

**BISPECIFIC CD19/CD20-TARGETED CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF**

This application claims the benefit of U.S. Provisional Patent Application No. 63/587,646, filed October 3, 2023, the disclosure of which is incorporated by reference herein in its entirety for all purposes.

**SEQUENCE LISTING**

This application contains an electronic Sequence Listing which has been submitted in XML file format with this application, the entire content of which is incorporated by reference herein in its entirety. The Sequence Listing XML file submitted with this application is entitled "14183-016-228\_SEQLISTING.xml", was created on September 30, 2024, and is 71,327 bytes in size.

**1. TECHNICAL FIELD**

**[0001]** Embodiments of the invention relate to bispecific chimeric antigen receptors (CARs) that target CD19 and CD20, cells comprising the CARs, and methods for treating B-lymphocyte antigen-related diseases (e.g., hematologic malignancies and autoimmune diseases) using such CARs and cells.

**2. BACKGROUND**

**[0002]** Hematologic malignancies represent some of the most common cancers occurring in both children and adults. For example, approximately 4,000 *de novo* cases of the aggressive B-cell lineage malignancy B-cell acute lymphoblastic leukemia (B-ALL) are diagnosed each year in the United States and represents the most common malignancy of childhood. Genetic mutations that induce aberrant arrest of normal lymphoid maturation, evasion of apoptosis and uncontrolled cellular proliferation result in over-production of B-cell lymphoblasts. In adults, over 6000 incident cases of acute lymphoblastic leukemia (ALL) occur each year. (Hanahan D. *et al.*, *Cell* (2000);100:57-70; Teitell M. *et al.*, *Annu Rev Pathol* (2009);4:175-198). Moreover, lymphomas (e.g., neoplasms of the lymphatic tissues) account for approximately 5% of all cases of cancer in the United States. The main classes are malignant neoplasms of the lymphocytes, a cell type present in both the lymph and the blood. In this way, lymphomas and leukemias are both malignancies (e.g., tumors) of the hematopoietic and lymphoid tissues. As lymphoproliferative disorders, lymphomas and lymphoid leukemias are closely related, to the

point that some of them are called by either name (for example adult T-cell leukemia/lymphoma).

**[0003]** Autoimmune disease is the third most prevalent disease in the US affecting some 50 million Americans. Autoimmune disease occurs when the immune system mistakenly attacks the body's own tissues, leading to inflammation and tissue damage. Three of the most well-known autoimmune diseases include multiple sclerosis (MS), systemic lupus erythematosus (SLE), and rheumatoid arthritis. In MS, the immune system targets the central nervous system, particularly the myelin sheath that protects nerve cells, leading to neurological impairment. In RA, the immune system primarily attacks the joints, causing chronic inflammation and joint destruction. SLE, on the other hand, is a systemic disease affecting multiple organs, driven by autoantibodies that target nuclear components within cells.

**[0004]** B-cells, a type of white blood cell, play a central role in the pathogenesis of these and other autoimmune diseases. In RA and SLE, B-cells produce autoantibodies that mistakenly target the body's tissues, exacerbating inflammation and tissue destruction. B cells also contribute to disease progression through antigen presentation and the production of pro-inflammatory cytokines, which amplify immune responses. In MS, B cells have been implicated in the production of antibodies that attack the central nervous system including the brain, contributing to demyelination and neurodegeneration. In other neurodegenerative diseases such as Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP) and multifocal motor neuropathy (MMN), B cells generate autoantibodies against peripheral nerves which attack the myelin sheaths of neurons in peripheral nerves such as those found in the limbs.

**[0005]** To address the role of B cells in autoimmune diseases, several therapies have been developed to target and deplete them. One of the most widely used therapies is rituximab, a monoclonal antibody that depletes B-cells by targeting the CD20 protein on their surface. This therapy has been effective in treating RA, and it is also used in some cases of MS and SLE. Other therapies include belimumab, which targets B-cell activating factor (BAFF), a protein necessary for B-cell survival, and newer CD20-targeting therapies like ofatumumab and obinutuzumab.

**[0006]** While these therapies have provided significant improvements in disease control, they also come with notable limitations. For example, adverse effects, such as increased susceptibility

to infections, occur due to the broad depletion of B-cells, weakening the immune system's ability to fight off infections. Additionally, lack of efficacy in certain patients remains a challenge, with some individuals showing incomplete responses or relapsing despite treatment. Further, long-term use of B-cell therapies may also lead to the development of treatment resistance or other immune complications. Thus there is a need for improved composition and therapies for the treatment of the aforementioned and other auto-immune diseases.

**[0007]** Cell-based immunotherapy is a therapy with curative potential for the treatment of cancer and autoimmune disease. T-cells and other immune cells may be modified to target tumor antigens through the introduction of genetic material coding for artificial or synthetic receptors for antigen, termed Chimeric Antigen Receptors (CARs), specific to selected antigens. Targeted T-cell therapy using CARs has shown recent clinical success in treating hematologic malignancies. However, despite positive results from early-phase trials, infusion of CD19-targeted CAR T cells into patients still results in a number of side effects of varying severity. These include tumor lysis syndrome (TLS), cytokine release syndrome (CRS) and macrophage activation syndrome, CNS trafficking, prolonged B-cell aplasia, and immune escape. Moreover, autoimmune disease progression cannot be stopped by current cell-based immunotherapy targeting B cells (e.g., via the CD20 antigen), perhaps in the case of MS and other related conditions, due to challenges in adequately accessing the central nervous system. Therefore, in view of the long-felt and unmet need described above, improved therapies for hematologic malignancies and autoimmune diseases are needed.

### **3. SUMMARY OF THE INVENTION**

**[0008]** Various embodiments of the invention provide chimeric antigen receptor (CAR)s that target CD19 and CD20, cells comprising the CARs, and uses of the cells, e.g., for treating B-lymphocyte antigen-related diseases or disorders.

**[0009]** In a first aspect, embodiments of the invention provide chimeric antigen receptors (CARs) that target CD19 and CD20. In certain embodiments, the CAR comprises: (a) an extracellular domain comprising (i) an antigen-binding domain that binds to CD19 and (ii) a second antigen-binding domain that binds to CD20; (b) a transmembrane domain comprising a first CD28 polypeptide; and (c) an intracellular domain comprising a modified CD3 $\zeta$  polypeptide that comprises a native ITAM1, an ITAM2 variant, and an ITAM3 variant, wherein each of the ITAM2 variant and the ITAM3 variant comprises two loss-of-function mutations.

**[0010]** In certain embodiments, the first anti-CD19 antigen-binding domain comprises: a first heavy chain variable region (V<sub>H</sub>) and a first light chain variable region (V<sub>L</sub>), wherein the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1, a V<sub>H</sub> CDR2, and a V<sub>H</sub> CDR3 of a V<sub>H</sub> comprising the amino acid sequence set forth in SEQ ID NO: 15. In certain embodiments, the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1, a V<sub>L</sub> CDR2, and a V<sub>L</sub> CDR3 of a V<sub>L</sub> comprising the amino acid sequence set forth in SEQ ID NO: 16.

**[0011]** In certain embodiments, the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof. In certain embodiments, the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14 or a conservative modification thereof.

**[0012]** In certain embodiments, the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11. In certain embodiments, the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14.

**[0013]** In certain embodiments, the first V<sub>H</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 15. In certain embodiments, the first V<sub>L</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 16.



[0014] In certain embodiments, the first  $V_H$  comprises the amino acid sequence set forth in SEQ ID NO: 15. In certain embodiments, the first  $V_L$  comprises the amino acid sequence set forth in SEQ ID NO: 16.

[0015] In certain embodiments, the first anti-CD19 antigen-binding domain comprises a first linker between the first  $V_H$  and the first  $V_L$ . In certain embodiments, the first linker comprises the amino acid sequence set forth in SEQ ID NO: 1. In certain embodiments, the first  $V_H$  and the first  $V_L$  are positioned from the N- to the C-terminus:  $V_L$ - $V_H$ .

[0016] In certain embodiments, the first anti-CD19 antigen-binding domain comprises a first single-chain variable fragment (scFv) that binds to anti-CD19. In certain embodiments, the first anti-CD19 scFv comprises the amino acid sequence set forth in SEQ ID NO: 17.

[0017] In certain embodiments, the second anti-CD20 antigen-binding domain comprises: a second  $V_H$  and a second  $V_L$ , wherein the second  $V_H$  comprises a  $V_H$  CDR1, a  $V_H$  CDR2, and a  $V_H$  CDR3 of a  $V_H$  comprising the amino acid sequence set forth in SEQ ID NO: 27. In certain embodiments, the second  $V_L$  comprises a  $V_L$  CDR1, a  $V_L$  CDR2, and a  $V_L$  CDR3 of a  $V_L$  comprising the amino acid sequence set forth in SEQ ID NO: 28.

[0018] In certain embodiments, the second  $V_H$  comprises a  $V_H$  CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof, a  $V_H$  CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22 or a conservative modification thereof, and a  $V_H$  CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23 or a conservative modification thereof. In certain embodiments, the second  $V_L$  comprises a  $V_L$  CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24 or a conservative modification thereof, a  $V_L$  CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25 or a conservative modification thereof, and a  $V_L$  CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26 or a conservative modification thereof.

[0019] In certain embodiments, the second  $V_H$  comprises a  $V_H$  CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a  $V_H$  CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a  $V_H$  CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23. In certain embodiments, the second  $V_L$  comprises a  $V_L$  CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24, a  $V_L$  CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a  $V_L$  CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26.

**[0020]** In certain embodiments, the second V<sub>H</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 27. In certain embodiments, the second V<sub>L</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 28.

**[0021]** In certain embodiments, the second V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 27. Also, in certain embodiments, the second V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 28.

**[0022]** In certain embodiments, the second anti-CD20 antigen-binding domain comprises a second linker between the second V<sub>H</sub> and the second V<sub>L</sub>. In certain embodiments, the second linker comprises the amino acid sequence set forth in SEQ ID NO: 2. In certain embodiments, the second V<sub>H</sub> and the second V<sub>L</sub> are positioned from the N- to the C-terminus: V<sub>H</sub>-V<sub>L</sub>.

**[0023]** In certain embodiments, the second anti-CD20 antigen-binding domain comprises a second anti-CD20 single-chain variable fragment (scFv) that binds to CD20. In certain embodiments, the second anti-CD20 scFv comprises the amino acid sequence set forth in SEQ ID NO: 29.

**[0024]** In certain embodiments, the V<sub>H</sub> CDR1, V<sub>H</sub> CDR2, V<sub>H</sub> CDR3, V<sub>L</sub> CDR1, V<sub>L</sub> CDR2, and V<sub>L</sub> CDR3 are identified according to the Kabat numbering system, the Chothia numbering system, the AbM numbering system, the Contact numbering system, or the IMGT Information System<sup>®</sup>. In certain embodiments, the V<sub>H</sub> CDR1, V<sub>H</sub> CDR2, V<sub>H</sub> CDR3, V<sub>L</sub> CDR1, V<sub>L</sub> CDR2, and V<sub>L</sub> CDR3 are identified according to the Kabat numbering system.

**[0025]** In certain embodiments, the CD19/CD20-targeted CAR further comprises a third linker between the first anti-CD19 antigen-binding domain and the second anti-CD20 antigen-binding domain. In certain embodiments, the third linker comprises the amino acid sequence set forth in SEQ ID NO: 3. In certain embodiments, the first anti-CD19 antigen-binding domain is positioned downstream of the second anti-CD20 antigen-binding domain.

**[0026]** In certain embodiments, a leader is covalently joined to the N-terminus of the second anti-CD20 antigen-binding domain. In certain embodiments, the leader comprises a CD8 polypeptide. In certain embodiments, the CD8 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 33.

**[0027]** In certain embodiments, the first CD28 polypeptide is a transmembrane domain sequence of CD28. In certain embodiments, the first CD28 polypeptide comprises amino acids 153 to 179 of SEQ ID NO: 35.

**[0028]** In certain embodiments, the CD19/CD20-targeted CAR further comprises a hinge domain. In certain embodiments, the hinge domain comprises a second CD28 polypeptide. In certain embodiments, the second CD28 polypeptide comprises amino acids 114 to 152 of SEQ ID NO: 35.

**[0029]** In certain embodiments, each of the two loss-of-function mutations is at a tyrosine amino acid residue. In certain embodiments, the ITAM2 variant comprises the amino acid sequence set forth in SEQ ID NO: 45. In certain embodiments, the ITAM3 variant comprises the amino acid sequence set forth in SEQ ID NO: 49. In certain embodiments, the native ITAM1 comprises the amino acid sequence set forth in SEQ ID NO: 39. In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 51.

**[0030]** In certain embodiments, the intracellular domain further comprises a co-stimulatory signaling region which may in one or more embodiments, comprise a third CD28 polypeptide. In certain embodiments, the third CD28 polypeptide is a signaling domain sequence of CD28. Also, in certain embodiments, the third CD28 polypeptide which in one or more embodiments, comprises amino acids 180 to 220 of SEQ ID NO: 35.

**[0031]** In certain embodiments, the CAR comprises the amino acid sequences set forth in SEQ ID NO: 54.

**[0032]** In another aspect, embodiments of the invention provide nucleic acids that encode the CD19/CD20-targeted CARs disclosed herein. In certain embodiments, the nucleic acid comprises the nucleotide sequence set forth in SEQ ID NO: 55.

**[0033]** In yet another aspect, embodiments of the invention provide vectors comprising the nucleic acids disclosed herein. In certain embodiments, the vector is a viral vector, which is a retroviral vector.

**[0034]** In still another aspect, embodiments of the invention provide cells comprising the CARs disclosed herein, the nucleic acids disclosed herein, or the vectors disclosed herein. In certain embodiments, the cell is transduced with the CAR, the nucleic acid, or the vector. In certain embodiments, the CAR is constitutively expressed on the surface of the cell.

**[0035]** In certain embodiments, the cell is an immunoresponsive cell or an immune effector cell. In certain embodiments, the cell is a cell of the lymphoid lineage or a cell of the myeloid lineage. In certain embodiments, the cell of lymphoid lineage is selected from the group consisting of T cells, Natural Killer (NK) cells, B cells, and stem cells from which lymphoid cells may be differentiated. In certain embodiments, the cell of the myeloid lineage is selected from the group consisting of monocytes, macrophages, neutrophils, dendritic cells, basophils, neutrophils, eosinophils, megakaryocytes, mast cell, erythrocyte, thrombocytes, and stem cells from which myeloid cells may be differentiated.

**[0036]** In certain embodiments, the cell is selected from the group consisting of a T cell, a B cell, a Natural Killer (NK) cell, a macrophage, an innate lymphoid cell (ILC), a cytokine induced killer (CIK) cell, a lymphokine activated killer (LAK) cell, a stem cell from which a lymphoid cell may be differentiated, a stem cell from which a myeloid cell may be differentiated, and combinations of the foregoing. In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is selected from the group consisting of helper T cells, cytotoxic T cells (CTLs), memory T cells, regulatory T cells, tumor-infiltrating lymphocyte (TIL), Natural Killer T cells, mucosal associated invariant T cells,  $\alpha\beta$  T cells, and  $\gamma\delta$  T cells. In certain embodiments, the T cell is a central memory T cell. In certain embodiments, the T cell is positive for CD45RO and CD62L.

**[0037]** In certain embodiments, the stem cell is a pluripotent stem cell. In certain embodiments, the pluripotent stem cell is an embryoid stem cell or an induced pluripotent stem cell.

**[0038]** In certain embodiments, the cell is a cytotoxic T lymphocyte (CTL). In certain embodiments, the cell is a viral antigen-sensitized CTL. In certain embodiments, the viral antigen is selected from the group consisting of Epstein-Barr virus (EBV), cytomegalovirus (CMV), B.K. virus (BKV), John Cunningham virus (JCV), picornavirus, hepadnavirus, hepacivirus, deltavirus, hepevirus, or any combination thereof. In certain embodiments, the cell is an EBV-sensitized CTL.

[0039] In yet another aspect, embodiments of the invention provide compositions comprising the cells disclosed herein for use in the treatment of various cancers, autoimmune and related diseases and disorders. In certain embodiments, the composition is a pharmaceutical composition further comprising: a pharmaceutically acceptable carrier. In certain embodiments, the composition comprises between about  $1 \times 10^6$  and about  $5 \times 10^8$  of the cells. In certain embodiments, the composition comprises between about  $1 \times 10^6$  and about  $1 \times 10^8$  of cells. In certain embodiments, the composition comprises between about  $1 \times 10^6$  and about  $5 \times 10^7$  of the cells. In certain embodiments, the composition comprises between about  $1 \times 10^6$  and about  $1 \times 10^7$  of the cells. In these and related embodiments, the dose of the cells can be titrated based on one or more of the patient weight, age and condition (e.g., leukemia, lymphoma, MS, SLE, etc.).

[0040] In still another aspect, embodiments of the invention provide methods of inhibiting growth of a target cell expressing at least one B-lymphocyte antigen. In certain embodiments, the method comprises contacting the target cell with the cell disclosed herein or the composition disclosed herein.

[0041] In certain embodiments, the at least one B-lymphocyte antigen is selected from the group consisting of CD19, CD20, and a combination thereof. In certain embodiments, the target cell expresses a detectable level of CD19 and a detectable level of CD20. In certain embodiments, the target cell expresses a detectable level of CD20 and a low or non-detectable level of CD19. In certain embodiments, the target cell expresses a detectable level of CD19 and a low or non-detectable level of CD20. In certain embodiments, the target cell is autologous to the CAR-expressing cell. In certain embodiments, the target cell is allogeneic to the CAR-expressing cell. In certain embodiments, the target cell is a tumor cell. In certain embodiments, the tumor is cancer. In certain embodiments, the cancer is a hematologic cancer. In certain embodiments, the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL). In certain embodiments, the target cell is a B cell. In certain embodiments, the B cell is  $CD19^+CD20^+$ ,  $CD19^+CD20^-$ , or  $CD19^-CD20^+$ . In certain embodiments, the target cell is a T cell. In certain

embodiments, the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the T cell is CD20<sup>+</sup>.

**[0042]** In certain embodiments, the cell or composition exhibits cytotoxic and/or cytolytic activity against the target cell. In certain embodiments, the cytotoxic and/or cytolytic activity is dose-dependent. In certain embodiments, the cytotoxic and/or cytolytic activity is long-term. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial contact of the target cell with the cell or composition. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 40 days after the initial contact of the target cell with the cell or composition. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 60 days after the initial contact of the target cell with the cell or composition.

**[0043]** In still yet another aspect, embodiments of the invention provide methods of treating a B-lymphocyte antigen-associated disease such as various hematological cancers and autoimmune diseases in a human or other subject. In certain embodiments, the method comprises administering to the subject the cells disclosed herein and/or a pharmaceutical composition comprising the cells disclosed herein.

**[0044]** In certain embodiments, the B-lymphocyte antigen is selected from the group consisting of CD19, CD20, and a combination thereof. In certain embodiments, the B-lymphocyte antigen is CD19 or CD20. In certain embodiments, the B-lymphocyte antigen-associated disease is a cancer. In certain embodiments, the cancer is a hematologic cancer. In certain embodiments, the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, diffuse large B cell lymphoma (DLBCL), mantle cell lymphoma (MCL), and follicular lymphoma (FL).

**[0045]** In certain embodiments, the B-lymphocyte antigen-associated disease is an autoimmune disease. In certain embodiments, the autoimmune disease is selected from the group consisting of multiple sclerosis, systemic lupus erythematosus (SLE), Sjogren's, AL amyloidosis, IgG4-related disease (IgG4-RD), inflammatory myopathies (e.g., myositis), scleroderma, pemphigus vulgaris, chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), stiff-person's syndrome, rheumatoid arthritis, neuromyelitis

optica spectrum disorder (NMOSD), myasthenia gravis, N-methyl-D-aspartate receptor (NMDAR) encephalitis, anti-myelin oligodendrocyte glycoprotein (MOG) syndrome, myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), membranous nephropathy (MN), IgA nephropathy (IgAN), Type 1 diabetes, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, thrombocytopenia purpura, hemolytic anemia (AIHA), multifocal motor neuropathy (MMN), inflammatory bowel disease (IBD), celiac disease, Crohn's disease, ulcerative colitis, and ankylosing spondylosis. In certain embodiments, the autoimmune disease is multiple sclerosis (MS). In certain embodiments, the autoimmune disease is systemic lupus erythematosus (SLE).

**[0046]** In certain embodiments, the cell or composition exhibits cytotoxic and/or cytolytic activity against a target cell in the subject, wherein the target cell is positive for the B-lymphocyte antigen. In certain embodiments, the target cell expresses: i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; or iii) a detectable level of CD19 and a low or non-detectable level of CD20.

**[0047]** In certain embodiments, the target cell is a tumor cell. In certain embodiments, the tumor is cancer. In certain embodiments, the cancer is a hematologic cancer. In certain embodiments, the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL). In certain embodiments, the target cell is a B cell. In certain embodiments, the B cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the target cell is a T cell. In certain embodiments, the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the T cell is CD20<sup>+</sup>.

**[0048]** In certain embodiments, the cytotoxic and/or cytolytic activity is dose-dependent. In certain embodiments, the cytotoxic and/or cytolytic activity is long-term. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial administration of the cell or composition. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 40 days after the initial administration of the cell or composition. In certain embodiments,

the cytotoxic and/or cytolytic activity lasts for about 60 days after the initial administration of the cell or composition. In certain embodiments, the cell or composition does not induce graft-versus-host disease.

**[0049]** In certain embodiments, the cell or composition induces no or minimal alloreactivity against a HLA-mismatched cell positive for the B-lymphocyte antigen in the subject. In certain embodiments, the cell or composition exhibit no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for the B-lymphocyte antigen. In certain embodiments, the off-target cell is selected from the group consisting of a CD19<sup>+</sup>CD20<sup>-</sup> T cell, a Natural Killer T cell (NK T cell), a Natural Killer cell (NK cell), and a combination thereof. In certain embodiments, the cell or composition induces cytokine secretion. In certain embodiments, the cytokine is selected from the group consisting of interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and a combination thereof.

**[0050]** In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is an EBV-sensitized CTL. In certain embodiments, the cell persists in the subject for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial administration of the cell or composition. In certain embodiments, the cell persists in the subject for about 40 days after the initial administration of the cell or composition. In certain embodiments, the cell persists in the subject for about 60 days after the initial administration of the cell or composition.

**[0051]** In another aspect, embodiments of the invention provide methods for treating a hematologic cancer in a subject. In certain embodiments, the method comprises administering to the subject the cell disclosed herein or the composition disclosed herein.

**[0052]** In certain embodiments, the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).

**[0053]** In certain embodiments, the cell or composition exhibits cytotoxic and/or cytolytic activity against a target cell in the subject, wherein the target cell expresses: i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-



detectable level of CD19; iii) a detectable level of CD19 and a low or non-detectable level of CD20. In certain embodiments, the target cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

**[0054]** In certain embodiments, the cytotoxic and/or cytolytic activity is dose-dependent. In certain embodiments, the cytotoxic and/or cytolytic activity is long-term. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for at least one month, or at least two months after the initial administration of the cell or composition. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 40 days after the initial administration of the cell or composition. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 60 days after the initial administration of the cell or composition. In certain embodiments, the cell or composition does not induce graft-versus-host disease.

**[0055]** In certain embodiments, the cell or composition induces no or minimal alloreactivity against a HLA-mismatched cell positive for CD19 and/or CD20 in the subject. In certain embodiments, the cell or composition exhibits no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for CD19 and CD20.

**[0056]** In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is an EBV-sensitized CTL. In certain embodiments, the cell persists in the subject for at least one month, or at least two months after the initial administration of the cell or composition. In certain embodiments, the cell persists in the subject for about 40 days after the initial administration of the cell or composition. In certain embodiments, the cell persists in the subject for about 60 days after the initial administration of the cell or composition.

**[0057]** In another aspect, embodiments of the invention provide methods for treating an autoimmune disease in a subject. In certain embodiments, the method comprises administering to the subject the cells disclosed herein and/or a pharmaceutical composition comprising the cells disclosed herein.

**[0058]** In certain embodiments, the autoimmune disease is selected from the group consisting of multiple sclerosis, systemic lupus erythematosus (SLE), Sjogren's, AL amyloidosis, Sjogren's, AL amyloidosis, IgG4-related disease (IgG4-RD), inflammatory myopathies (e.g., myositis), scleroderma, pemphigus vulgaris, chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), stiff-person's syndrome, rheumatoid arthritis, neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis, N-methyl-D-

aspartate receptor (NMDAR) encephalitis, anti-myelin oligodendrocyte glycoprotein (MOG) syndrome, myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), membranous nephropathy (MN), IgA nephropathy (IgAN), Type 1 diabetes, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, thrombocytopenia purpura, hemolytic anemia (AIHA), multifocal motor neuropathy (MMN), inflammatory bowel disease (IBD), celiac disease, Crohn's disease, ulcerative colitis, and ankylosing spondylosis. In certain embodiments, the autoimmune disease is multiple sclerosis. In certain embodiments, the autoimmune disease is systemic lupus erythematosus (SLE).

**[0059]** In certain embodiments, the cell or composition exhibits cytotoxic and/or cytolytic activity against a target cell in the subject, wherein the target cell expresses i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; or iii) a detectable level of CD19 and a low or non-detectable level of CD20. In certain embodiments, the target cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, wherein the target cell is a B cell. In certain embodiments, the B cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the target cell is a T cell. In certain embodiments, the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the T cell is CD20<sup>+</sup>. In certain embodiments, the cell or composition exhibits cytotoxic and/or cytolytic activity against a first target cell and a second target cell in the subject, wherein the first and second target cells each express i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; or iii) a detectable level of CD19 and a low or non-detectable level of CD20. In certain embodiments, the first and second target cells each are CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the first target cell is a B cell. In certain embodiments, the second target cell is a T cell. In certain embodiments, the T cell is CD20<sup>+</sup>. In certain embodiments, the cytotoxic and/or cytolytic activity is dose-dependent. In certain embodiments, the cytotoxic and/or cytolytic activity is long-term. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for at least 24 hours or at least 72 hours after the initial administration of the cell or composition. In certain embodiments, the cell or composition does not induce graft-versus-host disease.

**[0060]** In certain embodiments, the cell or composition induces no or minimal alloreactivity against a HLA-mismatched cell positive for CD19 and/or CD20 in the subject. In certain

embodiments, the cell or composition exhibit no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for CD19 and CD20. In certain embodiments, the off-target cell is selected from the group consisting of a CD19<sup>+</sup>CD20<sup>-</sup> T cell, a Natural Killer T cell (NK T cell), a Natural Killer cell (NK cell), and a combination thereof. In certain embodiments, the cell or composition induces cytokine secretion. In certain embodiments, the cytokine is selected from the group consisting of interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and a combination thereof. In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is an EBV-sensitized CTL.

[0061] In certain embodiments, the subject is a human subject. In certain embodiments, the cell is autologous to the subject. In certain embodiments, the cell is allogeneic to the subject.

[0062] In certain embodiments, the cell or composition disclosed herein is for use in a therapy. In certain embodiments, the cell or composition disclosed herein is for use in a method of inhibiting growth of a target cell expressing at least one B-lymphocyte antigen. In certain embodiments, the cell or composition disclosed herein is for use in a method of treating a B-lymphocyte antigen-associated disease in a subject. In certain embodiments, the cell or composition disclosed herein is for use in a method of treating a hematologic cancer in a subject. In certain embodiments, the cell or composition disclosed herein is for use in a method of treating an autoimmune disease in a subject.

[0063] In another aspect, embodiments of the invention provide methods for producing a cell/cells comprising a presently disclosed CD19/CD20-targeted CAR. In certain embodiments, the method comprises: introducing into a cell a presently disclosed CD19/CD20-targeted CAR, a nucleic acid that encodes a presently disclosed CD19/CD20-targeted CAR, or a vector disclosed herein.

#### **4. BRIEF DESCRIPTION OF THE DRAWINGS**

[0064] Figure 1 depicts the structures of various bispecific CD19/CD20-targeted CARs including the presently disclosed CAR. “TM” indicates the “transmembrane domain.”

[0065] Figures 2A-2E depict the CD19- and CD20-specific activities of the bispecific CD19/CD20-targeted CARs in serial challenge assays. The “20-19 1XX” CAR or “20-19 BB06z” CAR was transduced and expressed in allogeneic EBV-sensitized CTLs derived from Donor 40 using a proprietary manufacturing process MP1. Figure 2A shows the results of

antigen-specific cytolysis of CD19<sup>+</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. Figure 2B shows the results of antigen-specific cytolysis of CD19<sup>-</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 2C shows the results of antigen-specific cytolysis of CD19<sup>+</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 2D shows the results of antigen-specific cytolysis of CD19<sup>low</sup>CD20<sup>+</sup> Raji cells by the CAR-T cells. Figure 2E shows the results of antigen-specific cytolysis of wild type (WT) CD19<sup>-</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. “40 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. “40 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. “40 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 40 (not expressing a CAR). “Target only” represents the setting where no allogeneic EBV-sensitized CTLs were added to the assay, i.e., only the target cells exist as a negative control.

**[0066]** Figure 3 depicts an experimental layout of the *in vivo* xenograft mouse model with Burkitt’s Lymphoma (Raji cells).

**[0067]** Figures 4A-4C depict the *in vivo* tumor growth inhibition of 20-19 1XX CAR-T cells and 20-19 BB06z CAR-T cells in a treatment model of Burkitt’s Lymphoma (Raji cells). The mice received  $3 \times 10^6$  CAR-T cells. Figure 4A shows the tumor burden of the mice who received non-transduced allogeneic EBV-sensitized CTLs derived from Donor 40 (not expressing a CAR). Figure 4B shows the tumor burden of the mice who received  $3 \times 10^6$  allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. Figure 4C shows the tumor burden of the mice who received  $3 \times 10^6$  allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. PBS was used as a negative control. “40 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. “40 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. “40 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 40 (not expressing a CAR).

**[0068]** Figures 5A and 5B depict the association between the expansion of the CAR-T cells and the overall survival of the treated mice. Figure 5A shows the *in vivo* CAR-T expansion. Figure 5B shows the overall survival of the treated mice who received  $3 \times 10^6$  CAR-T cells. “40 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. “40 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. “40 NTD” represents non-transduced allogeneic EBV-

sensitized CTLs derived from Donor 40 (not expressing a CAR). “Tumor only” represents the setting where no allogeneic EBV-sensitized CTLs were added to the assay, i.e., only the target cells exist as a negative control.

**[0069]** Figures 6A-6E depict the CD19- and CD20-specific activities of bispecific CD19/CD20-targeted CARs in serial challenge assays. The “20-19 1XX” CAR or “20-19 BB06z” CAR was transduced and expressed in allogeneic EBV-sensitized CTLs derived from Donor 14 using a proprietary manufacturing process MP2. Figure 6A shows the results of antigen-specific cytotoxicity of CD19<sup>+</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. Figure 6B shows the results of antigen-specific cytotoxicity of CD19<sup>-</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 6C shows the results of antigen-specific cytotoxicity of CD19<sup>+</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 6D shows the results of antigen-specific cytotoxicity of CD19<sup>low</sup>CD20<sup>+</sup> Raji cells by the CAR-T cells. Figure 6E shows the results of antigen-specific cytotoxicity of wild type (WT) CD19<sup>-</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. “14 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 14 expressing the “20-19 1XX” CAR. “14 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 14 expressing the “20-19 BB06z” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 14 (not expressing a CAR). “Target only” represents the setting where no allogeneic EBV-sensitized CTLs were added to the assay, i.e., only the target cells exist as a negative control.

**[0070]** Figures 7A-7E depict the CD19- and CD20-specific activities of bispecific CD19/CD20-targeted CARs in serial challenge assays. The “20-19 1XX” CAR or “20-19 BB06z” CAR was transduced and expressed in allogeneic EBV-sensitized CTLs derived from Donor 40 using the proprietary manufacturing process MP2. Figure 7A shows the results of antigen-specific cytotoxicity of CD19<sup>+</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. Figure 7B shows the results of antigen-specific cytotoxicity of CD19<sup>-</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 7C shows the results of antigen-specific cytotoxicity of CD19<sup>+</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 7D shows the results of antigen-specific cytotoxicity of CD19<sup>low</sup>CD20<sup>+</sup> Raji cells by the CAR-T cells. Figure 7E shows the results of antigen-specific cytotoxicity of wild type (WT) CD19<sup>-</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. “40 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. “40 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. “40 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 40 (not

expressing a CAR). “Target only” represents the setting where no allogeneic EBV-sensitized CTLs were added to the assay, i.e., only the target cells exist as a negative control.

**[0071]** Figure 8 depicts the structure of the Benchmark CD19/CD20-targeted CAR tested in Example 4.

**[0072]** Figure 9 depicts an experimental layout of the *in vivo* xenograft model with Burkitt’s Lymphoma (Raji cells).

**[0073]** Figures 10A-10C depict the anti-tumor efficacy of allogeneic EBV-sensitized CTLs expressing the “20-19 1XX” CAR and autologous T cells expressing the Benchmark CD19/CD20-targeted CAR in challenging the *in vivo* lymphoma model. Figure 10A shows the tumor burden of the mice who received the tested T cells. “CD20/19 Auto Benchmark” represents autologous T cells expressing a Benchmark CD19/CD20-targeted CAR described in Example 4. Figure 10B shows the blood cell counts of the mice treated with the CAR-T cells. Figure 10C shows the vector copy number (VCN) per cell of the CAR-T cells.

**[0074]** Figures 11A-11C depict the CAR expression and T cell phenotypes of the allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR. “20-19 1XX” CAR T cells were produced from three different donors. Figure 11A shows the CAR expression and vector copy number (VCN) analyzed by flow cytometry. Figure 11B shows the CD4 and CD8 distribution in “20-19 1XX” products. Figure 11C shows the flow cytometry analyses of memory markers CD45RO and CD62L. Bars represent a standard error of the mean.

**[0075]** Figures 12A and 12B depict the CD19- and CD20-specific activity of the “20-19 1XX” CAR T cells in serial challenge assays. Serial stimulation assays were conducted against various Non-Hodgkin lymphoma (NHL) cell lines expressing CD19 and CD20, including Jeko-1 (Figure 12A) and Su-DHL-4 (Figure 12B), using a 5:1 effector ( $E^{CAR+}$ :target(T) (E:T) ratio. Repeated serial stimulations are denoted by vertical dashed lines.

**[0076]** Figures 13A and 13B depict the alloreactivities of the “20-19 1XX” CAR T cells. Alloreactive potential of “20-19 1XX” was tested on T cells, CD69<sup>+</sup>/4-1 BB<sup>+</sup> T cells, and the cytotoxicity of HLA-matched (autologous [auto]) and mismatched (allogeneic [allo]) CD20<sup>+</sup>/CD19<sup>+</sup> B-lymphoblastoid cell line (BLCL) targets. Figure 13A shows the activation of the T cells after two days in co-culture with the “20-19 1XX” CAR T cells. Figure 13B shows the cytotoxicity of BLCLs after two days in co-culture with the “20-19 1XX” CAR T cells. The maintenance of “20-19 1XX” EBV specificity was evaluated using flow cytometry. The target cell lysis was

measured using xCELLigence RTCA. Error bars represent SD. The sign “\*\*\*\*\*” indicates  $P < 0.0001$  by two-way analysis of variance (ANOVA).

**[0077]** Figures 14A-14E depict the *in vivo* anti-tumor activities of the “20-19 1XX” CAR T cells in dosage study. Figure 14A shows the tumor burden of the mice injected with  $1 \times 10^6$  of “20-19 1XX” or NTD EBV T cells. Figure 14B shows the tumor burden of the mice injected with  $3 \times 10^6$  of “20-19 1XX” or NTD EBV T cells. Figure 14C shows the tumor burden of the mice injected with  $9 \times 10^6$  of “20-19 1XX” or NTD EBV T cells. The vertical dotted lines indicate the injections. Tumor burdens were measured by bioluminescence imaging (BLI). Figure 14D shows the absolute count of circulating human hCD45<sup>+</sup> T cells. Figure 14E shows the measurement of vector copy numbers.

**[0078]** Figures 15A-15H depict the *in vivo* anti-tumor activity of the “20-19 1XX” CAR T cells at the dose of  $3 \times 10^6$  CAR T cells per mouse. NSG mice intravenously implanted with  $0.5 \times 10^6$  Raji CD19<sup>low</sup> cells were measured in geometric mean of BLI (Figure 15A), the mean absolute count of circulating human T cells (Figure 15B), the mean VCN (Figure 15C), and survival (Figure 15D). NSG mice intravenously implanted with  $0.5 \times 10^6$  Raji CD19<sup>high</sup> cells were measured in geometric mean of BLI (Figure 15E), the mean absolute count of circulating human T cells (Figure 15F), the mean VCN (Figure 15G), and survival (Figure 15H). The vertical dotted lines indicate the injections. Tumor burdens were measured by BLI. Error bars represent SD. The \* sign indicates  $P < 0.1$  and the \*\* sign indicates  $P < 0.01$ . One outlier from “20-19 1XX” and NTD were excluded from geometric mean calculation. Log rank tests were each tested against “20-19 1XX”.

**[0079]** Figure 16 depicts an experimental layout used for the single killing assay.

**[0080]** Figures 17A-17D depict the CD19- and CD20-specific activities of bispecific CD19/CD20-targeted CARs in single killing assays. Figure 17A shows the results of antigen-specific cytotoxicity of CD19<sup>+</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. Figure 17B shows the results of antigen-specific cytotoxicity of CD19<sup>-</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 17C shows the results of antigen-specific cytotoxicity of CD19<sup>+</sup>CD20<sup>+</sup> K562 cells by the CAR-T cells. Figure 17D shows the results of antigen-specific cytotoxicity of wild type (WT) CD19<sup>-</sup>CD20<sup>-</sup> K562 cells by the CAR-T cells. “20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 104 expressing the “20-19 1XX” CAR. “NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 104 (not expressing a CAR).

[0081] Figure 18 depicts an experimental layout of the single killing assay.

[0082] Figures 19A-19C depict the CD19- and CD20-specific activity of the bispecific CD19/CD20-targeted CARs in serial challenge assays. CAR- T cells and target cells were co-cultured at an effector ( $E^{CAR+}$ ):target(T) ratio of 5:1. The “20-19 1XX” CAR was transduced and expressed in allogeneic EBV-sensitized T cells derived from Donor 14 or Doner 23. Figure 19A shows the results of antigen-specific cytolysis of knockout (KO) CD19<sup>-</sup> Raji cells by the CAR-T cells. Figure 19B shows the results of antigen-specific cytolysis of CD19<sup>low</sup> Raji cells by the CAR-T cells. Figure 19C shows the results of antigen-specific cytolysis of CD19<sup>high</sup> Raji cells by the CAR-T cells. “14 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 14 expressing the “20-19 1XX” CAR. “23 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 23 expressing the “20-19 1XX” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 14 (not expressing a CAR). “23 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 23 (not expressing a CAR). “Target only” represents the setting where no allogeneic EBV-sensitized T cells were added to the assay, i.e., only the target cells existed as a negative control.

[0083] Figures 20A-20C depict the CD19- and CD20-specific activity of the bispecific CD19/CD20-targeted CARs in serial challenge assays. CAR- T cells and target cells were co-cultured at an effector ( $E^{CAR+}$ ):target(T) ratio of 1:1. The “20-19 1XX” CAR was transduced and expressed in allogeneic EBV-sensitized T cells derived from Donor 14 or Doner 23. Figure 20A shows the results of antigen-specific cytolysis of knockout (KO) CD19<sup>-</sup> Raji cells by the CAR-T cells. Figure 20B shows the results of antigen-specific cytolysis of CD19<sup>low</sup> Raji cells by the CAR-T cells. Figure 20C shows the results of antigen-specific cytolysis of CD19<sup>high</sup> Raji cells by the CAR-T cells. “14 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 14 expressing the “20-19 1XX” CAR. “23 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 23 expressing the “20-19 1XX” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 14 (not expressing a CAR). “23 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 23 (not expressing a CAR). “Target only” represents the setting where no allogeneic EBV-sensitized T cells were added to the assay, i.e., only the target cells existed as a negative control.



[0084] Figures 21A-21D depict the CD19- and CD20-specific activities of the bispecific CD19/CD20-targeted CARs in serial challenge assays against various NHL cell lines. CAR- T cells and target cells were co-cultured at an effector ( $E^{CAR+}$ ):target(T) ratio of 1:1. The “20-19 1XX” CAR was transduced and expressed in allogeneic EBV-sensitized T cells derived from Donor 14 or Doner 104. Figure 21A shows the results of antigen-specific cytolysis of Raji cells (Burkitt lymphoma) by the CAR-T cells. Figure 21B shows the results of antigen-specific cytolysis of Jeko-1 cells (mantle cell lymphoma (MCL)) by the CAR-T cells. Figure 21C shows the results of antigen-specific cytolysis of Su-DHL-4 cells (diffuse large B cell lymphoma (DLBCL)) by the CAR-T cells. Figure 21D shows the results of antigen-specific cytolysis of MEC-1 cells (chronic lymphocytic leukemia (CLL)) by the CAR-T cells. “14 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 14 expressing the “20-19 1XX” CAR. “104 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 104 expressing the “20-19 1XX” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 14 (not expressing a CAR). “104 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 104 (not expressing a CAR). “Target only” represents the setting where no allogeneic EBV-sensitized T cells were added to the assay, i.e., only the target cells existed as a negative control.

[0085] Figures 22A-22D depict the anti-tumor efficacy of allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR in challenging the *in vivo* Burkitt’s lymphoma model (Raji cells). “14 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 14 expressing the “20-19 1XX” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 14 (not expressing a CAR). “23 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 23 expressing the “20-19 1XX” CAR. “23 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 23 (not expressing a CAR). Figure 22A shows the tumor burden of the mice who received the tested T cells. Figure 22B shows the blood cell counts of the mice treated with the CAR-T cells. Figure 22C shows the vector copy number (VCN) per cell of the CAR-T cells. Figure 22D shows no severe toxicity detected in the mice treated with the tested CAR T cells.

[0086] Figure 23 depicts an experimental layout of the *in vivo* xenograft model with Mantle Cell Lymphoma (MCL) (Jeko-1 cells).

[0087] Figures 24A-24D depict the dose-dependent anti-tumor efficacy of allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR in challenging the *in vivo* mantle cell lymphoma (MCL) model (Jeko-1 cells). Figure 24A shows the tumor burden of the mice who received the tested CAR-T cells. Figure 24B shows the blood cell counts of the mice treated with the tested CAR-T cells. Figure 24C shows the vector copy number (VCN) per cell of the tested CAR-T cells. Figure 24D shows no severe toxicity detected in the mice treated with the tested CAR T cells.

[0088] Figures 25A and 25B depict the characterization of the peripheral blood mononuclear cells (PBMCs) from Multiple Sclerosis (MS) donors. Figure 25A shows the percentage of CD20<sup>+</sup> T-cell in the PBMCs from the MS donors. Figure 25B shows the percentage of CD19<sup>+</sup>/CD20<sup>+</sup> B cells in the PBMCs from the MS donors. \*\*<0.005 and \*<0.05 were obtained by two-way ANOVA.

[0089] Figure 26 depicts an experimental layout used for the *in vitro* cytotoxicity assays on multiple sclerosis (MS) B cells.

[0090] Figures 27A-27C depict the dose-dependent cytotoxic activities of T cells comprising “20-19 1XX” CAR against B cells of MS donors post 24 hours. Figure 27A shows the cytotoxicity of the CAR-T cells against B cells from MS Doner A. Figure 27B shows the cytotoxicity of the CAR-T cells against B cells from MS Doner B. Figure 27C shows the cytotoxicity of the CAR-T cells against B cells from MS Doner C.

[0091] Figures 28A-28D depict the cytotoxic activities of T cells comprising “20-19 1XX” CAR against B cells of MS donors over 72 hours. Figure 28A shows the cytotoxicity of the CAR-T cells against B cells from MS Doner A. Figure 28B shows the cytotoxicity of the CAR-T cells against B cells from MS Doner B. Figure 28C shows the cytotoxicity of the CAR-T cells against B cells from MS Doner C. Figure 28D shows the cytotoxicity of the CAR-T cells against B cells from MS Doners A, B and C measured at 48 hours.

[0092] Figures 29A-29I show the lack of off-target cytotoxic activities of T cell comprising “20-19 1XX” against non-B cell from the PBMCs of MS donors. Figures 29A, 29D, and 29G shows the lack of off-target cytolysis of the CAR-T cells against non-B cells of Donor A. Figures 29B, 29E, and 29H shows the lack of off-target cytolysis of the CAR-T cells against over 72 hours against non-B cells of Donor B. Figures 29C, 29F, and 29I shows the lack of off-target cytolysis over 48 hours of the CAR-T cells against non-B cells of Donor C.

**[0093]** Figures 30A and 30B depict the cytolytic activities of “20-19 1XX” CAR T cells on CD20<sup>+</sup> T cells from the PBMCs of MS donors. Figure 30A shows the cytotoxicity of the CAR-T cells against CD20<sup>+</sup> T cell of MS donors A, B, and C at 48 hours. Figure 30B shows the cytotoxicity of the CAR-T cells against CD20<sup>+</sup> T cells of MS donors A and B at 72 hours. Error bars represent SD. \*\*\*\*<0.0001, \*\*\*=0.0001 and \*\*<0.005 by two-way ANOVA.

**[0094]** Figures 31A and 31B depict the secretion of IFN- $\gamma$  and TNF- $\alpha$  of T cells comprising “20-19 1XX” CAR co-cultured with the PBMCs of MS donors. Figure 31A shows the secretion of IFN- $\gamma$  and TNF- $\alpha$  measured via cytometric bead array at 24 hours. Figure 31B shows the secretion of IFN- $\gamma$  and TNF- $\alpha$  measured via cytometric bead array at 72 hours. Error bars represent SD. \*\*\*=0.0001 by two-way ANOVA.

**[0095]** Figures 32A-32H depict the proliferation of T cells comprising “20-19 1XX” CAR co-cultured with the PBMCs of MS donors. Figures 32A and 32E shows the proliferation of the Donor 14 “20-19 1XX” CAR T cells and Donor 23 “20-19 1XX” CAR T cells, respectively, co-cultured with the PBMCs from MS Donor A over 48 hours. Figures 32B and 32F shows the proliferation of the Donor 14 “20-19 1XX” CAR T cells and Donor 23 “20-19 1XX” CAR T cells, respectively, co-cultured with the PBMCs from MS Donor B over 72 hours. Figures 32C and 32G shows the proliferation of the Donor 14 “20-19 1XX” CAR T cells and Donor 23 “20-19 1XX” CAR T cells, respectively, co-cultured with the PBMCs from MS Donor C over 72 hours. Figures 32D and 32H shows the proliferation of the Donor 14 “20-19 1XX” CAR T cells and Donor 23 “20-19 1XX” CAR T cells, respectively, co-cultured with NTD EBV T cells over 72 hours.

**[0096]** Figure 33 depicts an experimental layout used for the *in vitro* cytotoxicity assays on Systemic Lupus Erythematosus (SLE) B cells.

**[0097]** Figures 34A-34F depict the cytotoxic activities of T cells comprising “20-19 1XX” CAR against B cells from a SLE donor. Figure 34A shows the antigen-specific the cytotoxicity of the T cells comprising “20-19 1XX” CAR. Figures 34B and 34C show the cell count of the CTV<sup>+</sup> effector T cells. Figures 34D to 34F show the cell counts of CD56<sup>+</sup> NK cells (Figure 34D), CD56<sup>+</sup>/CD3<sup>+</sup> NK T cells (Figure 34E), and CD3<sup>+</sup> T cells (Figure 34F) in PBMCs of the SLE donor, detected via flow cytometry.

**[0098]** Figure 35 depicts the assessment of alloreactivity of allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR against HLA-mismatched targets.

## 5. DETAILED DESCRIPTION

[0099] Various embodiments of the invention provide CARs that target CD19 and CD20 (referred to as “bispecific CD19/CD20-targeted CARs”). Various embodiments of the invention also provide cells comprising such bispecific CARs. According one or more embodiments, the cells can be immunoresponsive cells and/or immune effector cells, e.g., genetically modified immunoresponsive cells (e.g., T cells or NK cells), or stem cells (e.g., pluripotent stem cells) that can differentiate into immunoresponsive cells and/or immune effector cells.

[00100] The presently disclosed subject matter is at least based on the discovery that the presently disclosed bispecific CD19/CD20-targeted CARs having a novel and unique structure, including, but not limited to, a G4S linker connecting the anti-CD19 antigen-binding domain and the anti-CD20 antigen-binding domain, a Whitlow linker connecting the anti-CD19 heavy chain variable region (V<sub>H</sub>) and the anti-CD19 light chain variable region (V<sub>L</sub>), and an intracellular domain comprising a modified CD3ζ signaling domain and a CD28 co-stimulatory signaling region, exhibited superior anti-tumor efficacy both *in vitro* and *in vivo* as compared to other bispecific CD19/20-targeted CARs having different structures.

[00101] Non-limiting embodiments of the present disclosure are described by the present specification and Examples.

[00102] For purposes of clarity of disclosure and not by way of limitation, the detailed description is divided into the following subsections:

- 5.1. Definitions;
- 5.2. B-lymphocyte Antigens;
- 5.3. Chimeric Antigen Receptors (CARs);
- 5.4. Cells;
- 5.5. Nucleic Acids and Vectors;
- 5.6. Formulations and Administration; and
- 5.7. Methods of Uses.

### 5.1. Definitions

[00103] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which the presently disclosed subject matter belongs.

**[00104]** As used herein, the term “about” or “approximately” refers to within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 3 or more than 3 standard deviations, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value.

**[00105]** As used herein, the term “immunoresponsive cell” refers to a cell that functions in an immune response or a progenitor, or progeny thereof. In certain embodiments, the immunoresponsive cell is a cell of lymphoid lineage. Non-limiting examples of cells of lymphoid lineage include T cells, Natural Killer (NK) cells, B cells, and stem cells from which lymphoid cells may be differentiated. In certain embodiments, the immunoresponsive cell is a cell of myeloid lineage.

**[00106]** As used herein, the term “activates an immunoresponsive cell” refers to induction of signal transduction or changes in protein expression in the cell resulting in initiation of an immune response. For example, when CD3 chains cluster in response to ligand binding and immunoreceptor tyrosine-based inhibition motifs (ITAMs) a signal transduction cascade is produced. In certain embodiments, when a CAR binds to an antigen, a formation of an immunological synapse occurs that includes clustering of many molecules near the bound receptor (e.g., CD4 or CD8, CD3 $\gamma/\delta/\epsilon/\xi$ , etc.). This clustering of membrane bound signaling molecules allows for ITAM motifs contained within the CD3 chains to become phosphorylated. This phosphorylation in turn initiates a T cell activation pathway ultimately activating transcription factors, such as NF- $\kappa$ B and AP-1. These transcription factors induce global gene expression of the T cell to increase IL-2 production for proliferation and expression of master regulator T cell proteins in order to initiate a T cell mediated immune response.

**[00107]** As used herein, the term “stimulates an immunoresponsive cell” refers to a signal that results in a robust and sustained immune response. In certain embodiments, this occurs after immunoresponsive cell (e.g., T cell) activation or concomitantly mediated through receptors including, but not limited to, CD28, 4-1BB, OX40, CD40 and ICOS. Receiving multiple

stimulatory signals can be important to mount a robust and long-term T-cell mediated immune response. T cells can quickly become inhibited and unresponsive to antigen. While the effects of these co-stimulatory signals may vary, they generally result in increased gene expression in order to generate long lived, proliferative, and anti-apoptotic T cells that robustly respond to antigen for complete and sustained eradication.

**[00108]** As used herein, the term “antigen-binding fragment” refers to any derivative of an antibody which is less than full-length. In exemplary embodiments, the antigen-binding fragment retains at least a significant portion of the full-length antibody’s specific binding ability. Examples of antigen-binding fragments include, but are not limited to, Fab, Fab’, F(ab’)<sub>2</sub>, scFv, Fv, dsFv diabody, Fc, and Fd fragments. The antigen-binding fragment may be produced by any means. For instance, the antigen-binding fragment may be enzymatically or chemically produced by fragmentation of an intact antibody, it may be recombinantly produced from a gene encoding the partial antibody sequence, or it may be wholly or partially synthetically produced. The antigen-binding fragment may optionally be a single chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multimolecular complex.

**[00109]** As used herein, the term “Fab fragment” refers to a fragment of an antibody comprising an antigen-binding site generated by cleavage of the antibody with the enzyme papain, which cuts at the hinge region N-terminally to the inter-H-chain disulfide bond and generates two Fab fragments from one antibody molecule.

**[00110]** As used herein, the term “F(ab’)<sub>2</sub> fragment” refers to a fragment of an antibody containing two antigen-binding sites, generated by cleavage of the antibody molecule with the enzyme pepsin which cuts at the hinge region C-terminally to the inter-H-chain disulfide bond.

**[00111]** As used herein, the term “Fc fragment” refers to the fragment of an antibody comprising the constant domain of its heavy chain.

**[00112]** As used herein, the term “Fv fragment” refers to the fragment of an antibody comprising the variable domains of its heavy chain and light chain.

**[00113]** The term “single chain variable fragment” or “scFv” refers to a variable fragment (Fv) fragment in which the heavy chain domain and the light chain domain are linked. The heavy chain domain and light chain domain are either joined directly or joined by a linker, which

connects the N-terminus of the heavy chain domain with the C-terminus of the light chain domain, or the C-terminus of the heavy chain domain with the N-terminus of the light chain domain. As used herein, the term “linker” is art-recognized and refers to a molecule or group of molecules connecting two compounds, such as two polypeptides. The linker may be comprised of a single linking molecule or may comprise a linking molecule and a spacer molecule, intended to separate the linking molecule and a compound by a specific distance. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility.

**[00114]** In certain embodiments, the linker is a Whitlow linker. In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1, which is provided below.

GSTSGSGKPGSGEGSTKG [SEQ ID NO: 1]

**[00115]** In certain embodiments, the linker is a G4S linker. In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 2, which is provided below.

GGGSGGGSGGGGS [SEQ ID NO: 2]

**[00116]** In certain embodiments, the linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3, which is provided below.

GGGSGGGSGGGSGGGSGGGGS [SEQ ID NO: 3]

**[00117]** In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 is set forth in SEQ ID NO: 4, which is provided below.

GGCTCCACGAGCGGCAGCGCAAACCCGGCAGCGCGAGGGCAGCACCAAGGGC [SEQ ID NO: 4]

**[00118]** In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2 is set forth in SEQ ID NO: 5, which is provided below.

GGTGGAGGGGGCTCTGGCGGTGGGGGGTCCGGCGGTGGCGGATCT [SEQ ID NO: 5]

**[00119]** In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 3 is set forth in SEQ ID NO: 6, which is provided below.

GGCGGTGGCGGGTCTGGCGGCGGTGGATCCGGTGGTGGTGGATCTGGAGGAGGGGGCTCCGGGG  
GCGGTGGCAGC [SEQ ID NO: 6]

**[00120]** As used herein, “complementarity determining regions” or “CDRs” are defined as the complementarity determining region amino acid sequences of an antibody which are the

hypervariable regions of immunoglobulin heavy and light chains. CDRs can be identified according to a number of known numbering systems. In certain embodiments, the CDRs are identified according to the Kabat numbering system. The Kabat CDRs are based on sequence variability and are the most commonly used (see e.g., Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The CDRs can be identified according to the Chothia numbering system. Chothia refers to the location of the structural loops (see e.g., Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987)). The CDRs can be identified according to the AbM numbering system. The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software (see e.g., Martin, in *Antibody Engineering*, Vol. 2, Chapter 3, Springer Verlag). The CDRs can be identified according to the Contact numbering system (see, e.g., MacCallum RM et al., 1996, *J Mol Biol* 5: 732-745). The Contact CDRs are based on an analysis of the available complex crystal structures. The CDRs can be identified according to the ImMunoGeneTics (IMGT)<sup>®</sup> Information System. A universal numbering system has been developed and widely adopted, IMGT<sup>®</sup> Information System (Lefranc et al., *Dev. Comp. Immunol.* 27(1):55-77 (2003)). IMGT<sup>®</sup> is an integrated information system specializing in immunoglobulins (IG), T cell receptors (TR) and major histocompatibility complex (MHC) of human and other vertebrates.

**[00121]** The term "amino acid sequence" refers to a list of abbreviations, letters, characters or words representing amino acid residues. The amino acid abbreviations used herein are conventional one letter codes for the amino acids and are expressed as follows: A, alanine; B, asparagine or aspartic acid; C, cysteine; D aspartic acid; E, glutamate, glutamic acid; F, phenylalanine; G, glycine; H histidine; I isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Z, glutamine or glutamic acid.

**[00122]** The terms "peptide," "protein," and "polypeptide" are used interchangeably to refer to a natural or synthetic molecule comprising two or more amino acids linked by the carboxyl group of one amino acid to the alpha amino group of another.

**[00123]** The terms "polypeptide fragment" or "fragment", when used in reference to a particular polypeptide, refers to a polypeptide in which amino acid residues are deleted as compared to the reference polypeptide itself, but where the remaining amino acid sequence is



usually identical to that of the reference polypeptide. Such deletions may occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both. Fragments typically are at least about 5, 6, 8 or 10 amino acids long, at least about 14 amino acids long, at least about 20, 30, 40 or 50 amino acids long, at least about 75 amino acids long, or at least about 100, 150, 200, 300, 500 or more amino acids long. A fragment can retain one or more of the biological activities of the reference polypeptide. In various embodiments, a fragment may comprise an enzymatic activity and/or an interaction site of the reference polypeptide. In certain embodiments, a fragment may have immunogenic properties.

**[00124]** As used herein, the term “substantially identical” or “substantially homologous” refers to a polypeptide or a nucleic acid molecule exhibiting at least about 50% identical or homologous to a reference amino acid sequence (for example, any of the amino acid sequences described herein) or a reference nucleotide sequence (for example, any of the nucleotide sequences described herein). In certain embodiments, such a sequence is at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99%, or at least about 100% identical or homologous to the amino acid sequence or the nucleotide sequence used for comparison.

**[00125]** The term “identity” refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleotide sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package, and can be used with, e.g., default setting. When BLASTP is used, the percent similarity is based on the BLASTP positives score and the percent sequence identity is based on the BLASTP identities score. BLASTP “Identities” shows the number and fraction of total residues in the high scoring sequence pairs which are identical; and BLASTP “Positives” shows the number and fraction of residues for which the alignment scores have positive values and which are similar to each other. Amino acid sequences having these degrees of identity or similarity or any intermediate degree of identity of similarity to the amino acid sequences

disclosed herein are contemplated and encompassed by this disclosure. The polynucleotide sequences of similar polypeptides are deduced using the genetic code and may be obtained by conventional means, in particular by reverse translating its amino acid sequence using the genetic code.

**[00126]** The term “nucleic acid” refers to a natural or synthetic molecule comprising a single nucleotide or two or more nucleotides linked by a phosphate group at the 3’ position of one nucleotide to the 5’ end of another nucleotide. The nucleic acid is not limited by length, and thus the nucleic acid can include deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

**[00127]** The term “operably linked to” refers to the functional relationship of a nucleic acid with another nucleic acid. Promoters, enhancers, transcriptional and translational stop sites, and other signal sequences are examples of nucleotide sequences operably linked to other sequences. For example, operable linkage of DNA to a transcriptional control element refers to the physical and functional relationship between the DNA and promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA.

**[00128]** As used herein, the term “signal sequence” or “leader sequence” refers to a peptide sequence (*e.g.*, 5, 10, 15, 20, 25 or 30 amino acids) present at the N-terminus of proteins that directs their entry to the secretory pathway.

**[00129]** As used herein, the term “a conservative sequence modification” refers to an amino acid modification that does not significantly affect or alter the binding characteristics of the presently disclosed bispecific CD19/CD20-targeted CARs. Conservative modifications can include amino acid substitutions, additions and deletions. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Amino acids can be classified into groups according to their physicochemical properties such as charge and polarity. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid within the same group. For example, amino acids can be classified by charge: positively-charged amino acids include lysine, arginine, histidine, negatively-charged amino acids include aspartic acid, glutamic acid, neutral charge amino acids include alanine, asparagine, cysteine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In addition, amino acids can be classified by polarity: polar amino acids include arginine (basic

polar), asparagine, aspartic acid (acidic polar), glutamic acid (acidic polar), glutamine, histidine (basic polar), lysine (basic polar), serine, threonine, and tyrosine; non-polar amino acids include alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine. Thus, one or more amino acid residues within a CDR region can be replaced with other amino acid residues from the same group and the altered antibody can be tested for retained function using the functional assays described herein. In certain embodiments, no more than one, no more than two, no more than three, no more than four, no more than five residues within a specified sequence or a CDR region are altered.

**[00130]** The term “vector” refers to a nucleic acid capable of transporting into a cell another nucleic acid to which the vector sequence has been linked. The term “expression vector” includes any vector, (e.g., a plasmid, cosmid or phage chromosome) comprising a gene construct in a form suitable for expression by a cell (e.g., linked to a transcriptional control element).

**[00131]** The term “pharmaceutically acceptable” refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

**[00132]** The term “treatment” refers to the medical management of a subject with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

**[00133]** An “individual” or “subject” herein is a vertebrate, such as a human or a non-human animal, for example, a mammal. Mammals include, but are not limited to, humans, primates, farm animals, sport animals, rodents and pets. Non-limiting examples of non-human animal

subjects include rodents such as mice, rats, hamsters, and guinea pigs, rabbits, dogs, cats, sheep, pigs, goats, cattle, horses, and non-human primates such as apes and monkeys. In certain embodiment, the subject is a mammal. In certain embodiment, the subject is a human.

**[00134]** As used herein, the term “B-lymphocyte antigen” refers to one or both of CD-19 and CD20. Further, the term “B-lymphocyte antigen” and, in particular, “CD19” or “CD20” is intended to include fragments, variants (e.g., allelic variants), and derivatives of the antigen molecule, e.g., the CD19 molecule or the CD20 molecule. For example and without limitation, in some embodiments, CD19 is a wildtype CD19 or a mutant CD19. For example and without limitation, in some embodiments, CD20 is a wildtype CD20 or a mutant CD20. In some such embodiments, B-lymphocyte antigen is expressed on the cell surface (e.g., on the surface of a pre-cancerous or malignant cell). Cells expressing the B-lymphocyte antigen may include without limitation, B cells, T cells, and tumor cells (e.g., cancer cells, e.g., hematologic cancer cells). In some embodiments, the cell expressing the B-lymphocyte antigen is a B cell. In some embodiments, the cell expressing the B-lymphocyte antigen is a T cell. In some embodiments, the cell expressing the B-lymphocyte antigen is a tumor cell. In some embodiments, the cell expressing the B-lymphocyte antigen is cancer cell. In some embodiments, the cell expressing the B-lymphocyte antigen is hematologic cancer cell.

**[00135]** Anti-CD19 antibodies (and scFv formats thereof) suitable for binding CD19 are well-known in the art and include, for example and without limitation, antibodies FMC63, SJ25C1 (JCAR015), and HD37 (blinatumomab).

**[00136]** Anti-CD20 antibodies (and scFv formats thereof) suitable for binding CD20 are well-known in the art and include, for example and without limitation, antibodies Leu16, Rituximab, Obinutuzumab, Ofatumumab, and TG-1101 (Ublituximab).

**[00137]** The terms “comprises”, “comprising”, and are intended to have the broad meaning ascribed to them in U.S. Patent Law and can mean “includes”, “including” and the like.

#### *5.1.1. Incorporation by Reference.*

**[00138]** Various patents, patent applications, publications, product descriptions, protocols, and sequence accession numbers are cited throughout this application, the disclosure of which are incorporated herein by reference in their entireties for all purposes.

### **5.2. B-lymphocyte Antigens**

#### *5.2.1 CD19*

**[00139]** In certain embodiments, the B-lymphocyte antigen is CD19. CD19 is also known as, for example, CD19 molecule (Cluster of Differentiation 19), B-lymphocyte surface antigen B4, T cell surface antigen Leu-12 and Common Variable Immunodeficiency 3 antigen (CVID3). CD19 is a 95 kDa Type I transmembrane glycoprotein in the immunoglobulin superfamily (IgSF). CD19 is expressed in all B lineage cells during B cell development. The majority of B cell malignancies express normal to high levels of CD19, making CD19 a promising tumor marker and a potential molecular target for therapeutic intervention in B cell cancers – notably hematologic cancers and lymphomas.

**[00140]** The presently disclosed CARs bind to CD19. In certain embodiments, the CD19 is a human CD19. In certain embodiments, the CD19 is a wild-type human CD19. In certain embodiments, the CD19 comprises or consists of the amino acid sequence with a Uniprot Reference No: P15391-1 (SEQ ID NO: 7), which is provided below.

MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLKGTSDGPTQQLTWSRESPLKPFLLKL  
SLGLPGLGIHMRPLAIWLFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGELFRWNVSD  
LGGLGCGLKNRSSEGPSSPSGKLMSPKLYVWAKDRPEIWEGEPPCLPPRDSLNSQLSQDLTMAP  
GSTLWLSCGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRPARDMWVMETGLLLPRATAQDA  
GKYYCHRGNLTMSFHLEITARPVLWHWLLRTGGWKVSAVTLAYLIFCLCSLVGILHLQRALVLR  
RKRKRMTDPTRRFFKVTPPPSSGPQNQYGNVLSLPTPTSGLGRAQRWAAGLGGTAPSYGNPSSD  
VQADGALGSRSPPGVGPEEEEEEGEGYEEDSEEDSEFYENDSNLGQDQLSQDGSQYENPEDEPLG  
PEDEDSFSNAESYENEDDELTQPVARTMDFLSPHGSADWPSREATSLGSQSYEDMRGILYAAPQ  
LRSIRGQPGPNHEEDADSYENMDNPDGPDPAWGGGGRMGWTWSTR [SEQ ID NO: 7]

**[00141]** In certain embodiments, the CD19 comprises or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identical to the amino acid sequence set forth in SEQ ID NO: 7 or a fragment thereof.

**[00142]** In certain embodiments, the CD19 comprises an extracellular domain, a transmembrane domain, and a cytoplasmic domain. In certain embodiments, the extracellular domain of the CD19 comprises amino acids 20 to 291 of SEQ ID NO: 7. In certain embodiments, the transmembrane domain of the CD19 comprises amino acids 292 to 313 of SEQ ID NO: 7. In certain embodiments, the cytoplasmic domain of the CD19 comprises amino acids 314 to 556 of SEQ ID NO: 7.

[00143] In certain embodiments, the presently disclosed CAR binds to a fragment of CD19 (e.g., a fragment of human CD19). In certain embodiments, the presently disclosed CAR binds to a fragment of the amino acid sequence set forth in SEQ ID NO: 7. In certain embodiments, the presently disclosed CAR binds to the extracellular domain of CD19 (e.g., human CD19). In certain embodiments, the presently disclosed CAR binds to amino acids 20 to 291 of SEQ ID NO: 7. In certain embodiments, the presently disclosed CAR binds to a fragment of amino acids 20 to 291 of SEQ ID NO: 7.

#### 5.2.2 *CD20*

[00144] In certain embodiments, the B-lymphocyte antigen is CD20. CD20 is also known as, for example, CD20 molecule (Cluster of Differentiation 20), Membrane Spanning 4-Domains A1 (MS4A1), B-lymphocyte surface antigen B1, Bp35 antigen, S7 antigen, FMC7 antigen, leukocyte surface antigen Leu-16, and Common Variable Immunodeficiency 5 antigen (CVID5). CD20 is a nonglycosylated 33–37 kDa phosphoprotein member of the MS4A family, which forms a tetraspan membrane-bound protein. CD20 is expressed on B cells throughout B cell differentiation prior to terminal differentiation of B cells to plasma cells. In particular, CD20 is found on B cell lymphomas, hairy cell leukemia, B cell chronic lymphocytic leukemia, and melanoma cancer stem cells, making CD20 a promising tumor marker and a potential therapeutic target for B cell malignancies – notably hematologic cancers and lymphomas.

[00145] The presently disclosed CARs bind to CD20. In certain embodiments, the CD20 is a human CD20. In certain embodiments, the CD20 is a wild-type human CD20. In certain embodiments, the CD20 comprises or consists of the amino acid sequence with a Uniprot Reference No: P11836-1 (SEQ ID NO: 8), which is provided below.

MTTPRNSVNGTFPAEPMKGPIAMQSGPKPLFRRMSSLVGPTQSFFMRRESKTLGAVQIMNGLFHI  
ALGGLLMIPAGIYAPICVTVWYPLWGGIMYIISGSLLAATEKNSRKCLVKGKMIMNSLSLFAAI  
SGMILSIMDILNIKISHFLKMESLNFIRAHTPYINIYNCEPANPSEKNSPSTQYCYSIQSLFLG  
ILSVMLIFAFFQELVIAGIVENEWKRTCSRPKSNIVLLSAEEKKEQTIEIKEEVVGLTETSSQP  
KNEEDIEIPIQEEEEETETNFPEPPQDQESSPIENDSSP [SEQ ID NO: 8]

[00146] In certain embodiments, the CD20 comprises or consists of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identical to the amino acid sequence set forth in SEQ ID NO: 8 or a fragment thereof.

[00147] In certain embodiments, the CD20 comprises four transmembrane domains, two extracellular domains, and intracellular N- and C-terminal domains. In certain embodiments, an extracellular domain of the CD20 comprises amino acids 79 to 84 of SEQ ID NO: 8. In certain embodiments, an extracellular domain of the CD20 comprises amino acids 142 to 188 of SEQ ID NO: 8. In certain embodiments, a transmembrane domain of the CD20 comprises amino acids 57 to 78 of SEQ ID NO: 8. In certain embodiments, a transmembrane domain of the CD20 comprises amino acids 85 to 105 of SEQ ID NO: 8. In certain embodiments, a transmembrane domain of the CD20 comprises amino acids 121 to 141 of SEQ ID NO: 8. In certain embodiments, a transmembrane domain of the CD20 comprises amino acids 189 to 209 of SEQ ID NO: 8. In certain embodiments, the N-terminal intracellular domain of the CD20 comprises amino acids 1 to 45 of SEQ ID NO: 8. In certain embodiments, the C-terminal intracellular domain of the CD20 comprises amino acids 210 to 297 of SEQ ID NO: 8.

[00148] In certain embodiments, the presently disclosed CAR binds to a fragment of CD20 (e.g., a fragment of human CD20). In certain embodiments, the presently disclosed CAR binds to a fragment of the amino acid sequence set forth in SEQ ID NO: 8. In certain embodiments, the presently disclosed CAR binds to an extracellular domain of CD20 (e.g., human CD20). In certain embodiments, the presently disclosed CAR binds to amino acids 79 to 84 of SEQ ID NO: 8. In certain embodiments, the presently disclosed CAR binds to a fragment of amino acids 79 to 84 of SEQ ID NO: 8. In certain embodiments, the presently disclosed CAR binds to amino acids 142 to 188 of SEQ ID NO: 8. In certain embodiments, the presently disclosed CAR binds to a fragment of amino acids 142 to 188 of SEQ ID NO: 8.

### 5.3. Chimeric Antigen Receptors (CARs)

[00149] The presently disclosed CAR binds to CD19 and CD20. CARs are engineered receptors, which graft or confer a specificity of interest onto an immune effector cell. CARs can be used to graft the specificity of a monoclonal antibody onto a cell, e.g., an immunoresponsive cell, e.g., a T cell or a NK cell; with transferring of their coding sequence, e.g., facilitated by retroviral vectors.

[00150] In certain embodiments, the presently disclosed CAR comprises an extracellular domain that comprises a first antigen-binding domain that targets CD19, a second antigen-binding domain that targets CD20, a transmembrane domain, and an intracellular domain. In certain embodiments, the transmembrane domain (TD) connects the extracellular domain (ED) to

the intracellular domain (ID) and resides within the cell membrane when expressed by a cell (*e.g.*, an immunoresponsive cell or an immune effector cell). In certain embodiments, the transmembrane domain comprises a first CD28 polypeptide. In certain embodiments, the intracellular domain comprises a signaling domain (SD) that transmits an activation signal to the cell after antigen recognition. A “signaling domain (SD)” generally comprises immunoreceptor tyrosine-based activation motifs (ITAMs) that activate a signaling cascade when the ITAM is phosphorylated. The SD may be a polypeptide selected from the group consisting of CD8, CD3 $\zeta$ , CD3 $\delta$ , CD3 $\gamma$ , CD3 $\epsilon$ , Fc $\gamma$ RI- $\gamma$ , Fc $\gamma$ RIII- $\gamma$ , Fc $\epsilon$ RI $\beta$ , Fc $\epsilon$ RI $\gamma$ , DAP10, DAP12, CD32, CD79a, CD79b, and mutants thereof. In certain embodiments, the SD comprises a modified CD3 $\zeta$  polypeptide. In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises a native ITAM1, an ITAM2 variant, and an ITAM3 variant. In certain embodiments, each of the ITAM2 variant and the ITAM3 variant comprises two loss-of-function mutations. In certain embodiments, the intracellular domain further comprises a co-stimulatory signaling region (CSR). The term “co-stimulatory signaling region (CSR)” refers to an intracellular signaling domain from a costimulatory protein receptor, such as CD28, 41BB, inducible co-stimulator (ICOS), CD80, CD86, OX40, DAP10, DAP12, MyD88, CD7, CD27, CD4, B2C, BTNL3, NKG2D, NKp30, and variants thereof, which are able to enhance the activation of an immunoresponsive cell or an immune effector cell (*e.g.*, a T cell).

**[00151]** In certain embodiments, the disclosed CAR is defined by the formula: LP–ED–HD–TM–CSR–ISD, wherein “LP” represents an optional leader peptide, wherein “ED” represents an extracellular domain that comprises a first antigen-binding domain that targets CD19 and a second antigen-binding domain that targets CD20, wherein “HD” represents an optional hinge domain, wherein “TM” represents a transmembrane domain, wherein “CSR” represents one or more co-stimulatory signaling regions, wherein “SD” represents a signaling domain, and wherein “–” represents a peptide bond or linker.

**[00152]** In certain embodiments, the first anti-CD19 antigen-binding domain is connected to the HG.

**[00153]** Additional CAR constructs are described, for example, in Fresnak et al., *Nat. Rev. Cancer* 16(9):566-81 (2016), which is incorporated by reference in its entirety for the teaching of these CAR models.



[00154] In certain embodiments, the CAR can be for example (and without limitation), a TRUCK, Universal CAR, a Self-driving CAR, an Armored CAR, a Self-destruct CAR, a Conditional CAR, or a Marked CAR.

[00155] TRUCKs (T cells redirected for universal cytokine killing) co-express a chimeric antigen receptor (CAR) and an antitumor cytokine. Cytokine expression may be constitutive or induced by T cell activation. Targeted by CAR specificity, localized production of pro-inflammatory cytokines recruits endogenous immune cells to tumor sites and may potentiate an antitumor response.

[00156] Universal, allogeneic CAR T cells are engineered to no longer express endogenous T cell receptor (TCR) and/or major histocompatibility complex (MHC) molecules, thereby preventing graft-versus-host disease (GVHD) or rejection, respectively.

[00157] Self-driving CARs co-express a CAR and a chemokine receptor, which binds to a tumor ligand, thereby enhancing tumor homing.

[00158] CAR T cells engineered to be resistant to immunosuppression (Armored CARs) may be genetically modified to no longer express various immune checkpoint molecules (e.g., cytotoxic T lymphocyte-associated antigen 4 (CTLA4) or programmed cell death protein 1 (PD-1)). Exemplary “Knockdown” and “Knockout” techniques include, but are not limited to, RNA interference (RNAi) (e.g., asRNA, miRNA, shRNA, siRNA, etc.) and CRISPR interference (CRISPRi) (e.g., CRISPR-Cas9). In certain embodiments, CAR T cells are engineered to express a dominant-negative form of a checkpoint molecule. In certain embodiments, the extracellular ligand-binding domain of the immune checkpoint molecule is fused to a transmembrane membrane in order to compete for ligand binding. For example, the extracellular ligand-binding domain of PD-1 may be fused to a CD8 transmembrane domain, thus competing for PD-1 ligand from the target cell. In certain embodiments, CAR T cells are engineered to express an immune checkpoint switch receptor to exploit the inhibitory immune checkpoint ligand present on a target cell. In certain embodiments, the extracellular ligand-binding domain of the immune checkpoint molecule is fused to a signaling, stimulatory, and/or co-stimulatory domain. For example, the extracellular ligand-binding domain of PD-1 may be fused to a CD28 domain, thus providing CD28 co-stimulation while blocking PD-1 signaling. In certain embodiments, the CAR T cells may be administered with an aptamer or a monoclonal antibody that blocks immune checkpoint signaling. In certain embodiments, the CAR T cells (e.g., CAR

T cell therapy) are combined with a PD-1 blockade method, such as administration with PD-1/PD-L1 antagonistic aptamers or anti-PD-1/PD-L1 antibodies. In certain embodiments, the CAR T cells and PD-1 pathway-blocking antibodies are administered conjointly. In certain embodiments, the CAR T cells are engineered to express or express and secrete an immune checkpoint-blocking antibody, such as anti-PD-1 or anti-PD-L1, or fragments thereof. In certain embodiments, the CAR T cells are administered with a vector (e.g., an engineered virus) that expresses an immune checkpoint-blocking molecule described herein.

**[00159]** A self-destruct CAR may be designed using RNA delivered by electroporation to encode the CAR. Alternatively, inducible apoptosis of the T cell may be achieved based on ganciclovir binding to thymidine kinase in gene-modified lymphocytes or the more recently described system of activation of human caspase 9 by a small-molecule dimerizer.

**[00160]** A conditional CAR T cell is by default unresponsive, or switched “off”, until the addition of a small molecule to complete the circuit, enabling full transduction of both signal 1 and signal 2, thereby activating the CAR T cell. Alternatively, T cells may be engineered to express an adaptor-specific receptor with affinity for subsequently administered secondary antibodies directed at target antigen.

**[00161]** Marked CAR T cells express a CAR plus a tumor epitope to which an existing monoclonal antibody agent binds. In the setting of intolerable adverse effects, administration of the monoclonal antibody clears the CAR T cells and alleviates symptoms with no additional off-tumor effects.

**[00162]** There are three generations of CARs. “First generation” CARs are typically composed of an extracellular antigen-binding domain (e.g., an scFv), which is fused to a transmembrane domain, which is fused to an intracellular domain. “First generation” CARs can provide *de novo* antigen recognition and cause activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells through their CD3ζ chain signaling domain in a single fusion molecule, independent of HLA-mediated antigen presentation. “Second generation” CARs add an intracellular signaling domain from a co-stimulatory molecule (e.g., CD28, 4-1BB, ICOS, OX40) to the intracellular domain of the CAR to provide additional signals to the cell. “Second generation” CARs comprise those that provide both co-stimulation (e.g., CD28 or 4-1BB) and activation (CD3ζ). “Third generation” CARs combine multiple signaling domains to further augment potency, e.g., are made by combining multiple signaling domains, such as CD3 ζ-CD28-OC40 or CD3ζ-CD28-4-

1BB, to augment potency with stronger cytokine production and killing ability. In certain embodiments, the presently disclosed CAR is a second-generation CAR. In certain embodiments, the presently disclosed CAR is a third-generation CAR.

#### 5.3.1. *Extracellular Domain of A CAR*

**[00163]** The extracellular domain of the presently disclosed CARs comprises a first antigen-binding domain that targets CD19 and a second antigen-binding domain that targets CD20. Such antigen-binding domains are typically derived from antibodies. In certain embodiments, the first and/or the second antigen-binding domains comprise or are functional antibody fragments or derivatives thereof (*e.g.*, single chain variable fragments (scFvs) or Fabs, or any suitable antigen-binding fragments of antibodies).

**[00164]** In certain embodiments, the first anti-CD19 antigen-binding domain comprises a first anti-CD19 scFv. In certain embodiments, the first anti-CD19 scFv is derived from a monoclonal antibody (mAb). The first anti-CD19 scFv can be a human scFv, a humanized scFv, or a murine scFv. In certain embodiments, the first anti-CD19 scFv is a murine scFv. In certain embodiments, the first anti-CD19 scFv is a mouse scFv. The first anti-CD19 scFv can be derived from fusing the heavy chain variable region ( $V_H$ ) and light chain variable region ( $V_L$ ) of an antibody. Alternatively or additionally, the first anti-CD19 scFv may be derived from Fab's (instead of from an antibody, *e.g.*, obtained from Fab libraries).

**[00165]** In certain embodiments, the second anti-CD20 antigen-binding domain comprises a second anti-CD20 scFv. In certain embodiments, the second anti-CD20 scFv is derived from a monoclonal antibody (mAb). The second anti-CD20 scFv can be a human scFv, a humanized scFv, or a murine scFv. In certain embodiments, the second anti-CD20 scFv is a murine scFv. In certain embodiments, the second anti-CD20 scFv is a mouse scFv. The second anti-CD20 scFv can be derived from fusing the  $V_H$  and  $V_L$  of an antibody. Alternatively or additionally, the second anti-CD20 scFv may be derived from Fab's (instead of from an antibody, *e.g.*, obtained from Fab libraries).

**[00166]** Alternatively, the first and/or the second antigen-binding domains can comprise or are Fabs or F(ab)<sub>2</sub>.

**[00167]** In certain embodiments, the first anti-CD19 antigen-binding domain comprises a first  $V_H$  and a first  $V_L$ . In certain embodiments, the first  $V_H$  comprises a  $V_H$  CDR1, a  $V_H$  CDR2, and a  $V_H$  CDR3 of a  $V_H$  comprising the amino acid sequence of SEQ ID NO: 15. In certain

embodiments, the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1, a V<sub>L</sub> CDR2, and a V<sub>L</sub> CDR3 of a V<sub>L</sub> comprising the amino acid sequence of SEQ ID NO: 16. SEQ ID NO: 15 and SEQ ID NO: 16 are disclosed in Table 1.

**[00168]** In certain embodiments, the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof. In certain embodiments, the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11. SEQ ID NOS: 9-11 are provided in Table 1.

**[00169]** In certain embodiments, the first V<sub>H</sub> comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 15. For example, the first V<sub>H</sub> comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 15. In certain embodiments, the first V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 15.

**[00170]** In certain embodiments, the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14 or a conservative modification thereof. In certain embodiments, the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14. SEQ ID NOS: 12-14 are provided in Table 1.

**[00171]** In certain embodiments, the first V<sub>L</sub> comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 16. For example, the first V<sub>L</sub>

comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 16. In certain embodiments, the first V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 16.

**[00172]** In certain embodiments, the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof; and the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14 or a conservative modification thereof. In certain embodiments, the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; and the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12; a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14.

**[00173]** In certain embodiments, the first V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 15, and the first V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 16. The first V<sub>H</sub> and first V<sub>L</sub> can be linked one after another, e.g., by a linker. In certain embodiments, the first V<sub>H</sub> and first V<sub>L</sub> are linked via a first linker. In certain embodiments, the first linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 1. The variable regions from the N- to the C-terminus can be V<sub>H</sub>-V<sub>L</sub> or V<sub>L</sub>-V<sub>H</sub>. In certain embodiments, the first V<sub>L</sub> is positioned at the N-terminus of the variable regions, i.e., the first V<sub>H</sub> and the first V<sub>L</sub> are positioned from the N- to the C-terminus as V<sub>L</sub>-V<sub>H</sub>.

**[00174]** In certain embodiments, the first anti-CD19 antigen-binding domain is a first anti-CD19 scFv. In certain embodiments, the first anti-CD19 scFv comprises a first V<sub>H</sub> disclosed

herein and a first V<sub>L</sub> disclosed herein. In certain embodiments, the first anti-CD19 scFv comprises or consists of the amino acid sequence set forth in SEQ ID NO: 17. SEQ ID NO: 17 is provided in Table 1. In certain embodiments, the first anti-CD19 scFv is derived from a mouse anti-CD19 antibody FCM63 that binds to human CD19. FCM63 is disclosed in Zola *et al.*, *Immunology and Cell Biology* (1991);69:411-422 and Nicholson *et al.*, *Molecular Immunology* (1997);34(16-17):1157-1165, which are incorporated by reference hereby in their entireties.

[00175] In certain embodiments, the CDRs are identified according to the Kabat numbering system.

**Table 1**

| CDRs                                                 | 1                                                                                                                                                                                                                                                                       | 2                               | 3                            |
|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|------------------------------|
| First V <sub>H</sub>                                 | DYGVS (SEQ ID NO: 9)                                                                                                                                                                                                                                                    | VIWGSETTYNSALKS (SEQ ID NO: 10) | HYYYGGSYAMDY (SEQ ID NO: 11) |
| First V <sub>L</sub>                                 | RASQDISKYLN (SEQ ID NO: 12)                                                                                                                                                                                                                                             | HTSRLHS (SEQ ID NO: 13)         | QQGNTLPYT (SEQ ID NO: 14)    |
| Full first V <sub>H</sub> Amino Acid                 | EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSE TTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSTVTSS (SEQ ID NO: 15)                                                                                                                                 |                                 |                              |
| Full first V <sub>L</sub> Amino Acid                 | DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNNWYQQKPDGTVKLLIYHTSRLH SGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEIT (SEQ ID NO: 16)                                                                                                                                            |                                 |                              |
| V <sub>L</sub> -V <sub>H</sub> first scFv Amino Acid | DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNNWYQQKPDGTVKLLIYHTSRLH SGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGST SGSGKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQP PRKGLEWLGVIWGSETTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYC AKHYYYGGSYAMDYWGQGTSTVTSS (SEQ ID NO: 17) |                                 |                              |

[00176] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 15 is set forth in SEQ ID NO: 18, which is provided below.

GAAGTAAACTGCAAGAGAGTGGCCCCGGTCTTGTGGCCCCCTTCTCAGAGCCTGAGCGTGACCT  
GCACCGTGAGCGGCGTGAGCCTGCCCGACTACGGCGTGAGCTGGATCAGACAGCCCCCTAGAAA  
GGGCCTGGAGTGGCTCGGTGTGATCTGGGGCAGCGAGACCACCTACTACAACAGCGCCCTGAAG  
AGCAGACTGACCATCATCAAGGACAACAGCAAGAGCCAAGTGTTCTGAAGATGAACAGCCTGC  
AGACCGACGACACCGCCATCTACTACTGTGCCAAGCATTACTACTACGGCGGCAGCTACGCCAT  
GGACTACTGGGGCCAAGGCACAAGCGTCACCGTCAGCAGC [SEQ ID NO: 18]

[00177] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 16 is set forth in SEQ ID NO: 19, which is provided below.

GATATACAGATGACACAGACCACAAGCAGCCTGAGCGCGTCGTTAGGTGACAGAGTGACCATCA  
 GCTGCCGCGCTAGCCAAGACATCAGCAAGTACCTGAACTGGTATCAGCAGAAGCCCCGACGGCAC  
 CGTGAAGCTGCTGATCTACCACACAAGCAGACTGCACAGCGGCGTACCATCTCGGTTCTCTGGC  
 AGCGGCTCTGGAACCGACTACTCGCTGACCATCAGTAATCTGGAGCAAGAGGACATCGCCACCT  
 ACTTCTGTCAGCAAGGCAACACCCTGCCCTACACATTTCGGCGGCGGTACGAAGCTGGAGATAAC  
 C [SEQ ID NO: 19]

[00178] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 17 is set forth in SEQ ID NO: 20, which is provided below.

GATATACAGATGACACAGACCACAAGCAGCCTGAGCGCGTCGTTAGGTGACAGAGTGACCATCA  
 GCTGCCGCGCTAGCCAAGACATCAGCAAGTACCTGAACTGGTATCAGCAGAAGCCCCGACGGCAC  
 CGTGAAGCTGCTGATCTACCACACAAGCAGACTGCACAGCGGCGTACCATCTCGGTTCTCTGGC  
 AGCGGCTCTGGAACCGACTACTCGCTGACCATCAGTAATCTGGAGCAAGAGGACATCGCCACCT  
 ACTTCTGTCAGCAAGGCAACACCCTGCCCTACACATTTCGGCGGCGGTACGAAGCTGGAGATAAC  
 CGGCTCCACGAGCGGCAGCGGCAACCCGGCAGCGGCGAGGGCAGCACCAAGGGCGAAGTAAAA  
 CTGCAAGAGAGTGCGCCCCGGTCTTGTGGCCCCCTTCTCAGAGCCTGAGCGTGACCTGCACCGTGA  
 GCGGCGTGAGCCTGCCCCGACTACGGCGTGAGCTGGATCAGACAGCCCCCTAGAAAGGGCCTGGA  
 GTGGCTCGGTGTGATCTGGGGCAGCGAGACCACCTACTACAACAGCGCCCTGAAGAGCAGACTG  
 ACCATCATCAAGGACAACAGCAAGAGCCAAGTGTTCTGAAGATGAACAGCCTGCAGACCGACG  
 ACACCGCCATCTACTACTGTGCCAAGCATTACTACTACGGCGGCAGCTACGCCATGGACTACTG  
 GGGCCAAGGCACAAGCGTCACCGTCAGCAGC [SEQ ID NO: 20]

[00179] In certain embodiments, the second anti-CD20 antigen-binding domain comprises a second V<sub>H</sub> and a second V<sub>L</sub>.

[00180] In certain embodiments, the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1, a V<sub>H</sub> CDR2, and a V<sub>H</sub> CDR3 of a V<sub>H</sub> comprising the amino acid sequence of SEQ ID NO: 27. In certain embodiments, the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1, a V<sub>L</sub> CDR2, and a V<sub>L</sub> CDR3 of a V<sub>L</sub> comprising the amino acid sequence of SEQ ID NO: 28. SEQ ID NO: 27 and SEQ ID NO: 28 are disclosed in Table 2.

[00181] In certain embodiments, the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23 or a conservative modification thereof. In certain embodiments, the second V<sub>H</sub> comprises a V<sub>H</sub>

CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23. SEQ ID NOs: 21-23 are provided in Table 2.

**[00182]** In certain embodiments, the second V<sub>H</sub> comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 27. For example, the second V<sub>H</sub> comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 27. In certain embodiments, the second V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 27.

**[00183]** In certain embodiments, the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25 or a conservative modification thereof, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26 or a conservative modification thereof. In certain embodiments, the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26. SEQ ID NOs: 24-26 are provided in Table 2.

**[00184]** In certain embodiments, the second V<sub>L</sub> comprises an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) identical or homologous to the amino acid sequence set forth in SEQ ID NO: 28. For example, the second V<sub>L</sub> comprises an amino acid sequence that is about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 28. In certain embodiments, the second V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 28.

**[00185]** In certain embodiments, the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof, a V<sub>H</sub> CDR2



comprising the amino acid sequence set forth in SEQ ID NO: 22 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23 or a conservative modification thereof; and the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25 or a conservative modification, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26 or a conservative modification thereof. In certain embodiments, the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23; and the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24; a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26.

**[00186]** In certain embodiments, the second V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 27, and the second V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 28.

**[00187]** The second V<sub>H</sub> and second V<sub>L</sub> can be linked one after another, e.g., by a linker. In certain embodiments, the second V<sub>H</sub> and second V<sub>L</sub> are linked via a second linker. In certain embodiments, the second linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 2. The variable regions from the N- to the C-terminus can be V<sub>H</sub>-V<sub>L</sub> or V<sub>L</sub>-V<sub>H</sub>. In certain embodiments, the second V<sub>H</sub> is positioned at the N-terminus of the variable regions, i.e., the second V<sub>H</sub> and the second V<sub>L</sub> are positioned from the N- to the C-terminus as V<sub>H</sub>-V<sub>L</sub>. In certain embodiments, the second anti-CD20 antigen-binding domain is a second anti-CD20 scFv that comprises a second V<sub>H</sub> disclosed herein and a second V<sub>L</sub> disclosed herein. In certain embodiments, the second anti-CD20 scFv comprises or consists of the amino acid sequence set forth in SEQ ID NO: 29. SEQ ID NO: 29 is provided in Table 2.

**[00188]** In certain embodiments, the second anti-CD20 scFv is derived from a mouse anti-CD20 antibody Leu16 that binds to human CD20. Leu16 is disclosed in Jensen *et al.*, *Biology of Blood Marrow Transplantation* (1998);4:75-83, which is incorporated by reference hereby in its entirety.

[00189] In certain embodiments, the CDRs are identified according to the Kabat numbering system.

Table 2

| CDRs                                                  | 1                                                                                                                                                                                                                                                              | 2                                 | 3                             |
|-------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|-------------------------------|
| Second V <sub>H</sub>                                 | SYNMH (SEQ ID NO: 21)                                                                                                                                                                                                                                          | AIYPGNGDTSYNQKFKG (SEQ ID NO: 22) | SNYYGSSYWFFDV (SEQ ID NO: 23) |
| Second V <sub>L</sub>                                 | RASSSVNYMD (SEQ ID NO: 24)                                                                                                                                                                                                                                     | ATSNLAS (SEQ ID NO: 25)           | QQWSFNPPT (SEQ ID NO: 26)     |
| Full second V <sub>H</sub> Amino Acid                 | EVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSNYYGSSYWFFDVWGAGTTVTVSS (SEQ ID NO: 27)                                                                                                                     |                                   |                               |
| Full second V <sub>L</sub> Amino Acid                 | DIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKPKGSSPKPWIYATSNLASGVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFGGGTKLEIK (SEQ ID NO: 28)                                                                                                                                     |                                   |                               |
| V <sub>H</sub> -V <sub>L</sub> second scFv Amino Acid | EVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSNYYGSSYWFFDVWGAGTTVTVSSGGGGSGGGGSDIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKPKGSSPKPWIYATSNLASGVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFGGGTKLEIK (SEQ ID NO: 29) |                                   |                               |

[00190] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 27 is set forth in SEQ ID NO: 30, which is provided below.

GAAGTGCAGCTTCAGCAGAGCGGCGCCGAAGTGGTGAAGCCCGGCGCTAGCGTGAAGATGAGCTGCAAGGCTAGCGGCTACACCTTCACAAGCTATAACATGCACTGGGTGAAGCAGACCCCCGGCCAAGGCCTGGAGTGGATCGGTGCCATCTACCCCGGCAACGGCGACACAAGCTACAATCAGAAGTTC AAGGGCAAGGCCACCCTGACCGCCGACAAGAGCAGCAGCACCGCCTACATGCAGCTGAGCAGCC TGACAAGCGAGGACAGCGCCGACTACTATTGCGCACGGAGCAACTACTACGGCAGCAGCTACTG GTTCTTCGACGTATGGGGCGCGGGCACCCACGGTCACCGTGAGCTCC [SEQ ID NO: 30]

[00191] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 28 is set forth in SEQ ID NO: 31, which is provided below.

GACATCGTGCTGACACAGAGCCCCGCCATCCTAAGCGCCTCCCCCGGTGAAAAAGTGACCATGACCTGCAGAGCTAGCAGCTCTGTGAACTACATGGACTGGTATCAGAAGAAACCTGGCAGCTCTCC AAAGCCGTGGATCTATGCCACAAGCAATCTGGCATCTGGCGTACCCGCTAGATTCAGCGGCAGC GGATCCGGCACATCATACTCCCTGACCATAAGCCGTGTGGAGGCCGAGGACGCCGCCACCTACT ACTGTCAGCAGTGGAGCTTCAACCCCCCTACATTCGAGGGGGCACCAAGCTGGAGATTAAA [SEQ ID NO: 31]

[00192] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 29 is set forth in SEQ ID NO: 32, which is provided below.

GAAGTGCAGCTTCAGCAGAGCGGCGCCGAACTGGTGAAGCCCGGCGCTAGCGTGAAGATGAGCT  
GCAAGGCTAGCGGCTACACCTTCACAAGCTATAACATGCACTGGGTGAAGCAGACCCCCGGCCA  
AGGCCTGGAGTGGATCGGTGCCATCTACCCCGGCAACGGCGACACAAGCTACAATCAGAAATTCT  
AAGGGCAAGGCCACCCTGACCGCCGACAAGAGCAGCAGCACCGCCTACATGCAGCTGAGCAGCC  
TGACAAGCGAGGACAGCGCCGACTACTATTGCGCACGGAGCAACTACTACGGCAGCAGCTACTG  
GTTCTTCGACGTATGGGGCGCGGGCACCACGGTCACCGTGAGCTCCGGTGGAGGGGGCTCTGGC  
GGTGGGGGGTCCGGCGGTGGCGGATCTGACATCGTGCTGACACAGAGCCCCGCCATCCTAAGCG  
CCTCCCCCGGTGAAAAAGTGACCATGACCTGCAGAGCTAGCAGCTCTGTGAACTACATGGACTG  
GTATCAGAAGAAACCTGGCAGCTCTCCAAAGCCGTGGATCTATGCCACAAGCAATCTGGCATCT  
GGCGTACCCGCTAGATTTCAGCGGCAGCGGATCCGGCACATCATACTCCCTGACCATAAGCCGTG  
TGGAGGCCGAGGACGCCGCCACCTACTACTGTGAGCAGTGGAGCTTCAACCCCCCTACATTCGG  
AGGGGGCACCAAGCTGGAGATTAAA [SEQ ID NO: 32]

[00193] The first V<sub>H</sub>, second V<sub>H</sub>, first V<sub>L</sub>, and/or second V<sub>L</sub> amino acid sequences consisting of at least about 80%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% (*e.g.*, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%) homology or identity to a specific sequence (*e.g.*, SEQ ID NO: 15, SEQ ID NO: 27, SEQ ID NO: 16, or SEQ ID NO: 28) may contain substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the specified sequence(s), but retain the ability to bind to a target antigen (*e.g.*, CD19 or CD20). In certain embodiments, a total of 1 to 10 amino acids are substituted, inserted and/or deleted in a specific sequence (*e.g.*, SEQ ID NO: 15, SEQ ID NO: 27, SEQ ID NO: 16, or SEQ ID NO: 28). In certain embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (*e.g.*, in the FRs) of the first and/or second antigen-binding domains.

[00194] In certain embodiments, the extracellular domain of the CAR comprises a third linker between the first anti-CD19 antigen-binding domain and the second anti-CD20 antigen-binding domain. In certain embodiments, the third linker comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3.

[00195] In addition, the CAR can comprise a leader peptide or a signal peptide that directs the CAR into the endoplasmic reticulum. The leader peptide or signal peptide can allow the CAR to be glycosylated and anchored in the cell membrane. The leader peptide or signal peptide can have a length of about 5, about 10, about 15, about 20, about 25, or about 30 amino acids. In certain embodiments, the leader peptide or signal peptide is covalently joined to the N-terminus of the extracellular domain. In certain embodiments, the leader peptide or signal peptide is covalently joined to the N-terminus of the second anti-CD20 antigen-binding domain. In certain embodiments, the leader peptide or signal peptide comprises a CD8 polypeptide. In certain embodiments, the leader peptide or signal peptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 33, which is provided below. In certain embodiments, the leader peptide or signal peptide is linked to the N-terminus of a V<sub>H</sub> disclosed herein. In certain embodiments, the leader peptide or signal peptide is linked to the N-terminus of a second V<sub>H</sub> disclosed herein. In certain embodiments, the leader peptide or signal peptide is linked to the N-terminus of a V<sub>L</sub> disclosed herein. In certain embodiments, the leader peptide or signal peptide is linked to the N-terminus of a second V<sub>L</sub> disclosed herein.

MALPVTALLLPLALLLHA (SEQ ID NO: 33)

[00196] In certain embodiments, an exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 33 is set forth in SEQ ID NO: 34, which is provided below.

ATGGCCCTGCCCGTCACCGCTCTGCTTCTGCCCCTGGCTCTGCTCTTACATGCC [SEQ ID NO: 34]

### 5.3.2. *Transmembrane Domain of a CAR*

[00197] In certain embodiments, the transmembrane domain of the CAR comprises a hydrophobic alpha helix that spans at least a portion of the membrane. Different transmembrane domains result in different receptor stability. After antigen recognition, receptors cluster and a signal are transmitted to the cell.

[00198] The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. For example, the transmembrane region may be derived from (*e.g.*, comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8 (*e.g.*, CD8 alpha, CD8 beta), CD9, CD16, CD22, CD27, CD33, CD37, CD40, CD64, CD80, CD86, CD134, CD137, CD154, ICAM-1,

KIRDS2, OX40, CD2, NKGD2, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), CD160, CD166, CD19, IL2R beta, IL2R gamma, IL7R  $\alpha$ , ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, and PAG/Cbp. In certain embodiments, the transmembrane domain of the CAR comprises a native or modified transmembrane domain of CD8, of CD28, of CD3 $\zeta$ , of CD4, of 4-1BB, of OX40, of ICOS, of CD84, of CD166, of CD8a, of CD8b, of ICAM-1, of CTLA-4, of CD27, of CD40, of NKGD2, or a combination thereof. Alternatively, the transmembrane domain may be synthetic, in which case it comprises predominantly hydrophobic residues such as leucine and valine. In some cases, a triplet of phenylalanine, tryptophan and valine can be found at each end of a synthetic transmembrane domain. A short oligo- or polypeptide linker, such as between 2 and 10 amino acids in length, may form the linkage between the transmembrane domain and the intracellular domain of the CAR.

**[00199]** In certain embodiments, the CAR comprises more than one transmembrane domain, which can be a repeat of the same transmembrane domain, or can be different transmembrane domains.

**[00200]** In certain embodiments, the transmembrane domain of the CAR comprises a CD28 polypeptide (e.g., a transmembrane domain of CD28 or a portion thereof). In certain embodiments, the transmembrane domain of the CAR comprises a transmembrane domain of human CD28 or a portion thereof. The CD28 polypeptide can comprise or consist of an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% identical or homologous to the sequence having a NCBI Reference No: NP\_006130 (SEQ ID NO: 35), or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 35, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, or at least about 60, and/or up to about 70, up to about 80, up to about 90, up to

about 100, up to about 150, up to about 200, or up to about 220 amino acids in length. In certain embodiments, the CD28 polypeptide comprises or consists of the amino acid sequence of amino acids 1 to 220, 1 to 50, 50 to 100, 100 to 150, 153 to 179, 150 to 200, 153 to 179, or 200 to 220 of SEQ ID NO: 35. In certain embodiments, the transmembrane domain of the CAR comprises a CD28 polypeptide comprising or consisting of the amino acid sequence of amino acids 153 to 179 of SEQ ID NO: 35. SEQ ID NO: 35 is provided below.

```
MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNLSCKYSYNLFSREFRASLHKGLDSAVE
VCVVGNYQQQLQVYSKTGFNC DGKLGNESVT FYLQNLVYNQTDIYFCKIEVMYPPPYLDNEKS
NGTIIHVKGKHLCPSP LFPGPSKPFWVLVVVGVLACYSLLVTVAFIIFWVRSKRSRLHSDYM
NMTPRRPGPTRKHYQPYAPPRDFAAYRS [SEQ ID NO: 35]
```

**[00201]** An exemplary nucleotide sequence encoding amino acids 153 to 179 of SEQ ID NO: 35 is set forth in SEQ ID NO: 36, which is provided below.

```
TTCTGGGTTCTGGTCGTGGTGGGCGGCGTCTGGCCTGCTACAGCCTGCTGGTGACCGTGGCCT
TCATCATCTTCTGGGTG [SEQ ID NO: 36]
```

**[00202]** In certain embodiments, the CAR further comprises a spacer/hinge domain that links the extracellular domain to the transmembrane domain. A hinge domain is a short sequence of amino acids that facilitates antibody flexibility (see, e.g., Woof et al., *Nat. Rev. Immunol.* 4(2): 89-99 (2004)). The hinge domain can be any suitable sequence derived or obtained from any suitable molecule. The hinge domain can be flexible enough to allow the extracellular domain to orient in different directions to facilitate antigen recognition while preserving the activating activity of the CAR.

**[00203]** The hinge/spacer domain of the CAR can comprise a native or modified hinge region of CD8, of CD28, of CD3 $\zeta$ , of CD40, of 4-1BB, of OX40, of CD84, of CD166, of CD8a, of CD8b, of ICOS, of ICAM-1, of CTLA-4, of CD27, of CD40, of NKGD2, or a combination thereof. The hinge/spacer region can be the hinge region from IgG1, or the CH<sub>2</sub>CH<sub>3</sub> region of immunoglobulin and portions of CD3, a portion of a CD28 polypeptide (e.g., a portion of SEQ ID NO: 35), a portion of a CD8 polypeptide, a variant of any of the foregoing which is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% identical or homologous thereto, or a synthetic spacer sequence.

**[00204]** In certain embodiments, the hinge domain of the CAR comprises a native or modified hinge region of CD28. In certain embodiments, the hinge domain of the CAR comprises a native

hinge region of CD28. In certain embodiments, the hinge domain of the CAR comprises amino acids 114 to 152 of SEQ ID NO: 35.

**[00205]** An exemplary nucleotide sequence encoding amino acids 114 to 152 of SEQ ID NO: 35 is set forth in SEQ ID NO: 37, which is provided below.

ATCGAGGTGATGTACCCCCCCCCCTACCTGGACAACGAGAAGAGCAACGGCACCATCATCCACG  
TGAAGGGCAAGCACCTGTGCCCTAGCCCCCTGTTCCCTGGCCCCCTCGAAGCCC [SEQ ID  
NO: 37]

### 5.3.3. Intracellular Domain of a CAR

**[00206]** In certain embodiments, the intracellular domain of the CAR transmits a signal to a cell comprising the CAR (e.g., an immunoresponsive cell or an immune effector cell) after antigen recognition, activating at least one of the normal effector functions of the cell. In certain embodiments, the effector function of a T cell, for example, is cytolytic activity or helper activity, including secretion of cytokines. Therefore, the intracellular domain can comprise the “intracellular signaling domain” of a T cell receptor (TCR) and optional co-receptors. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal.

**[00207]** Cytoplasmic signaling sequences that regulate primary activation of the TCR complex that act in a stimulatory manner may comprise signaling motifs, which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). Examples of ITAM-containing cytoplasmic signaling sequences include, but are not limited to, those derived from CD8, CD3 $\zeta$ , CD3 $\delta$ , CD3 $\gamma$ , CD3 $\epsilon$ , CD32 (Fc gamma RIIa), DAP10, DAP12, CD79a, CD79b, Fc $\gamma$ RI $\gamma$ , Fc $\gamma$ RIII $\gamma$ , Fc $\epsilon$ RI $\beta$  (FCERIB), and Fc $\epsilon$ RI $\gamma$  (FCERIG).

**[00208]** In certain embodiments, the intracellular domain is derived from CD3 zeta (CD3 $\zeta$ ) (TCR zeta). T-cell surface glycoprotein CD3 zeta (CD3 $\zeta$ ) chain, also known as T-cell receptor T3 zeta chain or CD247 (Cluster of Differentiation 247), is a protein that in humans is encoded by the *CD247* gene. Wild type (“native”) CD3 $\zeta$  comprises three functional ITAMs, three functional basic-rich stretch (BRS) regions (BRS1, BRS2 and BRS3). The intracellular tails of the CD3 molecules comprise a single ITAM, which is responsible for the signaling capacity of the TCR. The intracellular tail of the  $\zeta$  chain (CD3 $\zeta$ ) comprises three (3) ITAMs. The

intracellular domain of the CD3 $\zeta$ -chain is the primary transmitter of signals from endogenous TCRs.

**[00209]** In certain embodiments, the intracellular domain of the CAR comprises a native CD3 $\zeta$  polypeptide. In certain embodiments, the native CD3 $\zeta$  polypeptide comprises or consists of an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% identical or homologous to the amino acid sequence having a NCBI Reference No: NP\_932170 (SEQ ID NO: 38), or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the native CD3 $\zeta$  polypeptide comprises or consists of an amino acid sequence that is a consecutive portion of SEQ ID NO: 38, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to about 164 amino acids in length. In certain embodiments, the native CD3 $\zeta$  comprises or consists of the amino acid sequence of amino acids 1 to 164, 1 to 50, 50 to 100, 52 to 164, 100 to 150, or 150 to 164 of SEQ ID NO: 38. In certain embodiments, the intracellular domain of the CAR comprises a native CD3 $\zeta$  polypeptide comprising or consisting of amino acids 52 to 164 of SEQ ID NO: 38. SEQ ID NO: 38 is provided below.

MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAY  
QQGQNQLYNELNLGRREEYDVLDKRRGRDPENGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGM  
KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR [SEQ ID NO: 38]

**[00210]** In certain embodiments, the intracellular domain of the CAR comprises a modified CD3 $\zeta$  polypeptide. In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises a mutation, such as a point mutation, in at least one ITAM so as to render the ITAM non-functional. In certain embodiments, either the membrane-proximal ITAM (ITAM1), the membrane-distal ITAM (C-terminal third ITAM, ITAM3), or both are non-functional. In certain embodiments, either two membrane-proximal ITAMs (ITAM1 and ITAM2) or two membrane-distal ITAMs (ITAM2 and ITAM3) are non-functional. In certain embodiments, only ITAM2 is non-functional. In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises a deletion (e.g., a truncation) mutation such that at least one ITAM is missing. In certain embodiments, the modified CD3 $\zeta$  polypeptide is missing the membrane-proximal ITAM (ITAM1), the membrane-distal ITAM (ITAM3), or both. In other embodiments, the modified CD3 $\zeta$  polypeptide is missing either two membrane-proximal ITAMs (ITAM1 and ITAM2) or two membrane-distal



ITAMs (ITAM2 and ITAM3). In certain embodiments, the modified CD3 $\zeta$  polypeptide is missing ITAM2. Removing at least one ITAM from the introduced CAR may reduce CD3 $\zeta$ -mediated apoptosis. Alternatively, removing at least one ITAM from the introduced CAR can reduce its size without loss of function.

**[00211]** In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises one, two or three ITAMs. In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises a native ITAM1. In certain embodiments, the native ITAM1 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 39.

QNQLYNELNLGRREEYDVLDKR [SEQ ID NO: 39]

**[00212]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 39 is set forth in SEQ ID NO: 40, which is provided below.

CAGAATCAGCTGTACAACGAGCTGAACCTGGGCAGAAGAGAGGAGTACGACGTGCTGGACAAGA  
GA [SEQ ID NO: 40]

**[00213]** In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises an ITAM1 variant comprising one or more loss-of-function mutations. In certain embodiments, the ITAM1 variant comprises or consists of two loss-of-function mutations. In certain embodiments, each of the one or more (e.g., two) loss of function mutations comprises a mutation of a tyrosine residue in ITAM1. In certain embodiments, the ITAM1 variant consists of two loss-of-function mutations. In certain embodiments, the ITAM1 variant comprises or consists of the amino acid sequence set forth in SEQ ID NO: 41, which is provided below.

QNQLFNEELNLGRREEFDVLDKR [SEQ ID NO: 41]

**[00214]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 41 is set forth in SEQ ID NO: 42, which is provided below.

CAGAACCAGCTCTTTAACGAGCTCAATCTAGGACGAAGAGAGGAGTTCGATGTTTTGGACAAGA  
GA [SEQ ID NO: 42]

**[00215]** In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises a native ITAM2. In certain embodiments, the native ITAM2 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 43, which is provided below.

QEGLYNELQKDKMAEAYSEIGMK [SEQ ID NO: 43]

**[00216]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 43 is set forth in SEQ ID NO: 44, which is provided below.

CAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAA [SEQ ID NO: 44]

**[00217]** In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises an ITAM2 variant. In certain embodiments, the ITAM2 variant comprises or consists of one or more loss-of-function mutations. In certain embodiments, the ITAM2 variant comprises or consists of two loss-of-function mutations. In certain embodiments, each of the one or more (e.g., two) the loss of function mutations comprises a mutation of a tyrosine residue in ITAM2. In certain embodiments, the ITAM2 variant consists of two loss-of-function mutations. In certain embodiments, the ITAM2 variant comprises or consists of the amino acid sequence set forth in SEQ ID NO: 45, which is provided below.

QEGLFNELQKDKMAEAFSEIGMK [SEQ ID NO: 45]

**[00218]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 45 is set forth in SEQ ID NO: 46, which is provided below.

CAAGAGGGCCTGTTCAACGAGCTGCAGAAGGACAAGATGGCCGAGGCCTTCAGCGAGATCGGCA TGAAG [SEQ ID NO: 46]

**[00219]** In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises a native ITAM3. In certain embodiments, the native ITAM3 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 47, which is provided below.

HDGLYQGLSTATKDTYDALHMQ [SEQ ID NO: 47]

**[00220]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 47 is set forth in SEQ ID NO: 48, which is provided below.

CACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGC AG [SEQ ID NO: 48]

**[00221]** In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises an ITAM3 variant. In certain embodiments, the ITAM3 variant comprises or consists of two loss-of-function mutations. In certain embodiments, each of the one or more (e.g., two) the loss of function mutations comprises a mutation of a tyrosine residue in ITAM3. In certain embodiments, the ITAM3 variant comprises or consists of two loss-of-function mutations. In certain embodiments, the ITAM3 variant comprises or consists of the amino acid sequence set forth in SEQ ID NO: 49, which is provided below.

HDGLFQGLSTATKDTFDALHMQ [SEQ ID NO: 49]

**[00222]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 49 is set forth in SEQ ID NO: 50, which is provided below.

CACGACGGCCTGTTCCAAGGCCTGAGCACCGCCACCAAGGACACCTTCGACGCCCTGCACATGC  
AA [SEQ ID NO: 50]

**[00223]** Various modified CD3 $\zeta$  polypeptides and CARs comprising modified CD3 $\zeta$  polypeptides are disclosed in International Patent Application Publication No. WO2019/133969, which is incorporated by reference hereby in its entirety.

In certain embodiments, the intracellular domain of the CAR comprises a modified CD3 $\zeta$  polypeptide comprising a native ITAM1, an ITAM2 variant comprising or consisting of one or more (e.g., two) loss-of-function mutations, and an ITAM3 variant comprising or consisting of one or more (e.g., two) loss-of-function mutations. In certain embodiments, the intracellular domain of the CAR comprises a modified CD3 $\zeta$  polypeptide comprising a native ITAM1, an ITAM2 variant consisting of two loss-of-function mutations, and an ITAM3 variant consisting of two loss-of-function mutations. In certain embodiments, the intracellular domain of the CAR comprises a modified CD3 $\zeta$  polypeptide comprising a native ITAM1 consisting of the amino acid sequence set forth in SEQ ID NO: 39, an ITAM2 variant consisting of the amino acid sequence set forth in SEQ ID NO: 45, and an ITAM3 variant consisting of the amino acid sequence set forth in SEQ ID NO: 49. In certain embodiments, the modified CD3 $\zeta$  polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 51, which is designated as "1XX". SEQ ID NO: 51 is provided below.

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQEGLFNELQK  
DKMAEAFSEIGMKGERRRGKGGHDGLFQGLSTATKDTFDALHMQALPPR [SEQ ID NO: 51]

**[00224]** In certain embodiments, the intracellular domain of the CAR comprises a modified CD3 $\zeta$  polypeptide comprising or consisting of an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, at least about 100% identical to SEQ ID NO: 51 or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

**[00225]** An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 51 is set forth in SEQ ID NO: 52, which is provided below.

AGAGTGAAGTTCAGCAGAAGCGCCGACGCCCCCGCCTATCAGCAAGGGCAGAATCAGCTGTACA  
 ACGAGCTGAACCTGGGCAGAAGAGAGGAGTACGACGTGCTGGACAAGAGAAGAGGCAGAGACCC  
 CGAGATGGGCGGCAAGCCTAGAAGAAAGAACCCCCAAGAGGGCCTGTTCAACGAGCTGCAGAAG  
 GACAAGATGGCCGAGGCCTTCAGCGAGATCGGCATGAAGGGCGAGAGAAGAAGAGGCAAGGGCC  
 ACGACGGCCTGTTCCAAGGCCTGAGCACCGCCACCAAGGACACCTTCGACGCCCTGCACATGCA  
 AGCCCTGCCCCCTAGA [SEQ ID NO: 52]

**[00226]** In certain embodiments, the intracellular domain of the CAR further comprises at least one co-stimulatory signaling region (CSR). In certain embodiments, the at least one co-stimulatory region comprises a co-stimulatory molecule or a portion thereof. In certain embodiments, the at least one co-stimulatory region comprises an intracellular domain of at least one co-stimulatory molecule or a portion thereof.

**[00227]** As used herein, a “co-stimulatory molecule” refers to a cell surface molecule other than antigen receptor or its ligand that can provide an efficient response of lymphocytes to an antigen. A co-stimulatory molecule can provide optimal lymphocyte activation. The co-stimulatory molecule can bind to a co-stimulatory ligand, which is a protein expressed on cell surface that upon binding to its receptor produces a co-stimulatory response, *i.e.*, an intracellular response that effects the stimulation provided when the CAR binds to its target antigen(s) (e.g., CD19 and CD20 for the presently disclosed CAR). As one example, a 4-1BB ligand (*i.e.*, 4-1BBL) may bind to 4-1BB for providing an intracellular signal that in combination with a CAR signal induces an effector cell function of the CAR<sup>+</sup> T-cell.

**[00228]** Non-limiting examples of co-stimulatory molecules include CD28, 4-1BB (CD137), OX40, ICOS, DAP-10, CD27, CD28, CD30, CD40, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, CD8, CD4, B2C, CD80, CD86, DAP12, MyD88, BTNL3, NKG2D, TLR, IL-2R beta, IL-15R alpha, and combinations thereof.

**[00229]** In certain embodiments, the intracellular domain of the CAR comprises a co-stimulatory signaling region that comprises a CD28 polypeptide, e.g., an intracellular domain of CD28 or a portion thereof. In certain embodiments, the intracellular domain of the CAR comprises a co-stimulatory signaling region that comprises an intracellular domain of human CD28 or a portion thereof. In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region of the CAR comprise or consists of an amino acid sequence that is

at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, at least about 100% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 35, or a fragment thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consist of an amino acid sequence that is a consecutive portion of SEQ ID NO: 35, which is at least about 20, or at least about 30, or at least about 40, or at least about 50, and up to about 220 amino acids in length. Alternatively or additionally, in certain embodiments, the CD28 polypeptide comprised in the co-stimulatory signaling region comprises or consists of amino acids 1 to 220, 1 to 50, 50 to 100, 100 to 150, 114 to 220, 150 to 200, 180 to 220, or 200 to 220 of SEQ ID NO: 35. In certain embodiments, the intracellular domain of the CAR comprises a co-stimulatory signaling region that comprises a CD28 polypeptide comprising or consisting of amino acids 180 to 220 of SEQ ID NO: 35.

**[00230]** An exemplary nucleotide sequence encoding amino acids 180 to 220 of SEQ ID NO: 35 is set forth in SEQ ID NO: 53, which is provided below.

AGAAGCAAGAGAAGCAGACTGCTGCACAGCGACTACATGAACATGACCCCTAGAAGACCCGCCC  
CCACAAGAAAGCACTATCAGCCCTACGCCCCCCTAGAGACTTCGCCGCCTACAGAAGC [SEQ  
ID NO: 53]

#### 5.3.4. Exemplified CARs

##### **[00231]** “20-19 1XX” CAR

**[00232]** As shown in Figure 1, the “20-19 1XX” CAR comprises (a) an extracellular domain that comprise (i) a first anti-CD19 antigen-binding domain comprising a first V<sub>H</sub> that comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11, and a first V<sub>L</sub> that comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14, and (ii) a second anti-CD20 antigen-binding domain comprising a second V<sub>H</sub> that comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23, and a second V<sub>L</sub> that comprises a CDR1

comprising the amino acid sequence set forth in SEQ ID NO: 24, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26; (b) a hinge domain comprising a CD28 polypeptide (e.g., a hinge domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of amino acids 114 to 152 of SEQ ID NO: 35); (c) a transmembrane domain comprising a CD28 polypeptide (e.g., a transmembrane domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of amino acids 153 to 179 of SEQ ID NO: 35); and (d) an intracellular domain comprising (i) a CD3 $\zeta$  polypeptide (e.g., a modified human CD3 $\zeta$  polypeptide, e.g., one consisting of the amino acid sequence set forth in SEQ ID NO: 51), and (ii) a co-stimulatory signaling region comprising a CD28 polypeptide (e.g., an intracellular domain of human CD28 or a portion thereof, e.g., a CD28 polypeptide consisting of amino acids 180 to 220 of SEQ ID NO: 35). The first V<sub>H</sub> and first V<sub>L</sub> are linked via a first linker consisting of the amino acid sequence set forth in SEQ ID NO: 1. In certain embodiments, the first V<sub>H</sub> and the first V<sub>L</sub> are positioned from the N- to the C-terminus: V<sub>L</sub>-V<sub>H</sub>. In certain embodiments, the second V<sub>H</sub> and second V<sub>L</sub> are linked via a second linker consisting of the amino acid sequence set forth in SEQ ID NO: 2. In certain embodiments, the second V<sub>H</sub> and the second V<sub>L</sub> are positioned from the N- to the C-terminus: V<sub>H</sub>-V<sub>L</sub>. The CAR further comprises a third linker between the first anti-CD19 antigen-binding domain and the second anti-CD20 antigen-binding domain. The third linker consists of the amino acid sequence set forth in SEQ ID NO: 3. The first anti-CD19 antigen-binding domain is positioned downstream of the second anti-CD20 antigen-binding domain. In certain embodiments, the CAR further comprises a leader peptide that comprises a CD8 polypeptide (e.g., one consisting of the amino acid sequence set forth in SEQ ID NO: 33). In certain embodiments, the leader peptide is covalently joined to the N-terminus of the second anti-CD20 antigen-binding domain. In certain embodiments, the leader peptide is linked to the N-terminus of a second V<sub>H</sub>. In certain embodiments, the “20-19 1XX” CAR comprises the amino acid sequence set forth in SEQ ID NO: 54, which is provided below.

MALPVTALLLPLALLLHAARPEVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQ  
GLEWIGAIYPNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSNYYGSSYW  
FFDVWGAGTTTVTVSSGGGGSGGGSGGGGSDIVLTQSPAILSASPGEKVTMTCRASSSVNYMDW  
YQKKPGSSPKPWIYATSNLASGVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFG  
GGTKLEIKGGGGSGGGSGGGSGGGSGGGGSDIQMTQTSSLSASLGDRVTISCRASQDISK

YLNWYQOKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLP  
YTFGGGTKEITGSTSGSGKPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGV  
SWIRQPPRKGLEWLGVIWGSETTYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDBTAIYYCAKH  
YYYGGSYAMDYWGQTSVTVSSAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLPFGPSKP  
FWVLVVGGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFA  
AYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLFN  
ELQKDKMAEAFSEIGMKGERRRGKGHDLFQGLSTATKDTFDALHMQALPPR [SEQ ID NO:  
54]

[00233] An exemplary nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 54 is set forth in SEQ ID NO: 55, which is provided below.

ATGGCCCTGCCCGTCACCGCTCTGCTTCTGCCCTGGCTCTGCTCTTACATGCCGCTAGACCCG  
AAGTGCAGCTTCAGCAGAGCGGCGCCGAACTGGTGAAGCCCGGCGCTAGCGTGAAGATGAGCTG  
CAAGGCTAGCGGCTACACCTTCACAAGCTATAACATGCACTGGGTGAAGCAGACCCCCGGCCAA  
GGCCTGGAGTGGATCGGTGCCATCTACCCCGGCAACGGCGACACAAGCTACAATCAGAAGTTCA  
AGGGCAAGGCCACCCTGACCGCCGACAAGAGCAGCAGCACCCTACATGCAGCTGAGCAGCCT  
GACAAGCGAGGACAGCGCCGACTACTATTGCGCACGGAGCAACTACTACGGCAGCAGCTACTGG  
TTCTTCGACGTATGGGGCGCGGGCACACGGTCACCGTGAGCTCCGGTGGAGGGGGCTCTGGCG  
GTGGGGGGTCCGGCGGTGGCGGATCTGACATCGTGCTGACACAGAGCCCCGCCATCCTAAGCGC  
CTCCCCCGGTGAAAAGTGACCATGACCTGCAGAGCTAGCAGCTCTGTGAACCTACATGGACTGG  
TATCAGAAGAAACCTGGCAGCTCTCCAAAGCCGTGGATCTATGCCACAAGCAATCTGGCATCTG  
GCGTACCCGCTAGATTACAGCGGCAGCGGATCCGGCACATCATACTCCCTGACCATAAGCCGTGT  
GGAGGCCGAGGACGCCGCCACCTACTACTGTGAGCAGTGGAGCTTCAACCCCCCTACATTCCGA  
GGGGGCACCAAGCTGGAGATTAAAGCGGTGGCGGGTCTGGCGGCGGTGGATCCGGTGGTGGTG  
GATCTGGAGGAGGGGGCTCCGGGGGCGGTGGCAGCGATATACAGATGACACAGACCACAAGCAG  
CCTGAGCGCGTCGTTAGGTGACAGAGTGACCATCAGCTGCCGCGCTAGCCAAGACATCAGCAAG  
TACCTGAACCTGGTATCAGCAGAAGCCCGACGGCACCGTGAAGCTGCTGATCTACCACACAAGCA  
GACTGCACAGCGGCGTACCATCTCGGTTCTCTGGCAGCGGCTCTGGAACCGACTACTCGCTGAC  
CATCAGTAATCTGGAGCAAGAGGACATCGCCACCTACTTCTGTGAGCAAGGCAACACCCTGCCC  
TACACATTTCGGCGGCGGTACGAAGCTGGAGATAACCGGCTCCACGAGCGGCAGCGGCAAACCCG  
GCAGCGGCGAGGGCAGCACCAAGGGCGAAGTAAACTGCAAGAGAGTGCCCCCGGTCTTGTGGC  
CCCTTCTCAGAGCCTGAGCGTGACCTGCACCGTGAGCGGCGTGAGCCTGCCCCGACTACGGCGTG  
AGCTGGATCAGACAGCCCCCTAGAAAAGGGCTGGAGTGGCTCGGTGTGATCTGGGGCAGCGAGA

CCACCTACTACAACAGCGCCCTGAAGAGCAGACTGACCATCATCAAGGACAACAGCAAGAGCCA  
 AGTGTTCTGAAGATGAACAGCCTGCAGACCGACGACACCGCCATCTACTACTGTGCCAAGCAT  
 TACTACTACGGCGGCAGCTACGCCATGGACTACTGGGGCCAAGGCACAAGCGTCACCGTCAGCA  
 GCGCCGCGGCCATCGAGGTGATGTACCCCCCCCCCTACCTGGACAACGAGAAGAGCAACGGCAC  
 CATCATCCACGTGAAGGGCAAGCACCTGTGCCCTAGCCCCCTGTTCCCTGGCCCCCTGAAGCCC  
 TTCTGGGTTCTGGTTCGTGGTGGGCGGCGTCTGGCCTGCTACAGCCTGCTGGTGACCGTGGCCT  
 TCATCATCTTCTGGGTGAGAAGCAAGAGAAGCAGACTGCTGCACAGCGACTACATGAACATGAC  
 CCCTAGAAGACCCGGCCCCACAAGAAAGCACTATCAGCCCTACGCCCCCCTAGAGACTTCGCC  
 GCCTACAGAAGCAGAGTGAAGTTCAGCAGAAGCGCCGACGCCCCCGCCTATCAGCAAGGGCAGA  
 ATCAGCTGTACAACGAGCTGAACCTGGGCAGAAGAGAGGAGTACGACGTGCTGGACAAGAGAAG  
 AGGCAGAGACCCCGAGATGGGCGGCAAGCCTAGAAGAAAGAACCCCCAAGAGGGCCTGTTCAAC  
 GAGCTGCAGAAGGACAAGATGGCCGAGGCCTTCAGCGAGATCGGCATGAAGGGCGAGAGAAGAA  
 GAGGCAAGGGCCACGACGGCCTGTTCCAAGGCCTGAGCACCGCCACCAAGGACACCTTCGACGC  
 CCTGCACATGCAAGCCCTGCCCCCTAGATGA [SEQ ID NO: 55]

#### 5.4. Cells

[00234] The presently disclosed subject matter provides cells comprising a presently disclosed CD19/CD20-targeted CAR (e.g., one disclosed in Section 5.3). In certain embodiments, the cell is selected from the group consisting of cells of lymphoid lineage and cells of myeloid lineage. In certain embodiments, the cell is an immunoresponsive cell. In certain embodiments, the immunoresponsive cell is a cell of lymphoid lineage.

[00235] In certain embodiments, the cell is a cell of the lymphoid lineage. Cells of the lymphoid lineage can provide production of antibodies, regulation of cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. Non-limiting examples of cells of the lymphoid lineage include T cells, Natural Killer (NK) cells, B cells, dendritic cells, and stem cells from which lymphoid cells may be differentiated. In certain embodiments, the stem cell is a pluripotent stem cell (e.g., embryonic stem cell or an induced pluripotent stem cell).

[00236] In certain embodiments, the cell is an immune effector cell. Immune effector 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. Immune effector cells can be obtained from blood



collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. For example, cells from the circulating blood of an individual may be obtained by apheresis. In certain embodiments,, immune effector 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 immune effector cells can be further isolated by positive or negative selection techniques. For example, immune effector cells can be isolated using a combination of antibodies directed to surface markers unique to the positively selected cells, e.g., by incubation with antibody-conjugated beads for a time period sufficient for positive selection of the desired immune effector cells. Alternatively, enrichment of immune effector cells population can be accomplished by negative selection using a combination of antibodies directed to surface markers unique to the negatively selected cells.

**[00237]** In certain embodiments, the immune effector cells comprise any leukocyte involved in defending the body against infectious disease and foreign materials. For example, the immune effector cells can comprise lymphocytes, monocytes, macrophages, dendritic cells, mast cells, neutrophils, basophils, eosinophils, or any combinations thereof. For example, the immune effector cells can comprise T lymphocytes, preferably cytotoxic T lymphocytes (CTLs).

**[00238]** In certain embodiments, the cell is a T cell. T cells can be lymphocytes that mature in the thymus and are chiefly responsible for cell-mediated immunity. T cells are involved in the adaptive immune system. T cells or T lymphocytes can be distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. They are called T cells because they mature in the thymus (although some also mature in the tonsils). There are several subsets of T cells, each with a distinct function.

**[00239]** The T cells can be any type of T cells, including, but not limited to, helper T cells, cytotoxic T cells, memory T cells (including central memory T cells, stem-cell-like memory T cells (or stem-like memory T cells), and two types of effector memory T cells: *e.g.*, T<sub>EM</sub> cells and T<sub>EMRA</sub> cells), Regulatory T cells (also known as suppressor T cells or T<sub>reg</sub>), tumor-infiltrating lymphocyte (TIL), Natural killer T cells (NK-T cells), mucosal associated invariant T cells, αβ T cells, and γδ T cells. In certain embodiments, the T cell is a central memory T cell. Also, in certain embodiments, the T cell is positive for CD45RO and CD62L.

**[00240]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is a helper T cell or CD4<sup>+</sup> T cell. Helper T cells (or T helper cells, or T<sub>H</sub> cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. These cells are also known as CD4<sup>+</sup> T cells because they express the CD4 glycoprotein on their surface. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen-presenting cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response. These cells can differentiate into one of several subtypes, including T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>3, T<sub>H</sub>17, T<sub>H</sub>9, or T<sub>FH</sub>, which secrete different cytokines to facilitate a different type of immune response.

**[00241]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is a cytotoxic T cell or CD8<sup>+</sup> T cell. Cytotoxic T cells (TC cells, or CTLs) destroy virally infected cells and tumor cells and are also implicated in transplant rejection. These cells are also known as CD8<sup>+</sup> T cells since they express the CD8 glycoprotein at their surface. These cells recognize their targets by binding to antigen associated with MHC class I molecules, which are present on the surface of all nucleated cells. Through IL-10, adenosine and other molecules secreted by regulatory T cells, the CD8<sup>+</sup> cells can be inactivated to an anergic state, which prevents autoimmune diseases.

**[00242]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is a memory T cell. Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with “memory” against past infections. Memory cells may be either CD4<sup>+</sup> or CD8<sup>+</sup>. Memory T cells typically express the cell surface protein CD45RO.

**[00243]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is a regulatory T cell. Regulatory T cells (T<sub>reg</sub> cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus. Two major

classes of CD4<sup>+</sup> T<sub>reg</sub> cells have been described — naturally occurring T<sub>reg</sub> cells and adaptive T<sub>reg</sub> cells.

**[00244]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is a Natural killer T (NKT) cell. Natural killer T (NKT) cells (not to be confused with natural killer (NK) cells) bridge the adaptive immune system with the innate immune system. Unlike conventional T cells that recognize peptide antigens presented by major histocompatibility complex (MHC) molecules, NKT cells recognize glycolipid antigen presented by a molecule called CD1d.

**[00245]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is a  $\gamma\delta$  T cell.  $\gamma\delta$  T cells possess a distinct T-cell receptor (TCR) having one  $\gamma$  chain and one  $\delta$  chain instead of  $\alpha$  and  $\beta$  chains.

**[00246]** In certain embodiments, the cell comprising the presently disclosed CD19/CD20-targeted CAR is an innate lymphoid cell (ILC), a cytokine induced killer (CIK) cell, or a lymphokine activated killer (LAK) cell.

**[00247]** In certain embodiments, the cell is a T cell, and the presently disclosed CD19/CD20-targeted CAR is integrated at a locus within the genome of the T cell. Non-limiting examples of the loci include a TRAC locus, a TRBC locus, a TRDC locus, and a TRGC locus. In certain embodiments, the locus is a TRAC locus or a TRBC locus. Methods of targeting a CAR to a site within the genome of T cell are disclosed in WO2017180989 and Eyquem *et al.*, *Nature*. (2017 Mar 2); 543(7643): 113-117, both of which are incorporated by reference in their entireties.

**[00248]** In certain embodiments, the T cells comprise a mixture of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. In certain embodiments, the T cells are enriched for one or more subsets based on cell surface marker expression profile.

**[00249]** In certain embodiments, the cell is a Natural Killer (NK) cell. NK cells can be lymphocytes that are part of cell-mediated immunity and act during the innate immune response. NK cells do not require prior activation in order to perform their cytotoxic effect on target cells. Unlike cytotoxic CD8<sup>+</sup> T cells, NK cells launch cytotoxicity against tumor cells without the requirement for prior sensitization and can also eradicate MHC-I-negative cells. In certain embodiments, the cell is a genetically modified NK cell. In certain embodiments, the cell is an edited NK cell. In certain embodiments, the cell is a NK cell derived from a stem cell. In certain

embodiments, the cell is a NK cell derived from a pluripotent stem cell. In certain embodiments, the cell is an induced pluripotent stem cell (iPSC)-derived NK cell.

**[00250]** The presently disclosed cells (*e.g.*, T cells or NK cells) can be autologous (*e.g.*, the cells are derived/obtained from a subject receiving the CAR-expressing cells), non-autologous (*e.g.*, allogeneic) (*e.g.*, the cells are derived/obtained from a donor who is not the subject receiving the CAR-expressing cells), or derived *in vitro* from engineered progenitor or stem cells. In certain embodiments, the cells comprising a presently disclosed CD19/CD20-targeted CAR are obtained from the subject receiving the CAR-expressing cells (*e.g.*, a subject to be treated) (*i.e.* are autologous). In certain embodiments, the cells comprising a presently disclosed CD19/CD20-targeted CAR are allogeneic to the subject receiving the CAR-expressing cells (*e.g.*, a subject to be treated).

**[00251]** The cells of the presently disclosed subject matter can be cells of the myeloid lineage. Non-limiting examples of cells of the myeloid lineage include monocytes, macrophages, neutrophils, dendritic cells, basophils, neutrophils, eosinophils, megakaryocytes, mast cell, erythrocyte, thrombocytes, and stem cells from which myeloid cells may be differentiated. In certain embodiments, the stem cell is a pluripotent stem cell (*e.g.*, an embryonic stem cell or an induced pluripotent stem cell).

**[00252]** Epstein-Barr virus (EBV)-induced lymphoproliferative diseases (EBV-LPDs) and other EBV-associated cancers are a significant cause of morbidity and mortality for recipients of allogeneic hematopoietic cell transplantation (HCT) or solid organ transplants (SOT), particularly in those who have received certain T-cell reactive Abs to prevent or treat GVHD. Prophylaxis and treatment by the adoptive transfer of autologous or allogeneic EBV-specific cytotoxic T cells and the subsequent long-term restoration of immunity against EBV-associated lymphoproliferation have provided positive outcomes in the management of these uniformly fatal complications of allogeneic tissue transfer. Therefore, in some embodiments, the disclosed immune effector cells that comprise one or more of the CAR polypeptides of the present invention are allogeneic or autologous EBV-specific cytotoxic T lymphocytes (CTLs). For example, generation of EBV-specific cytotoxic T cells may involve isolating PBMCs from of an EBV-seropositive autologous or allogeneic donor and enriching them for T cells by depletion of monocytes and NK cells. EBV-specific cytotoxic T cells may also be produced by contacting donor PBMCs or purified donor T cells with a “stimulator” cell that expresses one or more EBV

antigen(s) and presents the EBV antigen(s) to unstimulated T cells, thereby causing stimulation and expansion of EBV-specific CTLs. EBV antigens include, for example, latent membrane protein (LMP) and EBV nuclear antigen (EBNA) proteins, such as LMP-1, LMP-2A, and LMP-2B and EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP. Cytotoxic T cells that comprise T cell receptor(s) which recognize one or more EBV-specific antigens are deemed to have been “sensitized” to those EBV antigen(s) and are therefore termed “EBV-sensitized cytotoxic T cells” herein. Known methods for generating allogeneic or autologous EBV-specific cytotoxic T cell populations that may comprise one or more of the CAR polypeptides of the present invention are described, for example, in Barker et al., *Blood* 116(23):5045-49 (2010); Doubrovina et al., *Blood* 119(11):2644-56 (2012); Koehne et al., *Blood* 99(5):1730-40 (2002); and Smith et al., *Cancer Res.* 72(5):1116-25 (2012), which are incorporated by reference for these teachings. Similarly, cytotoxic T cells may be “sensitized” to other viral antigens, including cytomegalovirus (CMV), papillomavirus (e.g., HPV), adenovirus, polyomavirus (e.g., BKV, JCV, and Merkel cell virus), retrovirus (e.g., HTLV-I, also including lentivirus such as HIV), picomavirus (e.g., Hepatitis A virus), hepadnavirus (e.g., Hepatitis B virus), hepacivirus (e.g., Hepatitis C virus), deltavirus (e.g., Hepatitis D virus), hepevirus (e.g., Hepatitis E virus), and the like. In certain embodiments, the T cells used for generating the CAR-T cells of the invention are polyfunctional T-cells, i.e., those T cells that are capable of inducing multiple immune effector functions, that provide a more effective immune response to a pathogen than do cells that produce, for example, only a single immune effector (e.g. a single biomarker such as a cytokine or CD107a). Less-polyfunctional, monofunctional, or even “exhausted” T cells may dominate immune responses during chronic infections, thus negatively impacting protection against virus-associated complications. In certain embodiments, the presently disclosed CAR-T cells are polyfunctional. In certain embodiments, at least 50% of the T cells used for generating the presently disclosed CAR-T cells are CD4<sup>+</sup> T cells. In certain embodiments, the T cells are less than 50% CD4<sup>+</sup> T cells. In certain embodiments, the T cells are predominantly CD4<sup>+</sup> T cells. In certain embodiments, at least 50% of the T cells used for generating the presently disclosed CAR-T cells are CD8<sup>+</sup> T cells. In certain embodiments, the T cells are less than 50% CD8<sup>+</sup> T cells. In certain embodiments, the T cells are predominantly CD8<sup>+</sup> T cells. In some embodiments, the T cells (e.g., the sensitized T cells and/or CAR-T cells described herein) are stored in a cell library or bank before they are administered to the subject.

[00253] In certain embodiments, the cells expressing the presently disclosed CD19/CD20-targeted CARs further express a dominant-negative mutation that effects immune checkpoint blockade. Non-limiting examples of immune checkpoint molecules include programmed death 1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), B- and T- lymphocyte attenuator (BTLA), T cell immunoglobulin mucin-3 (TIM-3), lymphocyte- activation protein 3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), leukocyte-associated immunoglobulin- like receptor 1 (LAIR1), natural killer cell receptor 2B4 (2B4), CD160, and transforming growth factor b (TGF-b) receptor. In certain embodiments, the immune checkpoint molecule is CTLA-4. In certain embodiments, the immune checkpoint molecule is PD-1. Methods of engineering CAR-T cells expressing a dominant negative form of an inhibitor of a cell-mediated immune response are described in International Patent Publication WO2017/040945, which is hereby incorporated by reference in its entirety.

[00254] In certain embodiments, the cells can be transduced with the presently disclosed CD19/CD20-targeted CAR such that the cells express the CD19/CD20-targeted CAR.

#### **5.5. Nucleic Acids and Vectors**

[00255] The presently disclosed subject matter provides nucleic acids encoding the presently disclosed CD19/CD20-targeted CARs and cells comprising such nucleic acids. In certain embodiments, the nucleic acid further comprises a promoter that is operably linked to the nucleic acid encoding a presently disclosed CD19/CD20-targeted CAR.

[00256] In certain embodiments, the promoter is endogenous or exogenous. In certain embodiments, the exogenous promoter is selected from the group consisting of an elongation factor (EF)-1 promoter, a cytomegalovirus immediate-early promoter (CMV) promoter, a simian virus 40 early promoter (SV40) promoter, a phosphoglycerate kinase (PGK) promoter, a metallothionein promoter, and Ubiquitin C promoter. In certain embodiments, the endogenous promoter is selected from a TCR alpha promoter, a TCR beta promoter, and a beta 2-microglobulin promoter. In certain embodiments, the promoter is an inducible promoter. In certain embodiment, the inducible promoter is selected from the group consisting of a NFAT transcriptional response element (TRE) promoter, a CD69 promoter, a CD25 promoter, an IL-2 promoter, a 4-1BB promoter, a PD1 promoter, and a LAG3 promoter.

[00257] The presently disclosed subject matter also provides vectors comprising the presently disclosed nucleic acids. In certain embodiments, the vector is an expression vector. In certain

embodiments, the vector is a viral vector. In certain embodiments, the vector is a retroviral vector.

**[00258]** The nucleic acids can be delivered into cells by art-known methods or as described herein. Genetic modification of a cell can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA construct. In certain embodiments, a retroviral vector (e.g., gammaretroviral vector or lentiviral vector) is employed for the introduction of the DNA construct into the cell. For example, a presently disclosed nucleic acid can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for a target cell type of interest.

**[00259]** Expression of nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide to a promoter and incorporating the construct into an expression vector. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

**[00260]** The disclosed nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

**[00261]** Further, the expression vector may be provided to a cell in the form of a viral vector. 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. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers. In some embodiments, the polynucleotide vectors are lentiviral or retroviral vectors.

**[00262]** 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. A selected gene 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 cells of the subject either *in vivo* or *ex vivo*.

**[00263]** One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor-1 $\alpha$  (EF-1 $\alpha$ ). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, MND (myeloproliferative sarcoma virus) promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. The promoter can alternatively be an inducible promoter. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

**[00264]** Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another.

**[00265]** In order to assess the expression of a CAR polypeptide or portions thereof, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes.

**[00266]** Reporter genes are 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. Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

**[00267]** Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

**[00268]** Physical methods for introducing a polynucleotide into a host 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).

**[00269]** Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells.

**[00270]** Chemical means for introducing a polynucleotide into a host 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 and in vivo is a liposome (e.g., an artificial membrane vesicle).

**[00271]** In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule

that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a “collapsed” structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes. Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine (“DMPC”) can be obtained from Sigma, St. Louis, Mo.; dicetyl phosphate (“DCP”) can be obtained from K & K Laboratories (Plainview, N.Y.); cholesterol (“Choi”) can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol (“DMPG”) and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.).

**[00272]** Any targeted genome editing methods can also be used to deliver a presently disclosed CD19/CD20-targeted CAR to a cell. In certain embodiments, a CRISPR system is used to deliver a presently disclosed CD19/CD20-targeted CAR. In certain embodiments, zinc-finger nucleases are used to deliver a presently disclosed CD19/CD20-targeted CAR. In certain embodiments, a TALEN system is used to deliver a presently disclosed CD19/CD20-targeted CAR.

**[00273]** Clustered regularly-interspaced short palindromic repeats (CRISPR) system is a genome editing tool discovered in prokaryotic cells. When utilized for genome editing, the system includes Cas9 (a protein able to modify DNA utilizing crRNA as its guide), CRISPR RNA (crRNA, contains the RNA used by Cas9 to guide it to the correct section of host DNA along with a region that binds to tracrRNA (generally in a hairpin loop form) forming an active complex with Cas9), trans-activating crRNA (tracrRNA, binds to crRNA and forms an active complex with Cas9), and an optional section of DNA repair template (DNA that guides the cellular repair process allowing insertion of a specific DNA sequence). CRISPR/Cas9 often employs a plasmid to transfect the target cells. The crRNA needs to be designed for each

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

**[00274]** A zinc-finger nuclease (ZFN) is an artificial restriction enzyme, which is generated by combining a zinc finger DNA-binding domain with a DNA-cleavage domain. A zinc finger domain can be engineered to target specific DNA sequences which allows a zinc-finger nuclease to target desired sequences within genomes. The DNA-binding domains of individual ZFNs typically contain a plurality of individual zinc finger repeats and can each recognize a plurality of base pairs. The most common method to generate new zinc-finger domain is to combine smaller zinc-finger "modules" of known specificity. The most common cleavage domain in ZFNs is the non-specific cleavage domain from the type IIs restriction endonuclease FokI. Using the endogenous homologous recombination (HR) machinery and a homologous DNA template carrying CAR expression cassette, ZFNs can be used to insert the CAR expression cassette into genome. When the targeted sequence is cleaved by ZFNs, the HR machinery searches for homology between the damaged chromosome and the homologous DNA template, and then copies the sequence of the template between the two broken ends of the chromosome, whereby the homologous DNA template is integrated into the genome.

**[00275]** Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. TALEN system operates on almost the same principle as ZFNs. They are generated by combining a transcription activator-like effectors DNA-binding domain with a DNA cleavage domain. Transcription activator-like effectors (TALEs) are composed of 33-34 amino acid repeating motifs with two variable positions that have a strong recognition for specific nucleotides. By assembling arrays of these TALEs, the TALE DNA-binding domain can be engineered to bind desired DNA sequence, and thereby guide the nuclease to cut at specific locations in genome. cDNA expression for use in polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), metallothionein promoters, or Ubiquitin C promoter), and regulated by any appropriate mammalian regulatory element or intron (e.g. the

elongation factor 1a enhancer/promoter/intron structure). For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

[00276] Methods for delivering the genome editing agents/systems can vary depending on the need. In certain embodiments, the components of a selected genome editing method are delivered as DNA constructs in one or more plasmids. In certain embodiments, the components are delivered via viral vectors. Common delivery methods include but is not limited to, electroporation, microinjection, gene gun, impalefection, hydrostatic pressure, continuous infusion, sonication, magnetofection, adeno-associated viruses, envelope protein pseudotyping of viral vectors, replication-competent vectors cis and trans-acting elements, herpes simplex virus, and chemical vehicles (e.g., oligonucleotides, lipoplexes, polymersomes, polyplexes, dendrimers, inorganic Nanoparticles, and cell-penetrating peptides).

#### 5.6. Formulations and Administration

[00277] The presently disclosed subject matter further provides compositions comprising the presently disclosed cells comprising a presently disclosed CD19/CD20-targeted CAR. In certain embodiments, the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable carrier.

[00278] The presently disclosed compositions can be conveniently provided as sterile liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof.

[00279] Compositions comprising the presently disclosed cells can be provided systemically or directly to a subject in need thereof. In certain embodiments, the presently disclosed compositions are directly injected into an organ of interest. Alternatively, the presently disclosed compositions are provided indirectly to the organ of interest, for example, by administration into the circulatory system (*e.g.*, the tumor vasculature). Expansion and differentiation agents can be provided prior to, during or after administration of compositions to increase production of cells *in vitro* or *in vivo*.

[00280] The quantity of cells comprising the CD19/CD20-targeted CAR comprised in the presently disclosed compositions can vary for the subject being treated. In certain embodiments, the presently disclosed composition comprises between about  $10^4$  and about  $10^{10}$ , between about  $10^4$  and about  $10^7$ , between about  $10^5$  and about  $10^7$ , between about  $10^5$  and about  $10^9$ , or between about  $10^6$  and about  $10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the presently disclosed composition comprises between about  $1 \times 10^6$  and about  $5 \times 10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the presently disclosed composition comprises between about  $1 \times 10^6$  and about  $1 \times 10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the presently disclosed composition comprises between about  $1 \times 10^6$  and about  $5 \times 10^7$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the presently disclosed composition comprises between about  $1 \times 10^6$  and about  $1 \times 10^7$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. More effective cells may be administered in even smaller numbers. Usually, at least about  $1 \times 10^5$  cells will be administered, eventually reaching about  $1 \times 10^{10}$  or more. In certain embodiments, the presently disclosed composition comprises at least about  $1 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ , about  $5 \times 10^6$ , about  $1 \times 10^7$ , about  $2.5 \times 10^7$ , about  $5 \times 10^7$ , about  $1 \times 10^8$ , about  $1.5 \times 10^8$ , about  $2 \times 10^8$ , or about  $5 \times 10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the presently disclosed composition comprises about  $1 \times 10^6$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. The precise determination of what would be considered an effective dose can be based on factors individual to each subject, including their size, age, sex, weight, and condition of the particular subject. Dosages can be readily ascertained by those skilled in the art from this

disclosure and the knowledge in the art. The compositions can be administered multiple times at these dosages.

[00281] The presently disclosed CD19/CD20-targeted CAR-modified cells may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2, IL-15, or other cytokines or cell populations. Briefly, pharmaceutical compositions may comprise a target cell population as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions for use in the disclosed methods are in some embodiments formulated for intravenous administration. The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

[00282] The administration of the disclosed compositions may be carried out in any convenient manner, including by injection, transfusion, or implantation. The presently disclosed compositions can be administered by any method known in the art including, but not limited to, intravenous administration, intradermal administration, subcutaneous administration, intranodal administration, intratumoral administration, intramedullary administration, intramuscular administration, intrathecal administration, intrapleural administration, intraosseous administration, intraperitoneal administration, pleural administration, and direct administration to the subject. In certain embodiments, the composition is administered to a subject intravenously. The compositions can be injected directly into a tumor, lymph node, or site of disease.

#### 5.7. Methods of Uses

[00283] The presently disclosed CD19/CD20-targeted CARs can elicit a therapeutically beneficial immune response against a B-lymphocyte antigen (e.g., CD19 and/or CD20). For example, an anti-tumor immune response elicited by the disclosed CAR-modified cells may be an active or a passive immune response. In addition, the CAR-mediated immune response may be part of an adoptive immunotherapy approach in which CAR-modified cells induce an immune response specific to at least one B-lymphocyte antigen, e.g., CD19 and/or CD20.

**[00284]** Adoptive transfer of cells expressing CARs is a promising anti-cancer therapeutic. Following the collection of a subject's cells, the cells may be genetically engineered to express the presently disclosed CD19/CD20-targeted CARs, and then infused back into the subject (i.e., autologous to the subject). Moreover, cells obtained from a donor other than the subject (i.e., allogeneic to the subject) may be genetically engineered to express the presently disclosed CD19/CD20-targeted CARs, then the CAR-containing cells are infused into the subject. In certain embodiments, the cells comprising the presently disclosed CD19/CD20-targeted CARs are allogeneic EBV-specific cytotoxic T cells (CTLs).

**[00285]** Embodiments of the invention provide methods of using the presently disclosed cells or compositions. According to one or more embodiments, the presently disclosed cells or compositions can be used in a therapy or medicament. For example, the presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions can be used for inducing and/or increasing an immune response in a subject in need thereof. Thus, the presently disclosed subject matter provides methods for inducing and/or increasing an immune response in a subject in need thereof. In certain embodiments, the method comprises administering to a subject in need thereof a presently disclosed cell comprising a presently disclosed CD19/CD20-targeted CAR or a composition comprising thereof.

**[00286]** Additionally, in various embodiments, the presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions can be used for treating a B-lymphocyte antigen-associated disease, disorder, or condition, including one or more symptoms of the disease, disorder, or condition. Thus, the presently disclosed subject matter provides methods of treating a B-lymphocyte antigen-associated disease, disorder, or condition. In certain embodiments, the method comprises administering to a subject in need thereof a presently disclosed cell comprising a presently disclosed CD19/CD20-targeted CAR or a composition comprising thereof.

**[00287]** Additionally, in various embodiments, the presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions can inhibit growth of a target cell expressing at least one B-lymphocyte antigen, for example, CD19, CD20, or a combination thereof. Thus, the presently disclosed subject matter provides methods of inhibiting growth of a target cell expressing at least one B-lymphocyte antigen (e.g., CD19, CD20, or a combination thereof). In certain embodiments, the method comprises contacting the target cell with a presently disclosed

CD19/CD20-targeted CAR-expressing cell or a composition comprising thereof. The target cell can express CD19 and/or CD20 at a different level. In certain embodiments, the target cell expresses a detectable level of CD19 and a detectable level of CD20. In certain embodiments, the target cell expresses a detectable level of CD20 and a low or non-detectable level of CD19. In certain embodiments, the target cell expresses a detectable level of CD19 and a low or non-detectable level of CD20. In certain embodiments, the subject suffers from or is diagnosed with a CD19/CD20-associated disease, disorder, or condition.

**[00288]** In certain embodiments, the target cell is a tumor cell such as a cancer tumor cell. In certain embodiments, the tumor is cancer. In certain embodiments, the cancer is a hematologic cancer. Non-limiting examples of hematologic cancer include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).

**[00289]** In certain embodiments, the target cell is a B cell. In certain embodiments, the B cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

**[00290]** In certain embodiments, the target cell is a T cell. In certain embodiments, the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>. In certain embodiments, the T cell is CD20<sup>+</sup>.

**[00291]** The presently disclosed cells or compositions exhibit cytotoxic and/or cytolytic activity against the target cell. In certain embodiments, the cytotoxic and/or cytolytic activity is dose-dependent. In certain embodiments, the cytotoxic and/or cytolytic activity is long-term. For example, the cytotoxic and/or cytolytic activity can last for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial contact of the target cell with the cells or compositions. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 40 days (e.g., 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, or 45 days) after the initial contact of the target cell with the cell or composition. In certain embodiments, the cytotoxic and/or cytolytic activity lasts for about 60 days (e.g., 55 days, 56 days, 57 days, 58 days, 59 days, 60 days, 61 days, 62 days, 63 days, 64 days, or 65 days) after the initial contact of the target cell with the cell or composition.

**[00292]** In certain embodiments, the cells are administered in an effective amount for treatment of one or more diseases or conditions such as cancers and autoimmune disease



described herein. As used herein, the term “effective amount” refers to an amount sufficient to affect a beneficial or desired clinical result upon treatment. An effective amount can be administered to a subject in one or more doses, one or a series of administrations, and/or in a bolus or by continuous perfusion. In certain embodiments, an effective amount can be an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the disease, or otherwise reduce the pathological consequences of the disease. The effective amount can be determined by a physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. The effective amount can be between about  $10^4$  and about  $10^{10}$ , between about  $10^4$  and about  $10^7$ , between about  $10^5$  and about  $10^7$ , between about  $10^5$  and about  $10^9$ , or between about  $10^6$  and about  $10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the effective amount is between about  $1 \times 10^6$  and about  $5 \times 10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the effective amount is between about  $1 \times 10^6$  and about  $1 \times 10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the effective amount is between about  $1 \times 10^6$  and about  $5 \times 10^7$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the effective amount is between about  $1 \times 10^6$  and about  $1 \times 10^7$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the effective amount is at least about  $1 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ , about  $5 \times 10^6$ , about  $1 \times 10^7$ , about  $2.5 \times 10^7$ , about  $5 \times 10^7$ , about  $1 \times 10^8$ , about  $1.5 \times 10^8$ , about  $2 \times 10^8$ , or about  $5 \times 10^8$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. In certain embodiments, the effective amount is about  $1 \times 10^6$  of the presently disclosed cells comprising the CD19/CD20-targeted CAR. These factors include age, sex and weight of the subject, the condition being treated, the severity of the condition and the form and effective concentration of the cells administered.

**[00293]** In certain embodiments, the B-lymphocyte antigen-associated disease or disorder expresses CD19, CD20, or both. In certain embodiments, the B-lymphocyte antigen-associated disease or disorder is a tumor. In certain embodiments, the B-lymphocyte antigen-associated disease or disorder is cancer. In certain embodiments, the B-lymphocyte antigen-associated disease or disorder is a hematologic cancer. In certain embodiments, the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia

(CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).

**[00294]** In certain embodiments, the B-lymphocyte antigen-associated disease can be any neoplasm or tumor of the hematopoietic and/or lymphatic tissues. Thus, the B-lymphocyte antigen-associated disease can be any malignancy affecting the blood, bone marrow, lymph, and/or lymphatic system; and any such disease resulting in unregulated myeloproliferation and/or lymphoproliferation. Non-limiting examples of B-lymphocyte antigen-associated diseases or disorders include leukemias (including, but not limited to, acute leukemias, chronic leukemias, lymphocytic leukemias, myelogenous leukemias), pre-leukemic conditions, and lymphomas (including, but not limited to, Hodgkin lymphoma, and non-Hodgkin lymphoma) such as, EBV-associated lymphoproliferative diseases, mature B cell neoplasms, mature T cell neoplasms, and natural killer (NK) cell neoplasms, precursor lymphoid neoplasms, and immunodeficiency-associated lymphoproliferative disorders.

**[00295]** In certain embodiments, the B-lymphocyte antigen-associated disease is selected from the group consisting of acute lymphoblastic leukemia (ALL), precursor B acute lymphoblastic leukemia, precursor T acute lymphoblastic leukemia, Burkitt's leukemia, acute biphenotypic leukemia, chronic lymphocytic leukemia (CLL), B-cell prolymphocytic leukemia, acute myelogenous leukemia (AML), acute promyelocytic leukemia (PML), acute myeloblastic leukemia, acute megakaryoblastic leukemia (AMKL), chronic myelogenous leukemia (CML), chronic myelomonocytic leukemia, hairy cell leukemia (HCL), T-cell prolymphocytic leukemia (T-PLL), large granular lymphocytic leukemia, adult T-cell leukemia, clonal eosinophilias, B-cell chronic lymphocytic leukemia/small cell lymphoma, lymphoplasmacytic lymphoma (such as Waldenstrom macroglobulinemia), splenic marginal zone lymphoma, plasma cell neoplasms such as plasma cell myeloma (multiple myeloma), plasmacytoma, monoclonal immunoglobulin deposition diseases, heavy chain diseases, extranodal marginal zone B cell lymphoma (MALT lymphoma), nodal marginal zone B cell lymphoma, follicular lymphoma (FL), primary cutaneous follicle center lymphoma, mantle cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL) (with or without chronic inflammation), Epstein-Barr virus-positive DLBCL of the elderly, lymphomatoid granulomatosis, primary mediastinal (thymic) large B-cell

lymphoma, intravascular large B-cell lymphoma, ALK<sup>+</sup> large B-cell lymphoma, plasmablastic lymphoma, primary effusion lymphoma, large B-cell lymphoma arising in HHV8-associated multicentric Castleman's disease, Burkitt's lymphoma, T-cell prolymphocytic leukemia, T-cell large granular lymphocyte leukemia, aggressive NK cell leukemia, adult T-cell leukemia/lymphoma, extranodal NK/T-cell lymphoma, enteropathy-associated T-cell lymphoma, hepatosplenic T-cell lymphoma, blastic NK cell lymphoma, mycosis fungoides / Sezary syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, peripheral T-cell lymphomas, angioimmunoblastic T cell lymphoma, anaplastic large cell lymphoma, B-lymphoblastic leukemia/lymphoma not otherwise specified, B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities, T-lymphoblastic leukemia/lymphoma; classical Hodgkin lymphomas such as nodular sclerosis form, mixed cellularity Hodgkin lymphoma, lymphocyte-rich Hodgkin lymphoma, lymphocyte depleted or not depleted Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, and immunodeficiency-associated lymphoproliferative disorders associated with a primary immune disorder, associated with the human immunodeficiency virus (HIV), associated with methotrexate therapy, post-transplant, and primary central nervous system lymphoma.

**[00296]** In certain embodiments, the subject is a human subject. The subjects can have an advanced form of disease, in which case the treatment objective can include mitigation or reversal of disease progression, and/or amelioration of side effects. The subjects can have a history of the condition, for which they have already been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk of recurrence.

**[00297]** As a consequence of surface expression of a presently disclosed CD19/CD20-targeted CAR, adoptively transferred cells are endowed with augmented and selective cytolytic activity at the tumor site. Furthermore, subsequent to their localization to tumor and their proliferation, the cells turn the tumor site into a highly conductive environment for a wide range of cells involved in the physiological anti-tumor response.

**[00298]** In certain embodiments, the presently disclosed cells and compositions are administered to a subject in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities. In certain embodiments, the presently disclosed cells and compositions can be used in combination with chemotherapy, radiation, immunosuppressive agents, immunoablative agents, cytoxin, and cytokines.

**[00299]** According to one or more embodiments, the presently disclosed cells and compositions can be used in combination with any compound, moiety or group which has a cytotoxic or cytostatic effect. Drug moieties include chemotherapeutic agents, which may function as microtubulin inhibitors, mitosis inhibitors, topoisomerase inhibitors, or DNA intercalators, and particularly those which are used for cancer therapy.

**[00300]** The presently disclosed cells and compositions can be used in combination with a checkpoint inhibitor. The two known inhibitory checkpoint pathways involve signaling through the cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed-death 1 (PD-1) receptors. These proteins are members of the CD28-B7 family of co-signaling molecules that play important roles throughout all stages of T cell function. The PD-1 receptor (also known as CD279) is expressed on the surface of activated T cells. Its ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), are expressed on the surface of APCs such as dendritic cells or macrophages. PD-L1 is the predominant ligand, while PD-L2 has a much more restricted expression pattern. When the ligands bind to PD-1, an inhibitory signal is transmitted into the T cell, which reduces cytokine production and suppresses T-cell proliferation. Checkpoint inhibitors include, but are not limited to, antibodies that block PD-1 (e.g., Nivolumab (BMS-936558 or MDX1106), CT-011, MK-3475), PD-L1 (e.g., MDX-1105 (BMS-936559), MPDL3280A, MSB0010718C), PD-L2 (e.g., rHlgM12B7), CTLA-4 (e.g., Ipilimumab (MDX-010), Tremelimumab (e.g., CP-675,206)), IDO, B7-H3 (e.g., MGA271), B7-H4, TIM3, LAG-3 (e.g., BMS-986016). Techniques for combining CARs with checkpoint inhibitors in immune effector cells and use thereof for the treatment of various disorders are described, for example, in WO 2017/040945, which is incorporated by reference herein.

**[00301]** T cell receptor activation plus co-stimulation can help generate optimal “killer” CD8 T cell responses, which can be provided through ligation of tumor necrosis factor receptor family members, including OX40 (CD134) and 4-1BB (CD137). OX40 is of particular interest as treatment with an activating (agonist) anti-OX40 mAb augments T cell differentiation and cytolytic function leading to enhanced anti-tumor immunity against a variety of tumors.

**[00302]** In certain embodiments, the presently disclosed method comprises administering to the subject a second therapeutic agent. In certain embodiments, the second therapeutic agent is an antimetabolite, such as methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, fludarabine, 5-fluorouracil, decarbazine, hydroxyurea, asparaginase, gemcitabine or cladribine.

**[00303]** In certain embodiments, the second therapeutic agent is an alkylating agent, such as mechlorethamine, thioepa, chlorambucil, melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, dacarbazine (DTIC), procarbazine, mitomycin C, cisplatin and other platinum derivatives, such as carboplatin.

**[00304]** In certain embodiments, the second therapeutic agent is an anti-mitotic agent, such as taxanes, for instance docetaxel, and paclitaxel, and vinca alkaloids, for instance vindesine, vincristine, vinblastine, and vinorelbine.

**[00305]** In certain embodiments, the second therapeutic agent is a topoisomerase inhibitor, such as topotecan or irinotecan, or a cytostatic drug, such as etoposide and teniposide.

**[00306]** In certain embodiments, the second therapeutic agent is a growth factor inhibitor, such as an inhibitor of ErbB1 (EGFR) (such as an EGFR antibody, e.g. zalutumumab, cetuximab, panitumumab or nimotuzumab or other EGFR inhibitors, such as gefitinib or erlotinib), another inhibitor of ErbB2 (HER2/neu) (such as a HER2 antibody, e.g. trastuzumab, trastuzumab-DM I or pertuzumab) or an inhibitor of both EGFR and HER2, such as lapatinib).

**[00307]** In certain embodiments, the second therapeutic agent is a tyrosine kinase inhibitor, such as imatinib (Glivec, Gleevec STI571) or lapatinib.

**[00308]** In certain embodiments, the second therapeutic agent is a cytokine, growth factor, chemokine, or a combination thereof. Non-limiting examples of cytokines and growth factors include IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, IL-23, IL-24, IL-27, IL-28a, IL-28b, IL-29, KGF, IFN- $\alpha$  (e.g., IFN $\alpha$ 2b), IFN- $\gamma$ , GM-CSF, CD40L, Flt3 ligand, stem cell factor, anacardim, and TNF $\alpha$ . Non-limiting examples of chemokines include Glu-Leu-Arg (ELR)-negative chemokines such as IP-10, MCP-3, MIG, and SDF-1 $\alpha$  from the human CXC and C-C chemokine families. Suitable cytokines include cytokine derivatives, cytokine variants, cytokine fragments, and cytokine fusion proteins.

**[00309]** In certain embodiments, the second therapeutic agent is a cell cycle control/apoptosis regulator (or “regulating agent”). A cell cycle control/apoptosis regulator may include molecules that target and modulate cell cycle control/apoptosis regulators such as (i) cdc-25 (such as NSC 663284), (ii) cyclin-dependent kinases that overstimulate the cell cycle (such as flavopiridol (L868275, HMR1275), 7-hydroxystaurosporine (UCN-01, KW-2401), and roscovitine (R-roscovitine, CYC202)), and (iii) telomerase modulators (such as BIBR1532, SOT-095, GRN163 and compositions described in for instance US 6,440,735 and US 6,713,055). Non-limiting

examples of molecules that interfere with apoptotic pathways include TNF-related apoptosis-inducing ligand (TRAIL)/apoptosis-2 ligand (Apo-2L), antibodies that activate TRAIL receptors, IFNs, and anti-sense Bcl-2.

**[00310]** In certain embodiments, the second therapeutic agent is a hormonal regulating agent, such as agents useful for anti-androgen and anti-estrogen therapy. Examples of such hormonal regulating agents are tamoxifen, idoxifene, fulvestrant, droloxifene, toremifene, raloxifene, diethylstilbestrol, ethinyl estradiol/estiny, an anti-androgen (such as flutamide/eulexin), a progestin (such as hydroxyprogesterone caproate, medroxy-progesterone/provera, megestrol acetate/megace), an adrenocorticosteroid (such as hydrocortisone, prednisone), luteinizing hormone-releasing hormone (and analogs thereof and other LHRH agonists such as buserelin and goserelin), an aromatase inhibitor (such as anastrozole/arimidex, aminoglutethimide/cytadren, exemestane) or a hormone inhibitor (such as octreotide/sandostatin).

**[00311]** In certain embodiments, the second therapeutic agent is an anti-cancer nucleic acid or an anti-cancer inhibitory RNA molecule.

**[00312]** Combined administration, as described above, may be simultaneous, separate, or sequential. For simultaneous administration the agents may be administered as one composition or as separate compositions, as appropriate.

**[00313]** In certain embodiments, the presently disclosed cells and compositions are administered in combination with radiotherapy. Radiotherapy may comprise radiation or associated administration of radiopharmaceuticals to a subject is provided. The source of radiation may be either external or internal to the patient being treated (radiation treatment may, for example, be in the form of external beam radiation therapy (EBRT) or brachytherapy (BT)). Radioactive elements that may be used in practicing such methods include, e.g., radium, cesium-137, iridium-192, americium-241, gold-198, cobalt-57, copper-67, technetium-99, iodine-123, iodine-131, and indium-111.

**[00314]** In certain embodiments, the presently disclosed cells and compositions are administered in combination with surgery.

**[00315]** According to various embodiments, the presently disclosed CAR-expressing cells (e.g., CAR-T cells) may be designed in several ways to achieve one or more of the following: enhance tumor cytotoxicity and specificity, evade tumor immunosuppression, avoid host

rejection, and prolong their therapeutic half-life. TRUCK (T-cells Redirected for Universal Cytokine Killing) T cells, for example, possess a CAR but are also engineered to release cytokine(s) (such as IL-12) that promote tumor killing. Because these cells are designed to release a molecular payload upon activation of the CAR once localized to the tumor environment, these CAR-T cells are sometimes also referred to as “armored CARs”. Several cytokines as cancer therapies are being investigated both pre-clinically and clinically, and may also prove useful when similarly incorporated into a TRUCK form of CAR-T therapy. Among these include IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, M-CSF, GM-CSF, IFN- $\alpha$ , IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ , TRAIL, FLT3 ligand, and Lymphotoxin. “Self-driving” or “homing” CAR-T cells are engineered to express a chemokine receptor in addition to their CAR. As certain chemokines can be upregulated in tumors, incorporation of a chemokine receptor aids in tumor trafficking to and infiltration by the adoptive T-cell, thereby enhancing both specificity and functionality of the CAR-T. Universal CAR-T cells also possess a CAR, but are engineered such that they do not express endogenous TCR (T-cell receptor) or MHC (major histocompatibility complex) proteins. Removal of these two proteins from the signaling repertoire of the adoptive T-cell therapy prevents graft-versus-host-disease and rejection, respectively. Armored CAR-T cells are additionally so named for their ability to evade tumor immunosuppression and tumor-induced CAR-T hypofunction. These particular CAR-Ts possess a CAR, and may be engineered to not express checkpoint inhibitors. Alternatively, these CAR-Ts can be co-administered with a monoclonal antibody (mAb) that blocks checkpoint signaling. Administration of an anti-PD-L1 antibody significantly restores the killing ability of CAR tumor infiltrating lymphocytes (TILs). While PD1-PDL1 and CTLA-4-CD80/CD86 signaling pathways have been investigated, it is possible to target other immune checkpoint signaling molecules in the design of an armored CAR-T including LAG-3, Tim-3, IDO-1, 2B4, and KIR. Other intracellular inhibitors of TILs include phosphatases (SHP1), ubiquitin-ligases (i.e., cbl-b), and kinases (i.e., diacylglycerol kinase). Armored CAR-Ts may also be engineered to express proteins or receptors that protect them against or make them resistant to the effects of tumor-secreted cytokines. For example, CTLs (cytotoxic T lymphocytes) transduced with the double negative form of the TGF- $\beta$  receptor are resistant to the immunosuppression by lymphoma secreted TGF- $\beta$ . These transduced cells showed notably increased antitumor activity in vivo when compared to their control counterparts.

**[00316]** Additionally, in certain embodiments, the B-lymphocyte antigen-associated disease is an autoimmune disease. Non-limiting examples of autoimmune diseases include multiple sclerosis, systemic lupus erythematosus (SLE), Sjogren's, AL amyloidosis, Sjogren's, AL amyloidosis, IgG4-related disease (IgG4-RD), inflammatory myopathies (e.g., myositis), scleroderma, pemphigus vulgaris, chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), stiff-person's syndrome, rheumatoid arthritis, neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis, N-methyl-D-aspartate receptor (NMDAR) encephalitis, anti-myelin oligodendrocyte glycoprotein (MOG) syndrome, myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), membranous nephropathy (MN), IgA nephropathy (IgAN), Type 1 diabetes, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, thrombocytopenia purpura, hemolytic anemia (AIHA), multifocal motor neuropathy (MMN), inflammatory bowel disease (IBD), celiac disease, Crohn's disease, ulcerative colitis, and ankylosing spondylitis. In certain embodiments, the autoimmune disease is multiple sclerosis (MS). In certain embodiments, the autoimmune disease is systemic lupus erythematosus (SLE).

**[00317]** Current cell-based immunotherapy targeting B cells has shown early promise in the treatment of autoimmune disease. In autoimmune disease, B cells may serve as immune regulators, for example, in multiple sclerosis (MS). MS is a demyelinating disease characterized by active neurodegeneration associated with lymphocyte infiltrates in the brain. While the cause of MS remains unknown, the results of B-cell depleting anti-CD20 therapy in MS highlight a central, antibody-independent role of B cells in MS attacks, specifically through the modulation of immune cell responses in both the periphery and within the CNS. (Li R, Bar-Or A, *Cold Spring Harb Perspect Med* (2019);9(4)). B cells contribute to the pathogenesis of MS through several mechanisms, which include releasing inflammatory cytokines/chemokines, activating peripheral T cells and driving chronic infiltration of pro-inflammatory myeloid cells and T cells into the CNS. As disease progresses, the proliferation of localized CNS-residing immune cells contributes to further neuroinflammation and demyelination (Li R, Bar-Or A, *Cold Spring Harb Perspect Med* (2019);9(4) Epub Date 2018/04/18).

**[00318]** Current immunomodulatory therapies targeting B cells via CD20 are effective at preventing relapse but are unable to stop disease progression, perhaps due to challenges in adequately accessing the CNS. Autologous chimeric antigen receptor (CAR) T-cell therapy



targeting CD19 has been highly effective in eliminating pathogenic B cells across B-cell driven autoimmune disorders with attenuation of disease activity and substantial improvement of symptoms in patients with systemic lupus erythematosus, systemic sclerosis, myositis and anti-synthetase syndrome. (Bergmann C, *et al.*, *Ann Rheum* (2023);82(8):1117–1120; Müller F, *et al.*, *N Engl J Med* (2024); 390(8):687-700; Pecher AC, *et al.* *JAMA* (2023);329(24):2154-2162). CD19-targeted CAR T cells have also shown early promise in the treatment of progressive MS; two patients treated with autologous CD19 CAR T cells showed expansion of CAR T in the cerebrospinal fluid (CSF) along with a reduction in oligoclonal bands (OCBs) and intrathecal antibodies in 1 patient. No CAR T cell-related neurotoxicity was reported in either patient (Fischbach F, *et al.* *Med* (2024);5(6):550-558.e552). CD19-directed CAR T cells may offer more complete B-cell depletion in the CNS compared to other B-cell targeting mAbs (e.g., anti-CD20 rituximab) due to their ability to access the blood brain barrier as well as target CD19-expressing plasmablasts that lose CD20 expression.

**[00319]** CD20<sup>+</sup> T cells, which were recently revealed to acquire CD20 from B cells via trogocytosis, were demonstrated to worsen disease course in animal models of MS (experimental autoimmune encephalomyelitis, EAE) (Ochs J, *et al.* *Sci Transl Med* (2022);14(638):eabi4632). Furthermore, a recent study has reported an association between a higher frequency of CD8<sup>+</sup>CD20<sup>+</sup> T cells with demyelination in PPMS patients (von Essen MR, *et al.*, *Neurol Neuroimmunol Neuroinflamm* (2023);10(5)). Recent reports indicate that CD20<sup>+</sup> T cells can be depleted by anti-CD20 antibody therapy in MS subjects (e.g. ocrelizumab and ofatumumab) (Gingele S, *et al.* *Cells* (2018);8(1); von Essen MR, *et al.*, *Neurol Neuroimmunol Neuroinflamm* (2022);9(4)). The potential role of CD20<sup>+</sup> T cells in the pathophysiology of MS supports an additional mechanism by which targeting CD20 is effective for treating an autoimmune disease (e.g., MS).

**[00320]** The present disclosure provides methods of treating an autoimmune disease in a subject using the presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions. The presently disclosed cells and compositions target both CD19 and CD20 to eliminate pathogenic B (e.g., killing or depleting CD19<sup>+</sup> B cells, CD20<sup>+</sup> B cells) and T cells (e.g., killing or depleting CD19<sup>+</sup> T cells, CD20<sup>+</sup> T cells) to provide therapeutic benefit in patients with autoimmune diseases (e.g., multiple sclerosis, and systemic lupus erythematosus).

[00321] The presently disclosed subject matter provides various methods of using the presently disclosed cells or compositions. The presently disclosed cells or compositions can be used in a therapy or medicament. For example, the presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions can be used for the manufacture of a medicament for inhibiting growth of a target cell expressing at least one B-lymphocyte antigen. The presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions can be used for the manufacture of a medicament for treating a hematologic cancer in a subject. The presently disclosed CD19/CD20-targeted CAR-expressing cells and compositions can be used for the manufacture of a medicament for treating an autoimmune disease in a subject. The various methods comprise administering to a subject in need thereof an effective amount of the presently disclosed cells or a presently disclosed composition. The presently disclosed cells and compositions do not induce graft-versus-host disease. The presently disclosed cells and compositions induce no or minimal alloreactivity against a HLA-mismatched cell positive for the B-lymphocyte antigen in the subject. Additionally or alternatively, the presently disclosed cells and compositions exhibit no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for the B-lymphocyte antigen. In certain embodiments, the off-target cell is negative for CD19 or CD20. In certain embodiments, the off-target cell is selected from the group consisting of a CD19<sup>+</sup>CD20<sup>-</sup> T cell, a Natural Killer T cell (NK T cell), a Natural Killer cell (NK cell), and a combination thereof. In certain embodiments, the presently disclosed cells and compositions induce cytokine secretion. In certain embodiments, the cytokine is interferon gamma (IFN- $\gamma$ ). In certain embodiments, the cytokine is tumor necrosis factor alpha (TNF- $\alpha$ ).

[00322] According to one or more embodiments, to better control CAR-T therapy and prevent against unwanted side effects, a variety of features can be engineered into embodiments of the CAR-T cells disclosed herein including one or more of off-switches, safety mechanisms, and conditional control mechanisms. Both self-destruct and marked/tagged CAR-T cells for example, are engineered to have an “off-switch” that promotes clearance of the CAR-expressing T-cell. A self-destruct CAR-T contains a CAR but is also engineered to express a pro-apoptotic suicide gene or “elimination gene” inducible upon administration of an exogenous molecule. A variety of suicide genes may be employed for this purpose, including HSV-TK (herpes simplex virus thymidine kinase), Fas, iCasp9 (inducible caspase 9), MYC TAG, and truncated epidermal

growth factor receptor (EGFRt) polypeptide (endothelial growth factor receptor). HSK for example, converts the prodrug ganciclovir (GCV) into GCV-triphosphate that incorporates itself into replicating DNA, ultimately leading to cell death. iCasp9 is a chimeric protein containing components of FK506-binding protein that binds the small molecule AP1903, leading to caspase 9 dimerization and apoptosis. In certain embodiments, the suicide gene is an EGFRt polypeptide. The EGFRt polypeptide can enable T-cell elimination by administering anti-EGFR monoclonal antibody (*e.g.*, cetuximab). EGFRt can be covalently joined to the upstream of the CD19/CD20-targeted CAR. The suicide gene can be included within the vector comprising nucleic acids encoding a presently disclosed CD19/CD20-targeted CAR. In this way, administration of a prodrug designed to activate the suicide gene (*e.g.*, a prodrug (*e.g.*, AP1903 that can activate iCasp-9) during malignant T-cell transformation (*e.g.*, GVHD) triggers apoptosis in the suicide gene-activated cells expressing the CD19/CD20-targeted CAR. The incorporation of a suicide gene into the a presently disclosed CD19/CD20-targeted CAR gives an added level of safety with the ability to eliminate the majority of receptor-expressing cells within a very short time period. A presently disclosed cell incorporated with a suicide gene can be preemptively eliminated at a given timepoint post the cell infusion, or eradicated at the earliest signs of toxicity.

**[00323]** According to one or more embodiments, the presently disclosed cells can be designed as marked/tagged CAR-T cells expressing a presently disclosed CD19/CD20-targeted CAR and a selection marker. Administration of a mAb against this selection marker may promote clearance of the CAR-T cell. Truncated EGFR is one such targetable antigen by the anti-EGFR mAb, and administration of cetuximab works to promotes elimination of the CAR-T cell. CARs created to have these features are also referred to as sCARs for “switchable CARs”, and RCARs for “regulatable CARs”.

**[00324]** Additionally, in various embodiments, the presently disclosed cells can further comprise an inhibitory CAR” (iCAR) that comprises an extracellular antigen-binding domain that binds to a normal tissue and one or more intracellular signaling domains of one or more inhibitory molecules (Fedorov, *et al.*, *Sci Transl Med.* (2013);5(215): 215). Non-limiting examples of inhibitory molecules include CTLA-3, PD-1, CD45, PD-L1, B7-1, CD160, PIH, 2B4, CEACAM (CEACAM-1, CEACAM-3, and/or CEACAM-5), LAG-3, TIGIT, BTLA, LAIR1, and TGFβ-R. In the presence of normal tissue, the iCAR can inhibit the presently

disclosed CD19/CD20-targeted CAR. The safety of CAR-T engineering enhances specificity of the CAR-T cell for tumor tissue, and is advantageous in situations where certain normal tissues may express very low levels of a tumor associated antigen that would lead to off-target effects with a standard CAR.

[00325] A conditional CAR-T cell expresses an extracellular antigen binding domain connected to an intracellular stimulatory domain and a separate, intracellular costimulatory domain. The costimulatory and stimulatory domain sequences are engineered in such a way that upon administration of an exogenous molecule the resultant proteins will come together intracellularly to complete the CAR circuit. In this way, CAR-T activation can be modulated, and possibly even “fine-tuned” or personalized to a specific patient. The stimulatory and costimulatory domains are physically separated when inactive in the conditional CAR; for this reason these too are also referred to as a “split CAR”.

## 6. EXAMPLES

[00326] The practice of the presently disclosed subject matter employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan.

[00327] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the presently disclosed cells and compositions, and are not intended to limit the scope of what the inventors regard as their invention.

### **Example 1. Generation of Bispecific CD19/CD20-targeted CAR-T Cells**

[00328] Two CARs designated as “20-19 1XX” and “20-19 BB06z” were constructed. The structure and sequence of the “20-19 1XX” CAR are described in Section 5.3.4. and illustrated in Figure 1. As shown in Figure 1, the “20-19 BB06z” CAR comprises: (i) an extracellular domain comprising (a) an anti-CD19 antigen-binding domain that is an scFv derived from the anti-CD19 antibody FMC63 and (b) an anti-CD20 antigen-binding domain that is an scFv derived from the anti-CD20 antibody Leu16, (ii) a CD8 hinge region, (iii) a CD8 transmembrane domain, and (iv) an intracellular domain comprising a native CD3 $\zeta$  polypeptide and a costimulatory signaling region comprising a mutant CD28 polypeptide designated as mut06, and a 4-1BB signaling

domain. Mut06 is disclosed in Boucher *et al.*, *Cancer Immunol Res.* (2021);9:62-74, which is incorporated by reference hereby in its entirety. To optimize CD28 function in CAR T cells, Boucher *et al.*, incorporated null mutations in CD28 ITAM subdomains (YMN, PRRP, or PYAP). The combined mutations of YMN to FMN and PRRP to ARRA (mut06) improved the persistence of function of CAR-T cells (Boucher *et al.*, (2021)). In addition, in order to enhance the persistence of CAR T-cells, Roselli *et al.*, combined mut06 costimulatory domain with 4-1BB costimulatory domain (which is known to increase persistence compared to CD28) and found that bispecific CD19/CD20-targeted CAR with 4-1BB and mut06 costimulatory domains had improved *in vivo* anti-tumor activity, increased persistence, and decreased exhaustion as compared to bispecific CD19/CD20-targeted CAR with mut06 costimulatory domain alone (Roselli *et al.*, *J. Immunother. Cancer* (2021);9:1-15). The “20-19 BB06z” CAR comprises a G4S linker (i.e., (G4S)<sub>3</sub>) consisting of the amino acid sequence set forth in SEQ ID NO: 2) connecting the V<sub>H</sub> and V<sub>L</sub> of the anti-CD20 scFv, and a G4S linker (i.e., (G4S)<sub>5</sub>) consisting of the amino acid sequence set forth in SEQ ID NO: 3) connecting the anti-CD20 scFv and the anti-CD19 scFv, and a Whitlow linker (i.e., one consisting of the amino acid sequence set forth in SEQ ID NO: 1) connecting V<sub>H</sub> and V<sub>L</sub> of the anti-CD19 scFv.

[00329] The “20-19 1XX” and “20-19 BB06z” CARs were introduced into the backbone of retroviral vector SFG-1xx-FMC63 for downstream lead construct determination. The “20-19 1XX” and “20-19 BB06z” CARs were successfully transduced into and expressed by host T cells. The binding specificity of these two CARs was assessed using an *in vitro* binding assay. The T cells comprising these two CARs were found to specifically bind to both CD19 and CD20 in the assay (data not shown).

**Example 2. In Vitro and In Vivo Activities of MPI-Generated Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs**

[00330] The *in vitro* and *in vivo* cytotoxicity activities of the “20-19 1XX” and “20-19 BB06z” CARs were assessed using serial stimulation assays.

[00331] Allogeneic cytotoxic T cells (CTLs) from donors (for example, “Doner 13,” “Doner 14,” “Doner 15,” and “Doner 40”) were transduced to express the “20-19 1XX” CAR or the “20-19 BB06z” CAR. The donor CTLs were stimulated to EBV antigens by co-culturing the CTLs with EBV-infected B-lymphoblastoid cell line (BLCL)s using a proprietary manufacturing process MPI. The CAR expression, vector copy number (VCN), and viability are shown in

Table 3. The CAR expression was measured with fluorescently labeled anti-idiotypic antibody against FMC63 scFv via flow cytometry. VCN was measured using droplet digital PCR. Viability was measured using cell countess machine. In Table 3, “40 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. “40 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. “40 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 40 (not expressing a CAR).

Table 3

| Group    | Manufacturing Process | CAR% | VCN  | Viability% |
|----------|-----------------------|------|------|------------|
| 40 NTD   | MP1                   | -    | -    | 69         |
| 40 BB06z | MP1                   | 23.8 | 0.95 | 70         |
| 40 1XX   | MP1                   | 15.7 | 0.51 | 62         |

[00332] Next, the *in vitro* cytotoxicity activities of the CAR-T cells against cells expressing CD19, CD20 or both were assessed in serial challenge assays. Briefly, CAR- T cells and target cells (K562 cells or Raji cells) were co-cultured at an effector ( $E^{CAR+}$ ):target(T) ratio of 5:1. K562 cells were artificially engineered to express human CD19, human CD20, or both. Raji B cell lymphoma line naturally expresses both CD19 and CD20. To further elucidate the impact of CD19 and CD20, a panel of Raji cells expressing varying levels of CD19 were used as target cells, including cells with no CD19 expression (Raji-CD19<sup>KO</sup>), with low levels (Raji-CD19<sup>Low</sup>), and high levels (Raji-CD19<sup>High</sup>) of CD19. Cells were transferred to a 96-well round bottom non-tissue culture plate for fluorescence-activated cell sorting (FACS) analysis. Phenotypic analysis was performed by FlowJo, and numbers of live target cells were counted. In addition, target cells were added to wells for re-stimulation. The results are shown in Table 4 and Figures 2A-2E. As shown in Table 4 and Figures 2A-2E, the allogeneic EBV-sensitized CTLs from Donor 40 expressing the “20-19 1XX” CAR surprisingly exhibited more potent cytotoxicity activity than the “20-19 BB06z” CAR.

Table 4

| Target Cells                               | Best-performed CAR construct in the <i>in vitro</i> testing |             |             |           |
|--------------------------------------------|-------------------------------------------------------------|-------------|-------------|-----------|
|                                            | Donor 13                                                    | Donor 14    | Donor 15    | Donor 40  |
| CD19 <sup>-</sup> CD20 <sup>-</sup> K562   | 20-19 1XX                                                   | 20-19 1XX   | 20-19 1XX   | 20-19 1XX |
| CD19 <sup>-</sup> CD20 <sup>+</sup> K562   | 20-19 BB06z                                                 | 20-19 1XX   | 20-19 1XX   | 20-19 1XX |
| CD19 <sup>+</sup> CD20 <sup>+</sup> K562   | 20-19 1XX                                                   | 20-19 1XX   | 20-19 BB06z | 20-19 1XX |
| CD19 <sup>low</sup> CD20 <sup>+</sup> Raji | 20-19 1XX                                                   | 20-19 BB06z | 20-19 1XX   | 20-19 1XX |

|                                             |           |             |           |           |
|---------------------------------------------|-----------|-------------|-----------|-----------|
| CD19 <sup>KO</sup> CD20 <sup>+</sup> Raji   | 20-19 1XX | 20-19 1XX   | 20-19 1XX | 20-19 1XX |
| CD19 <sup>high</sup> CD20 <sup>+</sup> Raji | 20-19 1XX | 20-19 BB06z | 20-19 1XX | 20-19 1XX |

[00333] Further, the *in vivo* anti-tumor activities of the CAR-T cells against Burkitt's lymphoma were assessed.

[00334] As shown in Figure 3, 6-7-week-old female NSG mice were transplanted intravenously with Raji CD19<sup>low</sup>-GFP/nanoluc tumor cells (Raji CD19<sup>low</sup>CD20<sup>+</sup> tumor cells) at an amount of  $5 \times 10^5$  cells per mouse. On Day 6 post-tumor engraftment, mice were injected intravenously with  $3 \times 10^6$  CAR-T cells per mouse. A non-transduced T cell designated as "NTD" was used as a negative control. Tumor growth was monitored for 10 weeks. In particular, tumor was measured twice weekly following tumor engraftment. Clinical symptoms and body weights were assessed at least twice a week. In addition, tumor burden was measured by bioluminescence (BLI, bioluminescence imaging) at indicated days since CAR-T cell injection. Mouse survival days post CAR-T cell treatment were shown by Kaplan-Meier plot.

[00335] The results are shown in Figures 4A-4C and 5A-5B. As shown in Figures 4A-4C and 5B, the allogeneic EBV-sensitized CTLs from Donor 40 expressing the "20-19 1XX" CAR demonstrated surprisingly greater tumor growth inhibition than T cells comprising the "20-19 BB06z" CAR. Further, Figures 5A and 5B show that the "20-19 1XX" CAR-T cell expansion was correlated with its anti-tumor activity and led to a statistically significant extension in the overall survival of treated mice as compared to the "20-19 BB06z" CAR-T cells.

[00336] In summary, the "20-19 1XX" CAR demonstrated higher tumor growth inhibition in serial re-challenge assays and tumor-bearing animals than the "20-19 BB06z" CAR. In addition, the "20-19 1XX" CAR-T cells showed antigen-specific cytolysis of CD19<sup>low</sup> and CD19<sup>KO</sup> targets, which suggests that CD20 CAR-mediated mechanism of action required for treating B cell tumors with CD19 antigen loss (e.g., Relapsed/refractory (R/R) B cell tumors).

**Example 3. In Vitro Activities of MP2-Generated Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs**

[00337] Allogeneic CTLs from Donor 14 and Donor 40 were transduced to express the "20-19 1XX" CAR or the "20-19 BB06z" CAR. In this example, the donor CTLs were stimulated to EBV antigens by co-culturing the CTLs with EBV-infected BLCLs using a different proprietary manufacturing process MP2. The CAR expression, vector copy number (VCN), and viability for the CAR-T cells from Donor 14 are shown in Table 5. CAR expression, vector copy number

(VCN), and viability for the CAR-T cells from Donor 14 are shown in Table 6. The CAR expression was measured with fluorescently labeled anti-idiotypic antibody against FMC63 scFv via flow cytometry. VCN was measured using droplet digital PCR. Viability was measured using cell countess machine. In Table 5, “14 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 14 expressing the “20-19 1XX” CAR. “14 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 14 expressing the “20-19 BB06z” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 14 (not expressing a CAR). In Table 6, “40 1XX” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 1XX” CAR. “40 BB06z” represents allogeneic EBV-sensitized CTLs derived from Donor 40 expressing the “20-19 BB06z” CAR. “40 NTD” represents non-transduced allogeneic EBV-sensitized CTLs derived from Donor 40 (not expressing a CAR).

Table 5

| Group    | Manufacturing Process | CAR% | VCN  | Viability% |
|----------|-----------------------|------|------|------------|
| 14 NTD   | MP2                   | -    | -    | 83         |
| 14 BB06z | MP2                   | 19.7 | 0.98 | 62         |
| 14 1XX   | MP2                   | 37.8 | 1.25 | 74         |

Table 6

| Group    | Manufacturing Process | CAR% | VCN  | Viability% |
|----------|-----------------------|------|------|------------|
| 40 NTD   | MP2                   | -    | -    | 70         |
| 40 BB06z | MP2                   | 46.7 | 1.47 | 68         |
| 40 1XX   | MP2                   | 64.6 | 1.89 | 76         |

[00338] Next, the *in vitro* cytotoxicity activities of the CAR-T cells against cells expressing CD19, CD20 or both were assessed in serial challenge assays following the same protocol used in Example 2. The results are shown in Table 7 and Figures 6A-6E and 7A-7E. As shown in Table 7 and Figures 6A-6E and 7A-7E, the allogeneic EBV-sensitized CTLs from both Donor 14 and Donor 40 expressing the “20-19 1XX” CAR surprisingly exhibited more potent cytotoxicity activity than other tested T cells.

Table 7

| Target Cells                             | Best-performed CAR construct |           |
|------------------------------------------|------------------------------|-----------|
|                                          | Donor 14                     | Donor 40  |
| CD19 <sup>-</sup> CD20 <sup>-</sup> K562 | 20-19 1XX                    | 20-19 1XX |
| CD19 <sup>-</sup> CD20 <sup>+</sup> K562 | 20-19 1XX                    | 20-19 1XX |
| CD19 <sup>+</sup> CD20 <sup>+</sup> K562 | 20-19 1XX                    | 20-19 1XX |



|                                            |           |           |
|--------------------------------------------|-----------|-----------|
| CD19 <sup>low</sup> CD20 <sup>+</sup> Raji | 20-19 1XX | 20-19 1XX |
|--------------------------------------------|-----------|-----------|

[00339] In summary, similar to the results seen in Example 2, the allogeneic “20-19 1XX” CAR-T cells demonstrated higher tumor growth inhibition in serial re-challenge assays than the “20-19 BB06z” CAR-T cells. In addition, the “20-19 1XX” CAR-T cells showed antigen-specific cytolysis of CD19<sup>+</sup> or CD20<sup>+</sup> targets, confirming CD19 and CD20-specific CAR-mediated mechanism of action is required for treating B cell tumors as an earlier line therapy and preventing relapse due to CD19 antigen escape.

**Example 4. In Vivo Anti-tumor Activities of CD19/CD20-targeted CAR-T cells**

[00340] In this Example, the *in vivo* anti-tumor efficacy of allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR was compared to that of autologous T cells expressing a Benchmark CD19/CD20-targeted CAR. The structure of the Benchmark CD19/CD20-targeted CAR is shown in Figure 8. As shown in Figure 8, the Benchmark CD19/CD20-targeted CAR comprises: (i) an extracellular domain comprising (a) an anti-CD19 antigen-binding domain that is an scFv derived from the anti-CD19 antibody FMC63 and (b) an anti-CD20 antigen-binding domain that is an scFv derived from the anti-CD20 antibody Leu16, (ii) a CD28 hinge region, (iii), a CD28 transmembrane domain, and (iv) an intracellular domain comprising a native CD3ζ polypeptide and a costimulatory signaling region comprising a 4-1BB signaling domain. The Benchmark CD19/CD20-targeted CAR comprises a GS18 linker (i.e., one consisting of the amino acid sequence set forth in SEQ ID NO: 56) connecting the V<sub>H</sub> and V<sub>L</sub> of the anti-CD20 scFv, and a rigid EAAAK linker (i.e., (EAAAK)<sub>3</sub> consisting of the amino acid sequence set forth in SEQ ID NO: 57) connecting the anti-CD20 scFv and the anti-CD19 scFv, and a Whitlow linker (i.e., one consisting of the amino acid sequence set forth in SEQ ID NO: 1) connecting V<sub>H</sub> and V<sub>L</sub> of the anti-CD19 scFv. SEQ ID NO: 56 and SEQ ID NO: 57 are provided below. Zah *et al.*, disclose CD19/CD20-targeted CARs having similar structures to the Benchmark CD19/CD20-targeted CAR were effective to treat B cell malignancy (Zah *et al.*, *Cancer Immunol Res.* (2016);4(6):498-508).

GSTSGGGSGGGSGGGSS [SEQ ID NO: 56]

EAAAKEAAKEAAAK [SEQ ID NO: 57]

[00341] The allogeneic EBV-sensitized T cells were generated from Doner 14, and the donor CTLs were stimulated to EBV antigens by co-culturing the CTLs with EBV-infected BLCLs using MP2.

[00342] As shown in Figure 9, 6-7-week-old female NSG mice were transplanted intravenously with Raji CD19<sup>low</sup>CD20<sup>+</sup> tumor cells at an amount of  $5 \times 10^5$  cells per mouse. On Day 6 post-tumor engraftment, mice were injected intravenously with  $3 \times 10^6$  CAR-T cells per mouse. A non-transduced T cell designated as “NTD” was used as a negative control. Tumor growth was monitored for 10 weeks. In particular, tumor was measured twice weekly following tumor engraftment. Clinical symptoms and body weights were assessed at least twice a week. In addition, tumor burden was measured by bioluminescence (BLI, bioluminescence imaging) at indicated days since CAR-T cell injection. Mouse survival days post CAR-T cell treatment were shown by Kaplan–Meyer plot.

[00343] The results are shown in Figures 10A-10C. As shown in Figure 10A, the allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR demonstrated surprisingly superior anti-tumor efficacy as compared to the autologous Benchmark CAR-T cells in challenging the *in vivo* lymphoma model. The blood cells of the treated mice were quantified by flow cell using BD TruCount Absolute Counting beads (BD Biosciences), and the results are shown in Figure 10B. The vector copy number (VCN) per cell was also measured for the CAR-T cells, as shown in Figure 10C.

**Example 5. Safety of the Presently Disclosed CAR-T cells in *in vivo* Treatment**

[00344] The safety of the allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR or the “20-19 BB06z” CAR for *in vivo* treatment was assessed by monitoring relative body weight changes of the treated mice as compared to the Day 0 treatment. 6-7-week-old female NSG mice were transplanted intravenously with Raji CD19<sup>low</sup>CD20<sup>+</sup> tumor cells. On Day 6 post-tumor engraftment, mice were injected intravenously with  $1 \times 10^6$  CAR-T cells,  $3 \times 10^6$  CAR-T cells, or  $9 \times 10^6$  CAR-T cells per mouse. The body weight of the various mice employed in the *in vivo* experiments remained consistent over time in both the treated and untreated groups, thereby demonstrating that no *in vivo* safety issues were observed.

**Example 6. Characterization of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs**

[00345] “20-19 1XX” CAR T cells were produced according to the manufacturing process MP2 from three different donors. Flow cytometry analyses of the CAR expression on “20-19 1XX” products from three different donors demonstrated successful and stable transduction of EBV T cells with “20-19 1XX” retroviral vector, as shown in Figure 11A. Vector copy number

(VCN) analyses showed consistently lower than 5 vector copies per genome in vector copy number (VCN) analyses. Further, CD4 and CD8 distribution in “20-19 1XX” products demonstrated predominantly CD8<sup>+</sup> T cells, as shown in Figure 11B. As indicated in Figure 11C, flow cytometry analyses of memory markers CD45RO and CD62L showed “20-19 1XX” products maintained a robust central memory population compared with autologous benchmark CART cells.

[00346] Further, in studies evaluating research lots of “20-19 1XX” (n=10), T cells contained a high frequency of CD3<sup>+</sup> lymphocytes (median, 95.8%) with a heterogeneous number of CD8<sup>+</sup> T cells, ranging from 41.1% to 90.4% (median, 85.1%) and CD4<sup>+</sup> T cells, ranging from 1.6% to 53.3% (median, 7.78%). Generation of “20-19 1XX” demonstrated a median cumulative fold expansion of 243.5 compared to 377.1 for non-transduced (NTD) controls, indicating minimal impact of CAR transduction on cell expansion (data not shown).

[00347] Additional assessments were performed to further characterize the immunophenotype of research lots of “20-19 1XX”. These studies showed “20-19 1XX” to be enriched for central memory populations, with high double positive populations of CD45RO and CD62L ranging from 76.7% to 92.6%, indicative of antigen-experienced central memory T cell populations.

[00348] Surface CAR expression across 10 research lots of “20-19 1XX” yielded a range of 57.6-84.6% of cells expressing the CAR protein. Analysis of CAR integration via droplet digital PCR measured a vector copy number range of 2.48-4.41 copies per cell (data not shown).

**Example 7. In Vitro Activities of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs**

[00349] To test the function of “20-19 1XX” *in vitro*, serial stimulation assay was conducted against various Non-Hodgkin lymphoma (NHL) cell lines expressing CD19 and CD20. CD19 and CD20 specific anti-tumor activity of “20-19 1XX” was detected against each cell line. Specific functional activity of “20-19 1XX” against CD20- and/or CD19-expressing target cell lines was measured in a serial stimulation assay using a 5:1 effector (E<sup>CAR+</sup>):target(T) (E:T) ratio co-cultured with Jeko-1 (Figure 12A) and Su-DHL-4 (Figure 12B).

[00350] Further, the functional activity of “20-19 1XX” was measured through assessments of CD19 and/or CD20-directed cytotoxicity and cytokine secretion of the CAR-T cells against antigen positive target cells K562. The “20-19 1XX” effectors and luciferase-expressing target cells were incubated together at a 1:1 E:T ratio. Lysis of the target cells was measured 24 hours later.

[00351] Enumeration of target cells following repeated serial stimulation (denoted by vertical dashed lines) demonstrated durable tumor killing and CD20- and/or CD19-specific functional activity against various non-Hodgkin lymphoma cell lines, as shown in Figures 12A and 12B. NTD EBV T cells from each product lot did not show specific activity against CD19 and/or CD20-positive or negative targets tested.

**Example 8. Alloreactivities of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs**

[00352] Alloreactive potential of “20-19 1XX” was measured through the activation of T cells, CD69<sup>+</sup>/4-1 BB<sup>+</sup> T cells, and the cytotoxicity of HLA-matched (autologous [auto]) and mismatched (allogeneic [allo]) CD20<sup>+</sup>/CD19<sup>+</sup> B-lymphoblastoid cell line (BLCL) targets after 2 days in co-culture. The maintenance of “20-19 1XX” EBV specificity was evaluated using flow cytometry.

[00353] “20-19 1XX” demonstrated minimal alloreactivity against HLA-mismatched targets and showed HLA-independent activity against CD20<sup>+</sup>/CD19<sup>+</sup> targets *in vitro*. The results show that autologous EBV<sup>+</sup>/CD20<sup>+</sup>/CD19<sup>+</sup> BLCLs were lysed by both “20-19 1XX” and NTD EBV T cells via CD20, CD19, and/or HLA-restricted EBV recognition; but allogeneic HLA-mismatched EBV<sup>+</sup>/CD20<sup>+</sup>/CD19<sup>+</sup> BLCLs were lysed only by “20-19 1XX” via HLA-independent CD20/CD19 CAR-directed lysis. Through the EBV specificity assay *in vitro*, it was demonstrated that “20-19 1XX” killing was modulated via CAR and that “20-19 1XX” demonstrated minimal alloreactivity against HLA-mismatched targets. In Figure 13A, while “20-19 1XX” showed robust activation against HLA-mismatched BLCL targets, NTD cells were not activated without HLA-match in target BLCLs. Similarly in Figure 13B, “20-19 1XX” killing was confirmed to be acted through HLA-independent recognition by the CAR. Without CAR, NTD cells only targeted HLA-matched autologous BLCL targets.

**Example 9. In Vivo Anti-tumor Activities of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs in Dosage Study**

[00354] NOD scid gamma (NSG) mice were intravenously implanted with  $0.5 \times 10^6$  Raji CD19<sup>low</sup> cells on day 0. Mice were randomized into different groups by bioluminescence imaging (BLI) and treated with a single IV injections of  $1 \times 10^6$ ,  $3 \times 10^6$ , or  $9 \times 10^6$  freshly thawed “20-19 1XX” or NTD EBV T cells, as indicated by the vertical dotted lines. BLI radiance for

each mouse (n=6) was measured post tumor implantation. The absolute count of circulating human T-cells and vector copy number were measured.

[00355] “20-19 1XX” dosage study demonstrated potent tumor rejection after a single low dose without exogenous cytokine support. As shown in Figures 14A-14C, “20-19 1XX” showed a potent tumor rejection even in a low dosage against CD19<sup>low</sup> Raji tumor model. Figures 14A-14C showed dose-dependent anti-tumor activity of “20-19 1XX”. The lowest dosage of  $1 \times 10^6$  CAR T cells per mouse still had some therapeutic effects. As shown in Figures 14D and 14E, the detection of hCD45<sup>+</sup> cells and VCN in peripheral blood indicated persistence of CAR T cells until the end of study at day 62 post-therapy. No treatment-related toxicities were observed following all dosages (data not shown).

**Example 10. In Vivo Anti-tumor Activities of Allogeneic EBV-Sensitized T Cells**

**Expressing CD19/CD20-targeted CARs**

[00356] A sub-therapeutic dosage of  $3 \times 10^6$  of “20-19 1XX” CAR T cells per mouse was identified in the previous examples. “20-19 1XX” was then compared to autologous benchmark dual CAR T cells in *in vivo* studies using CD19<sup>low</sup> Raji model and CD19<sup>high</sup> Raji model. NSG mice were intravenously implanted with  $0.5 \times 10^6$  Raji CD19<sup>low</sup> cells or with  $0.5 \times 10^6$  Raji CD19<sup>high</sup> cells on day 0. Mice were randomized into different groups by BLI and treated with single IV injections of  $3 \times 10^6$  freshly thawed “20-19 1XX” or NTD EBV T-cells, as indicated by the vertical dashed lines. BLI radiance for each mouse (n=5) was measured post tumor implantation. The geometric mean of BLI, the mean absolute count of circulating human T cells, the mean VCN, and survival for each group were measured.

[00357] “20-19 1XX” demonstrated superior *in vivo* anti-tumor activity and functional persistence compared to autologous benchmark CAR T cells. Figures 15A-15D show that “20-19 1XX” had superior tumor control than autologous benchmark CAR T cells in 2 donors even when the CD19 level was low. In Figures 15E-15H, “20-19 1XX” showed complete control of tumor growth while autologous benchmark CAR T cells had varied levels of tumor growth control. Both CAR T cells persisted until the end of the study. Animals treated with autologous benchmark CAR T cells showed alloreactive expansion of non-CAR T cells after about 40 days. No treatment-related toxicities were observed following all dosages (data not shown).

**Example 11. In Vitro Activities of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs in Single Killing Assays**

[00358] The “20-19 1XX” CAR was transduced and expressed in allogeneic EBV-sensitized CTLs derived from Donor 104 using a proprietary manufacturing process MP3. *In vitro* activities of “20-19 1XX” were assessed in a single killing assay. The CAR expression and vector copy number (VCN) are shown in Table 8. In Table 8, “104 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 104 expressing the “20-19 1XX” CAR. “104 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 104 (not expressing a CAR).

Table 8

| Group         | Manufacturing Process | CAR% | VCN  |
|---------------|-----------------------|------|------|
| 104 NTD       | MP3                   | -    | -    |
| 104 20-19 1XX | MP3                   | 77.4 | 4.19 |

[00359] Next, the *in vitro* cytotoxicity activities of the CAR-T cells against cells expressing CD19, CD20 or both were assessed. Briefly, CAR-T cells and target cells (K562 cells) were co-cultured at an effector ( $E^{CAR+}$ :target(T) serial ratios of 1:4, 1:2, 1:1, 2:1, and 4:1. K562 cells were artificially engineered to express human CD19, human CD20, or both. As shown in Figure 16, on day 0, a set number of tumor cells were co-cultured with a set number of “20-19 1XX” CAR-T cells. On day 1 or day 2, each set up was analyzed for live number of T cells and tumor cells on flow cytometry. As shown in Figures 17A-17D, the allogeneic EBV-sensitized T cells from Donor 104 expressing the “20-19 1XX” CAR exhibited CD19 and CD20-specific activity.

**Example 12. In Vitro Activities of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs in Serial Stimulation Assays**

[00360] The “20-19 1XX” CAR was transduced and expressed in allogeneic EBV-sensitized CTLs derived from Donor 14 and Donor 23 using the manufacturing process MP3. *In vitro* activities of “20-19 1XX” were assessed in serial challenge assays. The CAR expression and vector copy number (VCN) are shown in Table 9. In Table 9, “14 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 14 expressing the “20-19 1XX” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 14 (not expressing a CAR). “23 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 23 expressing the “20-19 1XX” CAR. “23 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 23 (not expressing a CAR).

Table 9

| Group        | Manufacturing Process | CAR% | VCN  |
|--------------|-----------------------|------|------|
| 14 NTD       | MP3                   | -    | -    |
| 14 20-19 1XX | MP3                   | 84.6 | 5.21 |
| 23 NTD       | MP3                   | -    | -    |
| 23 20-19 1XX | MP3                   | 72.5 | 3.53 |

[00361] Next, the *in vitro* cytotoxicity activities of the CAR-T cells against cells expressing CD19, CD20 or both were assessed in serial challenge assays. As shown in Figure 18, on day 0, a set number of tumor cells were co-cultured with a set number of “20-19 1XX” CAR-T cells. On day 2 to day 10, every 2-3 days, each set up was analyzed for live number of T cells and tumor cells, and a set number of tumor cells were added to the culture. CAR- T cells and target cells were co-cultured at an effector ( $E^{CAR+}$ :target(T)) ratio of 5:1 or 1:1. To further elucidate the impact of CD19 and CD20, a panel of Raji cells expressing varying levels of CD19 were used as target cells, including cells with no CD19 expression (Raji-CD19<sup>KO</sup>), with low levels (Raji-CD19<sup>Low</sup>), and high levels (Raji-CD19<sup>High</sup>) of CD19. As shown in Figures 19A-19C and Figures 20A-20C, the allogeneic EBV-sensitized T cells from Donor 14 or Doner 23 expressing the “20-19 1XX” CAR both exhibited CD19 and CD20-specific activity against Raji cells with various level of CD19 and CD20-positive expression.

[00362] Further, the *in vitro* cytotoxicity activities of the CAR-T cells against various non-Hodgkin’s lymphoma (NHL) cells were assessed in serial challenge assays. CAR- T cells and target cells were co-cultured at an effector ( $E^{CAR+}$ :target(T)) ratio of 1:1. Various NHL cell lines were used as the target cells, including Raji cells (Burkitt lymphoma), Jeko-1 cells (mantle cell lymphoma (MCL)), Su-DHL-4 cells (diffuse large B cell lymphoma (DLBCL)), and MEC-1 cells (chronic lymphocytic leukemia (CLL)). As shown in Figures 21A-21D, the allogeneic EBV-sensitized T cells from Donor 14 or Doner 104 expressing the “20-19 1XX” CAR both exhibited CD19 and CD20-specific activity against these NHL cells.

**Example 13. In Vivo Anti-tumor Activities of CD19/CD20-targeted CAR-T cells**

[00363] The *in vivo* anti-tumor efficacy of these CAR-T cells was assessed. Tumor growth was monitored through bioluminescent tumor cells, detectable by IVIS imaging machine. Blood was collected and hCD45<sup>+</sup> cells detected in blood was used to indicate the presence of injected human cells. Injected cell growth and persistence were analyzed by flow cytometry. Mouse body weight was monitored as an indication of severe toxicity (lower body weight with toxicity).

“14 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 14 expressing the “20-19 1XX” CAR. “14 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 14 (not expressing a CAR). “23 20-19 1XX” represents allogeneic EBV-sensitized T cells derived from Donor 23 expressing the “20-19 1XX” CAR. “23 NTD” represents non-transduced allogeneic EBV-sensitized T cells derived from Donor 23 (not expressing a CAR).

[00364] The *in vivo* anti-tumor efficacy of allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR was assessed in the Burkitt’s lymphoma model with Raji CD19<sup>low</sup>/CD20 tumor cells injected. The results are shown in Figures 22A-22C. As shown in Figure 22A, the allogeneic EBV-sensitized T cells from Donor 14 or Donor 23 expressing the “20-19 1XX” CAR both demonstrated anti-tumor efficacy in challenging the *in vivo* Burkitt’s lymphoma model (Raji CD19<sup>low</sup>/CD20 cells). The blood cells of the treated mice were quantified by flow cell using BD TruCount Absolute Counting beads (BD Biosciences), and the results are shown in Figure 22B. The vector copy number (VCN) per cell was also measured for the CAR-T cells, as shown in Figure 22C. The allogeneic EBV-sensitized T cells derived from donors expressing the “20-19 1XX” CAR demonstrated potent tumor growth inhibition against Burkitt’s Lymphoma tumor model (low CD19-expressing Burkitt’s Lymphoma) in NSG mice, and persisted until end of study at about day 40 for Donor 14. No severe toxicity related to “20-19 1XX” treatment was observed (Figure 22D).

[00365] Further, the *in vivo* anti-tumor efficacy of allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR was assessed in the Mantle Cell Lymphoma (MCL) model with Jeko-1 CD19/CD20 tumor cells injected. As shown in Figures 24A-24C, the allogeneic EBV-sensitized T cells expressing the “20-19 1XX” CAR demonstrated potent dosage-dependent anti-tumor efficacy in *in vivo* Jeko-1 MCL model and persisted until end of study at about day 40. No severe toxicity related to “20-19 1XX” treatment was observed (Figure 24D).

**Example 14. Characterization of PBMCs from Multiple Sclerosis Donors**

[00366] To investigate the effectiveness of targeting CD 20 for treatment of Multiple Sclerosis (MS), the CD20<sup>+</sup> T cell population from Peripheral blood mononuclear cells (PBMCs) was characterized from 4 untreated MS donors and 10 healthy donors. PBMCs collected from healthy donors (HD; n=7) and untreated MS donors (n=4) were characterized for CD20<sup>+</sup> T cells



and CD19<sup>+</sup>/CD20<sup>+</sup> B cells. CD20<sup>+</sup> T-cell % was calculated out of total CD3<sup>+</sup> T cells and CD19<sup>+</sup>/CD20<sup>+</sup> B-cell % was calculated out of total live cells.

[00367] As shown in Figures 25A and 25B, PBMCs from MS donors had a significantly higher percentage of CD20<sup>+</sup> T cells compared to PBMCs from healthy donors. Thus, the inventors investigated the therapeutic potential of the presently disclosed CD19/CD20-targeted CAR T cells to treat MS by eliminating pathogenic B and T cells.

**Example 15. In Vitro Activities of MP3-Generated Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs for Treating Multiple Sclerosis**

[00368] The *in vitro* cytotoxicity activities of the “20-19 1XX” CAR were assessed by co-culturing PBMCs from MS patients and “20-19 1XX” CAR T cells. The assay schematic is shown in Figure 26. Briefly, the killing activity against B cells from MS donors of the CAR-T cells was measured. CAR T cells were labeled with cell trace violet dye (CTV) that emits signal on flow cytometry. The PBMCs of MS donors were co-cultured with CTV labeled CAR T cells at an effector (E<sup>CAR<sup>+</sup></sup>):target(T) ratio (CAR<sup>+</sup> T cells:CD19<sup>+</sup> B cells) of 1:1 or 3:1. Live cell count for CTV labeled CAR T cells and MS patient PBMCs were monitored for 72 hours. Cytolysis was detected via flow cytometry and measured via percentage of B cell cytolysis. IFN- $\gamma$  and TNF- $\alpha$  secretion by the CAR-T cells was measured to indicate T-cell activation. CD19 or CD20 targeted cytolysis, the “20-19 1XX” CAR T cell proliferation, and cytokine secretion were measured after 24-, 48- and 72-hours co-culture at the 1:1 ratio using 3 different MS donor PBMCs (Donor A, Doner B, and Doner C). For the set-up of the 3:1 ratio, data were only collected at 24 hours.

[00369] The “20-19 1XX” CAR T cells demonstrated dose-dependent B cell killing post 24 hours. As shown in Figures 27A-27C, “20-19 1XX” demonstrated antigen-specific B-cell cytolysis over 24 hours in response to CD19<sup>+</sup> B cells from the PBMCs of MS donors (Donor A, Doner B, and Doner C) at E:T ratios of 1:1 or 3:1, normalized to the number of B cells. Higher levels of B-cell cytotoxicity observed with increased E:T ratio demonstrated CAR-mediated responses following exposure to CD19 and CD20 on B cells of the MS donors. Further, the “20-19 1XX” CAR T cells continued to target B cells over 72 hours. “20-19 1XX” demonstrated antigen-specific B-cell cytolysis over 48 hours, as shown in Figures 28A and 28D, or 72 hours, as shown in Figures 28B and 28C, in response to CD19<sup>+</sup> B cells from the PBMCs of MS donors (n=3) at an E:T ratio of 1:1, normalized to the number of B cells. As shown in Figure 28D,

calculation of the percent lysis of target B cells demonstrated CAR-mediated killing of CD19 and CD20 positive B cells, while no consistent lysis was observed against CD19 and CD20 negative T cells, NK T cells and NK cells, as shown in Figures 29A-29I. “20-19 1XX” demonstrated no or minimal off-target non-B cell cytotoxicity over 48 hours (Figures 29C, 29F, and 29I) or 72 hours (Figures 29A, 29D, and 29G; and Figures 29B, 29E, and 29H) in response to non-B cells from the PBMCs of MS donors (Doner A, B, and C) at an E:T ratio of 1:1, normalized to the number of B cells.

**[00370]** Next, the cytolytic activities of “20-19 1XX” CAR T cells on CD20<sup>+</sup> T cells were investigated. Depletion of CD20<sup>+</sup> T cells was detected via flow cytometry at 48 hours or 72 hours in response to CD20<sup>+</sup> T cells from the PBMCs of MS donors (n=3) at an E:T ratio of 1:1, normalized to the number of B cells. As shown in Figures 30A and 30B, the lysis of CD20<sup>+</sup> T cells was observed, indicating that CD20<sup>+</sup> T cell population was targeted by “20-19 1XX” CAR T cells. NTD EBV T cells did not show consistent or dose-dependent specific activity against CD19 and/or CD20-positive or negative targets.

**[00371]** Further, IFN- $\gamma$  and TNF- $\alpha$  secretion by “20-19 1XX” CAR T cells was assessed. The co-culture supernatants were harvested at 24 hours and 72 hours and measured cytokine using a bead-based immunoassay (n=3). As shown in Figures 31A and 31B, levels of pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  were elevated following incubation of MS PBMCs with “20-19 1XX” CAR T cells compared to NTD EBV-T cell controls, demonstrating CAR-mediated responses following exposure to CD19 and CD20 on B cells of MS donors. In particular, the results show that “20-19 1XX” released a high level of IFN- $\gamma$ , confirming CAR-mediated T-cell activation following exposure to CD19 and CD20 on MS B cells.

**[00372]** Next, the proliferation of “20-19 1XX” CAR T cells was assessed. As shown in Figures 32A-32H, CTV+ “20-19 1XX” cells demonstrated a robust proliferation over 48 hours (Figures 32A and 32E) or 72 hours Figures 32B, 32F, 32C, and 32G) in response to CD19 and CD20<sup>+</sup> B cells from the PBMCs of MS donors (n=3) at an E:T ratio of 1:1, normalized to the number of B cells. CTV+ NTD EBV-T cell negative control did not demonstrate a robust proliferation over 72 hours (Figures 32D and 32H) in response to CD19 and CD20<sup>+</sup> B cells from their party MS patient PBMCs (n=3; representative figure) at an E:T ratio of 1:1, normalized to the number of B cells.

**Example 16. In Vitro Activities of MP2-Generated Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs for Treating Systemic Lupus Erythematosus**

[00373] The *in vitro* cytotoxicity activities of the “20-19 1XX” CAR were assessed by co-culturing PBMCs from a Systemic Lupus Erythematosus (SLE) donor and “20-19 1XX” CAR T cells. The assay schematic is shown in Figure 33. Briefly, the killing capability of the CAR-T Cells against B cells of the PBMCs of the SLE donor was measured. CAR-T cells were labeled with cell trace violet dye (CTV) that emits signal on flow cytometry. The PBMCs of the SLE donor were co-cultured with CTV labeled CAR T cells at an effector ( $E^{CAR+}$ ):target(T) ratio (CAR+ T cells:CD19<sup>+</sup> B cells) of 1:1. Live cell count for CTV labeled CAR T cells and SLE patient PBMCs were monitored for 48 hours.

[00374] The results show that the “20-19 1XX” CAR-T cells continued to target B cells over 48 hours with minimal off-target non-B cell targeting over time. Figure 34A shows that “20-19 1XX” CAR-T cells demonstrated no or minimal off-target non-B cell cytolysis over 48 hours in response to non-B cells from third-party SLE patient PBMC at an E:T ratio of 1:1, normalized to the number of B cells. Figures 34D to 34F show no or minimal off-target killing, e.g., no or minimal killing of non-B cells including CD56<sup>+</sup> NK cells (Figure 34D), CD56<sup>+</sup>/CD3<sup>+</sup> NKT cells (Figure 34E), and CD3<sup>+</sup> T cells (Figure 34F), in the PBMCs of the SLE donor.

**Example 17. Alloreactivities of Allogeneic EBV-Sensitized T Cells Expressing CD19/CD20-targeted CARs against HLA-mismatched Targets**

[00375] In addition to CD19/CD20 bispecific-CAR mediated function, *in vitro* cytotoxicity of chromium-labeled HLA-matched and HLA-mismatched targets was measured following co-culture with “20-19 1XX” CAR T cells. The potential alloreactivity of “20-19 1XX” research lots from 4 donors was assessed via chromium release of labeled targets.

[00376] As shown in Figure 35, “20-19 1XX” from four research lots demonstrated equal to or greater than 30% specific lysis against autologous CD19/CD20-positive BLCL target cells. None of the research lots showed cytolytic activity greater than 30% against autologous phytohemagglutinin blasts (PHAb). Co-incubation with complete HLA-mismatched PHA blasts from 8 different donors also did not yield cytolytic activity above 30%.

**[00377]** Conclusion

**[00378]** While various embodiments of the present disclosure have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will be apparent to those skilled in the art without departing from the disclosure. For example, various embodiments of the CARs and CAR T-cell compositions and associated methods of use (e.g., for treating cancer or an autoimmune disease) may be adapted for various pediatric and veterinary applications, the former including various pediatric leukemias and fibromas (e.g., AML, MDS, CML and JMML as is known in the oncology arts), the latter including one or more of felines, canines and equines. Specific adaptations for the preceding can include one or more of the specific CARs, CAR-T cells and their intended target cells (e.g., B-cells or T-cells) in the therapeutic composition administered to the patient and the dose of cells in the composition along with the carrier solution and various excipients used in the carrier solution. They can also be adapted for treatment of any number B-cell mediated cancers including one or more of leukemia, lymphoma, as well as a number of B and/or t-cell mediated and other autoimmune diseases including one more of multiple sclerosis, Guilian Barrie Syndrome, CIDP, MFMN, systemic lupus erythematosus (SLE), rheumatoid arthritis, IBD, Celiac Disease, Crohn's disease, ulcerative colitis and ankylosing spondylosis.

**[00379]** Elements, characteristics, or acts from one embodiment can be readily recombined or substituted with one or more elements, characteristics or acts from other embodiments to form numerous additional embodiments within the scope of the disclosure. Moreover, elements that are shown or described as being combined with other elements, characteristics, steps or acts, can, in various embodiments, exist as stand-alone elements, characteristics, steps or acts. Further, various embodiments expressly contemplate the negative recitation of any element, characteristic, step or act etc. that is/are shown or described in one or more embodiments. Hence, the scope of the present disclosure is not limited to the specifics of the described embodiments, but is instead limited solely by the appended claims.

**WHAT IS CLAIMED IS:**

1. A bi-specific chimeric antigen receptor (CAR) that binds to CD19 and CD20, the CAR comprising:
  - a) an extracellular domain that comprises (i) a first antigen-binding domain that binds to CD19 and (ii) a second antigen-binding domain that binds to CD20;
  - b) a transmembrane domain comprising a first CD28 polypeptide; and
  - c) an intracellular domain comprising a modified CD3 $\zeta$  polypeptide that comprises a native ITAM1, an ITAM2 variant, and an ITAM3 variant, wherein each of the ITAM2 variant and the ITAM3 variant comprises two loss-of-function mutations.
2. The CAR of claim 1, wherein the first anti-CD19 antigen-binding domain comprises: a first heavy chain variable region (V<sub>H</sub>) and a first light chain variable region (V<sub>L</sub>), wherein the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1, a V<sub>H</sub> CDR2, and a V<sub>H</sub> CDR3 of a V<sub>H</sub> comprising the amino acid sequence set forth in SEQ ID NO: 15, and/or the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1, a V<sub>L</sub> CDR2, and a V<sub>L</sub> CDR3 of a V<sub>L</sub> comprising the amino acid sequence set forth in SEQ ID NO: 16.
3. The CAR of claim 2, wherein the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9 or a conservative modification thereof, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 or a conservative modification thereof; and/or the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 13 or a conservative modification thereof, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14 or a conservative modification thereof.
4. The CAR of claim 2 or 3, wherein the first V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 9, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 10, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; and/or the first V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 12, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth

in SEQ ID NO: 13, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 14.

5. The CAR of any one of claims 2-4, wherein the first V<sub>H</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 15; and/or the first V<sub>L</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 16.

6. The CAR of any one of claims 2-5, wherein the first V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 15; and/or the first V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 16.

7. The CAR of any one of claims 1-6, wherein the first anti-CD19 antigen-binding domain comprises a first linker between the first V<sub>H</sub> and the first V<sub>L</sub>.

8. The CAR of claim 7, wherein the first linker comprises the amino acid sequence set forth in SEQ ID NO: 1.

9. The CAR of any one of claims 2-8, wherein the first V<sub>H</sub> and the first V<sub>L</sub> are positioned from the N- to the C-terminus: V<sub>L</sub>-V<sub>H</sub>.

10. The CAR of any one of claims 1-9, wherein the first anti-CD19 antigen-binding domain comprises a first single-chain variable fragment (scFv) that binds to anti-CD19.

11. The CAR of claim 10, wherein the first anti-CD19 scFv comprises the amino acid sequence set forth in SEQ ID NO: 17.

12. The CAR of claim 1, wherein the second anti-CD20 antigen-binding domain comprises: a second V<sub>H</sub> and a second V<sub>L</sub>, wherein the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1, a V<sub>H</sub> CDR2, and a V<sub>H</sub> CDR3 of a V<sub>H</sub> comprising the amino acid sequence set forth in SEQ ID NO: 27, and/or the

second V<sub>L</sub> comprises a V<sub>L</sub> CDR1, a V<sub>L</sub> CDR2, and a V<sub>L</sub> CDR3 of a V<sub>L</sub> comprising the amino acid sequence set forth in SEQ ID NO: 28.

13. The CAR of claim 12, wherein the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21 or a conservative modification thereof, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22 or a conservative modification thereof, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23 or a conservative modification thereof; and/or the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24 or a conservative modification thereof, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25 or a conservative modification thereof, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26 or a conservative modification thereof.

14. The CAR of claim 12 or 13, wherein the second V<sub>H</sub> comprises a V<sub>H</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a V<sub>H</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a V<sub>H</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23; and/or the second V<sub>L</sub> comprises a V<sub>L</sub> CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24, a V<sub>L</sub> CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a V<sub>L</sub> CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26.

15. The CAR of any one of claims 12-14, wherein the second V<sub>H</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 27; and/or the second V<sub>L</sub> comprises an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical or homologous to the amino acid sequence set forth in SEQ ID NO: 28.

16. The CAR of any one of claims 12-15, wherein the second V<sub>H</sub> comprises the amino acid sequence set forth in SEQ ID NO: 27; and/or the second V<sub>L</sub> comprises the amino acid sequence set forth in SEQ ID NO: 28.
17. The CAR of any one of claims 12-16, wherein the second anti-CD20 antigen-binding domain comprises a second linker between the second V<sub>H</sub> and the second V<sub>L</sub>.
18. The CAR of claim 17, wherein the second linker comprises the amino acid sequence set forth in SEQ ID NO: 2.
19. The CAR of any one of claims 12-18, wherein the second V<sub>H</sub> and the second V<sub>L</sub> are positioned from the N- to the C-terminus: V<sub>H</sub>-V<sub>L</sub>.
20. The CAR of any one of claims 1-19, wherein the second anti-CD20 antigen-binding domain comprises a second anti-CD20 single-chain variable fragment (scFv) that binds to CD20.
21. The CAR of claim 20, wherein the second anti-CD20 scFv comprises the amino acid sequence set forth in SEQ ID NO: 29.
22. The CAR of claim 2 or 12, wherein the V<sub>H</sub> CDR1, V<sub>H</sub> CDR2, V<sub>H</sub> CDR3, V<sub>L</sub> CDR1, V<sub>L</sub> CDR2, and V<sub>L</sub> CDR3 are identified according to the Kabat numbering system, the Chothia numbering system, the AbM numbering system, the Contact numbering system, or the IMGT Information System®.
23. The CAR of claim 22, wherein the V<sub>H</sub> CDR1, V<sub>H</sub> CDR2, V<sub>H</sub> CDR3, V<sub>L</sub> CDR1, V<sub>L</sub> CDR2, and V<sub>L</sub> CDR3 are identified according to the Kabat numbering system.
24. The CAR of any one of claims 1-23, further comprising: a third linker between the first anti-CD19 antigen-binding domain and the second anti-CD20 antigen-binding domain.
25. The CAR of claim 24, wherein the third linker comprises the amino acid sequence set forth in SEQ ID NO: 3.
26. The CAR of any one of claims 1-25, wherein the first anti-CD19 antigen-binding domain is positioned downstream of the second anti-CD20 antigen-binding domain.



27. The CAR of any one of claims 1-26, wherein a leader is covalently joined to the N-terminus of the second anti-CD20 antigen-binding domain.
28. The CAR of claim 27, wherein the leader comprises a CD8 polypeptide.
29. The CAR of claim 28, wherein the CD8 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 33.
30. The CAR of any one of claims 1-29, wherein the first CD28 polypeptide is a transmembrane domain sequence of CD28.
31. The CAR of any one of claims 1-30, wherein the first CD28 polypeptide comprises amino acids 153 to 179 of SEQ ID NO: 35.
32. The CAR of any one of claims 1-31, further comprising: a hinge region.
33. The CAR of claim 32, wherein the hinge region comprises a second CD28 polypeptide.
34. The CAR of claim 33, wherein the second CD28 polypeptide comprises amino acids 114 to 152 of SEQ ID NO: 35.
35. The CAR of any one of claims 1-34, wherein each of the two loss-of-function mutations is at a tyrosine amino acid residue.
36. The CAR of any one of claims 1-35, wherein the ITAM2 variant comprises the amino acid sequence set forth in SEQ ID NO: 45.
37. The CAR of any one of claims 1-36, wherein the ITAM3 variant comprises the amino acid sequence set forth in SEQ ID NO: 49.
38. The CAR of any one of claims 1-37, wherein the native ITAM1 comprises the amino acid sequence set forth in SEQ ID NO: 39.
39. The CAR of any one of claims 1-38, wherein the modified CD3 $\zeta$  polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 51.

40. The CAR of any one of claims 1-39, wherein the intracellular signaling domain further comprises a co-stimulatory signaling region.
41. The CAR of claim 40, wherein the co-stimulatory signaling region comprises a third CD28 polypeptide.
42. The CAR of claim 41, wherein the third CD28 polypeptide is a signaling domain sequence of CD28.
43. The CAR of claim 42, wherein the third CD28 polypeptide comprises amino acids 180 to 220 of SEQ ID NO: 35.
44. The CAR of any one of claims 1-43, comprising the amino acid sequences set forth in SEQ ID NO: 54.
45. A nucleic acid encoding the CAR of any one of claims 1-44.
46. The nucleic acid of claim 45, comprising the nucleotide sequence set forth in SEQ ID NO: 55.
47. A vector comprising the nucleic acid of claim 45 or 46.
48. The vector of claim 47, wherein the vector is a viral vector.
49. The vector of claim 48, wherein the viral vector is a retroviral vector.
50. A cell comprising the CAR of any one of claims 1-44, the nucleic acid of claim 44 or 45, or the vector of any one of claims 47-49.
51. The cell of claim 50, wherein the cell is transduced with the CAR, the nucleic acid, or the vector.
52. The cell of claim 50 or claim 51, wherein the CAR is constitutively expressed on the surface of the cell.
53. The cell of any one of claims 50-52, wherein the cell is an immunoresponsive cell or an immune effector cell.

54. The cell of any one of claims 50-53, wherein the cell is a cell of the lymphoid lineage or a cell of the myeloid lineage.
55. The cell of claim 54, wherein the cell of lymphoid lineage is selected from the group consisting of T cells, Natural Killer (NK) cells, B cells, and stem cells from which lymphoid cells may be differentiated.
56. The cell of claim 54, wherein the cell of the myeloid lineage is selected from the group consisting of monocytes, macrophages, neutrophils, dendritic cells, basophils, neutrophils, eosinophils, megakaryocytes, mast cell, erythrocyte, thrombocytes, and stem cells from which myeloid cells may be differentiated.
57. The cell of any one of claims 50-56, wherein the cell is selected from the group consisting of a T cell, a B cell, a Natural Killer (NK) cell, a macrophage, an innate lymphoid cell (ILC), a cytokine induced killer (CIK) cell, a lymphokine activated killer (LAK) cell, a stem cell from which a lymphoid cell may be differentiated, a stem cell from which a myeloid cell may be differentiated, and combinations of the foregoing.
58. The cell of claim 57, wherein the cell is a T cell.
59. The cell of any one of claims 55, 57, and 58, wherein the T cell is selected from the group consisting of helper T cells, cytotoxic T cells (CTLs), memory T cells, regulatory T cells, tumor-infiltrating lymphocyte (TIL), Natural Killer T cells, mucosal associated invariant T cells,  $\alpha\beta$  T cells, and  $\gamma\delta$  T cells.
60. The cell of claim 59, wherein the memory T cell is a central memory T cells.
61. The cell of any one of claims 58-60, wherein the T cell is positive for CD45RO and CD62L.
62. The cell of any one of claims 55-57, wherein the stem cell is a pluripotent stem cell.
63. The cell of claim 62, wherein the pluripotent stem cell is an embryoid stem cell or an induced pluripotent stem cell.

64. The cell of any one of claims 50-61, wherein the cell is a cytotoxic T lymphocyte (CTL).
65. The cell of any one of claims 50-61 and 64, wherein the cell is a viral antigen-sensitized CTL.
66. The cell of any one of claims 50-61, 64, and 65, wherein the viral antigen is selected from the group consisting of Epstein-Barr virus (EBV), cytomegalovirus (CMV), B.K. virus (BKV), John Cunningham virus (JCV), picornavirus, hepadnavirus, hepacivirus, deltavirus, hepevirus, or any combination thereof.
67. The cell of claim 66, wherein the cell is an EBV-sensitized CTL.
68. A composition comprising cells of any one of claims 50-67.
69. The composition of claim 68, wherein the composition is a pharmaceutical composition further comprising: a pharmaceutically acceptable carrier.
70. The composition of claim 68 or 69, comprising between about  $1 \times 10^6$  and about  $5 \times 10^8$  of the cells.
71. The composition of any one of claims 68-70, comprising between about  $1 \times 10^6$  and about  $1 \times 10^8$  of the cells.
72. The composition of any one of claims 68-71, comprising between about  $1 \times 10^6$  and about  $5 \times 10^7$  of the cells.
73. The composition of any one of claims 68-72, comprising between about  $1 \times 10^6$  and about  $1 \times 10^7$  of the cells.
74. A method of inhibiting growth of a target cell expressing at least one B-lymphocyte antigen, the method comprising: contacting the target cell with the cell of any one of claims 50-67 or the composition of any one of claims 68-73.
75. The method of claim 74, wherein the at least one B-lymphocyte antigen is selected from the group consisting of CD19, CD20, and a combination thereof.

76. The method of claim 74 or 75, wherein the target cell expresses a detectable level of CD19 and a detectable level of CD20.
77. The method of claim 74 or 75, wherein the target cell expresses a detectable level of CD20 and a low or non-detectable level of CD19.
78. The method of claim 74 or 75, wherein the target cell expresses a detectable level of CD19 and a low or non-detectable level of CD20.
79. The method of any one of claims 74-78, wherein the target cell is autologous to the cell.
80. The method of any one of claims 74-78, wherein the target cell is allogeneic to the cell.
81. The method of any one of claims 74-80, wherein the target cell is a tumor cell.
82. The method of claim 81, wherein the tumor is cancer.
83. The method of claim 82, wherein the cancer is a hematologic cancer.
84. The method of claim 83, wherein the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).
85. The method of any one of claims 74-80, wherein the target cell is a B cell.
86. The method of claim 85, wherein the B cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.
87. The method of any one of claims 74-80, wherein the target cell is a T cell.
88. The method of claim 87, wherein the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.
89. The method of claim 87 or 88, wherein the T cell is CD20<sup>-</sup>.

90. The method of any one of claims 74-89, wherein the cell or composition exhibits cytotoxic and/or cytolytic activity against the target cell.
91. The method of claim 90, wherein the cytotoxic and/or cytolytic activity is dose-dependent.
92. The method of claim 90 or 91, wherein the cytotoxic and/or cytolytic activity is long-term.
93. The method of any one of claims 90-92, wherein the cytotoxic and/or cytolytic activity lasts for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial contact of the target cell with the cell or composition.
94. The method of any one of claims 90-92, wherein the cytotoxic and/or cytolytic activity lasts for about 40 days after the initial contact of the target cell with the cell or composition.
95. The method of any one of claims 90-92, wherein the cytotoxic and/or cytolytic activity lasts for about 60 days after the initial contact of the target cell with the cell or composition.
96. A method of treating a B-lymphocyte antigen-associated disease in a subject, the method comprising: administering to the subject the cell of any one of claims 50-67 or the composition of any one of claims 68-73.
97. The method of claim 96, wherein the B-lymphocyte antigen is selected from the group consisting of CD19, CD20, and a combination thereof.
98. The method of claim 96 or 97, wherein the B-lymphocyte antigen-associated disease is a cancer.
99. The method of claim 98, wherein the cancer is a hematologic cancer.
100. The method of claim 99, wherein the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell

lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).

101. The method of claim 96 or 97, wherein the B-lymphocyte antigen-associated disease is an autoimmune disease.

102. The method of claim 101, wherein the autoimmune disease is selected from the group consisting of multiple sclerosis, systemic lupus erythematosus (SLE), Sjogren's, AL amyloidosis, Sjogren's, AL amyloidosis, IgG4-related disease (IgG4-RD), inflammatory myopathies (e.g., myositis), scleroderma, pemphigus vulgaris, chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), stiff-person's syndrome, rheumatoid arthritis, neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis, N-methyl-D-aspartate receptor (NMDAR) encephalitis, anti-myelin oligodendrocyte glycoprotein (MOG) syndrome, myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), membranous nephropathy (MN), IgA nephropathy (IgAN), Type 1 diabetes, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, thrombocytopenia purpura, hemolytic anemia (AIHA), multifocal motor neuropathy (MMN), inflammatory bowel disease (IBD), celiac disease, Crohn's disease, ulcerative colitis, and ankylosing spondylosis.

103. The method of claim 102, wherein the autoimmune disease is multiple sclerosis.

104. The method of claim 102, wherein the autoimmune disease is systemic lupus erythematosus (SLE).

105. The method of any one of claims 96-104, wherein the cell or composition exhibits cytotoxic and/or cytolytic activity against a target cell in the subject, wherein the target cell is positive for the B-lymphocyte antigen.

106. The method of any one of claims 96-105, wherein the target cell expresses i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; or iii) a detectable level of CD19 and a low or non-detectable level of CD20.

107. The method of claim 105 or 106, wherein the target cell is a tumor cell.

108. The method of any one of claims 105-107, wherein the target cell is a cancer cell.
109. The method of claim 108, wherein the cancer is a hematologic cancer.
110. The method of claim 109, wherein the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).
111. The method of claim 105 or 106, wherein the target cell is a B cell.
112. The method of claim 111, wherein the B cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.
113. The method of claim 105 or 106, wherein the target cell is a T cell.
114. The method of claim 113, wherein the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.
115. The method of claim 113 or 114, wherein the T cell is CD20<sup>+</sup>.
116. The method of any one of claims 105-115, wherein the cytotoxic and/or cytolytic activity is dose-dependent.
117. The method of any one of claims 105-116, wherein the cytotoxic and/or cytolytic activity is long-term.
118. The method of any one of claims 105-117, wherein the cytotoxic and/or cytolytic activity lasts for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial administration of the cell or composition.
119. The method of any one of claims 105-118, wherein the cytotoxic and/or cytolytic activity lasts for about 40 days after the initial administration of the cell or composition.



120. The method of any one of claims 105-119, wherein the cytotoxic and/or cytolytic activity lasts for about 60 days after the initial administration of the cell or composition.

121. The method of any one of claims 96-120, wherein the cell or composition does not induce graft-versus-host disease.

122. The method of any one of claims 96-121, wherein the cell or composition induces no or minimal alloreactivity against a HLA-mismatched cell positive for the B-lymphocyte antigen in the subject.

123. The method of any one of claims 96-122, wherein the cell or composition exhibit no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for the B-lymphocyte antigen.

124. The method of claim 123, wherein the off-target cell is selected from the group consisting of a CD19<sup>+</sup>CD20<sup>-</sup> T cell, a Natural Killer T cell (NK T cell), a Natural Killer cell (NK cell), and a combination thereof.

125. The method of any one of claims 96-124, wherein the cell or composition induces cytokine secretion.

126. The method of claim 125, wherein the cytokine is selected from the group consisting of interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and a combination thereof.

127. The method of any one of claims 96-126, wherein the cell is a T cell.

128. The method of claim 127, wherein the T cell is an EBV-sensitized CTL.

129. The method of any one of claims 96-128, wherein the cell persists in the subject for at least 24 hours, at least 72 hours, at least one week, at least one month, or at least two months after the initial administration of the cell or composition.

130. The method of any one of claims 96-129, wherein the cell persists in the subject for about 40 days after the initial administration of the cell or composition.

131. The method of any one of claims 96-130, wherein the cell persists in the subject for about 60 days after the initial administration of the cell or composition.

132. A method of treating a hematologic cancer in a subject, the method comprising: administering to the subject the cell of any one of claims 50-67 or the composition of any one of claims 68-73.

133. The method of claim 132, wherein the hematologic cancer is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, mantle cell lymphoma (MCL), and follicular lymphoma (FL).

134. The method of claim 132 or 133, wherein the cell or composition exhibits cytotoxic and/or cytolytic activity against a target cell in the subject, wherein the target cell expresses i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; iii) a detectable level of CD19 and a low or non-detectable level of CD20.

135. The method of claim 134, wherein the target cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

136. The method of claim 134 or 135, wherein the cytotoxic and/or cytolytic activity is dose-dependent.

137. The method of any one of claims 134-136, wherein the cytotoxic and/or cytolytic activity is long-term.

138. The method of any one of claims 134-137, wherein the cytotoxic and/or cytolytic activity lasts for at least one month, or at least two months after the initial administration of the cell or composition.

139. The method of any one of claims 134-138, wherein the cytotoxic and/or cytolytic activity lasts for about 40 days after the initial administration of the cell or composition.

140. The method of any one of claims 134-139, wherein the cytotoxic and/or cytolytic activity lasts for about 60 days after the initial administration of the cell or composition.
141. The method of any one of claims 132-140, wherein the cell or composition does not induce graft-versus-host disease.
142. The method of any one of claims 132-141, wherein the cell or composition induces no or minimal alloreactivity against a HLA-mismatched cell positive for CD19 and/or CD20 in the subject.
143. The method of any one of claims 132-142, wherein the cell or composition exhibit no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for CD19 and CD20.
144. The method of any one of claims 132-143, wherein the cell is a T cell.
145. The method of claim 144, wherein the T cell is an EBV-sensitized CTL.
146. The method of any one of claims 132-145, wherein the cell persists in the subject for at least one month, or at least two months after the initial administration of the cell or composition.
147. The method of any one of claims 132-146, wherein the cell persists in the subject for about 40 days after the initial administration of the cell or composition.
148. The method of any one of claims 132-147, wherein the cell persists in the subject for about 60 days after the initial administration of the cell or composition.
149. A method of treating an autoimmune disease in a subject, the method comprising: administering to a subject the cell of any one of claims 50-67 or the composition of any one of claims 68-73.
150. The method of claim 149, wherein the autoimmune disease is selected from the group consisting of multiple sclerosis, systemic lupus erythematosus (SLE), Sjogren's, AL amyloidosis, Sjogren's, AL amyloidosis, IgG4-related disease (IgG4-RD), inflammatory myopathies (e.g., myositis), scleroderma, pemphigus vulgaris, chronic inflammatory

demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), stiff-person's syndrome, rheumatoid arthritis, neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis, N-methyl-D-aspartate receptor (NMDAR) encephalitis, anti-myelin oligodendrocyte glycoprotein (MOG) syndrome, myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), membranous nephropathy (MN), IgA nephropathy (IgAN), Type 1 diabetes, anti-neutrophil cytoplasmic antibody (ANCA) vasculitis, thrombocytopenia purpura, hemolytic anemia (AIHA), multifocal motor neuropathy (MMN), inflammatory bowel disease (IBD), celiac disease, Crohn's disease, ulcerative colitis, and ankylosing spondylosis.

151. The method of claim 150, wherein the autoimmune disease is multiple sclerosis.

152. The method of claim 150, wherein the autoimmune disease is systemic lupus erythematosus (SLE).

153. The method of any one of claims 149-152, wherein the cell or composition exhibits cytotoxic and/or cytolytic activity against a target cell in the subject, wherein the target cell expresses i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; or iii) a detectable level of CD19 and a low or non-detectable level of CD20.

154. The method of claim 153, wherein the target cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

155. The method of claim 153 or 154, wherein the target cell is a B cell.

156. The method of claim 155, wherein the B cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

157. The method of claim 153 or 154, wherein the target cell is a T cell.

158. The method of claim 157, wherein the T cell is CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

159. The method of claim 157 or 158, wherein the T cell is CD20<sup>+</sup>.

160. The method of any one of claims 149-159, wherein the cell or composition exhibits cytotoxic and/or cytolytic activity against a first target cell and a second target cell in the subject, wherein the first and second target cells each express i) a detectable level of CD19 and a detectable level of CD20; ii) a detectable level of CD20 and a low or non-detectable level of CD19; or iii) a detectable level of CD19 and a low or non-detectable level of CD20.

161. The method of claim 160, wherein the first and second target cells each are CD19<sup>+</sup>CD20<sup>+</sup>, CD19<sup>+</sup>CD20<sup>-</sup>, or CD19<sup>-</sup>CD20<sup>+</sup>.

162. The method of claim 160 or 161, wherein the first target cell is a B cell.

163. The method of any one of claims 160-162, wherein the second target cell is a T cell.

164. The method of claim 163, wherein the T cell is CD20<sup>+</sup>.

165. The method of any one of claims 153-164, wherein the cytotoxic and/or cytolytic activity is dose-dependent.

166. The method of any one of claims 153-165, wherein the cytotoxic and/or cytolytic activity is long-term.

167. The method of any one of claims 153-166, wherein the cytotoxic and/or cytolytic activity lasts for at least 24 hours or at least 72 hours after the initial administration of the cell or composition.

168. The method of any one of claims 153-17, wherein the cell or composition does not induce graft-versus-host disease.

169. The method of any one of claims 153-168, wherein the cell or composition induces no or minimal alloreactivity against a HLA-mismatched cell positive for CD19 and/or CD20 in the subject.

170. The method of any one of claims 153-169, wherein the cell or composition exhibit no or minimal cytotoxic and/or cytolytic activity against an off-target cell in the subject, wherein the off-target cell is negative for CD19 and CD20.

171. The method of claim 170, wherein the off-target cell is selected from the group consisting of a CD19<sup>+</sup>CD20<sup>-</sup> T cell, a Natural Killer T cell (NK T cell), a Natural Killer cell (NK cell), and a combination thereof.
172. The method of any one of claims 149-171, wherein the cell or composition induces cytokine secretion.
173. The method of claim 172, wherein the cytokine is selected from the group consisting of interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and a combination thereof.
174. The method of any one of claims 149-173, wherein the cell is a T cell.
175. The method of claim 174, wherein the T cell is an EBV-sensitized CTL.
176. The method of any one of claims 96-175, wherein the subject is a human subject.
177. The method of claim 176, wherein the cell is autologous to the subject.
178. The method of claim 176, wherein the cell is allogeneic to the subject.
179. The cell of any one of claims 50-67 or the composition of any one of claims 68-73 for use in a therapy.
180. The cell of any one of claims 50-67 or the composition of any one of claims 68-73 for use in a method of inhibiting growth of a target cell expressing at least one B-lymphocyte antigen.
181. The cell of any one of claims 50-67 or the composition of any one of claims 68-73 for use in a method of treating a B-lymphocyte antigen-associated disease in a subject.
182. The cell of any one of claims 50-67 or the composition of any one of claims 68-73 for use in a method of treating a hematologic cancer in a subject.
183. The cell of any one of claims 50-67 or the composition of any one of claims 68-73 for use in a method of treating an autoimmune disease in a subject.

184. Use of the cell of any one of claims 50-67 or the composition of any one of claims 68-73 for the manufacture of a medicament for inhibiting growth of a target cell expressing at least one B-lymphocyte antigen.

185. Use of the cell of any one of claims 50-67 or the composition of any one of claims 68-73 for the manufacture of a medicament for treating a B-lymphocyte antigen-associated disease in a subject.

186. Use of the cell of any one of claims 50-67 or the composition of any one of claims 68-73 for the manufacture of a medicament for treating a hematologic cancer in a subject.

187. Use of the cell of any one of claims 50-67 or the composition of any one of claims 68-73 for the manufacture of a medicament for treating an autoimmune disease in a subject.

188. A method for producing the cell of any one of claims 50-67, the method comprising: introducing into a cell the CAR of any one of claims 1-44, the nucleic acid of claim 45 or 46, or the vector of any one of claims 47-49.