The peptide linker may have a naturally occurring sequence, or a non-naturally occurring sequence. For example, a sequence derived from the hinge region of heavy chain only antibodies may be used as the linker. *See,* for example, WO1996/34103. In some embodiments, the peptide linker is a flexible linker. Exemplary flexible linkers include glycine polymers $(G)_n$, glycine-serine polymers (including, for example, $(GS)_n$, $(GSGGS)_n$, $(GGGS)_n$, and $(GGGGS)_n$, where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers known in the art. In some embodiments, the peptide linker comprises the amino acid sequence GGGGS (SEQ ID NO: 208), $(GGGGS)_2$ (SEQ ID NO: 209), $(GGGS)_4$ (SEQ ID NO: 210), GGGGSGGGGSGGGGGGSGSGGGGS (SEQ ID NO: 211),

GGGGSGGGGSGGGGGGSGSGGGGSGGGGSGGGGS (SEQ ID NO: 212), $(GGGGS)_3$ (SEQ ID NO: 213), $(GGGGS)_4$ (SEQ ID NO: 214), or $(GGGGS)_3$ (SEQ ID NO: 215).

Transmembrane domain

[0308] The CARs of the present application comprise a transmembrane domain that can be directly or indirectly fused to the extracellular antigen binding domain. The transmembrane domain may be derived either from a natural or from a synthetic source. As used herein, a "transmembrane domain" refers to any protein structure that is thermodynamically stable in a cell membrane, preferably a eukaryotic cell membrane. Transmembrane domains compatible for use in the CARs described herein may be obtained from a naturally occurring protein. Alternatively, it can be a synthetic, non-naturally occurring protein segment, *e.g.*, a hydrophobic protein segment that is thermodynamically stable in a cell membrane.

[0309] 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.

[0310] 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.

[0311] In some embodiments, the transmembrane domain of the CAR comprises a transmembrane domain chosen from the transmembrane domain of an alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, KIRDS2, OX40, CD2, CD27, LFA-1 (CDl la, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRFl), CD160, CD19, IL-2R beta, IL-2R gamma, IL-7R a, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDl ld, ITGAE, CD103, ITGAL, CDl la, LFA-1, ITGAM, CDl lb, ITGAX, CDl lc, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CDIOO (SEMA4D), SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, and/or NKG2C. In some embodiments, the transmembrane domain is derived from a molecule selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1.

[0312] In some embodiments, the transmembrane domain is derived from CD28. In some embodiments, the transmembrane domain is a transmembrane domain of CD28 comprising the amino acid sequence of SEQ ID NO: 194. In some embodiments, the transmembrane domain of CD28 is encoded by the nucleic acid sequence of SEQ ID NO: 203.

[0313] In some embodiments, the transmembrane domain is derived from CD8α. In some embodiments, the transmembrane domain is a transmembrane domain of CD8α comprising the amino acid sequence of SEQ ID NO: 193. In some embodiments, the transmembrane domain of CD8 is encoded by the nucleic acid sequence of SEQ ID NO: 202.

[0314] 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, the relevant disclosures of which are incorporated by reference herein.

[0315] 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.

[0316] 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

[0317] The CARs of the present application comprise an intracellular signaling domain. The intracellular signaling domain is responsible for activation of at least one of the normal effector functions of the immune effector cell expressing the CARs. The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term "cytoplasmic signaling domain" refers to the portion of a protein which transduces the effector function signal

and directs the cell to perform a specialized function. While usually the entire cytoplasmic signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the cytoplasmic signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term cytoplasmic signaling domain is thus meant to include any truncated portion of the cytoplasmic signaling domain sufficient to transduce the effector function signal.

[0318] In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell. In some embodiments, the CAR comprises an intracellular signaling domain consisting essentially of a primary intracellular signaling domain of an immune effector cell. "Primary intracellular signaling domain" refers to cytoplasmic signaling sequence that acts in a stimulatory manner to induce immune effector functions. In some embodiments, the primary intracellular signaling domain contains a signaling motif known as immunoreceptor tyrosine-based activation motif, or ITAM. An "ITAM," as used herein, is a conserved protein motif that is generally present in the tail portion of signaling molecules expressed in many immune cells. The motif may comprises two repeats of the amino acid sequence YxxL/I separated by 6-8 amino acids, wherein each x is independently any amino acid, producing the conserved motif YxxL/Ix(6-8)YxxL/I. ITAMs within signaling molecules are important for signal transduction within the cell, which is mediated at least in part by phosphorylation of tyrosine residues in the ITAM following activation of the signaling molecule. ITAMs may also function as docking sites for other proteins involved in signaling pathways. Exemplary ITAM-containing primary cytoplasmic signaling sequences include those derived from CD3ζ, FcR gamma(FCER1G), FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

[0319] In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain consists of the cytoplasmic signaling domain of CD3ζ. In some embodiments, the primary intracellular signaling domain is a cytoplasmic signaling domain of wildtype CD3ζ. In some embodiments, the primary intracellular signaling domain of wildtype CD3ζ comprises the amino acid sequence of SEQ ID NO: 197. In some embodiments, the primary intracellular signaling domain is a functional mutant of the cytoplasmic signaling domain of CD3ζ containing one or more mutations, such as Q65K. In some embodiments, the primary intracellular signaling domain of mutant

CD3ζ comprises the amino acid sequence of SEQ ID NO: 198. In some embodiments, the primary intracellular signaling domain is encoded by the nucleic acid sequence of SEQ ID NO: 206 or 207.

Co-stimulatory signaling domain

[0320] 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.

[0321] In some embodiments, the intracellular signaling domain comprises a single co-stimulatory signaling domain. In some embodiments, the intracellular signaling domain comprises two or more (such as about any of 2, 3, 4, or more) co-stimulatory signaling domains. In some embodiments, the intracellular signaling domain comprises two or more of the same co-stimulatory signaling domains, for example, two copies of the co-stimulatory signaling domain of CD28. In some embodiments, the intracellular signaling domain comprises two or more co-stimulatory signaling domains from different co-stimulatory proteins, such as any two or more co-stimulatory proteins described herein. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3ζ) and one or more co-stimulatory signaling domains. In some embodiments, the one or more co-stimulatory signaling domains and the primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3ζ) are fused to each other via optional peptide linkers. The primary intracellular signaling domain, and the one or more co-stimulatory signaling domains may be arranged in any suitable order. In some embodiments, the one or more co-

stimulatory signaling domains are located between the transmembrane domain and the primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3ζ). Multiple co-stimulatory signaling domains may provide additive or synergistic stimulatory effects.

[0322] Activation of a co-stimulatory signaling domain in a host cell (*e.g.*, an immune cell) may induce the cell to increase or decrease the production and secretion of cytokines, phagocytic properties, proliferation, differentiation, survival, and/or cytotoxicity. The co-stimulatory signaling domain of any co-stimulatory molecule may be compatible for use in the CARs described herein. The type(s) of co-stimulatory signaling domain is selected based on factors such as the type of the immune effector cells in which the effector molecules would be expressed (*e.g.*, T cells, NK cells, macrophages, neutrophils, or eosinophils) and the desired immune effector function (*e.g.*, ADCC effect). Examples of co-stimulatory signaling domains for use in the CARs can be the cytoplasmic signaling domain of co-stimulatory proteins, including, without limitation, members of the B7/CD28 family (*e.g.*, B7-1/CD80, B7-2/CD86, B7-H1/PD-L1, B7-H2, B7-H3, B7-H4, B7-H6, B7-H7, BTLA/CD272, CD28, CTLA-4, Gi24/VISTA/B7-H5, ICOS/CD278, PD- 1, PD-L2/B7-DC, and PDCD6); members of the TNF superfamily (*e.g.*,4-1BB/TNFSF9/CD137, 4-1BB Ligand/TNFSF9, BAFF/BLyS/TNFSF13B, BAFF R/TNFRSF13C, CD27/TNFRSF7, CD27 Ligand/TNFSF7, CD30/TNFRSF8, CD30 Ligand/TNFSF8, CD40/TNFRSF5, CD40/TNFSF5, CD40 Ligand/TNFSF5, DR3/TNFRSF25, GITR/TNFRSF18, GITR Ligand/TNFSF18, HVEM/TNFRSF14, LIGHT/TNFSF14, Lymphotoxin-alpha/TNF-beta, OX40/TNFRSF4, OX40 Ligand/TNFSF4, RELT/TNFRSF19L, TACI/TNFRSF13B, TL1A/TNFSF15, TNF-alpha, and TNF RII/TNFRSF1B); members of the SLAM family (*e.g.*, 2B4/CD244/SLAMF4, BLAME/SLAMF8, CD2, CD2F-10/SLAMF9, CD48/SLAMF2, CD58/LFA-3, CD84/SLAMF5, CD229/SLAMF3, CRACC/SLAMF7, NTB-A/SLAMF6, and SLAM/CD150); and any other co-stimulatory molecules, such as CD2, CD7, CD53, CD82/Kai-1, CD90/Thy1, CD96, CD160, CD200, CD300a/LMIR1, HLA Class I, HLA- DR, Ikaros, Integrin alpha 4/CD49d, Integrin alpha 4 beta 1, Integrin alpha 4 beta 7/LPAM-1, LAG-3, TCL1A, TCL1B, CRTAM, DAP12, Dectin-1/CLEC7A, DPPIV/CD26, EphB6, TIM-1/KIM-1/HAVCR, TIM-4, TSLP, TSLP R, lymphocyte function associated antigen-1 (LFA-1), and NKG2C.

[0323] In some embodiments, the one or more co-stimulatory signaling domains are selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, CD3, lymphocyte

function-associated antigen-1(LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and ligands that specially bind to CD83.

[0324] In some embodiments, the intracellular signaling domain in the CAR of the present application comprises a co-stimulatory signaling domain derived from CD28. In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3ζ and a co-stimulatory signaling domain of CD28. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain of CD28 comprising the amino acid sequence of SEQ ID NO: 195. In some embodiments, the co-stimulatory signaling domain of CD28 is encoded by the nucleic acid sequence of SEQ ID NO: 204.

[0325] In some embodiments, the intracellular signaling domain in the CAR of the present application comprises a co-stimulatory signaling domain derived from CD137 (*i.e.*, 4-1BB). In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3ζ and a co-stimulatory signaling domain of CD137. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain of CD137 comprising the amino acid sequence of SEQ ID NO: 196. In some embodiments, the co-stimulatory signaling domain of CD137 is encoded by the nucleic acid sequence of SEQ ID NO: 205.

[0326] In some embodiments, the intracellular signaling domain in the CAR of the present application comprises a co-stimulatory signaling domain of CD28 and a co-stimulatory signaling domain of CD137. In some embodiments, the intracellular signaling domain comprises a cytoplasmic signaling domain of CD3ζ, a co-stimulatory signaling domain of CD28, and a co-stimulatory signaling domain of CD137. In some embodiments, the intracellular signaling domain comprises a polypeptide comprising from the N-terminus to the C-terminus: a co-stimulatory signaling domain of CD28, a co-stimulatory signaling domain of CD137, and a cytoplasmic signaling domain of CD3ζ. In some embodiments, the co-stimulatory signaling domain of CD28 comprising the amino acid sequence of SEQ ID NO: 195. In some embodiments, the co-stimulatory signaling domain of CD137 comprising the amino acid sequence of SEQ ID NO: 196.

[0327] 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

[0328] The CARs of the present application may comprise a hinge domain that is located between the extracellular antigen binding domain and the transmembrane domain. A hinge domain is an amino acid segment that is generally found between two domains of a protein and may allow for flexibility of the protein and movement of one or both of the domains relative to one another. Any amino acid sequence that provides such flexibility and movement of the extracellular antigen binding domain relative to the transmembrane domain of the effector molecule can be used.

[0329] 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.

[0330] In some embodiments, the hinge domain is a hinge domain of a naturally occurring protein. Hinge domains of any protein known in the art to comprise a hinge domain are compatible for use in the chimeric receptors described herein. In some embodiments, the hinge domain is at least a portion of a hinge domain of a naturally occurring protein and confers flexibility to the chimeric receptor. In some embodiments, the hinge domain is derived from CD8α. In some embodiments, the hinge domain is a portion of the hinge domain of CD8α, *e.g.*, a fragment containing at least 15 (*e.g.*, 20, 25, 30, 35, or 40) consecutive amino acids of the hinge domain of CD8α. In some embodiments, the hinge domain of CD8α comprises the amino acid sequence of SEQ ID NO: 192. In some embodiments, the hinge domain of CD8α is encoded by the nucleic acid sequence of SEQ ID NO: 201.

[0331] 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.

[0332] Non-naturally occurring peptides may also be used as hinge domains for the chimeric receptors described herein. In some embodiments, the hinge domain between the C-terminus of the extracellular ligand-binding domain of an Fc receptor and the N- terminus of the transmembrane domain is a peptide linker, such as a (GxS)n linker, wherein x and n, independently can be an integer between 3 and 12, including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more.

Signal peptide

[0333] The CARs of the present application may comprise a signal peptide (also known as a signal sequence) at the N-terminus of the polypeptide. In general, signal peptides are peptide sequences that target a polypeptide to the desired site in a cell. In some embodiments, the signal peptide targets the effector molecule to the secretory pathway of the cell and will allow for integration and anchoring of the effector molecule into the lipid bilayer. Signal peptides including signal sequences of naturally occurring proteins or synthetic, non-naturally occurring signal sequences, which are compatible for use in the CARs described herein will be evident to one of skill in the art. In some embodiments, the signal peptide is derived from a molecule selected from the group consisting of CD8α, GM-CSF receptor α, and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8α. In some embodiments, the signal

peptide of CD8α comprises the amino acid sequence of SEQ ID NO: 191. In some embodiments, the signal peptide of CD8α is encoded by the nucleic acid sequence of SEQ ID NO: 199 or 200.

IV. Engineered immune effector cells

[0334] Further provided in the present application are host cells (such as immune effector cells) comprising any one of the CARs described herein.

[0335] Thus, in some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a multivalent CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety specifically binding to a first epitope of BCMA, and a second BCMA binding moiety specifically binding to a second epitope of BCMA; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope and the second epitope are different.

[0336] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a multivalent CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb specifically binding to a first epitope of BCMA, and a second anti-BCMA sdAb specifically binding to a second epitope of BCMA; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the first epitope and the second epitope are different. In some embodiments, the first anti-BCMA sdAb and/or the second anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the first anti-BCMA and the second anti-BCMA are fused to each other via a peptide bond or a peptide linker. In some embodiments, the peptide linker is no more than about 50 (such as no more than about any one of 35, 25, 20, 15, 10, or 5) amino acids long. In some embodiments, the transmembrane domain is selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the multivalent CAR further comprises a hinge

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

[0337] In some embodiments, there is provided an engineered immune effector cell (such as T cell) comprising a BCMA CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain, wherein the anti-BCMA sdAb comprises any one of the following: (1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77; (2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78; (3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; (4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80; (5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81; (6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82; (7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83; (8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2

comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84; (9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85; (10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; (11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87; (12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88; (13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89; (14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90; (15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; (16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92; (17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93; (18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; (19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95; (20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; (21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97; (22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of

SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98; (23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99; (24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100; (25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101; (26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102; (27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103; (28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104; (29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105; (30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106; (31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107; (32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108; (33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109; (34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110; (35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111; (36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2

comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112; (37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or (38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114. In some embodiments, the extracellular antigen binding domain comprises at least two anti-BCMA sdAbs. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 115-152. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell (such as T cell). In some embodiments, the primary intracellular signaling domain is derived from CD3ζ. In some embodiments, the intracellular signaling domain comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof. In some embodiments, the BCMA CAR further comprises a hinge domain (such as a CD8α hinge domain) located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the BCMA CAR further comprises a signal peptide (such as a CD8α signal peptide) located at the N-terminus of the polypeptide. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen-binding domain, a CD8α hinge domain, a CD28 transmembrane domain, a first co-stimulatory signaling domain derived from CD28, a second co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ. In some embodiments, the polypeptide comprises from the N-terminus to the C-terminus: a CD8α signal peptide, the extracellular antigen binding domain, a CD8α hinge domain, a CD8α transmembrane domain, a co-stimulatory signaling domain derived from CD137, and a primary intracellular signaling domain derived from CD3ζ.In some embodiments, the BCMA CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 216-256, 298-335. 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.

[0338] 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 VHH-based CARs are illustrated in FIG. 15E. 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.

[0339] 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, which are incorporated herein by reference in their entirety.

Vectors

[0340] The present application provides vectors for cloning and expressing any one of the CARs described herein. In some embodiments, the vector is suitable for replication and integration in eukaryotic cells, such as mammalian cells. In some embodiments, the vector is a viral vector. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, lentiviral vector, retroviral vectors, vaccinia vector, herpes simplex viral vector, and derivatives thereof. Viral vector technology is well known in the art and is described, for example, in Sambrook *et al.* (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals.

[0341] 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 CARs 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.

[0342] In some embodiments, the vector is a non-viral vector. In some embodiments, the vector is a transposon, such as a Sleeping Beauty (SB) transposon system, or a PiggyBac transposon system. In some embodiments, the vector is a polymer-based non-viral vector, including for example, poly(lactic-co-glycolic acid) (PLGA) and poly lactic acid (PLA), poly(ethylene imine) (PEI), and dendrimers. In some embodiments, the vector is a cationic-lipid based non-viral vector, such as cationic liposome, lipid nanoemulsion, and solid lipid nanoparticle (SLN). In some embodiments, the vector is a peptide-based gene non-viral vector, such as poly-L-lysine. Any of the known non-viral vectors suitable for genome editing can be used for introducing the CAR-encoding nucleic acids to the engineered immune effector cells. *See*, for example, Yin H. et al. *Nature Rev. Genetics* (2014) 15:521-555; Aronovich EL *et al.* "The Sleeping Beauty transposon system: a non-viral vector for gene therapy." *Hum. Mol. Genet.* (2011) R1: R14-20; and Zhao S. *et al.* "PiggyBac transposon vectors: the tools of the human gene editing." *Transl. Lung Cancer Res.* (2016) 5(1): 120-125, which are incorporated herein by reference. In some embodiments, any one or more of the nucleic acids encoding a CAR is introduced to the engineered immune effector cells by a physical method, including, but not limited to electroporation, sonoporation, photoporation, magnetofection, hydroporation.

[0343] In some embodiments, the vector comprises any one of the nucleic acids encoding a CAR described herein. The nucleic acid can be cloned into the vector using any known molecular cloning methods in the art, including, for example, using restriction endonuclease sites and one or more selectable markers. In some embodiments, the nucleic acid is operably linked to

a promoter. Varieties of promoters have been explored for gene expression in mammalian cells, and any of the promoters known in the art may be used in the present invention. Promoters may be roughly categorized as constitutive promoters or regulated promoters, such as inducible promoters.

[0344] In some embodiments, the nucleic acid encoding the CAR is operably linked to a constitutive promoter. Constitutive promoters allow heterologous genes (also referred to as transgenes) to be expressed constitutively in the host cells. Exemplary constitutive promoters contemplated herein include, but are not limited to, Cytomegalovirus (CMV) promoters, human elongation factors-1alpha (hEF1α), ubiquitin C promoter (UbiC), phosphoglycerokinase promoter (PGK), simian virus 40 early promoter (SV40), and chicken β-Actin promoter coupled with CMV early enhancer (CAGG). The efficiencies of such constitutive promoters on driving transgene expression have been widely compared in a huge number of studies. For example, Michael C. Milone *et al* compared the efficiencies of CMV, hEF1α, UbiC and PGK to drive CAR expression in primary human T cells, and concluded that hEF1α promoter not only induced the highest level of transgene expression, but was also optimally maintained in the CD4 and CD8 human T cells (Molecular Therapy, 17(8): 1453-1464 (2009)). In some embodiments, the nucleic acid encoding the CAR is operably linked to a hEF1α promoter.

[0345] In some embodiments, the nucleic acid encoding the CAR is operably linked to an inducible promoter. Inducible promoters belong to the category of regulated promoters. The inducible promoter can be induced by one or more conditions, such as a physical condition, microenvironment of the engineered immune effector cell, or the physiological state of the engineered immune effector cell, an inducer (*i.e.*, an inducing agent), or a combination thereof. In some embodiments, the inducing condition does not induce the expression of endogenous genes in the engineered mammalian cell, and/or in the subject that receives the pharmaceutical composition. In some embodiments, the inducing condition is selected from the group consisting of: inducer, irradiation (such as ionizing radiation, light), temperature (such as heat), redox state, tumor environment, and the activation state of the engineered mammalian cell.

[0346] 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.

[0347] 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: 385.

Immune effector cells

[0348] "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.

[0349] In some embodiments, the immune effector cells are T cells. In some embodiments, the T cells are CD4+/CD8-, CD4-/CD8+, CD4+/CD8+, CD4-/CD8-, or combinations thereof. In some embodiments, the T cells produce IL-2, TFN, and/or TNF upon expressing the CAR and binding to the target cells, such as CD20+ or CD19+ tumor cells. In some embodiments, the CD8+ T cells lyse antigen-specific target cells upon expressing the CAR and binding to the target cells.

[0350] 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.

[0351] 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.

[0352] The engineered immune effector cells are prepared by introducing the CARs into the immune effector cells, such as T cells. In some embodiments, the CAR is introduced to the immune effector cells by transfecting any one of the isolated nucleic acids or any one of the vectors described in Section III. In some embodiments, the CAR is introduced to the immune effector cells by inserting proteins into the cell membrane while passing cells through a

microfluidic system, such as CELL SQUEEZE® (*see*, for example, U.S. Patent Application Publication No. 20140287509).

[0353] 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.

[0354] Physical methods for introducing the vector into an immune effector cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. *See*, for example, Sambrook *et al.* (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. In some embodiments, the vector is introduced into the cell by electroporation.

[0355] 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.

[0356] 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).

[0357] In some embodiments, RNA molecules encoding any of the CARs described herein may be prepared by a conventional method (*e.g.*, *in vitro* transcription) and then introduced into the immune effector cells via known methods such as mRNA electroporation. See, *e.g.*, Rabinovich *et al.*, Human Gene Therapy 17:1027-1035.

[0358] In some embodiments, the transduced or transfected immune effector cell is propagated ex vivo after introduction of the vector or isolated nucleic acid. In some embodiments, the transduced or transfected immune effector cell is cultured to propagate for at least about any of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, or 14 days. In some embodiments, the transduced or transfected immune effector cell is further evaluated or screened to select the engineered mammalian cell.

[0359] 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.

[0360] 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

[0361] Prior to expansion and genetic modification of the T cells, a source of T cells is obtained from an individual. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In some embodiments, any number of T cell lines available in the art, may be used. In some embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as FICOLL™ separation. In some embodiments, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In some embodiments, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In some embodiments, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Again, surprisingly, initial activation steps in the absence of calcium lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics

Cell Saver 5) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca^{2+}-free, Mg^{2+}-free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

[0362] 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.

[0363] Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In certain embodiments, it may be desirable to enrich for or positively select for regulatory T cells which typically express CD4+, CD25+, CD62Lhi, GITR+, and FoxP3+. Alternatively, in certain embodiments, T regulatory cells are depleted by anti-C25 conjugated beads or other similar method of selection.

[0364] 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.

[0365] In some embodiments, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (*e.g.*, particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute

concentrations. In some embodiments, the concentration of cells used is 5×10^6/ml. In some embodiments, the concentration used can be from about 1×10^5/ml to 1×10^6/ml, and any integer value in between.

[0366] 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.

[0367] 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.

[0368] 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.

[0369] Also contemplated in the present application is the collection of blood samples or apheresis product from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as T cells, isolated and frozen for later use in T cell therapy for any number of diseases or conditions that would benefit from T cell therapy, such as those described herein. In one embodiment a blood sample or an apheresis is taken from a generally healthy subject. In certain embodiments, a blood sample or an apheresis is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet developed a disease, and the cells of interest are isolated and frozen for later use. In certain embodiments, the T cells may be expanded, frozen, and used at a later time. In certain embodiments, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but

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

[0370] 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

[0371] 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.

[0372] Generally, T cells can be expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the T cells. In particular, T cell populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (*e.g.*, bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4+ T cells or CD8+ T cells, an anti-CD3 antibody and an anti-CD28 antibody. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, Besancon, France) can be used as can other methods commonly known in the art (Berg et al., Transplant Proc. 30(8):3975-3977, 1998; Haanen et al., J. Exp. Med. 190(9):13191328, 1999; Garland et al., J. Immunol Meth. 227(1-2):53-63, 1999).

[0373] 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.

[0374] 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.

[0375] By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3×28 beads) to contact the T cells. In one embodiment the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, preferably PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (i.e., 100%) may comprise the target cell of interest. Accordingly, any cell number is within the context of the present invention. In certain embodiments, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in one embodiment, a concentration of about 2 billion cells/ml is used. In another embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells. Such populations of cells may have therapeutic value and would be desirable to obtain in certain embodiments. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[0376] In some embodiments, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may

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

[0377] 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

[0378] Further provided by the present application are pharmaceutical compositions comprising any one of the anti-BCMA single-domain antibodies, or any one of the engineered immune effector cells comprising any one of the CARs (such as BCMA CARs) as described herein, and a pharmaceutically acceptable carrier. Pharmaceutical compositions can be prepared by mixing a sdAb, or a plurality of engineered immune effector cells having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions.

[0379] 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.

[0380] 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.

[0381] 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.

[0382] 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.

[0383] Additional excipients include agents which can serve as one or more of the following: (1) bulking agents, (2) solubility enhancers, (3) stabilizers and (4) and agents preventing denaturation or adherence to the container wall. Such excipients include: polyhydric sugar alcohols (enumerated above); amino acids such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, threonine, etc.; organic sugars or sugar alcohols such as sucrose, lactose, lactitol, trehalose, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (*e.g.*, inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thio sulfate; low molecular weight proteins such as human serum albumin, bovine serum albumin, gelatin or other immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides (*e.g.*, xylose, mannose, fructose, glucose; disaccharides (*e.g.*, lactose, maltose, sucrose); trisaccharides such as raffinose; and polysaccharides such as dextrin or dextran.

[0384] 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.

[0385] Suitable non-ionic surfactants include polysorbates (20, 40, 60, 65, 80, etc.), polyoxamers (184, 188, etc.), PLURONIC® polyols, TRITON®, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.), lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, sucrose fatty acid ester, methyl celluose and carboxymethyl cellulose. Anionic detergents that can be used include sodium lauryl sulfate, dioctyle sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents include benzalkonium chloride or benzethonium chloride.

[0386] 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.

[0387] 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.

[0388] 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.

[0389] 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.

[0390] 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-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 18th edition.

VI. Methods of treatment

[0391] The present application further relates to methods and compositions for use in 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 anti-BCMA sdAbs, CARs, and engineered immune effector cells (such as CAR-T 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 methods and compositions described herein may be used for treating other diseases that are associated with the antigens specifically recognized by the single-domain antibodies or CARs, including, for example, autoimmune diseases.

[0392] In some embodiments, there is provided a method of treating a cancer (such as multiple myeloma, *e.g.*, relapsed or refractory multiple myeloma) 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety specifically binding to a first epitope of BCMA, and a second BCMA binding moiety specifically binding to a second epitope of BCMA; (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 embodiments, there is provided a method of treating a cancer (such as multiple myeloma, *e.g.*, relapsed or refractory multiple myeloma) 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first anti-BCMA sdAb specifically binding to a first epitope of BCMA, and a second anti-BCMA sdAb specifically binding to a second epitope of BCMA; (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 embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic. In some embodiments, the engineered immune effector cells are CAR-T cells. 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 refractory or relapsed multiple myeloma. In some embodiments, the engineered immune effector cell is administered at a dose of about 10^5 to about 10^7 cells/kg, such

as about $3x10^5$ to about $7x10^6$ cells/kg, or about $3x10^6$ cells/kg. In some embodiments, the engineered immune effector cell is administered by intravenous injection. In some embodiments, the engineered immune effector cell is administered in three split doses over about a week.

[0393] In some embodiments, there is provided a method of treating a cancer (such as multiple myeloma, *e.g.*, relapsed or refractory multiple myeloma) 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 BCMA CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising an anti-BCMA sdAb; (b) a transmembrane domain; and (c) an intracellular signaling domain; and (2) a pharmaceutically acceptable carrier, wherein the anti-BCMA sdAb comprises any one of the following: (1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77; (2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78; (3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; (4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80; (5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81; (6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82; (7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83; (8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84; (9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85; (10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48;

and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; (11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87; (12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88; (13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89; (14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90; (15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; (16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92; (17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93; (18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; (19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95; (20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; (21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97; (22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98; (23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99; (24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3

comprising the amino acid sequence of SEQ ID NO:100; (25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101; (26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102; (27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103; (28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104; (29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105; (30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106; (31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107; (32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108; (33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109; (34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110; (35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111; (36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112; (37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or (38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid

sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114. In some embodiments, the extracellular antigen binding domain comprises at least two anti-BCMA sdAbs. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises a V_HH domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 115-152. In some embodiments, the BCMA CAR comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 216-256 and 298-335. 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 refractory or relapsed multiple myeloma. In some embodiments, the engineered immune effector cell is administered at a dose of about 10^5 to about 10^7 cells/kg, such as about $3x10^5$ to about $7x10^6$ cells/kg, or about $3x10^6$ cells/kg. In some embodiments, the engineered immune effector cell is administered by intravenous injection. In some embodiments, the engineered immune effector cell is administered in three split doses over about a week.

[0394] In some embodiments, there is provided a method of obtaining partial or complete clinical remission in an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (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 embodiments, the engineered immune effector cell is autologous. In some embodiments, the engineered immune effector cell is allogenic. In some embodiments, the engineered immune effector cells are CAR-T cells. 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 refractory or relapsed multiple myeloma. In some embodiments, the engineered immune effector cell is administered at a dose of about 10^5 to about 10^7 cells/kg, such as about $3x10^5$ to about $7x10^6$

cells/kg, or about $3x10^6$ cells/kg. In some embodiments, the engineered immune effector cell is administered by intravenous injection. In some embodiments, the engineered immune effector cell is administered in three split doses over about a week.

[0395] In some embodiments, there is provided a method of treating a cancer (such as multiple myeloma, *e.g.*, relapsed or refractory multiple myeloma) in an individual (such as a human individual), comprising administering to the individual an effective amount of a pharmaceutical composition comprising an anti-BCMA sdAb and a pharmaceutically acceptable carrier, wherein the anti-BCMA sdAb comprises any one of the following: (1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77; (2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78; (3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; (4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80; (5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81; (6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82; (7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83; (8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84; (9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85; (10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; (11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of

SEQ ID NO:87; (12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88; (13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89; (14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90; (15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; (16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92; (17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93; (18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; (19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95; (20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; (21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97; (22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98; (23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99; (24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100; (25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101; (26) a

CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102; (27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103; (28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104; (29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105; (30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106; (31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107; (32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108; (33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109; (34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110; (35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111; (36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112; (37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or (38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114. In some embodiments, the anti-BCMA sdAb is camelid, chimeric, human, or humanized. In some embodiments, the anti-BCMA sdAb comprises an amino acid sequence

selected from the group consisting of SEQ ID NO: 115-152. 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 refractory or relapsed multiple myeloma.

[0396] 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.

[0397] In some embodiments, the cancer is multiple myeloma. In some embodiments, the cancer is stage I, stage II or stage III, and/or stage A or stage B multiple myeloma based on the Durie-Salmon staging system. In some embodiments, the cancer is stage I, stage II or stage III multiple myeloma based on the International staging system published by the International Myeloma Working Group (IMWG). In some embodiments, the cancer is monoclonal gammopathy of undetermined significance (MGUS). In some embodiments, the cancer is asymptomatic (smoldering/indolent) myeloma. In some embodiments, the cancer is symptomatic or active myeloma. In some embodiments, the cancer is refractory multiple myeloma. In some embodiments, the cancer is metastatic multiple myeloma. In some embodiments, the individual did not respond to a previous treatment for multiple myeloma. In some embodiments, the individual has progressive disease after a previous treatment of multiple myeloma. In some embodiments, the individual has previously received at least about any one of 2, 3, 4, or more treatment for multiple myeloma. In some embodiments, the cancer is relapsed multiple myeloma.

[0398] In some embodiments, the individual has active multiple myeloma. In some embodiments, the individual has clonal bone marrow plasma cells of at least 10%. In some embodiments, the individual has a biopsy-proven bony or extramedullary plasmacytoma. In some embodiments, the individual has evidence of end organ damage that can be attributed to the underlying plasma cell proliferative disorder. In some embodiments, the individual has hypercalcemia, *e.g.*, serum calcium >0.25 mmol/L (>1mg/dL) higher than the upper limit of

normal or >2.75 mmol/L (>11mg/dL). In some embodiments, the individual has renal insufficiency, *e.g.*, creatinine clearance <40 mL per minute or serum creatinine >177μmol/L (>2mg/dL). In some embodiments, the individual has anemia, *e.g.*, hemoglobin value of >20g/L below the lowest limit of normal, or a hemoglobin value <100g/L. In some embodiments, the individual has one or more bone lesions, *e.g.*, one or more osteolytic lesion on skeletal radiography, CT, or PET/CT. In some embodiments, the individual has one or more of the following biomarkers of malignancy (MDEs): (1) 60% or greater clonal plasma cells on bone marrow examination; (2) serum involved / uninvolved free light chain ratio of 100 or greater, provided the absolute level of the involved light chain is at least 100mg/L; and (3) more than one focal lesion on MRI that is at least 5mm or greater in size.

[0399] 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.

[0400] 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 application 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.

[0401] In some embodiments, wherein the pharmaceutical composition comprises any one of the sdAbs described herein, the pharmaceutical composition is administered at ta 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.

[0402] In some embodiments, wherein the pharmaceutical composition comprises any one of the engineered immune cells described herein, 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 about10^8, 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. In some embodiments, the pharmaceutical composition is administered at a dose of at least about any $1x10^5$, $2x10^5$, $3x10^5$, $4x10^5$, $5x10^5$, $6x10^5$, $7x10^5$, $8x10^5$, $9x10^5$, $1x10^6$, $2x10^6$, $3x10^6$, $4x10^6$, $5x10^6$, $6x10^6$, $7x10^6$, $8x10^6$, $9x10^6$, $1x10^7$ cells/kg or more. In some embodiments, the pharmaceutical composition is administered at a dose of about $3x10^5$ to about $7x10^6$ cells/kg, or about $3x10^6$ cells/kg.

[0403] 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.

[0404] Moreover, dosages may be administered by one or more separate administrations, or by continuous infusion. In some embodiments, the pharmaceutical composition is administered in split doses, such as about any one of 2, 3, 4, 5, or more doses. In some embodiments, the split doses are administered over about a week. In some embodiments, the dose is equally split. In some embodiments, the split doses are about 20%, about 30% and about 50% of the total dose. In some embodiments, the interval between consecutive split doses is about 1 day, 2 days, 3 days or longer. 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.

[0405] In some embodiments, the amount of the pharmaceutical composition is effective to cause an objective clinical response in the individual. In some embodiments, there is provided a method of obtaining an objective clinical response in an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (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 embodiments, Stringent Clinical Response (sCR) is obtained in the individual.

[0406] In some embodiments, the amount of the pharmaceutical composition is effective to cause disease remission (partial or complete) in the individual. In some embodiments, there is provided a method of causing disease remission (partial or complete) in an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA

binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (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 the clinical remission is obtained after no more than about any one of 6 months, 5 months, 4 months, 3 months, 2 months, 1 months or less after the individual receives the pharmaceutical composition.

[0407] In some embodiments, the amount of the pharmaceutical composition is effective to prevent relapse or disease progression of the cancer in the individual. In some embodiments, there is provided a method of preventing relapse or disease progression in an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (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 embodiments, the relpase or disease progression is prevented for at least about 6 months, 1 year, 2 years, 3 years, 4 years, 5 years or more.

[0408] In some embodiments, the amount of the pharmaceutical composition is effective to prolong survival (such as disease free survival) in the individual. In some embodiments, the survival is prolonged for at least about 2, 3, 4, 5, 6, 12, or 24 months. In some embodiments, there is provided a method of prolonging survival of an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of

BCMA; (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.

[0409] In some embodiments, the pharmaceutical composition is effective to improve quality of life in the individual. In some embodiments, there is provided a method of improving quality of life of an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (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.

[0410] In some embodiments, the amount of the pharmaceutical composition is effective to inhibit growth or reducing the size of a solid or lymphatic tumor. In some embodiments, the size of the solid or lymphatic tumor is reduced for at least about 10% (including for example at least about any of 20%, 30%, 40%, 60%, 70%, 80%, 90%, or 100%). In some embodiments, a method of inhibiting growth or reducing the size of a solid or lymphatic tumor in an individual is provided.

[0411] In some embodiments, the amount of the pharmaceutical composition is effective to inhibit tumor metastasis in the individual. In some embodiments, at least about 10% (including for example at least about any of 20%, 30%, 40%, 60%, 70%, 80%, 90%, or 100%) metastasis is inhibited. In some embodiments, there is provided a method of inhibiting tumor metastasis of an individual having multiple myeloma (*e.g.*, relapsed or refractory multiple myeloma), 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 CAR comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising a first BCMA binding moiety (such as a first anti-BCMA sdAb) specifically binding to a first epitope of BCMA, and a second BCMA binding moiety (such as a second anti-BCMA sdAb) specifically binding to a second epitope of BCMA; (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 embodiments, a method of inhibiting metastasis to lymph node is provided. In some embodiments, a method of inhibiting metastasis to the lung is provided. In some embodiments, a method of inhibiting metastasis to the liver is provided. Metastasis can be assessed by any known methods in the art, such as by blood tests, bone scans, x-ray scans, CT scans, PET scans, and biopsy.

VII. Kits and articles of manufacture

[0412] Further provided are kits, unit dosages, and articles of manufacture comprising any of the single-domain antibodies, the chimeric antigen receptors, or the engineered immune effector cells described herein. In some embodiments, a kit is provided which contains any one of the pharmaceutical compositions described herein and preferably provides instructions for its use.

[0413] The kits of the present application 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 application thus also provides articles of manufacture, which include vials (such as sealed vials), bottles, jars, flexible packaging, and the like.

[0414] 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.

[0415] 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.

[0416] The examples and exemplary embodiments 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 exemplary embodiments are offered by way of illustration and not by way of limitation.

VIII. Exemplary embodiments

[0417] The invention provides the following embodiments:

[0418] Embodiment 1. An anti-BCMA single-domain antibody (sdAb) comprising any one of the following: (1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77; (2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78; (3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79; (4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80; (5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81; (6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82; (7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83; (8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84; (9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9;

a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85; (10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86; (11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87; (12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88; (13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89; (14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90; (15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91; (16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92; (17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93; (18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94; (19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95; (20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96; (21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97; (22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98; (23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino

acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99; (24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100; (25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101; (26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102; (27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103; (28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104; (29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105; (30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106; (31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107; (32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108; (33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109; (34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110; (35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111; (36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112; (37) a CDR1 comprising the amino acid sequence of SEQ ID

NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or (38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114.

[0419] Embodiment 2. The anti-BCMA sdAb of embodiment 1, wherein the anti-BCMA sdAb comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152.

[0420] Embodiment 3. An anti-BCMA antibody that competes with the anti-BCMA sdAb of embodiment 1 or 2.

[0421] Embodiment 4. The anti-BCMA antibody of embodiment 3, wherein the anti-BCMA antibody is an sdAb.

[0422] Embodiment 5. The anti-BCMA sdAb of any one of embodiments 1, 2 and 4, wherein the anti-BCMA sdAb is a camelid antibody.

[0423] Embodiment 6. The anti-BCMA sdAb of any one of embodiments 1, 2 and 4, wherein the anti-BCMA sdAb is a chimeric antibody.

[0424] Embodiment 7. The anti-BCMA sdAb of any one of embodiments 1, 2 and 4, wherein the anti-BCMA sdAb is humanized.

[0425] Embodiment 8. The anti-BCMA sdAb of any one of embodiments 1-2 and 4-7, wherein the anti-BCMA sdAb is a V_HH fragment.

[0426] Embodiment 9. A chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising the anti-BCMA sdAb of any one of embodiments 1-2 and 4-7; (b) a transmembrane domain; and (c) an intracellular signaling domain.

[0427] Embodiment 10. The CAR of embodiment 9, wherein the extracellular antigen binding domain comprises at least two anti-BCMA sdAbs.

[0428] Embodiment 11. A multivalent chimeric antigen receptor (CAR) comprising a polypeptide comprising: (a) an extracellular antigen binding domain comprising at least two BCMA binding moieties; (b) a transmembrane domain; and (c) an intracellular signaling domain.

[0429] Embodiment 12. The multivalent CAR of embodiment 11, wherein the extracellular antigen binding domain comprises a first BCMA binding moiety and a second BCMA binding moiety.

[0430] Embodiment 13. The multivalent CAR of embodiment 12, wherein one or more of the first BCMA binding moiety and the second BCMA binding moiety is a sdAb.

[0431] Embodiment 14. The multivalent CAR of embodiment 12 or 13, wherein the first BCMA binding moiety is a first anti-BCMA sdAb and the second BCMA binding moiety is a second anti-BCMA sdAb.

[0432] Embodiment 15. The multivalent CAR of embodiment 12 or 13, wherein the first BCMA binding moiety is an anti-BCMA sdAb and the second BCMA binding moiety is derived from a human antibody.

[0433] Embodiment 16. The multivalent CAR of embodiment 12 or 13, wherein the first BCMA binding moiety is an anti-BCMA sdAb and the second BCMA binding moiety is a polypeptide ligand of BCMA.

[0434] Embodiment 17. The multivalent CAR of any one of embodiments 12-16, wherein the first BCMA binding moiety and the second BCMA binding moiety specifically bind to the same epitope on BCMA.

[0435] Embodiment 18. The multivalent CAR of any one of embodiments 12-16, wherein the first BCMA binding moiety and the second BCMA binding moiety specifically bind to different epitopes on BCMA.

[0436] Embodiment 19. The multivalent CAR of any one of embodiments 12-18, wherein the first BCMA binding moiety and/or the second BCMA binding moiety specifically binds to an epitope on BCMA derived from an amino acid sequence selected from SEQ ID NOs: 388-394.

[0437] Embodiment 20. The multivalent CAR of any one of embodiments 12-19, wherein one or more of the first BCMA binding moiety and the second BCMA binding moiety is the anti-BCMA sdAb of embodiment 1.

[0438] Embodiment 21. The multivalent CAR of any one of embodiments 12-20, wherein the first BCMA binding moiety is located at the N-terminus of the second BCMA binding moiety.

[0439] Embodiment 22. The multivalent CAR of any one of embodiments 12-20, wherein the first BCMA binding moiety is located at the C-terminus of the second BCMA binding moiety.

[0440] Embodiment 23. The multivalent CAR of any one of embodiments 12-22, wherein the first BCMA binding moiety and the second BCMA binding moiety are fused to each other via a peptide linker.

[0441] Embodiment 24. The multivalent CAR of embodiment 23, wherein the peptide linker is no more than about 50 amino acids long.

[0442] Embodiment 25. The multivalent CAR of embodiment 24, wherein the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 208-215.

[0443] Embodiment 26. The CAR or multivalent CAR of any one of embodiments 9-25, wherein the transmembrane domain is derived from a molecule selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152 and PD1.

[0444] Embodiment 27. The CAR or multivalent CAR of embodiment 26, wherein the transmembrane domain is derived from CD8α or CD28.

[0445] Embodiment 28. The CAR or multivalent CAR of embodiment 27, wherein the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 193 or 194.

[0446] Embodiment 29. The CAR or multivalent CAR of any one of embodiments 9-28, wherein the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell.

[0447] Embodiment 30. The CAR or multivalent CAR of embodiment 29, wherein the primary intracellular signaling domain is derived from CD3ζ.

[0448] Embodiment 31. The CAR or multivalent CAR of embodiment 30, wherein the primary intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 197 or 198.

[0449] Embodiment 32. The CAR or multivalent CAR of any one of embodiments 9-31, wherein the intracellular signaling domain comprises a co-stimulatory signaling domain.

[0450] Embodiment 33. The CAR or multivalent CAR of embodiment 32, wherein 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, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, Ligands of CD83 and combinations thereof.

[0451] Embodiment 34. The CAR or multivalent CAR of embodiment 33, the co-stimulatory signaling domain comprises a cytoplasmic domain of CD28 and/or a cytoplasmic domain of CD137.

[0452] Embodiment 35. The CAR or multivalent CAR of embodiment 34, wherein the co-stimulatory signaling domain comprises the amino acid sequence of SEQ ID NO: 195 and/or SEQ ID NO: 196.

[0453] Embodiment 36. The CAR or multivalent CAR of any one of embodiments 32-35, wherein the intracellular signaling domain comprises at least two co-stimulatory signaling domains.

[0454] Embodiment 37. The CAR or multivalent CAR of any one of embodiments 9-36, further comprising a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain.

[0455] Embodiment 38. The CAR or multivalent CAR of embodiment 37, wherein the hinge domain is derived from CD8α.

[0456] Embodiment 39. The CAR or multivalent CAR of embodiment 38, wherein the hinge domain comprises the amino acid sequence of SEQ ID NO: 192.

[0457] Embodiment 40. The CAR or multivalent CAR of any one of embodiments 9-39, further comprising a signal peptide located at the N-terminus of the polypeptide.

[0458] Embodiment 41. The CAR or multivalent CAR of embodiment 40, wherein the signal peptide is derived from CD8α.

[0459] Embodiment 42. The CAR or multivalent CAR of embodiment 41, wherein the signal peptide comprises the amino acid sequence of SEQ ID NO: 191.

[0460] Embodiment 43. A chimeric antigen receptor comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 216-256 and 298-335.

[0461] Embodiment 44. An isolated nucleic acid comprising a nucleic acid sequence encoding the CAR or multivalent CAR of any one of embodiments 11-43.

[0462] Embodiment 45. The isolated nucleic acid of claim 44, comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 257-297 and 336-373.

[0463] Embodiment 46. The isolated nucleic acid of embodiment 45, further comprising 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.

[0464] Embodiment 47. The isolated nucleic acid of embodiment 46, the self-cleaving peptide is selected from the group consisting of T2A, P2A, and F2A.

[0465] Embodiment 48. The isolated nucleic acid of embodiment 47, the third nucleic acid sequence is SEQ ID NO: 385.

[0466] Embodiment 49. The isolated nucleic acid of any one of embodiments 44-48, the isolated nucleic acid is an RNA molecule.

[0467] Embodiment 50. A vector comprising the isolated nucleic acid of any one of embodiments 44-49.

[0468] Embodiment 51. The vector of embodiment 50, wherein the vector is an expression vector.

[0469] Embodiment 52. The vector of embodiment 50 or 51, wherein the vector is a viral vector.

[0470] Embodiment 53. The vector of embodiment 52, wherein the vector is a lentiviral vector.

[0471] Embodiment 54. The vector of embodiment 50 or 51, wherein the vector is a non-viral vector.

[0472] Embodiment 55. An engineered immune effector cell, comprising the CAR or multivalent CAR of any one of embodiments 9-43, the isolated nucleic acid of any one of embodiments 44-49, or the vector of any one of embodiments 50-54.

[0473] Embodiment 56. The engineered immune effector cell of embodiment 55, wherein 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.

[0474] Embodiment 57. The engineered immune effector cell of embodiment 56, wherein the immune effector cell is a T cell.

[0475] Embodiment 58. A pharmaceutical composition, comprising the engineered immune effector cell of any one of embodiments 55-57, and a pharmaceutically acceptable carrier.

[0476] Embodiment 59. A method of treating cancer in an individual, comprising administering to the individual an effective amount of the pharmaceutical composition of embodiment 58.

[0477] Embodiment 60. The method of embodiment 59, wherein the cancer is multiple myeloma.

[0478] Embodiment 61. The method of embodiment 60, wherein the cancer is refractory or relapsed multiple myeloma.

[0479] Embodiment 62. The method of any one of embodiments 59-61, wherein the engineered immune effector cell is autologous.

[0480] Embodiment 63. The method of any one of embodiments 59-61, wherein the engineered immune effector cell is allogenic.

[0481] Embodiment 64. The method of any one of embodiments 59-63, wherein the individual is human.

[0482] Embodiment 65. The method of any one of embodiments 59-64, wherein the pharmaceutical composition is administered intravenously.

[0483] Embodiment 66. The method of any one of embodiments 59-65, wherein the pharmaceutical composition is administered at a dose of about 1×10^5 to about 1×10^7 cells/kg.

[0484] Embodiment 67. The method of any one of embodiments, 59-66, wherein the pharmaceutical composition is administered in 3 split doses over about one week.

[0485] Embodiment 68. Use of the pharmaceutical composition of embodiment 58 for treating cancer in an individual.

[0486] Embodiment 69. Use of the pharmaceutical composition of embodiment 58 in the preparation of a medicament for treating cancer in an individual.

[0487] Embodiment 70. A pharmaceutical composition, comprising the anti-BCMA sdAb of any one of embodiments 1-8.

[0488] Embodiment 71. A method of treating a disease in an individual, comprising administering to the individual an effective amount of the pharmaceutical composition of embodiment 70.

[0489] Embodiment 72. The method of embodiment 71, wherein the disease is cancer.

EXAMPLES

[0490] The examples discussed below are intended to be purely exemplary of the invention and should not be considered to limit the invention in any way. The examples 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 anti-BCMA sdAbs

[0491] To develop sdAbs with high binding affinity to BCMA, llamas were immunized with a recombinant BCMA antigen. A phage-display library was then constructed to identify V_HH 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. An exemplary protocol is described below. Other protocols for preparing sdAbs 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

[0492] An immunogen comprising a recombinant human BCMA protein having a C-terminal Fc tag (ACRO Biosystems, Cat No.:BC7-H5254) was mixed with adjuvant or PBS and injected to llamas. 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 sdAbs. 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

[0493] 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® Protein G HP column was then loaded onto a 1 ml HITRAP® 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

[0494] 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_HH phage display library construction

2.1 RNA extraction

[0495] Total RNA was extracted from the isolated lymphocytes (from 1.1.1) using TRIZOL® 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_HH amplification

[0496] Total RNA was reverse transcribed into cDNA with an oligo$(dT)_{20}$ primer using PRIMESCRIPT™ 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_HH fragments, which had two BglI restriction sites introduced. The V_HH fragments were amplified according to GenScript's standard operating procedure as described below.

[0497] The variable regions of the heavy-chain immunoglobulins (*i.e.*, V_HHs) 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: 374) and CALL002 (SEQ ID NO: 375). 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: 376), BACK-2 (SEQ ID NO: 377) and PMCF (SEQ ID NO: 378). The amplified second PCR products containing

V_HH PCR fragments were gel purified and enzyme digested, and then inserted into phagemid plasmids. The recombinant plasmids with V_HH gene fragments were electro-transferred into *E.coli* cells in order to generate the phage display V_HH immune library.

[0498] 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

[0499] The V_HH 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

[0500] The constructed V_HH 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} = 0.4~0.6) for output titration.

3.2 Phage ELISA

[0501] 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.

[0502] 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. After panning, ELISA positive phage clones were randomly selected and DNA was prepared from output phage using a plasmid extraction kit. The anti-BCMA V_HHs in the plasmids were sequenced.

Example 2. Preparation of exemplary monovalent BCMA chimeric antigen receptors

[0503] A nucleic acid sequence encoding a CAR backbone polypeptide comprising from the N-terminus to the C-terminus: a CD8α hinge domain, a CD28 transmembrane domain, a CD28 cytoplasmic domain, a CD137 cytoplasmic domain, and a CD3ζ cytoplasmic domain was

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-8281373." Multi-cloning sites (MCS) in the vector allowed insertion of a nucleic acid sequence comprising a Kozak sequence (SEQ ID NO:379) operably linked to a nucleic acid sequence encoding a CD8α signal peptide fused to the N-terminus of a V_HH fragment into the PLLV-hEF1α-8281373 vector, upstream and operably linked to the CAR backbone sequence.

[0504] To construct a monospecific CAR having a single V_HH domain using the PLLV-hEF1α-8281373 backbone, the nucleic acid sequence encoding the V_HH domain was operably linked to the 3' of the nucleic acid sequence encoding the CD8α signal peptide. The fusion nucleic acid sequence was chemically synthesized and cloned into the PLLV-hEF1α-8281373 CAR backbone via the EcoRI (SEQ ID NO: 380: 5'-GAATTC-3') and SpeI (SEQ ID NO: 381: 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 CARs.

[0505] For ease of further inserting additional sequences, such as a nucleotide encoding a second V_HH, when designing a monospecific CAR construct, restriction sites including HpaI (SEQ ID NO: 382: 5'-GTTAAC-3'), MluI (SEQ ID NO: 383: 5'-ACGCGT-3'), NsiI (SEQ ID NO: 384: 5'-ATGCAT-3') sites were included between the CD8α signal peptide nucleic acid sequence and the V_HH nucleic acid sequence.

[0506] The lentivirus packaging plasmid mixture including pCMV-ΔR-8.74 and pMD2.G (Addgene#12259) was pre-mixed with the vectors PLLV-hEF1α-8281373 having V_HH fragments 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_2 cell incubator. The supernatants were collected after centrifugation at 4°C, 500 g for 10 min.

[0507] The virus-containing supernatants were filtered through a 0.45μm 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

[0508] Leukocytes were collected from healthy donors by apheresis, and cell concentration was adjusted to 5×10^6 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

[0509] 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.

[0510] 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.

***In vitro* cytotoxicity assay**

[0511] 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.

[0512] 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 human multiple myeloma cell line RPMI8226.Luc, human cell line K562.BCMA.Luc cells which recombinantly expressed BCMA, K562.CD19. Luc cell line which recombinantly expressed CD19, or human glioblastoma cell line U87MG.Luc cells. All of the cell lines were 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 the target 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 the 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)).

[0513] Exemplary monovalent CAR targeting BCMA (CD269) were selected and tested in the cytotoxicity assay. As shown in FIG. 1A, among a first group of monovalent BCMA CARs tested, the selected clones exhibited different levels of cytotoxicity against multiple myeloma cell line RPMI8226.Luc cells, with over 60% monovalent V_HH-based CAR-Ts showing >50% cytotoxicity against RPMI8226.Luc cells. Clones 269A37346, 269A37348, 269A37353, and 269A37355 based CAR-T were selected for further testing. In particular, clones 269A37346, 269A37348, 267A37353 and 269A37355 based CAR-T exhibited potent cytotoxicity against 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 against human glioblastoma cell line U87MG.Luc cells (see FIG. 1B). No significant cytotoxicity effects were detected against U87MG.Luc by these monovalent V_HH-based CAR-T cells as compared to UnT controls. The observation above indicated that some of these clones might be target specific and potent against BCMA positive cells.

[0514] A second group of exemplary monovalent BCMA CARs were assessed for *in vitro* cytotoxicity. GSS005, a CAR comprising an anti-BMCA scFv, served as a positive control. GSI026, a CAR comprising an anti-EGFRvIII scFv, served as a negative control. As shown in FIGs. 2A-2B, the selected clones exhibited different levels of cytotoxicity against the multiple myeloma cells RPMI8226.Luc, and BCMA over-expressing stable cell line K562.BCMA.Luc. No clones showed potent cytotoxicity against BCMA negative cell line K562.CD19.Luc (FIG. 2C). Among these clones, 269B005S, 269B028S, 269B030S, 269B054S, 269B060S, 269B069S, 269B093S, 269B094S, 269B104S, 269B109S, 269B110S and 269B129S based CAR-T were most potent according to the cytotoxicity data.

IFNgamma release

[0515] Additionally, supernatants from the *in vitro* co-culture assays were collected to assess CAR-induced cytokine release, *e.g.*, interferon gamma (*i.e*, IFNγ) release. As shown in FIG. 3, T cells expressing selected monovalent BCMA CARs released high levels of IFNγ upon co-culturing with BCMA-expressing target cells K562.BCMA.Luc. Nonspecific CAR-Ts, such as GSI026, or untransduced T cells (UnT) did not induce release of IFNγ in the co-culture. The cytokine release data is consistent with the *in vitro* cytotoxicity data.

Example 3. Preparation of exemplary multivalent BCMA chimeric antigen receptors

[0516] Multivalent V_HH-based CARs can be constructed by cloning a nucleic acid sequence encoding multiple copies of a V_HH, or multiple different V_HHs fused to each other via peptide linkers into a CAR signal domain backbone vector. Exemplary multivalent BCMA CAR constructs are shown in Table 5. These constructs were prepared by fusing 2-3 anti-BCMA V_HHs by Glycine-serine peptide linkers followed by directly synthesizing this fusion sequence in combination with a Kozak-CD8α signal peptide nucleic acid sequence, and cloning into the PLLV-hEF1α-81373 CAR backbone via EcoRI and SpeI restriction sites. Monovalent BCMA

CAR constructs were also cloned into the same PLLV-hEF1α-81373 CAR backbone to serve as controls (*e.g.*, GSI5011, GSI5019, and GSI5020, Table 4).

[0517] Lentiviral vectors carrying CAR genes 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.

***In vitro* cytotoxicity assay**

[0518] On day 3 post transduction, transduced T cells were harvested and co-incubated with tumor cells. To assay the cytotoxicity of CAR-T on tumor cells, ONE-GLO™ luminescent luciferase assay reagents were added to the co-cultured cells and the specific cytotoxicity for each CAR-T was measured as described in Example 2.

[0519] In a first experiment, monovalent BCMA CAR (GSI5011), bivalent BCMA CAR (GSI5014), and trivalent BCMA CAR (GSI5015) expressing T cells were co-cultured with RPMI8226.Luc cells at an effector to target ratio of 20:1 for 20 hours. All three CAR constructs comprise anti-BCMA VHH domains of clone 269A37346. As shown in FIG. 4A, 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). The BCMA CARs tested having different antigen binding modalities had potent antitumor activity against BCMA positive cells.

[0520] In a second experiment, exemplary bivalent BCMA CARs (GSI5021-GSI5026) having two different BCMA binding moieties 269A37353 and 269A37917 were tested. Engineered T cells expressing each bivalent BCMA CAR was co-cultured with RPMI8226.Luc cells at an effector to target ratio of 20:1 for 20 hours. Monovalent BCMA CARs, GSI5019 and GSI5020 were also tested for comparison. As shown in FIG. 4B, the specific percentage of lysis of RPMI8226.Luc cells were 88.21±1.29% by GSI5019-expressing CAR-T cells,93.84±1.13% by GSI5020-expressing CAR-T cells, 71.45±1.79% by GSI5021-expressing CAR-T cells,

99.80±0.45% by GSI5022-expressing CAR-T cells, 97.46±0.50% by GSI5023-expressing CAR-T cells, 81.29±1.27% by GSI5024-expressing CAR-T cells, 93.50±0.47% by GSI5025-expressing CAR-T cells, 87.83±0.23% by GSI5026-expressing CAR-T cells, respectively, as compared to 13.49%±1.75% by untransduced control T cells (UnT). Also, as depicted in FIG. 4C, the specific percentage of lysis of BCMA-negative cell line U87MG.Luc was 2.84±7.41% by GSI5019-expressing CAR-T cells, 15.50±2.24% by GSI5020-expressing CAR-T cells, 6.74±3.37% by GSI5021-expressing CAR-T cells, 8.03±2.36% by GSI5022-expressing CAR-T cells, 9.00±1.88% by GSI5023-expressing CAR-T cells, 17.03±2.27% by GSI5024-expressing CAR-T cells, 16.81±1.98% by GSI5025-expressing CAR-T cells, -11.55±5.43% by GSI5026-expressing CAR-T cells, as compared to 12.49%±3.79% by untransduced control T cells (UnT). The data suggests that the bivalent CARs with different antigen-binding modalities had potent antitumor activity against BCMA positive cells, but not against BCMA negative cells.

[0521] In a third experiment, exemplary bivalent BCMA CARs (*i.e.*, BCAR001-BCAR008) having two different BCMA binding moieties were constructed, and engineered CAR-T cells expressing the bivalent BCMA CARs were prepared from primary T cells obtained from R/R MM patient donor #13. In-house developed firefly luciferase expressing cell lines including RPMI8226 (human multiple myeloma cell line), A549 (human lung cancer cell line), U87-MG (human glioblastoma cell line) and Raji (human Burkitt's lymphoma cell line) were used as target cells and co-cultured with each group of transduced T cells side by side (with Effector: target cell ratio of 20:1 or 5:1) for 20 hours in a 37°C/5% CO_2 cell incubator. Upon completion of co-culture, the remaining luciferase activities (relative light unit, RLU) were assayed with ONE-GLO™ luminescent luciferase assay kit (Promega) to assess the cytotoxicity of each CAR-T. As shown in FIGs. 5A-5E, the bivalent BCMA CAR-Ts had dose-dependent cytotoxicity against RPMI8226.Luc, K562.BCMA.Luc and Raji.Luc cells, but little cytotoxicity against BCMA negative A549.Luc and U87-MG.Luc cells. Data in FIG. 5F demonstrate that the cytotoxicity of the bivalent BCMA CAR-T cells against tumor cells are BCMA-specific as the CAR-T cells were not cytotoxic against K562.C38.Luc cells that were $CD38^+/BCMA^-$. K562.BCMA.Luc treated with BCAR001-BCAR008 CAR-T cells only showed limited residual Luciferase activities (*i.e.*, viable cells) as compared to UnT treated target cells (2.88±0.45%, 12.84±1.67%, 2.22±0.56%, 1.77±0.14%, 2.59±0.28%, 6.58±1.19%, 2.47±0.20%, 6.61±1.47% for BCAR001-BCAR008 respectively, as compared to UnT of 100 ± 3.95%, mean ± standard

error). These results demonstrate potent cytotoxicity of the bivalent BCMA CAR-T cells against K562.BCMA.Luc cells. BCAR001-BCAR008 CAR-T cells did not have significant cytotoxicity against K562.CD38.Luc cells as no significant decrease in Luciferase activity was detected as compared to UnT-treated target cells (111.82±5.11%, 111.72±3.43%, 104.74±0.24%, 95.04±2.70%, 93.93±7.23%, 97.72±1.86%, 111.90±2.01%, 108.33±4.05%, for BCAR001-BCAR008 respectively, as compared to UnT of 100 ±6.58%, mean ± standard error). These data suggested the cytotoxicity of the bivalent BCMA CAR-T is BCMA dependent.

IFNgamma release

[0522] Additionally, supernatants from *in vitro* co-culture assays were collected to assess CAR-induced cytokine release (*e.g.*, interferon gamma, IFNγ release). As shown in FIGs. 6A-6B, IFNγ release in the co-culture assays was CAR-dependent and BCMA-specific, which is consistent with the *in vitro* cytotoxicity data (Table 6).

TABLE 6. IFN gamma release in co-culture assays by bivalent BCMA CAR-T

	RPMI8226.Luc		A549.Luc		K562.CD38.Luc		Raji.Luc	
	Mean, pg/ml	s.e.	Mean, pg/ml	s.e.	Mean, pg/ml	s.e.	Mean, pg/ml	s.e.
BCAR001	1097.23	61.87	89.18	42.19	135.81	5.87	795.87	7.29
BCAR002	4651.22	1.13	503.63	130.73	361.87	49.68	3613.30	34.04
BCAR003	3569.84	108.19	243.82	1.31	265.08	3.24	3348.66	49.80
BCAR004	3077.41	110.82	161.70	12.97	128.50	17.08	2931.11	120.31
BCAR005	2850.34	20.16	170.90	8.27	141.20	17.54	2976.27	67.22
BCAR006	2023.71	37.61	223.96	6.21	215.00	17.87	1588.54	77.96
BCAR007	1912.98	2.28	239.43	1.93	289.72	1.94	1472.87	49.76
BCAR008	1798.90	76.85	258.71	19.39	171.89	6.51	1526.93	66.70
UnT	281.75	20.55	143.70	10.46	85.65	1.98	328.61	6.69

Copy numbers of integrated CAR genes

[0523] The copy numbers of integrated CAR genes for each transduced T cell group was determined by a semi-quantitative 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: 398: 5'-GTCCTTCTCCTGTCACTGGTTAT-3'; and reverse primer 137P2R, SEQ ID NO: 399:

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.

[0524] As shown in Table 7, a high copy number of CAR vector was integrated into the genome of the T cells in each CAR-T preparation.

TABLE 7. Genome integration copy numbers.

CAR-T cells with constructs	Copies/ng gDNA
GSI5019	35091.6
GSI5020	27627.2
GSI5021	24926.8
GSI5022	26393.6
GSI5023	32376.3
GSI5024	39319. 8
GSI5025	22269. 3
GSI5026	34790. 4
UnT	26.6

Example 4. Epitope mapping and differential epitope binding of two V_HH domains in LCAR-B38M

[0525] The epitopes of the four anti-BCMA V_HH domains were mapped. An exemplary bivalent BCMA CAR having two different anti-BCMA V_HH domains that specifically bind to different epitopes of BCMA was constructed. Bivalent/bi-epitope CAR comprising V_HH1 and V_HH2, named LCAR-B38M CAR, is one multivalent BCMA CAR listed in Table 5.

Surface Plasmon Resonance (SPR) assay

[0526] Each of four exemplary anti-BCMA V_HH sequences was cloned into a vector containing a human IgG1 Fc fragment (hIgG1Fc) sequence to facilitate recombinant expression of BCMA V_HH-hIgG1Fc. Recombinant proteins were obtained and purified for SPR assays.

[0527] The affinity of each V_HH-hIgG1Fc for BCMA was determined by SPR using a BIACORE® 2000 analytical system (GE Healthcare). Briefly, each V_HH-hIgG1Fc protein was covalently coupled to a CM5(s) sensor chip using 4 μg/ml V_HH-hIgG1Fc. Recombinant BCMA-His protein (ACRO Biosystems, Cat#BCA-H522y) was serially diluted in running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20, pH 7.4) and injected at a flowrate of 10 μl/min followed by dissociation. The association and dissociation rate constants were determined using the BIACORE® 2000 evaluation software version 3.0 (Langmuir binding, local fit, 1: 1 binding model). The binding affinities of the four V_HH-hIgG1Fcs are shown in Table 8.

TABLE 8. Binding affinities of V_HH-hIgG1Fcs to human BCMA-His protein.

Ligand	k_a (1/Ms)	k_d (1/s)	K_D (M)
V_HH1-hIgG1Fc	5.5E+04	<1.0E-05*	<1.8E-10*
V_HH2-hIgG1Fc	1.9E+06	1.5E-02	7.8E-09
V_HH3-hIgG1Fc	3.4E+04	1.7E-04	5.1E-09
V_HH4-hIgG1Fc	2.1E+06	9.4E-04	4.6E-10

Binding of Recombinant V_HH-His proteins to target cells

[0528] Recombinant anti-BCMA V_HH-His proteins were constructed by fusing the anti-BCMA V_HH sequence to a human albumin signal peptide sequence (N'-MKWVTFISLLFLFSSAYS-C'; SEQ ID NO: 386) at the N terminus, and a 6xHis-tag (N'-GSGHHHHHH-C'; SEQ ID NO: 387) at the C terminus. The codons were further optimized for optimal expression in mammalian host cells. The obtained nucleotide sequences were then cloned into a mammalian expression vector pTT5 via 5'-XbaI and 3'-HindIII restriction sites to provide plasmids, pTT5-LAB001 (for V_HH1), pTT5-LAB002 (for V_HH2) and pTT5-LAB003 (for V_HH1xV_HH2).

[0529] In order to obtain recombinant BCMA V_HH proteins, HEK293T cells were transiently transfected with the plasmids. Briefly, $5x10^6$ HEK293T cells were seeded in 10cm cell culture dishes one day prior to the transfection. On the next day, the cells were transfected with each plasmid using LIPOFECTAMINE™ 2000 Reagent (Thermofisher Scientific, Cat.No.: 11668-019) following the manufacturer's manual. Four days after transfection, supernatant was harvested and the expression levels of the antibodies were detected by ELSIA using HRP anti-His Tag (Biolegend, Cat.No.: 652504). The expression levels of LAB001, LAB002 and LAB003 were 109.31ng/ml, 152.48ng/ml and 396.62ng/ml respectively.

[0530] Binding affinities of LAB001, LAB002 and LAB003 anti-BCMA V_HH-His proteins were determined using cell-based assays. Briefly, serially diluted anti-BCMA V_HH-His proteins were incubated with $1x10^5$ target cells (either K562.BCMA.Luc or K562.CD38.Luc cells, which were in-house developed cell lines stably expressing BCMA or CD39 respectively) at 4°C for 2 hours. Afterwards, cells were centrifuged at 300g for 10min, and the supernatant was discarded. The cell pellets were re-suspended with DPBS. The cell pellets were washed, centrifuged, and the supernantant was discarded for 2 more times. Then cell pellets were subsequently re-suspended with detection antibody (THE™ His tag Antibody [FITC], GenScript Cat: A01620) containing buffers for 45min at 4 °C for 2 hours. Afterwards, cells were centrifuged at 300g for

10min, and the supernatant was discarded. The cell pellets were washed, centrifuged, and the supernantant was discarded for 2 more times. Binding affinities of LAB001, LAB002 and LAB003 to either K562.BCMA.Luc or K562.CD38.Luc cells were determined using an ATTUNE™ Nxt flow cytometer. Data was fitted by GraphPad PRISM™ version 6.0 using a "One site - Specific binding with Hill slope".

[0531] As shown in FIG. 7A-7C, LAB001, LAB002 and LAB003 specifically bind to K562.BCMA.Luc cells in a dose-dependent manner. The binding affinities are 0.079 nM, 0.035 nM and 0.0047 nM respectively. None of the antibodies showed significant binding to BCMA negative cell line K562.CD38.Luc. Moreover, LAB003 (V_HH1xV_HH2) showed significantly higher binding affinity (0.0047nM) than either LAB001 (V_HH1) or LAB002 (V_HH2).

Epitope binding

[0532] BCMA (NP_001183, UniProt#Q02223) is a transmembrane protein of 184 amino acids long. Human BCMA consists of an extracellular domain (ECD, amino residue number 1-54), a transmembrane domain (TM, amino residue number 55-77) and a cytoplasmic domain (CD, amino residue number 78-184). In addition, sequence analysis suggests that BCMA has no recognizable signal peptide at its N terminus (Laabi Y et al. (1992) EMBO J 11:3897–3904; Laabi Y et al. (1994) Nucleic Acids Res 22:1147–1154; Gras M P (1995) Int Immunol 7:1093–1106; Hong-Bing Shu and Holly Johnson (2000): Proc. Natl. Acad. Sci. USA, 10.1073).

[0533] As also illustrated by the online database UniProt (worldwide web.uniprot.org/uniprot/Q02223), 3 disulfide bonds (Cys-Cys) are located in the ECD of BCMA, which are at positions 8 ↔ 21, 24 ↔ 37 and 28 ↔ 41 (Table 9). The secondary structure of the BCMA ECD from the N-terminus to the C-terminus consists of a beta strand (aa12-15), a turn (aa16-19), a beta strand (aa20-23), a helix (aa 24-27), a beta strand (aa30-32), a helix (aa35 – 37), a turn (aa38-40), and a turn (aa42-44). A structure of the BCMA ECD is shown in FIG. 8A, which is replicated from the structure from the PDB database worldwide web.ebi.ac.uk/pdbe/entry/pdb/2kn1/.

TABLE 9. Protein sequences of human BCMA.

Position(s)	Description	Length	AA sequence
1 – 54	Extracellular domain	54	MLQMAGQCSQNEYFDSLLHACIPCQ LRCSSNTPPLTCQRYCNASVTNSVKG TNA (SEQ ID NO: 395)
55 – 77	Transmembrane domain	23	ILWTCLGLSLIISLAVFVLMFLL (SEQ ID NO: 396)

78 – 184	Cytoplasmic domain	107	RKINSEPLKDEFKNTGSGLLGMANID LEKSRTGDEIILPRGLEYTVEECTCED CIKSKPKVDSDHCFPLPAMEEGATIL VTTKTNDYCKSLPAALSATEIEKSISA R (SEQ ID NO: 397)

[0534] BCMA epitope peptides (269EP001-269EP007) were designed as shown in FIG. 8B and TABLE 10, and chemically synthesized and biotinylated at the N-terminus. Binding affinities of V_HH1-hIgG1Fc or V_HH2-hIgG1Fc were determined using ELISA. Briefly, peptides described above were coated on MAXISORP™ ELISA plate at 1μM overnight at 4°C. The next day, the plates were washed with PBST (add 0.5% TWEEN-20) twice, followed by plate blocking with 0.5% BSA at room temperature for 1h. The plates were then washed with PBST twice, followed by addition of serially diluted V_HH1-hIgG1Fc or V_HH2-hIgG1Fc at 10nM in triplicates, then incubated at 4 °C for 2h. The plates were then washed for 3 times with cold PBST, after which goat anti-Llama-HRP (1:1500, Bethyl Lab# A160) was added to each well and further incubated at room temperature for 1h. The plates were then washed for 4 times with PBST, and TMB substrates were added to each well and incubated at room temperature for 10-30 min. The plates were then read on TECAN® 10M microplate reader with absorbance at 450nm.

TABLE 10. BCMA epitope peptide sequences.

Epitope	Positions	Amino acid residue sequence	Length
269EP001	1-10	MLQMAGQCSQ (SEQ ID NO: 388)	10
269EP002	8-21	CSQNEYFDSLLHAC (SEQ ID NO: 389)	14
269EP003	11-23	NEYFDSLLHACIP (SEQ ID NO: 390)	13
269EP004	20-30	ACIPCQLRCSS (SEQ ID NO: 391)	11
269EP005	24-42	CQLRCSSNTPPLTCQRYCN (SEQ ID NO: 392)	19
269EP006	36-43	LTCQRYCNAS (SEQ ID NO: 393)	10
269EP007	43-54	ASVTNSVKGTNA (SEQ ID NO: 394)	12

[0535] As shown in FIG. 9A, V_HH1 showed strongest binding to the 269EP005 peptide followed by the 269EP004 peptide. However, binding of V_HH1 to 269EP003 and 269EP006 was relatively weak compared to 269EP005 and 269EP004. V_HH1 tends to bind an epitope located in the 269EP005 peptide (*i.e.*, amino acid 24-36 of BCMA ECD), which contains the secondary structures of a helix (aa24-27), a beta strand (aa30-32), and a helix (aa35 – 37).

[0536] As shown in FIG. 9B, V_HH2 showed strongest binding to the 269EP002 peptide followed by the 269EP003 peptide. However, binding of V_HH1 to 269EP001 and 269EP004 was relatively weak compared to 269EP002 and 269EP003. While the first beta strand (aa12-15) and the beta strand (aa20-23) of the BCMA ECD are mainly located in the sequence covered by 269EP002 (aa8-21) and 269EP003 (aa11-23), V_HH2 tends to bind to an epitope located in the first two beta strands.

Competitive binding assay

[0537] Differential epitope binding of V_HH1 and V_HH2 was further validated by a cell-based competitive binding assay. A stable CHO cell line overexpressing human BCMA ("CHO-BCMA") was used in the assay.

[0538] Briefly, $0.5x10^6$ CHO-BCMA cells were pre-incubated with 12.5 ng/ml LAB001 (which contains 6xHis tag at the C terminus) at 4 °C for 0.5h in duplicates. Then serially diluted V_HH2-hIgG1Fc recombinant antibody was added to each well of the plate and further incubated at 4 °C for another 1h. After incubation, cells were washed with 500μl DPBS and centrifuged at 300g for 10min. The cell pellets were re-suspended with DPBS containing anti-His tag-FITC (1:200, GenScript Cat: A16020), then cells were washed with 500μl DPBS and centrifuged at 300g for 10min. The cell pellets were re-suspended with DPBS, and were then subject to FACS analysis on an ATTUNE™ Nxt flow cytometer. As an assay control, serially diluted V_HH2-hIgG1Fc was directly incubated with CHO-BCMA cells without the presence of LAB001 following identical procedures as above side-by-side. Goat anti- Human IgG (Fc specific)-FITC antibody (Sigma Aldrich Cat:F9512) was used to detect binding of V_HH2-hIgG1Fc to the CHO-BCMA cells. As shown in FIG. 10, V_HH2-hIgG1Fc alone binds to CHO-BCMA in a dose-dependent manner. However, V_HH2-hIgG1Fc was not able to compete with V_HH1-His binding to CHO-BCMA cells, which indicates different binding sites of V_HH1 and V_HH2 on BCMA.

Example 5. *In vivo* efficacy of LCAR-B38M CAR-T in tumor xenograft mice

[0539] *In vivo* anti-tumor efficacy of LCAR-B38M CAR-T cells was evaluated in a NCG mouse model (NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/NjuCrl) having a multiple myeloma tumor xenograft. LCAR-B38M CAR is a bivalent BCMA CAR having two anti-BCMA V_HH domains that target different BCMA epitopes.

[0540] The NCG mouse model was created by sequential CRISPR/Cas9 editing of the Prkdc and Il2rg loci in the NOD/Nju mouse, providing a mouse coisogenic to the NOD/Nju. The NOD/Nju mouse carries a mutation in the Sirpa (SIRP α) gene that allows for engrafting of foreign hematopoietic stem cells. The Prkdc knockout generates a SCID-like phenotype lacking proper T-cell and B-cell formation. The knockout of the Il2rg gene further exacerbates the SCID-like phenotype while additionally resulting in a decrease of NK cell production. Thus, the NCG mouse is a "triple-immunodeficient" mouse strain that is more immunocompromised than commonly used immunodeficient mouse strains including SCID and nude mice.

[0541] *Prkdc* and *Il2rg* are part of the SCID (severe combined immunodeficiency) family of genes affecting maturation and formation of T cells, B cells, NK cells and, to a lesser degree, dendritic cells. *Prkdc* encodes the catalytic subunit of the DNA-dependent protein kinase enzyme, which is required for V(D)J recombination, a necessary process to propagate antibody diversity in maturing T and B cells. *Il2rg* encodes the common gamma subunit found in IL-2 and multiple IL receptors (IL-4, IL-7, IL-9, IL-15 and IL-21), which are required to induce cytokine-mediated signaling for maturation of immature lymphocytes (*e.g.*, T, B and NK cells) and other leukocytes.

[0542] LCAR-B38M CAR-T cells were prepared using T cells from various donors to screen for T cell source yielding CAR-T with the highest efficacy of killing RPMI8226.Luc cells *in vitro* (FIG.11). Based on the results of FIG. 11, LCAR-B38M CAR-T cells were prepared using T cells of the selected donor for *in vivo* animal assays. FIG. 12A shows dose-dependent *in vitro* cytotoxicity of this batch of LCAR-B38M CAR-T cells. To create the tumor xenograft, NCG mice were injected intravenously with RPMI8226.Luc cells. 14 days later, tumor engrafted mice were treated with the LCAR-B38M CAR-T cells or un-transduced T cells, followed by *in vivo* bioluminescence imaging (BLI).

[0543] As shown in FIGs. 12B-12C, LCAR-B38M CAR-T cells were efficient to eradicate the engrafted RPMI8226.Luc tumor cells in NCG mice and rescue the mice, while most mice in the control (UnT) group died within 4 weeks. Interestingly, during autopsy, numerous metastatic tumors were observed in the liver of all mice in the UnT group. This observation was further validated by assessing *ex vivo* luciferase activities from the tumor samples (FIGs. 12D-12E). In contrast, the LCAR-B38M CAR-T treated mice did not have metastatic tumors in the livers. In summary, the *in vivo* study demonstrates the potency of LCAR-B38M CAR-T cells in eradication of multiple myeloma cells (*e.g.*, RPMI8226.Luc) from NCG mice.

Example 6. Safety study of LCAR-B38M CAR-T treatment on non-human primates

[0544] The *in vivo* safety of the LCAR-B38M CAR-T cells was evaluated in a cynomolgus monkey model. PBMC was obtained from peripheral blood samples of two monkeys (NHP#120117 and NHP#120545, both male, around 4kg), and prepared by density gradient centrifugation. Cynomolgus monkey T cells were isolated from PBMC using non-human primate Pan T Cell Isolation Kit (Miltenyi#130-091-993) according to the manufacturer's manual. The prepared monkey T cells were pre-activated with non-human primate T Cell Activation/ Expansion Kit (Miltenyi#130-092-919), human IL-2, and autologous monkey serum for 3 days. Afterwards, the pre-activated T cells were transduced with the LCAR-B38M lentivirus, followed by expansion for 10 additional days.

[0545] 3 days prior to the infusion of autologous CAR-T cells, the monkeys were pre-treated with Cyclophosphamide at a dose of 22mg/kg body weight by intravenous infusion. On the day of autologous infusion, cells were thawed in a 37°C water bath by gentle swirling and immediately infused to the animals intravenously within 5 minutes. Monkey NHP#120117 was infused with 5×10^6/kg CAR-T cells, and monkey NHP#120545 was infused with 4×10^7/kg CAR-T cells.

[0546] The monkeys were monitored after the T-cell administration for fever, respiratory distress, change in appetite, diarrhea, and weight loss. Pre- and post-administration blood samples were obtained and examined for CBC, serum chemistry, and cytokine levels. As shown in FIGs. 13A-13F, the CAR-T cells had no significant toxicity in the monkeys.

Example 7. A clinical study of LCAR-B38M CAR-T in human patients with refractory/relapsed multiple myeloma

[0547] A single-arm, open-label, multi-center, phase 1/2 clinical study was conducted to determine the safety and efficacy of LCAR-B38M CAR-T cells in treating human patients diagnosed with refractory or relapsed multiple myeloma ("r/r MM"). Information of the clinical trial can be found on worldwide web.clinicaltrials.gov, with identifier NCT03090659.

[0548] In the study, refractory/relapsed multiple myeloma patients were treated with LCAR-B38M CAR-T cells derived from autologous T cells of the patients. A total dose of 0.5×10^6 - 5×10^6 cells/kg body weight was administered to each patient by intravenous injection in three split doses (20%, 30%, and 50% respectively) over the duration of a week (*e.g.*, on Days 0, 2, and 6). During Days 1-30 of the study, patients were monitored for adverse events, and patient

samples were obtained for laboratory assessment. All patients are followed up for at least 36 months after the CAR-T administration.

[0549] The primary outcome of the study measures occurrence of treatment related adverse events as assessed by Common Terminology Criteria for Adverse Events (CTCAE) v4.0 within 1-30 days after injection of the LCAR-B38M CAR-T cells. Secondary outcome assesses CAR-T induced anti-myeloma responses, *e.g.*, by determining aberrant immunoglobulin levels in the serum, and number of multiple myeloma cells in the bone marrow of the patients before and after administration of the LCAR-B38M CAR-T cells. Efficacy objectives of the study include pathological Complete Response proportion, 3-year Disease Free Survival, 3-year Progression Free Survival.

[0550] Patients 18-75 years old are eligible for the study if: (1) the patient has a confirmed prior diagnosis of active multiple myeloma as defined by the updated IMWG criteria; (2) Clear BCMA expression is detected on malignant plasma cells from either bone marrow or a plasmacytoma by flow cytometry or immunohistochemistry; and (3) the patient has refractory multiple myeloma as defined by having received at least 3 prior treatment regimens including bortezomib, or otherwise identified by clinical doctors; or the patient has relapsed multiple myeloma as defined by in the NCCN clinical practice guidelines in Oncology: Multiple Myeloma (2016 V2).

[0551] The following patients are excluded from the study: (1) women of child-bearing potential or who are pregnant or breastfeeding; (2) patients who have any active and uncontrolled infection: hepatitis B, hepatitis C, HIV, or other fatal viral and bacterial infection; (3) patients who have received systemic corticosteroid steroid therapy of greater than 5 mg/day of prednisone or equivalent dose of another corticosteroid are not allowed within 2 weeks prior to either the required leukapheresis or the initiation of the conditioning chemotherapy regimen; (4) patients with any uncontrolled intercurrent illness or serious uncontrolled medical disorder; (5) patients with CNS metastases or symptomatic CNS involvement (including cranial neuropathies or mass lesions and spinal cord compression); (6) patients with a history of allogeneic stem cell transplantation, who have active acute or chronic graft-versus-host-disease (GVHD), or require immunosuppressant medications for GVHD, within 6 months of enrollment; or (7) patients with active autoimmune skin diseases such as psoriasis or other active autoimmune diseases such as rheumatoid arthritis.

[0552] At an interim analysis in May 2017, 35 patients with relapsed or treatment resistant (refractory) multiple myeloma received LCAR-B38M CAR-T treatment. First signs of treatment efficacy appeared as early as 10 days after initial injection of the CAR-T cells. Overall, the objective response rate was 100% and 33 out of 35 (94%) patients had an evident clinical remission of myeloma (complete response or very good partial response) within two months of receiving the CAR-T cells.

[0553] By the time of the analysis, 19 patients were followed for more than four months, a pre-set time for full efficacy assessment by the International Myeloma Working Group (IMWG) consensus criteria. One patient reached partial response and four patients achieved very good partial remission criteria (VgPR) in efficacy. No patients who reached the Stringent Complete Response ("sCR") criteria relapsed. Five patients who had been followed for over a year (12-14 months) remained at the sCR status and were free of minimal residual disease (*i.e.*, no detectable cancer cells in the bone marrow).

[0554] Cytokine release syndrome ("CRS") is a common and potentially dangerous side effect of CAR T-cell therapy. Only transient CRS was experience by 85% of the 35 patients. CRS symptoms include fever, low blood pressure, difficulty of breathing, and problems with multiple organs. In a majority of the patients, CRS symptoms were mild and manageable. Only two patients experienced severe CRS (grade 3), but recovered upon receiving tocilizumab (an inflammation-reducing treatment commonly used to manage CRS in clinical trials of CAR-T cell therapy). No patients experienced neurologic side effects, another common and serious complication from CAR T-cell therapy.

[0555] The interim clinical trial data demonstrate potent efficacy and safety of the LCAR-B38M CAR-T treatment on patients with refractory/relapsed multiple myeloma.

[0556] In a pilot clinical study, 3 patients were treated with autologous T cells expressing a monovalent BCMA CAR, *i.e.*, LCAR-B27S CAR-T cells. The LCAR-B27S CAR (one monovalent BCMA CAR listed in Table 4) has an antigen binding domain containing a single V_HH fragment that recognizes a single epitope of the BCMA molecule. This V_HH domain is identical to the second V_HH domain of the bi-epitope/bivalent LCAR-B38M CAR.

[0557] In an in vitro cytotoxicity assay, LCAR-B27S CAR-T cells were prepared from three multiple myeloma patients respectively, and LCAR-B38M CAR-T cells were also prepared respectively from the same three multiple myeloma patients as a control. Both CAR-T cells were

co-cultured with RPMI8226.Luc cells at an effector to target ratio (E/T ratio) of 20:1 and 5:1 for 20 hours. As shown in FIG.14A, CAR-T cells were prepared using T cells from patient A. The percentage of remaining viable cells, as assessed by remaining luciferase activity in RPMI8226.Luc cells, were 3.97±0.75% for LCAR-B38M and 3.17±0.57% for LCAR-B27S, when E/T ratio was 20:1. However, when E/T ratio was 5:1, LCAR-B38M showed higher potencies of killing RPMI8226.Luc cells as compared with LCAR-B27S (33.37±0.75% remaining viable cells for LCAR-B38M, 68.60±1.60% for LCAR-B27S). As shown in FIG.14B, CAR-T cells were prepared using T cells from patient B. The percentage of remaining viable cells, as assessed by remaining luciferase activity in RPMI8226.Luc cells, were 4.45±0.57% for LCAR-B38M and 9.32±1.16% for LCAR-B27S, when E/T ratio was 20:1. However, when E/T ratio was 5:1, LCAR-B38M again showed higher potencies of killing RPMI8226.Luc cells as compared with LCAR-B27S (40.92±3.00% remaining viable cells for LCAR-B38M, 84.05±1.56% for LCAR-B27S). As shown in FIG.14C, CAR-T cells were prepared using T cells from patient C. The percentage of remaining viable cells, as assessed by remaining luciferase activity in RPMI8226.Luc cells, were 2.56±0.88% for LCAR-B38M and 10.12±1.83% for LCAR-B27S, when E/T ratio was 20:1. However, when E/T ratio was 5:1, LCAR-B38M again showed higher potencies of killing RPMI8226.Luc cells as compared with LCAR-B27S (29.99±3.13% remaining viable cells for LCAR-B38M, 100.93±9.25% for LCAR-B27S).

[0558] In the pilot clinical study, 3 patients were treated with the LCAR-B27S CAR-T cells, in which all the preconditioning, injection and follow-up protocols were identical to those of the clinical study with the bivalent LCAR-B38M CAR. A total dose of 3×10^6 cells/kg (patient A), 5×10^6 cells/kg (patient B) and 7×10^6 cells/kg (patient C) body weight of autologous LCAR-B27S modified CAR-T cells were administered to each patient respectively by intravenous injection in three split doses (20%, 30%, and 50% respectively) over the duration of a week (*e.g.*, on Days 0, 2, and 6). During Days 1-30 of the study, patients were monitored for adverse events, and patient samples were obtained for laboratory assessment. All patients were followed up for at least 36 months after the CAR-T administration.

[0559] 2 patients among the three patients reached very good partial response (VgPR), but both patients relapsed within 6 months after the CAR-T infusion. The third patient did not show any clinical response. The pilot study with LCAR-B27S was consequently terminated by the IRB with no further patient enrollment.

[0560] Objective response rate, complete remission rate and relapse rate from this pilot monovalent BCMA CAR study (LCAR-B27S) and the bivalent BCMA CAR study (LCAR-B38M CAR-T) are shown in Table 11 below. The bivalent BCMA CAR-T had superior clinical efficacy in comparison to the monovalent BCMA CAR-T.

TABLE 11: Comparable clinical data of monovalent and bivalent/bi-epitope BCMA CAR-T therapies

Agent	N	Dose (cells/kg)	Clinical trial	Objective Response Rate (ORR)	Complete Remission (CR) rate	Relapse Rate (>6 mon)
Monovalent BCMA CAR-T (LCAR-B27S)	3	3, 5, 7×10^6 respectively	Pilot study	67%	0%	67%
Bivalent/Bi-epitope BCMA CAR-T (LCAR-B38M)	35	0.3×10^6 -5.6×10^6 (median=2.9×10^6)	NCT03090659	100%	57.1%	7.5%

CLAIMS

1. A multivalent chimeric antigen receptor (CAR) comprising a polypeptide comprising:

(a) an extracellular antigen binding domain comprising a first BCMA binding moiety and a second BCMA binding moiety, wherein the first BCMA binding moiety is a first anti-BCMA single domain antibody (sdAb), and the second BCMA binding moiety is 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 are each independently selected from a group of sdAbs comprising:

(1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77;

(2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78;

(3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86;

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87;

(12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88;

(13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89;

(14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90;

(15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91;

(16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92;

(17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93;

(18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94;

(19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95;

(20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96;

(21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97;

(22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98;

(23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99;

(24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100;

(25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101;

(26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102;

(27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103;

(28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104;

(29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105;

(30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106;

(31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107;

(32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108;

(33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109;

(34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110;

(35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111;

(36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112;

(37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; and

(38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114.

2. The multivalent CAR of claim 1, wherein:

(i) the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 1; a CDR2 comprising the amino acid sequence of SEQ ID NO: 39; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 77; and

(ii) the second anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 1; a CDR2 comprising the amino acid sequence of SEQ ID NO: 39; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 77.

3. The multivalent CAR of claim 2, wherein the first anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 115, and the second anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 115.

4. The multivalent CAR of claim 1, wherein:

(i) the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 10; a CDR2 comprising the amino acid sequence of SEQ ID NO: 48; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 86; and

(ii) the second anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 3; a CDR2 comprising the amino acid sequence of SEQ ID NO: 41; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 79.

5. The multivalent CAR of claim 4, wherein the first anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 124, and the second anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 117.

6. The multivalent CAR of claim 1, wherein:

(i) the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 10; a CDR2 comprising the amino acid sequence of SEQ ID NO: 48; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 86; and

(ii) the second anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 11; a CDR2 comprising the amino acid sequence of SEQ ID NO: 49; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 87.

7. The multivalent CAR of claim 6, wherein the first anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 124, and the second anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 125.

8. The multivalent CAR of claim 1, wherein:

(i) the first anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 7; a CDR2 comprising the amino acid sequence of SEQ ID NO: 45; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 83; and

(ii) the second anti-BCMA sdAb comprises a CDR1 comprising the amino acid sequence of SEQ ID NO: 11; a CDR2 comprising the amino acid sequence of SEQ ID NO: 49; and a CDR3 comprising the amino acid sequence of SEQ ID NO: 87.

9. The multivalent CAR of claim 8, wherein the first anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 121, and the second anti-BCMA sdAb comprises the amino acid sequence of SEQ ID NO: 125.

10. An anti-BCMA single-domain antibody (sdAb) comprising any one of the following:

(1) a CDR1 comprising the amino acid sequence of SEQ ID NO:1; a CDR2 comprising the amino acid sequence of SEQ ID NO:39; and a CDR3 comprising the amino acid sequence of SEQ ID NO:77;

(2) a CDR1 comprising the amino acid sequence of SEQ ID NO:2; a CDR2 comprising the amino acid sequence of SEQ ID NO:40; and a CDR3 comprising the amino acid sequence of SEQ ID NO:78;

(3) a CDR1 comprising the amino acid sequence of SEQ ID NO:3; a CDR2 comprising the amino acid sequence of SEQ ID NO:41; and a CDR3 comprising the amino acid sequence of SEQ ID NO:79;

(4) a CDR1 comprising the amino acid sequence of SEQ ID NO:4; a CDR2 comprising the amino acid sequence of SEQ ID NO:42; and a CDR3 comprising the amino acid sequence of SEQ ID NO:80;

(5) a CDR1 comprising the amino acid sequence of SEQ ID NO:5; a CDR2 comprising the amino acid sequence of SEQ ID NO:43; and a CDR3 comprising the amino acid sequence of SEQ ID NO:81;

(6) a CDR1 comprising the amino acid sequence of SEQ ID NO:6; a CDR2 comprising the amino acid sequence of SEQ ID NO:44; and a CDR3 comprising the amino acid sequence of SEQ ID NO:82;

(7) a CDR1 comprising the amino acid sequence of SEQ ID NO:7; a CDR2 comprising the amino acid sequence of SEQ ID NO:45; and a CDR3 comprising the amino acid sequence of SEQ ID NO:83;

(8) a CDR1 comprising the amino acid sequence of SEQ ID NO:8; a CDR2 comprising the amino acid sequence of SEQ ID NO:46; and a CDR3 comprising the amino acid sequence of SEQ ID NO:84;

(9) a CDR1 comprising the amino acid sequence of SEQ ID NO:9; a CDR2 comprising the amino acid sequence of SEQ ID NO:47; and a CDR3 comprising the amino acid sequence of SEQ ID NO:85;

(10) a CDR1 comprising the amino acid sequence of SEQ ID NO:10; a CDR2 comprising the amino acid sequence of SEQ ID NO:48; and a CDR3 comprising the amino acid sequence of SEQ ID NO:86;

(11) a CDR1 comprising the amino acid sequence of SEQ ID NO:11; a CDR2 comprising the amino acid sequence of SEQ ID NO:49; and a CDR3 comprising the amino acid sequence of SEQ ID NO:87;

(12) a CDR1 comprising the amino acid sequence of SEQ ID NO:12; a CDR2 comprising the amino acid sequence of SEQ ID NO:50; and a CDR3 comprising the amino acid sequence of SEQ ID NO:88;

(13) a CDR1 comprising the amino acid sequence of SEQ ID NO:13; a CDR2 comprising the amino acid sequence of SEQ ID NO:51; and a CDR3 comprising the amino acid sequence of SEQ ID NO:89;

(14) a CDR1 comprising the amino acid sequence of SEQ ID NO:14; a CDR2 comprising the amino acid sequence of SEQ ID NO:52; and a CDR3 comprising the amino acid sequence of SEQ ID NO:90;

(15) a CDR1 comprising the amino acid sequence of SEQ ID NO:15; a CDR2 comprising the amino acid sequence of SEQ ID NO:53; and a CDR3 comprising the amino acid sequence of SEQ ID NO:91;

(16) a CDR1 comprising the amino acid sequence of SEQ ID NO:16; a CDR2 comprising the amino acid sequence of SEQ ID NO:54; and a CDR3 comprising the amino acid sequence of SEQ ID NO:92;

(17) a CDR1 comprising the amino acid sequence of SEQ ID NO:17; a CDR2 comprising the amino acid sequence of SEQ ID NO:55; and a CDR3 comprising the amino acid sequence of SEQ ID NO:93;

(18) a CDR1 comprising the amino acid sequence of SEQ ID NO:18; a CDR2 comprising the amino acid sequence of SEQ ID NO:56; and a CDR3 comprising the amino acid sequence of SEQ ID NO:94;

(19) a CDR1 comprising the amino acid sequence of SEQ ID NO:19; a CDR2 comprising the amino acid sequence of SEQ ID NO:57; and a CDR3 comprising the amino acid sequence of SEQ ID NO:95;

(20) a CDR1 comprising the amino acid sequence of SEQ ID NO:20; a CDR2 comprising the amino acid sequence of SEQ ID NO:58; and a CDR3 comprising the amino acid sequence of SEQ ID NO:96;

(21) a CDR1 comprising the amino acid sequence of SEQ ID NO:21; a CDR2 comprising the amino acid sequence of SEQ ID NO:59; and a CDR3 comprising the amino acid sequence of SEQ ID NO:97;

(22) a CDR1 comprising the amino acid sequence of SEQ ID NO:22; a CDR2 comprising the amino acid sequence of SEQ ID NO:60; and a CDR3 comprising the amino acid sequence of SEQ ID NO:98;

(23) a CDR1 comprising the amino acid sequence of SEQ ID NO:23; a CDR2 comprising the amino acid sequence of SEQ ID NO:61; and a CDR3 comprising the amino acid sequence of SEQ ID NO:99;

(24) a CDR1 comprising the amino acid sequence of SEQ ID NO:24; a CDR2 comprising the amino acid sequence of SEQ ID NO:62; and a CDR3 comprising the amino acid sequence of SEQ ID NO:100;

(25) a CDR1 comprising the amino acid sequence of SEQ ID NO:25; a CDR2 comprising the amino acid sequence of SEQ ID NO:63; and a CDR3 comprising the amino acid sequence of SEQ ID NO:101;

(26) a CDR1 comprising the amino acid sequence of SEQ ID NO:26; a CDR2 comprising the amino acid sequence of SEQ ID NO:64; and a CDR3 comprising the amino acid sequence of SEQ ID NO:102;

(27) a CDR1 comprising the amino acid sequence of SEQ ID NO:27; a CDR2 comprising the amino acid sequence of SEQ ID NO:65; and a CDR3 comprising the amino acid sequence of SEQ ID NO:103;

(28) a CDR1 comprising the amino acid sequence of SEQ ID NO:28; a CDR2 comprising the amino acid sequence of SEQ ID NO:66; and a CDR3 comprising the amino acid sequence of SEQ ID NO:104;

(29) a CDR1 comprising the amino acid sequence of SEQ ID NO:29; a CDR2 comprising the amino acid sequence of SEQ ID NO:67; and a CDR3 comprising the amino acid sequence of SEQ ID NO:105;

(30) a CDR1 comprising the amino acid sequence of SEQ ID NO:30; a CDR2 comprising the amino acid sequence of SEQ ID NO:68; and a CDR3 comprising the amino acid sequence of SEQ ID NO:106;

(31) a CDR1 comprising the amino acid sequence of SEQ ID NO:31; a CDR2 comprising the amino acid sequence of SEQ ID NO:69; and a CDR3 comprising the amino acid sequence of SEQ ID NO:107;

(32) a CDR1 comprising the amino acid sequence of SEQ ID NO:32; a CDR2 comprising the amino acid sequence of SEQ ID NO:70; and a CDR3 comprising the amino acid sequence of SEQ ID NO:108;

(33) a CDR1 comprising the amino acid sequence of SEQ ID NO:33; a CDR2 comprising the amino acid sequence of SEQ ID NO:71; and a CDR3 comprising the amino acid sequence of SEQ ID NO:109;

(34) a CDR1 comprising the amino acid sequence of SEQ ID NO:34; a CDR2 comprising the amino acid sequence of SEQ ID NO:72; and a CDR3 comprising the amino acid sequence of SEQ ID NO:110;

(35) a CDR1 comprising the amino acid sequence of SEQ ID NO:35; a CDR2 comprising the amino acid sequence of SEQ ID NO:73; and a CDR3 comprising the amino acid sequence of SEQ ID NO:111;

(36) a CDR1 comprising the amino acid sequence of SEQ ID NO:36; a CDR2 comprising the amino acid sequence of SEQ ID NO:74; and a CDR3 comprising the amino acid sequence of SEQ ID NO:112;

(37) a CDR1 comprising the amino acid sequence of SEQ ID NO:37; a CDR2 comprising the amino acid sequence of SEQ ID NO:75; and a CDR3 comprising the amino acid sequence of SEQ ID NO:113; or

(38) a CDR1 comprising the amino acid sequence of SEQ ID NO:38; a CDR2 comprising the amino acid sequence of SEQ ID NO:76; and a CDR3 comprising the amino acid sequence of SEQ ID NO:114.

11. The anti-BCMA sdAb of claim 10, wherein the anti-BCMA sdAb comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 115-152.

12. The anti-BCMA sdAb of claim 10, wherein the anti-BCMA sdAb is a camelid antibody, or wherein the anti-BCMA sdAb is a chimeric antibody.

13. The anti-BCMA sdAb of claim 10, wherein the anti-BCMA sdAb is a V_HH fragment.

14. The multivalent CAR of any one of claims 1-9, wherein the transmembrane domain is from a molecule selected from the group consisting of CD8α, CD4, CD28, CD137, CD80, CD86, CD152, and PD1.

15. The multivalent CAR of claim 14, wherein the transmembrane domain is from CD8α or CD28.

16. The multivalent CAR of any one of claims 1-9, 14 or 15, wherein the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell.

17. The multivalent CAR of claim 16, wherein the primary intracellular signaling domain is from CD3ζ.

18. The multivalent CAR of any one of claims 1-9 or 14-17, wherein the intracellular signaling domain comprises a co-stimulatory signaling domain.

19. The multivalent CAR of claim 18, wherein the co-stimulatory signaling domain is from a co-stimulatory molecule selected from the group consisting of CD27, CD28, CD137, OX40, CD30, CD40, CD3, LFA-1, ICOS, CD2, CD7, LIGHT, NKG2C, B7-H3, ligands of CD83, and combinations thereof.

20. The multivalent CAR of claim 19, wherein the co-stimulatory signaling domain comprises a cytoplasmic domain of CD28 and/or a cytoplasmic domain of CD137.

21. The multivalent CAR of any one of claims 1-9 or 14-20, further comprising a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain.

22. The multivalent CAR of claim 21, wherein the hinge domain is from CD8α.

23. The multivalent CAR of any one of claims 1-9 or 14-22, further comprising a signal peptide located at the N-terminus of the polypeptide.

24. The multivalent CAR of claim 23, wherein the signal peptide is from CD8α.

25. A multivalent chimeric antigen receptor (CAR) comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 298, 300-315, 317-321, 323-327, 329-333, and 335.

26. An isolated nucleic acid comprising a nucleic acid sequence encoding the multivalent CAR of any one of claims 1-9 or 14-25.

27. A vector comprising the isolated nucleic acid of claim 26.

28. An engineered immune effector cell, comprising the multivalent CAR of any one of claims 1-9 or 14-25, the isolated nucleic acid of claim 26, or the vector of claim 27.

29. The engineered immune effector cell of claim 28, wherein the immune effector cell is a T cell.

30. A pharmaceutical composition, comprising the engineered immune effector cell of claims 28 or 29, or the anti-BCMA sdAb of any one of claims 10-13, and a pharmaceutically acceptable carrier.

31. Use of the engineered immune effector cell of claims 28 or 29, or the anti-BCMA sdAb of any one of claims 10-13 in the manufacture of a medicament for treating cancer in an individual, wherein the cancer comprises a cell expressing BCMA.

32. The use of claim 31, wherein the cancer is multiple myeloma.

33. The the use of claim 32, wherein the cancer is refractory or relapsed multiple myeloma.