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**(71) Applicant: BLUEBIRD BIO, INC.** [US/US]; 150 Second Street, Cambridge, Massachusetts 02141 (US).

**(72) Inventors: MORGAN, Richard**; PO Box 1254, Center Harbor, New Hampshire 03226-1254 (US). **FRIEDMAN, Kevin**; 215 Harvard Street, Unit 34, Medford, Massachusetts 02155 (US).

**(74) Agents: MCDONALD, Michael J.** et al.; Cooley LLP, 1299 Pennsylvania Avenue, NW, Suite 700, Washington, District of Columbia 20004 (US).

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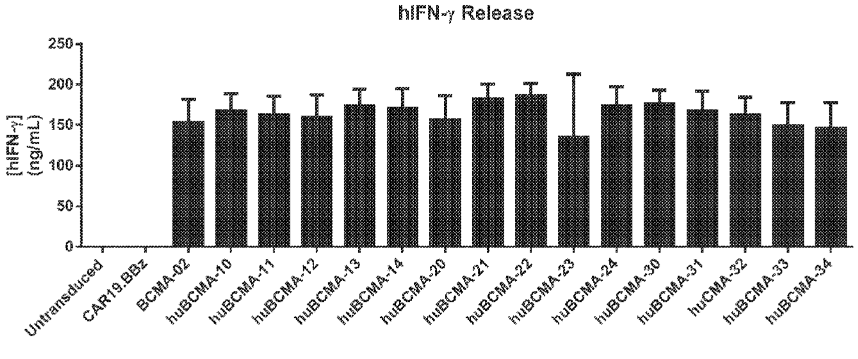


*FIG. 2*

**(57) Abstract**: The invention provides improved compositions for adoptive T cell therapies for B cell related conditions.

# BCMA CHIMERIC ANTIGEN RECEPTORS

## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/028,664, filed July 24, 2014, U.S. Provisional Application No. 62/044,103, filed August 29, 2014, and U.S. Provisional Application No. 62/152,575, filed April 24, 2015, each of which is incorporated by reference herein in its entirety.

## STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is BLBD_037_03WO_ST25.txt. The text file is 124 KB, was created on July 23, 2015, and is being submitted electronically via EFS-Web, concurrent with the filing of the specification.

## BACKGROUND

### Technical Field

The present invention relates to improved compositions and methods for treating B cell related conditions. More particularly, the invention relates to improved chimeric antigen receptors (CARs) comprising humanized anti-BCMA antibodies or antigen binding fragments thereof, immune effector cells genetically modified to express these CARs, and use of these compositions to effectively treat B cell related conditions.

### Description of the Related Art

Several significant diseases involve B lymphocytes, *i.e.*, B cells. Abnormal B cell physiology can also lead to development of autoimmune diseases including, but not limited to systemic lupus erythematosus (SLE). Malignant transformation of B cells leads to cancers including, but not limited to lymphomas, *e.g.,* multiple myeloma and non-Hodgkin’s lymphoma.

The large majority of patients having B cell malignancies, including non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM), are significant contributors to cancer mortality. The response of B cell malignancies to various forms of treatment is mixed. Traditional methods of treating B cell malignancies, including chemotherapy and radiotherapy, have limited utility due to toxic side effects. Immunotherapy with anti-CD19, anti-CD20, anti-CD22, anti-CD23, anti-CD52,anti-CD80, and anti-HLA-DR therapeutic antibodies have provided limited success, due in part to poor pharmacokinetic profiles, rapid elimination of antibodies by serum proteases and filtration at the glomerulus, and limited penetration into the tumor site and expression levels of the target antigen on cancer cells. Attempts to use genetically modified cells expressing chimeric antigen receptors (CARs) have also met with limited success due to poor *in vivo* expansion of CAR T cells, rapid disappearance of the cells after infusion, and disappointing clinical activity.

## BRIEF SUMMARY

The invention generally provides improved vectors for generating T cell therapies and methods of using the same. More particularly, the invention provides humanized anti-BCMA CAR molecules and their use in treating, preventing, or ameliorating B cell related conditions.

Without wishing to be bound to any particular theory, the inventors have discovered that certain humanized anti-BCMA CARs elicit antigen independent ("tonic") cytokine release, a characteristic that would make humanized anti-BCMA CARs unsuitable for use in T cell therapies. Surprisingly, the inventors have discovered that tonic cytokine release by certain humanized anti-BCMA CAR T cells can be made antigen dependent by altering one or more characteristics of at least the transmembrane domain of the humanized anti-BCMA CARs.

In various embodiments, a chimeric antigen receptor (CAR) is provided comprising: an extracellular domain that comprises a humanized anti-BCMA (B cell maturation antigen) antibody or antigen binding fragment thereof that binds one or more epitopes of a human BCMA polypeptide; a transmembrane domain, one or more intracellular co-stimulatory signaling domains, and a primary signaling domain.

In particular embodiments, the humanized anti-BCMA antibody or antigen binding fragment that binds the human BCMA polypeptide is selected from the group consisting of: a Camel Ig, Ig NAR, Fab fragments, Fab' fragments, F(ab)'2 fragments, F(ab)'3 fragments, Fv, single chain Fv antibody ("scFv"), bis-scFv, (scFv)2, minibody, diabody, triabody, tetrabody, disulfide stabilized Fv protein ("dsFv"), and single-domain antibody (sdAb, Nanobody).

In additional embodiments, the humanized anti-BCMA antibody or antigen binding fragment that binds the human BCMA polypeptide is an scFv.

In some embodiments, the humanized anti-BCMA antibody or antigen binding fragment thereof comprises one or more CDRs as set forth in any one of SEQ ID NOs: 1-3.

In particular embodiments, the humanized anti-BCMA antibody or antigen binding fragment thereof comprises one or more CDRs as set forth in any one of SEQ ID NOs: 4-6.

In certain embodiments, the humanized anti-BCMA antibody or antigen binding fragment thereof comprises a variable light chain sequence as set forth in any one of SEQ ID NOs: 7-9.

In particular embodiments, the variable light chain sequence comprises the CDR sequences set forth in SEQ ID NOs: 1-3.

In other embodiments, the humanized anti-BCMA antibody or antigen binding fragment thereof comprises a variable heavy chain sequence as set forth in any one of SEQ ID NOs: 10-14.

In additional embodiments, the variable heavy chain sequence comprises the CDR sequences set forth in SEQ ID NOs: 4-6.

In further embodiments, the transmembrane domain is from a polypeptide selected from the group consisting of: alpha, beta or zeta chain of the T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD 154, and PD1.

In some embodiments, the transmembrane domain is from a polypeptide selected from the group consisting of: CD8α; CD4, CD45, PD1, and CD152.

In some embodiments, the transmembrane domain is from PD1.

In some embodiments, the transmembrane domain is from CD152.

In certain embodiments, the transmembrane domain is from CD8α.

In particular embodiments, the one or more co-stimulatory signaling domains are from a co-stimulatory molecule selected from the group consisting of: CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70.

In particular embodiments, the one or more co-stimulatory signaling domains are from a co-stimulatory molecule selected from the group consisting of: CD28, CD134, and CD137.

In additional embodiments, the one or more co-stimulatory signaling domains are from a co-stimulatory molecule selected from the group consisting of: CD28, CD134, and CD137.

In additional embodiments, the one or more co-stimulatory signaling domains is from CD28.

In particular embodiments, the one or more co-stimulatory signaling domains is from CD134.

In other embodiments, the one or more co-stimulatory signaling domains is from CD137.

In certain embodiments, a CAR comprises a hinge region polypeptide.

In further embodiments, the hinge region is from a polypeptide selected from the group consisting of: CD8α, PD1, and CD152.

In further embodiments, the hinge region polypeptide comprises a hinge region of PD1

In further embodiments, the hinge region polypeptide comprises a hinge region of CD152.

In further embodiments, the hinge region polypeptide comprises a hinge region of CD8α.

In further embodiments, the CAR comprises a hinge region polypeptide and a transmembrane domain from PD1

In further embodiments, the CAR comprises a hinge region polypeptide and a transmembrane domain from CD152.

In further embodiments, the CAR comprises a hinge region polypeptide and a transmembrane domain from CD8α.

In some embodiments, a CAR comprises a spacer region.

In additional embodiments, the spacer region polypeptide comprises CH2 and CH3 regions of IgG1 or IgG4.

In particular embodiments, a CAR comprises a signal peptide.

In further embodiments, the signal peptide comprises an IgG1 heavy chain signal polypeptide, a CD8α signal polypeptide, or a human GM-CSF receptor alpha signal peptide.

In one embodiment, a CAR comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 15-29, 71, and 73.

In another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 15.

In a particular embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 16.

In a certain embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 17.

In an additional embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 18.

In a further embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 19.

In one particular embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 20.

In one embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 21.

In another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 22.

In yet another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 23.

In still yet another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 24.

In one particular embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 25.

In one certain embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 26.

In one additional embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 27.

In one further embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 28.

In another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 29.

In another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 71.

In another embodiment, a CAR comprises an amino acid sequence as set forth in SEQ ID NO: 73.

In various embodiments, a polynucleotide encoding a CAR contemplated herein, is provided.

In various particular embodiments, a polynucleotide encoding a CAR is provided, wherein the polynucleotide sequence is set forth in any one of SEQ ID NOs: 30-44, 70, and 72.

In various certain embodiments, a vector comprising a polynucleotide encoding a CAR contemplated herein or as set forth in any one of SEQ ID NOs: 30-44, 70, and 72 is provided.

In certain embodiments, the vector is an expression vector.

In additional embodiments, the vector is an episomal vector.

In particular embodiments, the vector is a viral vector.

In further embodiments, the vector is a retroviral vector.

In other embodiments, the vector is a lentiviral vector.

In additional embodiments, the lentiviral vector is selected from the group consisting essentially of: human immunodeficiency virus 1 (HIV-1); human immunodeficiency virus 2 (HIV-2), visna-maedi virus (VMV) virus; caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

In particular embodiments, a vector comprises a left (5') retroviral LTR, a Psi (Ψ) packaging signal, a central polypurine tract/DNA flap (cPPT/FLAP), a retroviral export element; a promoter operably linked to the polynucleotide encoding a CAR contemplated herein; and a right (3') retroviral LTR.

In other embodiments, a CAR comprises a heterologous polyadenylation sequence.

In some embodiments, a CAR comprises a hepatitis B virus posttranscriptional regulatory element (HPRE) or woodchuck post-transcriptional regulatory element (WPRE).

In certain embodiments, the promoter of the 5' LTR is replaced with a heterologous promoter.

In further embodiments, the heterologous promoter is a cytomegalovirus (CMV) promoter, a Rous Sarcoma Virus (RSV) promoter, or an Simian Virus 40 (SV40) promoter.

In particular embodiments, the 5' LTR or 3' LTR is a lentivirus LTR.

In particular embodiments, the 3' LTR comprises one or more modifications.

In some embodiments, the 3' LTR comprises one or more deletions.

In certain embodiments, the 3' LTR is a self-inactivating (SIN) LTR.

In some embodiments, the polyadenylation sequence is a bovine growth hormone polyadenylation or signal rabbit β-globin polyadenylation sequence.

In additional embodiments, a polynucleotide encoding a CAR contemplated herein comprises an optimized Kozak sequence.

In further embodiments, the promoter operably linked to the polynucleotide encoding a CAR contemplated herein is selected from the group consisting of: a cytomegalovirus immediate early gene promoter (CMV), an elongation factor 1 alpha promoter (EF1-α), a phosphoglycerate kinase-1 promoter (PGK), a ubiquitin-C promoter (UBQ-C), a cytomegalovirus enhancer/chicken beta-actin promoter (CAG), polyoma enhancer/herpes simplex thymidine kinase promoter (MC1), a beta actin promoter (β-ACT), a simian virus 40 promoter (SV40), and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter.

In various embodiments, an immune effector cell is provided comprising a vector contemplated herein.

In further embodiments, the immune effector cell is selected from the group consisting of: a T lymphocyte and a natural killer (NK) cell.

In various embodiments, a composition is provided comprising an immune effector cell contemplated herein and a physiologically acceptable excipient.

In various embodiments, a method of generating an immune effector cell comprising a CAR contemplated herein is provided, comprising introducing into an immune effector cell a vector comprising a polynucleotide encoding the CAR.

In additional embodiments, the method further comprises stimulating the immune effector cell and inducing the cell to proliferate by contacting the cell with antibodies that bind CD3 and antibodies that bind to CD28; thereby generating a population of immune effector cells.

In particular embodiments, the immune effector cell is stimulated and induced to proliferate before introducing the vector.

In certain embodiments, the immune effector cells comprise T lymphocytes.

In particular embodiments, the immune effector cells comprise NK cells.

In various embodiments, a method of treating a B cell related condition in a subject in need thereof is provided, comprising administering to the subject a therapeutically effect amount of a composition comprising anti-BCMA CAR T cells contemplated herein and optionally, a pharmaceutically acceptable excipient.

In other embodiments, the B cell related condition is multiple myeloma, non-Hodgkin’s lymphoma, B cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, post-transplant lymphoproliferative disorder, an immunoregulatory disorder, rheumatoid arthritis, myasthenia gravis, idiopathic thrombocytopenia purpura, anti-phospholipid syndrome, Chagas' disease, Grave's disease, Wegener's granulomatosis, poly-arteritis nodosa, Sjogren's syndrome, pemphigus vulgaris, scleroderma, multiple sclerosis, anti-phospholipid syndrome, ANCA associated vasculitis, Goodpasture's disease, Kawasaki disease, autoimmune hemolytic anemia, and rapidly progressive glomerulonephritis, heavy-chain disease, primary or immunocyte-associated amyloidosis, or monoclonal gammopathy of undetermined significance.

In further embodiments, the B cell related condition is a B cell malignancy.

In certain embodiments, the B cell malignancy is multiple myeloma (MM) or non-Hodgkin's lymphoma (NHL).

In certain embodiments, the MM is selected from the group consisting of: overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

In some embodiments, the NHL is selected from the group consisting of: Burkitt lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma.

In particular embodiments, the B cell related condition is a plasma cell malignancy.

In further embodiments, the B cell related condition is an autoimmune disease.

In additional embodiments, the autoimmune disease is systemic lupus erythematosus.

In certain embodiments, the B cell related condition is rheumatoid arthritis.

In particular embodiments, the B cell related condition is idiopathic thrombocytopenia purpura, or myasthenia gravis, or autoimmune hemolytic anemia.

## BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

**Figure 1** shows a schematic of humanized anti-B cell maturation antigen (anti-BCMA) CAR constructs.

**Figure 2** shows the amount of IFNγ released from anti-BCMA CAR T cells after the cells were co-cultured for 24 hours with K562 cells expressing BCMA.

**Figure 3** shows the cytolytic activity of anti-BCMA CAR T cells co-cultured for four hours with K564- cells expressing BCMA.

**Figure 4** shows anti-BCMA CAR expression in T cells transduced with mouse (anti-BCMA-02) or humanized (anti-BCMAanti-BCMA-10, anti-anti-BCMA-11, anti-anti-BCMA-31) CAR sequences.

**Figure 5** shows the amount of IFNγ released from anti-BCMA CAR T cells after the cells were co-cultured for 24 hours with K562 BCMA cells expressing low or high amounts of BCMA, multiple myeloma cell lines (RPMI-8226, NCI-H929), or BCMA-negative cell lines (K562, HDLM-2).

**Figure 6** shows the cytolytic activity of T cells expressing anti-BCMA CARs co-cultured with K564-BCMA cells for four hours.

**Figure 7** shows antigen independent cytokine release by T cells expressing the humanized anti-BCMA-10 CAR construct cultured in media containing human serum but lacking BCMA.

**Figure 8** shows antigen independent cytokine release by T cells expressing the humanized anti-BCMA-10 CAR construct cultured in media containing human serum but lacking BCMA.

**Figure 9** shows a schematic of the anti-BCMA-02, anti-BCMA-10, anti-BCMA-10.2 and anti-BCMA-10.5 CAR constructs.

**Figure 10** shows the amount of IFNγ released from anti-BCMA-10, anti-BCMA-10.2, or anti-BCMA-10.5 CAR T cells after the cells were co-cultured for 24 hours with K562 cells expressing BCMA.

**Figure 11** shows a comparison of the amount of tonic IFNγ release from T cells expressing mouse anti-BCMA-02 CAR, or expressing the humanized anti-BCMA-10, anti-BCMA-10.2 or anti-BCMA-10.5 CAR after 24 hours in culture.

## BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

**SEQ ID NOs: 1-3** set forth amino acid sequences of exemplary light chain CDR sequences for anti-BCMA CARs contemplated herein.

**SEQ ID NOs: 4-6** set forth amino acid sequences of exemplary heavy chain CDR sequences for anti-BCMA CARs contemplated herein.

**SEQ ID NOs: 7-9** set forth amino acid sequences of exemplary light chain sequences for anti-BCMA CARs contemplated herein.

**SEQ ID NOs: 10-14** set forth amino acid sequences of exemplary heavy chain sequences for anti-BCMA CARs contemplated herein.

**SEQ ID NOs: 15-29, 71, and 73** set forth amino acid sequences of exemplary anti-BCMA CARs contemplated herein.

**SEQ ID NOs: 30-44, 70, and 72** set forth polynucleotide sequences that encode exemplary anti-BCMA CARs contemplated herein.

**SEQ ID NO: 45** sets for the amino acid sequence of human BCMA.

**SEQ ID NOs: 46-56** set for the amino acid sequence of various linkers.

**SEQ ID NOs: 57-69** set for the amino acid sequence of protease cleavage sites and self-cleaving polypeptide cleavage sites.

## DETAILED DESCRIPTION

### A. OVERVIEW

The invention generally relates to improved compositions and methods for treating B cell related conditions. As used herein, the term “B cell related conditions” relates to conditions involving inappropriate B cell activity and B cell malignancies.

In particular embodiments, the invention relates to improved adoptive cell therapy of B cell related conditions using genetically modified immune effector cells. Genetic approaches offer a potential means to enhance immune recognition and elimination of cancer cells. One promising strategy is to genetically engineer immune effector cells to express chimeric antigen receptors (CAR) that redirect cytotoxicity toward cancer cells. However, existing adoptive cell immunotherapies for treating B cell disorders present a serious risk of compromising humoral immunity because the cells target antigens expressed on all of, or the majority of, B cells. Accordingly, such therapies are not clinically desirable and thus, a need in the art remains for more efficient therapies for B cell related conditions that spare humoral immunity.

The improved compositions and methods of adoptive cell therapy disclosed herein, provide genetically modified immune effector cells that can readily be expanded, exhibit long-term persistence *in vivo*, and reduce impairment of humoral immunity by targeting B cells expression B cell maturation antigen (BCMA, also known as CD269 or tumor necrosis factor receptor superfamily, member 17; TNFRSF17).

BCMA is a member of the tumor necrosis factor receptor superfamily (see, *e.g.,* Thompson *et al., J. Exp. Medicine*, 192(1): 129-135, 2000, and Mackay *et al., Annu. Rev.Immunol*, 21: 231-264, 2003. BCMA binds B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) (*see, e.g.,* Mackay *et al.*, 2003 and Kalled *et al., Immunological Reviews*, 204: 43-54, 2005). Among nonmalignant cells, BCMA has been reported to be expressed mostly in plasma cells and subsets of mature B-cells (*see, e.g.,* Laabi *et al., EMBO J.*, 77(1 ): 3897-3904, 1992; Laabi *et al., Nucleic Acids Res.*, 22(7): 1147-1154,, 1994; Kalled *et al.*, 2005; O'Connor *et al., J. Exp. Medicine,* 199(1): 91-97, 2004; and Ng *et al., J. Immunol.*, 73(2): 807-817, 2004. Mice deficient in BCMA are healthy and have normal numbers of B cells, but the survival of long-lived plasma cells is impaired (*see, e.g.,* O'Connor *et al.*, 2004; Xu *et al., Mol. Cell. Biol,* 21(12): 4067-4074, 2001; and Schiemann *et al., Science,* 293(5537): 2 111-21 14, 2001). BCMA RNA has been detected universally in multiple myeloma cells and in other lymphomas, and BCMA protein has been detected on the surface of plasma cells from multiple myeloma patients by several investigators (*see, e.g.,* Novak *et al., Blood,* 103(2): 689-694, 2004; Neri *et al., Clinical Cancer Research*, 73(19): 5903-5909, 2007; Bellucci *et al., Blood*, 105(10): 3945-3950, 2005; and Moreaux *et al., Blood*, 703(8): 3148-3157, 2004.

In various embodiments, CARs comprising humanized anti-BCMA antibody sequences are highly efficacious compared to anti-BCMA CARs comprising mouse antibody sequences; undergo robust *in vivo* expansion; and recognize human B cells expressing BCMA and show cytotoxic activity against the BCMA expressing B cells. The present inventors have unexpectedly discovered that substantially modifying portions of the mouse anti-human BCMA antibody sequences, including structural and minor antigen recognition domains, did not substantially alter the function of humanized anti-BCMA CAR T cells, and that the cytokine release properties of the humanized anti-BCMA CAR T cells can be increased or decreased compared to a T cell expressing a reference humanized anti-BCMA CAR by altering at least the transmembrane domain of the humanized anti-BCMA CAR.

In particular embodiments, a humanized anti-BCMA CAR leads to surprisingly high antigen independent or tonic cytokine release. In particular embodiments, the transmembrane domain of a humanized anti-BCMA CAR that generates antigen independent cytokine release is altered such that the signaling properties (*e.g.,* cytokine release) of the altered humanized anti-BCMA CAR T cells become substantially antigen dependent compared to a T cell expressing the unaltered humanized anti-BCMA CAR.

In certain embodiments, the transmembrane domain of a humanized anti-BCMA CAR that generates antigen independent cytokine release is altered such that the altered humanized anti-BCMA CAR T cells become substantially antigen dependent or releases substantially less cytokine in the absence of antigen compared to a T cell expressing the unaltered humanized anti-BCMA CAR.

In one embodiment, a CAR comprising a humanized anti-BCMA antibody or antigen binding fragment, a transmembrane domain, and one or more intracellular signaling domains is provided.

In one embodiment, an immune effector cell is genetically modified to express a CAR contemplated herein is provided. T cells expressing a CAR are referred to herein as CAR T cells or CAR modified T cells.

In various embodiments, the genetically modified immune effector cells contemplated herein, are administered to a patient with a B cell related condition, *e.g.,* an autoimmune disease associated with B cells or a B cell malignancy.

The practice of the invention will employ, unless indicated specifically to the contrary, conventional methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, and cell biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.,* Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982); Ausubel *et al.*, *Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford, 1985); Anand, T*echniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); T*ranscription and Translation* (B. Hames & S. Higgins, Eds., 1984); Perbal, *A Practical Guide to Molecular Cloning* (1984); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998) *Current Protocols in Immunology* Q. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, eds., 1991); *Annual Review of Immunology*; as well as monographs in journals such as *Advances in Immunology*.

## B. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred embodiments of compositions, methods and materials are described herein. For the purposes of the present invention, the following terms are defined below.

The articles “a,” “an,” and “the” are used herein to refer to one or to more than one (*i.e.,* to at least one, or to one or more) of the grammatical object of the article. By way of example, “an element” means one element or one or more elements.

The use of the alternative (*e.g.,* “or”) should be understood to mean either one, both, or any combination thereof of the alternatives.

The term “and/or” should be understood to mean either one, or both of the alternatives.

As used herein, the term “about” or “approximately” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the term “about” or “approximately” refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length ± 15%, ± 10%, ± 9%, ± 8%, ± 7%, ± 6%, ± 5%, ± 4%, ± 3%,

± 2%, or ± 1% about a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By “consisting of” is meant including, and limited to, whatever follows the phrase “consisting of.” Thus, the phrase “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present. By “consisting essentially of” is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase “consisting essentially of” indicates that the listed elements are required or mandatory, but that no other elements are present that materially affect the activity or action of the listed elements.

Reference throughout this specification to “one embodiment,” “an embodiment,” “a particular embodiment,” “a related embodiment,” “a certain embodiment,” “an additional embodiment,” or “a further embodiment” or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. It is also understood that the positive recitation of a feature in one embodiment, serves as a basis for excluding the feature in a particular embodiment.

## C. CHIMERIC ANTIGEN RECEPTORS

In various embodiments, genetically engineered receptors that redirect cytotoxicity of immune effector cells toward B cells are provided. These genetically engineered receptors referred to herein as chimeric antigen receptors (CARs). CARs are molecules that combine antibody-based specificity for a desired antigen (*e.g.,* BCMA) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-BCMA cellular immune activity. As used herein, the term, “chimeric,” describes being composed of parts of different proteins or DNAs from different origins.

CARs contemplated herein, comprise an extracellular domain (also referred to as a binding domain or antigen-specific binding domain) that binds to BCMA, a transmembrane domain, and an intracellular signaling domain. Engagement of the anti-BCMA antigen binding domain of the CAR with BCMA on the surface of a target cell results in clustering of the CAR and delivers an activation stimulus to the CAR-containing cell. The main characteristic of CARs are their ability to redirect immune effector cell specificity, thereby triggering proliferation, cytokine production, phagocytosis or production of molecules that can mediate cell death of the target antigen expressing cell in a major histocompatibility (MHC) independent manner, exploiting the cell specific targeting abilities of monoclonal antibodies, soluble ligands or cell specific co-receptors.

In various embodiments, a CAR comprises an extracellular binding domain that comprises a humanized BCMA-specific binding domain; a transmembrane domain; one or more intracellular co-stimulatory signaling domains; and a primary signaling domain.

In particular embodiments, a CAR comprises an extracellular binding domain that comprises a humanized anti-BCMA antibody or antigen binding fragment thereof; one or more hinge domains or spacer domains; a transmembrane domain including; one or more intracellular co-stimulatory signaling domains; and a primary signaling domain.

### *1.* ***BINDING DOMAIN***

In particular embodiments, CARs contemplated herein comprise an extracellular binding domain that comprises a humanized anti-BCMA antibody or antigen binding fragment thereof that specifically binds to a human BCMA polypeptide expressed on a B cell. As used herein, the terms, “binding domain,” “extracellular domain,” “extracellular binding domain,” “antigen-specific binding domain,” and “extracellular antigen specific binding domain,” are used interchangeably and provide a CAR with the ability to specifically bind to the target antigen of interest, *e.g.,* BCMA. The binding domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source.

The terms “specific binding affinity” or “specifically binds” or “specifically bound” or “specific binding” or “specifically targets” as used herein, describe binding of an anti-BCMA antibody or antigen binding fragment thereof (or a CAR comprising the same) to BCMA at greater binding affinity than background binding. A binding domain (or a CAR comprising a binding domain or a fusion protein containing a binding domain) “specifically binds” to a BCMA if it binds to or associates with BCMA with an affinity or $K_a$ (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) of, for example, greater than or equal to about $10^5$ $M^{-1}$. In certain embodiments, a binding domain (or a fusion protein thereof) binds to a target with a Ka greater than or equal to about $10^6$ $M^{-1}$, $10^7$ $M^{-1}$, $10^8$ $M^{-1}$, $10^9$ $M^{-1}$, $10^{10}$ $M^{-1}$, $10^{11}$ $M^{-1}$, $10^{12}$ $M^{-1}$, or $10^{13}$ $M^{-1}$. “High affinity” binding domains (or single chain fusion proteins thereof) refers to those binding domains with a $K_a$ of at least $10^7$ $M^{-1}$, at least $10^8$ $M^{-1}$, at least $10^9$ $M^{-1}$, at least $10^{10}$ $M^{-1}$, at least $10^{11}$ $M^{-1}$, at least $10^{12}$ $M^{-1}$, at least $10^{13}$ $M^{-1}$, or greater.

Alternatively, affinity may be defined as an equilibrium dissociation constant ($K_d$) of a particular binding interaction with units of M (*e.g.,* $10^{-5}$ M to $10^{-13}$ M, or less). Affinities of binding domain polypeptides and CAR proteins according to the present disclosure can be readily determined using conventional techniques, *e.g.,* by competitive ELISA (enzyme-linked immunosorbent assay), or by binding association, or displacement assays using labeled ligands, or using a surface-plasmon resonance device such as the Biacore T100, which is available from Biacore, Inc., Piscataway, NJ, or optical biosensor technology such as the EPIC system or EnSpire that are available from Corning and Perkin Elmer respectively (*see also*, *e.g.,* Scatchard *et al.* (1949) Ann. N.Y. Acad. Sci. 51:660; and U.S. Patent Nos. 5,283,173; 5,468,614, or the equivalent) .

In one embodiment, the affinity of specific binding is about 2 times greater than background binding, about 5 times greater than background binding, about 10 times greater than background binding, about 20 times greater than background binding, about 50 times greater than background binding, about 100 times greater than background binding, or about 1000 times greater than background binding or more.

In particular embodiments, the extracellular binding domain of a CAR comprises an antibody or antigen binding fragment thereof. An “antibody” refers to a binding agent that is a polypeptide comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen, such as a peptide, lipid, polysaccharide, or nucleic acid containing an antigenic determinant, such as those recognized by an immune cell.

An “antigen (Ag)” refers to a compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions (such as one that includes a cancer-specific protein) that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous antigens, such as the disclosed antigens. In particular embodiments, the target antigen is an epitope of a BCMA polypeptide.

An “epitope” or “antigenic determinant” refers to the region of an antigen to which a binding agent binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5, about 9, or about 8-10 amino acids in a unique spatial conformation.

Antibodies include antigen binding fragments thereof, such as Camel Ig, Ig NAR, Fab fragments, Fab' fragments, F(ab)'$_2$ fragments, F(ab)'$_3$ fragments, Fv, single chain Fv proteins (“scFv”), bis-scFv, (scFv)$_2$, minibodies, diabodies, triabodies, tetrabodies, disulfide stabilized Fv proteins (“dsFv”), and single-domain antibody (sdAb, Nanobody) and portions of full length antibodies responsible for antigen binding. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies (such as, bispecific antibodies) and antigen binding fragments thereof. See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., Immunology, 3rd Ed., W. H. Freeman & Co., New York, 1997.

As would be understood by the skilled person and as described elsewhere herein, a complete antibody comprises two heavy chains and two light chains. Each heavy chain consists of a variable region and a first, second, and third constant region, while each light chain consists of a variable region and a constant region. Mammalian heavy chains are classified as $\alpha$, $\delta$, $\varepsilon$, $\gamma$, and $\mu$. Mammalian light chains are classified as $\lambda$ or $\kappa$. Immunoglobulins comprising the $\alpha$, $\delta$, $\varepsilon$, $\gamma$, and $\mu$ heavy chains are classified as immunoglobulin (Ig)A, IgD, IgE, IgG, and IgM. The complete antibody forms a “Y” shape. The stem of the Y consists of the second and third constant regions (and for IgE and IgM, the fourth constant region) of two heavy chains bound together and disulfide bonds (inter-chain) are formed in the hinge. Heavy chains γ, α and δ have a constant region composed of three tandem (in a line) Ig domains, and a hinge region for added flexibility; heavy chains μ and ε have a constant region composed of four immunoglobulin domains. The second and third constant regions are referred to as “CH2 domain” and “CH3 domain”, respectively. Each arm of the Y includes the variable region and first constant region of a single heavy chain bound to the variable and constant regions of a single light chain. The variable regions of the light and heavy chains are responsible for antigen binding.

Light and heavy chain variable regions contain a “framework” region interrupted by three hypervariable regions, also called “complementarity-determining regions” or “CDRs.” The CDRs can be defined or identified by conventional methods, such as by sequence according to Kabat *et al* (Wu, TT and Kabat, E. A., *J Exp Med.* 132(2):211-50, (1970); Borden, P. and Kabat E. A., *PNAS*, 84: 2440-2443 (1987); (see, Kabat *et al.*, *Sequences of Proteins of Immunological Interest,* U.S. Department of Health and Human Services, 1991, which is hereby incorporated by reference), or by structure according to Chothia *et al* (Chothia, C. and Lesk, A.M., *J Mol. Biol.*, 196(4): 901-917 (1987), Chothia, C. *et al*, *Nature*, 342: 877 - 883 (1989)).

The sequences of the framework regions of different light or heavy chains are relatively conserved within a species, such as humans. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space. The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, the CDRs located in the variable domain of the heavy chain of the antibody are referred to as CDRH1, CDRH2, and CDRH3, whereas the CDRs located in the variable domain of the light chain of the antibody are referred to as CDRL1, CDRL2, and CDRL3. Antibodies with different specificities (*i.e.,* different combining sites for different antigens) have different CDRs. Although it is the CDRs that vary from antibody to antibody, only a limited number of amino acid positions within the CDRs are directly involved in antigen binding. These positions within the CDRs are called specificity determining residues (SDRs). Illustrative examples of light chain CDRs that are suitable for constructing humanized anti-BCMA CARs contemplated herein include, but are not limited to the CDR sequences set forth in SEQ ID NOs: 1-3. Illustrative examples of heavy chain CDRs that are suitable for constructing humanized anti-BCMA CARs contemplated herein include, but are not limited to the CDR sequences set forth in SEQ ID NOs: 4-6.

References to "$V_H$" or "VH" refer to the variable region of an immunoglobulin heavy chain, including that of an antibody, Fv, scFv, dsFv, Fab, or other antibody fragment as disclosed herein. References to "$V_L$" or "VL" refer to the variable region of an immunoglobulin light chain, including that of an antibody, Fv, scFv, dsFv, Fab, or other antibody fragment as disclosed herein.

A "monoclonal antibody" is an antibody produced by a single clone of B lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized monoclonal antibodies.

A "chimeric antibody" has framework residues from one species, such as human, and CDRs (which generally confer antigen binding) from another species, such as a mouse. In particular preferred embodiments, a CAR contemplated herein comprises antigen-specific binding domain that is a chimeric antibody or antigen binding fragment thereof.

In preferred embodiments, the antibody is a humanized antibody (such as a humanized monoclonal antibody) or fragment thereof that specifically binds to a human BCMA polypeptide. A "humanized" antibody is an immunoglobulin including a human framework region and one or more CDRs from a non-human (for example a mouse, rat, or synthetic) immunoglobulin. The non-human immunoglobulin providing the CDRs is termed a "donor," and the human immunoglobulin providing the framework is termed an "acceptor." In one embodiment, all the CDRs are from the donor immunoglobulin in a humanized immunoglobulin. Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, *i.e.*, at least about 85-90%, such as about 95% or more identical.

Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. Humanized or other monoclonal antibodies can have additional conservative amino acid substitutions, which have substantially no effect on antigen binding or other immunoglobulin functions. Humanized antibodies can be constructed by means of genetic engineering (see for example, U.S. Patent No. 5,585,089).

In particular embodiments, a humanized anti-BCMA antibody or antigen binding fragment thereof, includes but is not limited to a Camel Ig (a camelid antibody (VHH)), Ig NAR, Fab fragments, Fab' fragments, F(ab)'2 fragments, F(ab)'3 fragments, Fv, single chain Fv antibody (“scFv”), bis-scFv, (scFv)2, minibody, diabody, triabody, tetrabody, disulfide stabilized Fv protein (“dsFv”), and single-domain antibody (sdAb, Nanobody).

“Camel Ig” or “camelid VHH” as used herein refers to the smallest known antigen-binding unit of a heavy chain antibody (Koch-Nolte, *et al*, FASEB J., 21: 3490-3498 (2007)). A “heavy chain antibody” or a “camelid antibody” refers to an antibody that contains two VH domains and no light chains (Riechmann L. *et al*, J. Immunol. Methods 231:25–38 (1999); WO94/04678; WO94/25591; U.S. Patent No. 6,005,079).

“IgNAR” of “immunoglobulin new antigen receptor” refers to class of antibodies from the shark immune repertoire that consist of homodimers of one variable new antigen receptor (VNAR) domain and five constant new antigen receptor (CNAR) domains. IgNARs represent some of the smallest known immunoglobulin-based protein scaffolds and are highly stable and possess efficient binding characteristics. The inherent stability can be attributed to both (i) the underlying Ig scaffold, which presents a considerable number of charged and hydrophilic surface exposed residues compared to the conventional antibody VH and VL domains found in murine antibodies; and (ii) stabilizing structural features in the complementary determining region (CDR) loops including inter-loop disulphide bridges, and patterns of intra-loop hydrogen bonds.

Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab′)2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three hypervariable regions (HVRs) of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab′ fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab′-SH is the designation herein for Fab′ in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab′)2 antibody fragments originally were produced as pairs of Fab′ fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO 1993/01161; Hudson *et al.*, Nat. Med. 9:129-134 (2003); and Hollinger *et al.*, PNAS USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson *et al.*, Nat. Med. 9:129-134 (2003).

"Single domain antibody" or "sdAb" or "nanobody" refers to an antibody fragment that consists of the variable region of an antibody heavy chain (VH domain) or the variable region of an antibody light chain (VL domain) (Holt, L., *et al*, Trends in Biotechnology, 21(11): 484-490).

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain and in either orientation (*e.g.,* VL-VH or VH-VL). Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, *e.g.,* Pluckthün, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315.

In preferred embodiments, a CAR contemplated herein comprises antigen-specific binding domain that is an scFv and may be a murine, human or humanized scFv. Single chain antibodies may be cloned form the V region genes of a hybridoma specific for a desired target. The production of such hybridomas has become routine. A technique which can be used for cloning the variable region heavy chain ($V_H$) and variable region light chain ($V_L$) has been described, for example, in Orlandi *et al., PNAS,* 1989; 86: 3833-3837.

In particular embodiments, the antigen-specific binding domain that is a humanized scFv that binds a human BCMA polypeptide. Illustrative examples of variable heavy chains that are suitable for constructing anti-BCMA CARs contemplated herein include, but are not limited to the amino acid sequences set forth in SEQ ID NOs: 10-14. Illustrative examples of variable light chains that are suitable for constructing anti-BCMA CARs contemplated herein include, but are not limited to the amino acid sequences set forth in SEQ ID NOs: 7-9.

An exemplary humanized anti-BCMA-specific binding domain is an immunoglobulin variable region specific for BCMA that comprises at least one human framework region. A "human framework region" refers to a wild type (*i.e.*, naturally occurring) framework region of a human immunoglobulin variable region, an altered framework region of a human immunoglobulin variable region with less than about 50% (*e.g.,* preferably less than about 45%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, or 1%) of the amino acids in the region are deleted or substituted (*e.g.,* with one or more amino acid residues of a nonhuman immunoglobulin framework region at corresponding positions), or an altered framework region of a nonhuman immunoglobulin variable region with less than about 50% (*e.g.,* less than 45%, 40%, 30%, 25%, 20%, 15%, 10%, or 5%) of the amino acids in the region deleted or substituted (*e.g.,* at positions of exposed residues and/or with one or more amino acid residues of a human immunoglobulin framework region at corresponding positions) so that, in one aspect, immunogenicity is reduced.

In certain embodiments, a human framework region is a wild type framework region of a human immunoglobulin variable region. In certain other embodiments, a human framework region is an altered framework region of a human immunoglobulin variable region with amino acid deletions or substitutions at one, two, three, four, five , six, seven, eight, nine, ten or more positions. In other embodiments, a human framework region is an altered framework region of a non-human immunoglobulin variable region with amino acid deletions or substitutions at one, two, three, four, five , six, seven, eight, nine, ten or more positions.

In particular embodiments, a BCMA-specific binding domain comprises at least one, two, three, four, five, six, seven or eight human framework regions (FR) selected from human light chain FR1, human heavy chain FR1, human light chain FR2, human heavy chain FR2, human light chain FR3, human heavy chain FR3, human light chain FR4, and human heavy chain FR4.

Human FRs that may be present in a BCMA-specific binding domains also include variants of the exemplary FRs provided herein in which one, two, three, four, five , six, seven, eight, nine, ten or more amino acids of the exemplary FRs have been substituted or deleted.

In certain embodiments, a humanized anti-BCMA-specific binding domain comprises (a) a humanized light chain variable region that comprises a human light chain FR1, a human light chain FR2, a human light chain FR3, and a human light chain FR4, and (b) a humanized heavy chain variable region that comprises a human heavy chain FR1, a human heavy chain FR2, a human heavy chain FR3, and a human heavy chain FR4.

BCMA-specific binding domains provided herein also comprise one, two, three, four, five, or six CDRs. Such CDRs may be nonhuman CDRs or altered nonhuman CDRs selected from CDRL1, CDRL2 and CDRL3 of the light chain and CDRH1, CDRH2 and CDRH3 of the heavy chain. In certain embodiments, a BCMA-specific binding domain comprises (a) a light chain variable region that comprises a light chain CDRL1, a light chain CDRL2, and a light chain CDRL3, and (b) a heavy chain variable region that comprises a heavy chain CDRH1, a heavy chain CDRH2, and a heavy chain CDRH3.

### 2. *LINKERS*

In certain embodiments, the CARs contemplated herein may comprise linker residues between the various domains, *e.g.,* , added for appropriate spacing and conformation of the molecule. In particular embodiments the linker is a variable region linking sequence. A "variable region linking sequence," is an amino acid sequence that connects the $V_H$ and $V_L$ domains and provides a spacer function compatible with interaction of the two sub-binding domains so that the resulting polypeptide retains a specific binding affinity to the same target molecule as an antibody that comprises the same light and heavy chain variable regions. CARs contemplated herein, may comprise one, two, three, four, or five or more linkers. In particular embodiments, the length of a linker is about 1 to about 25 amino acids, about 5 to about 20 amino acids, or about 10 to about 20 amino acids, or any intervening length of amino acids. In some embodiments, the linker is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more amino acids long.

Illustrative examples of linkers include glycine polymers $(G)_n$; glycine-serine polymers $(G_{1-5}S_{1-5})_n$, where n is an integer of at least one, two, three, four, or five; glycine-alanine polymers; alanine-serine polymers; and other flexible linkers known in the art. Glycine and glycine-serine polymers are relatively unstructured, and therefore may be able to serve as a neutral tether between domains of fusion proteins such as the CARs described herein. Glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (*see* Scheraga, *Rev. Computational Chem.* 11173-142 (1992)). The ordinarily skilled artisan will recognize that design of a CAR in particular embodiments can include linkers that are all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired CAR structure.

Other exemplary linkers include, but are not limited to the following amino acid sequences: GGG; DGGGS (SEQ ID NO: 46); TGEKP (SEQ ID NO: 47) (see, *e.g.,* Liu *et al.*, PNAS 5525-5530 (1997)); GGRR (SEQ ID NO: 48) (Pomerantz *et al.*

1995, supra); (GGGGS)$_n$ wherein = 1, 2, 3, 4 or 5 (SEQ ID NO: 49) (Kim *et al.*, PNAS 93, 1156-1160 (1996.); EGKSSGSGSESKVD (SEQ ID NO: 50) (Chaudhary *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1066-1070); KESGSVSSEQLAQFRSLD (SEQ ID NO: 51) (Bird *et al.*, 1988, Science 242:423-426), GGRRGGGS (SEQ ID NO: 52); LRQRDGERP (SEQ ID NO: 53); LRQKDGGGSERP (SEQ ID NO: 54); LRQKd(GGGS)$_2$ ERP (SEQ ID NO: 55). Alternatively, flexible linkers can be rationally designed using a computer program capable of modeling both DNA-binding sites and the peptides themselves (Desjarlais & Berg, *PNAS* 90:2256-2260 (1993), *PNAS* 91:11099-11103 (1994) or by phage display methods. N one embodiment, the linker comprises the following amino acid sequence: GSTSGSGKPGSGEGSTKG (SEQ ID NO: 56) (Cooper *et al., Blood*, 101(4): 1637-1644 (2003)).

### *3. SPACER DOMAIN*

In particular embodiments, the binding domain of the CAR is followed by one or more "spacer domains," which refers to the region that moves the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation (Patel *et al.*, *Gene Therapy*, 1999; 6: 412-419). The hinge domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. In certain embodiments, a spacer domain is a portion of an immunoglobulin, including, but not limited to, one or more heavy chain constant regions, *e.g.,* CH2 and CH3. The spacer domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

In one embodiment, the spacer domain comprises the CH2 and CH3 domains of IgG1 or IgG4.

### *4. HINGE DOMAIN*

The binding domain of the CAR is generally followed by one or more "hinge domains," which plays a role in positioning the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation. A CAR generally comprises one or more hinge domains between the binding domain and the transmembrane domain (TM). The hinge domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. The hinge domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

An "altered hinge region" refers to (a) a naturally occurring hinge region with up to 30% amino acid changes (*e.g.,* up to 25%, 20%, 15%, 10%, or 5% amino acid substitutions or deletions), (b) a portion of a naturally occurring hinge region that is at least 10 amino acids (*e.g.,* at least 12, 13, 14 or 15 amino acids) in length with up to 30% amino acid changes (*e.g.,* up to 25%, 20%, 15%, 10%, or 5% amino acid substitutions or deletions), or (c) a portion of a naturally occurring hinge region that comprises the core hinge region (which may be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15, or at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids in length). In certain embodiments, one or more cysteine residues in a naturally occurring immunoglobulin hinge region may be substituted by one or more other amino acid residues (*e.g.,* one or more serine residues). An altered immunoglobulin hinge region may alternatively or additionally have a proline residue of a wild type immunoglobulin hinge region substituted by another amino acid residue (*e.g.,* a serine residue).

Other illustrative hinge domains suitable for use in the CARs described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD8α, CD4, CD28, PD1, CD152, and CD7, which may be wild-type hinge regions from these molecules or may be altered. In another embodiment, the hinge domain comprises a , PD1, CD152, or CD8α hinge region.

### *5. TRANSMEMBRANE (TM) DOMAIN*

The "transmembrane domain" is the portion of the CAR that fuses the extracellular binding portion and intracellular signaling domain and anchors the CAR to the plasma membrane of the immune effector cell. The TM domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. The TM domain may be derived from (*i.e.*, comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD 154, and PD1. In a particular embodiment, the TM domain is synthetic and predominantly comprises hydrophobic residues such as leucine and valine.

In one embodiment, the CARs contemplated herein comprise a TM domain derived from, PD1, CD152, or CD8α. In another embodiment, a CAR contemplated herein comprises a TM domain derived from, PD1, CD152, or CD8α and a short oligo- or polypeptide linker, preferably between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length that links the TM domain and the intracellular signaling domain of the CAR. A glycine-serine based linker provides a particularly suitable linker.

### *6. INTRACELLULAR SIGNALING DOMAIN*

In particular embodiments, CARs contemplated herein comprise an intracellular signaling domain. An "intracellular signaling domain," refers to the part of a CAR that participates in transducing the message of effective anti-BCMA CAR binding to a human BCMA polypeptide into the interior of the immune effector cell to elicit effector cell function, *e.g.,* activation, cytokine production, proliferation and cytotoxic activity, including the release of cytotoxic factors to the CAR-bound target cell, or other cellular responses elicited with antigen binding to the extracellular CAR domain.

The term "effector function" refers to a specialized function of an immune effector cell. Effector function of the T cell, for example, may be cytolytic activity or help or activity including the secretion of a cytokine. Thus, the term "intracellular signaling domain" refers to the portion of a protein which transduces the effector function signal and that directs the cell to perform a specialized function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire domain. To the extent that a truncated portion of an intracellular signaling domain is used, such truncated portion may be used in place of the entire domain as long as it transduces the effector function signal. The term intracellular signaling domain is meant to include any truncated portion of the intracellular signaling domain sufficient to transducing effector function signal.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of intracellular signaling domains: primary signaling domains that initiate antigen-dependent primary activation through the TCR (*e.g.,* a TCR/CD3 complex) and co-stimulatory signaling domains that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. In preferred embodiments, a CAR contemplated herein comprises an intracellular signaling domain that comprises one or more “co-stimulatory signaling domain” and a “primary signaling domain.”

Primary signaling domains regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

Illustrative examples of ITAM containing primary signaling domains that are of particular use in the invention include those derived from TCRζ, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d. In particular preferred embodiments, a CAR comprises a CD3ζ primary signaling domain and one or more co-stimulatory signaling domains. The intracellular primary signaling and co-stimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain.

CARs contemplated herein comprise one or more co-stimulatory signaling domains to enhance the efficacy and expansion of T cells expressing CAR receptors. As used herein, the term, “co-stimulatory signaling domain,” or “co-stimulatory domain”, refers to an intracellular signaling domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. Illustrative examples of such co-stimulatory molecules include CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70. In one embodiment, a CAR comprises one or more co-stimulatory signaling domains selected from the group consisting of CD28, CD137, and CD134, and a CD3ζ primary signaling domain.

In another embodiment, a CAR comprises CD28 and CD137 co-stimulatory signaling domains and a CD3ζ primary signaling domain.

In yet another embodiment, a CAR comprises CD28 and CD134 co-stimulatory signaling domains and a CD3ζ primary signaling domain.

In one embodiment, a CAR comprises CD137 and CD134 co-stimulatory signaling domains and a CD3ζ primary signaling domain.

In one embodiment, a CAR comprises a CD137 co-stimulatory signaling domain and a CD3ζ primary signaling domain.

In one embodiment, a CAR comprises a CD134 co-stimulatory signaling domain and a CD3ζ primary signaling domain.

In one embodiment, a CAR comprises a CD28 co-stimulatory signaling domain and a CD3ζ primary signaling domain.

In particular embodiments, CARs contemplated herein comprise a humanized anti-BCMA antibody or antigen binding fragment thereof that specifically binds to a BCMA polypeptide expressed on B cells.

In one embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a transmembrane domain derived from a polypeptide selected from the group consisting of: alpha, beta or zeta chain of the T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD 154, and PD1; and one or more intracellular co-stimulatory signaling domains from a co-stimulatory molecule selected from the group consisting of: CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70; and a primary signaling domain from TCRζ, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

In one embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain selected from the group consisting of: IgG1 hinge/CH2/CH3, IgG4 hinge/CH2/CH3, a PD1 hinge, a CD152 hinge, and a CD8α hinge; a transmembrane domain derived from a polypeptide selected from the group consisting of: alpha, beta or zeta chain of the T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD 154, and PD1; and one or more intracellular co-stimulatory signaling domains from a co-stimulatory molecule selected from the group consisting of: CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70; and a primary signaling domain from TCRζ, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

In one embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain selected from the group consisting of: IgG1 hinge/CH2/CH3, IgG4 hinge/CH2/CH3, a PD1 hinge, a CD152 hinge, and a CD8α hinge; a transmembrane domain derived from a polypeptide selected from the group consisting of: alpha, beta or zeta chain of the T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD 154, and PD1; a short oligo- or polypeptide linker, preferably between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length that links the TM domain to the intracellular signaling domain of the CAR; and one or more intracellular co-stimulatory signaling domains from a co-stimulatory molecule selected from the group consisting of: CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70; and a primary signaling domain from TCRζ, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising an IgG1 hinge/CH2/CH3 polypeptide and a CD8α polypeptide; a CD8α transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD137 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a CD8α polypeptide; a CD8α transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD134 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a CD8α polypeptide; a CD8α transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD28 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a PD1 hinge, polypeptide; a PD1 or CD152 transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD137 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a PD1 hinge, polypeptide; a PD1 or CD152 transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD134 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a PD1 hinge, polypeptide; a PD1 or CD152 transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD28 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a CD152 hinge, polypeptide; a PD1 or CD152 transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD137 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a CD152 hinge, polypeptide; a PD1 or CD152 transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD134 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

In a particular embodiment, a CAR comprises a humanized anti-BCMA scFv that binds a BCMA polypeptide; a hinge domain comprising a CD152 hinge, polypeptide; a PD1 or CD152 transmembrane domain comprising a polypeptide linker of about 3 to about 10 amino acids; a CD28 intracellular co-stimulatory signaling domain; and a CD3ζ primary signaling domain.

Moreover, the design of the CARs contemplated herein enable improved expansion, long-term persistence, and cytotoxic properties in T cells expressing the CARs compared to non-modified T cells or T cells modified to express other CARs.

## D. POLYPEPTIDES

The present invention contemplates, in part, CAR polypeptides and fragments thereof, cells and compositions comprising the same, and vectors that express polypeptides. In preferred embodiments, a polypeptide comprising one or more CARs as set forth in any one of SEQ ID NOs: 15-29, 71, and 73 is provided.

"Polypeptide," "polypeptide fragment," "peptide" and "protein" are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.,* as a sequence of amino acids. Polypeptides are not limited to a specific length, *e.g.,* they may comprise a full length protein sequence or a fragment of a full length protein, and may include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. In various embodiments, the CAR polypeptides contemplated herein comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. Illustrative examples of suitable signal sequences useful in CARs disclosed herein include, but are not limited to the IgG1 heavy chain signal polypeptide, a CD8α signal polypeptide, or a human GM-CSF receptor alpha signal polypeptide. Polypeptides can be prepared using any of a variety of well known recombinant and/or synthetic techniques. Polypeptides contemplated herein specifically encompass the CARs of the present disclosure, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acid of a CAR as disclosed herein.

An "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to *in vitro* isolation and/or purification of a peptide or polypeptide molecule from a cellular environment, and from association with other components of the cell, *i.e.*, it is not significantly associated with *in vivo* substances. Similarly, an "isolated cell" refers to a cell that has been obtained from an *in vivo* tissue or organ and is substantially free of extracellular matrix.

Polypeptides include "polypeptide variants." Polypeptide variants may differ from a naturally occurring polypeptide in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences.

For example, in particular embodiments, it may be desirable to improve the binding affinity and/or other biological properties of the CARs by introducing one or more substitutions, deletions, additions and/or insertions into a binding domain, hinge, TM domain, co-stimulatory signaling domain or primary signaling domain of a CAR polypeptide. Preferably, polypeptides of the invention include polypeptides having at least about 65%, 70%, 75%, 85%, 90%, 95%, 98%, or 99% amino acid identity thereto.

Polypeptides include "polypeptide fragments." Polypeptide fragments refer to a polypeptide, which can be monomeric or multimeric, that has an amino-terminal deletion, a carboxyl-terminal deletion, and/or an internal deletion or substitution of a naturally-occurring or recombinantly-produced polypeptide. In certain embodiments, a polypeptide fragment can comprise an amino acid chain at least 5 to about 500 amino acids long. It will be appreciated that in certain embodiments, fragments are at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 150, 200, 250, 300, 350, 400, or 450 amino acids long. Particularly useful polypeptide fragments include functional domains, including antigen-binding domains or fragments of antibodies. In the case of a humanized anti-BCMA antibody, useful fragments include, but are not limited to: a CDR region, a CDR3 region of the heavy or light chain; a variable region of a heavy or light chain; a portion of an antibody chain or variable region including two CDRs; and the like.

The polypeptide may also be fused in-frame or conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.,* poly-His), or to enhance binding of the polypeptide to a solid support.

As noted above, polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of a reference polypeptide can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985, *Proc. Natl. Acad. Sci. USA*. 82: 488-492), Kunkel *et al.*, (1987, *Methods in Enzymol*, 154: 367-382), U.S. Pat. No. 4,873,192, Watson, J. D. *et al.*, (*Molecular Biology of the Gene*, Fourth Edition, Benjamin/Cummings, Menlo Park, Calif., 1987) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.*, (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.).

In certain embodiments, a variant will contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Modifications may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, variant polypeptide of the invention, one skilled in the art, for example, can change one or more of the codons of the encoding DNA sequence, *e.g.,* according to Table 1.

**TABLE 1- Amino Acid Codons**

| **Amino Acids** | **One letter code** | **Three letter code** | **Codons** | | | | | |
|---|---|---|---|---|---|---|---|---|
| Alanine | A | Ala | GCA | GCC | GCG | GCU | | |
| Cysteine | C | Cys | UGC | UGU | | | | |
| Aspartic acid | D | Asp | GAC | GAU | | | | |
| Glutamic acid | E | Glu | GAA | GAG | | | | |
| Phenylalanine | F | Phe | UUC | UUU | | | | |
| Glycine | G | Gly | GGA | GGC | GGG | GGU | | |
| Histidine | H | His | CAC | CAU | | | | |
| Isoleucine | I | Iso | AUA | AUC | AUU | | | |
| Lysine | K | Lys | AAA | AAG | | | | |
| Leucine | L | Leu | UUA | UUG | CUA | CUC | CUG | CUU |
| Methionine | M | Met | AUG | | | | | |
| Asparagine | N | Asn | AAC | AAU | | | | |
| Proline | P | Pro | CCA | CCC | CCG | CCU | | |
| Glutamine | Q | Gln | CAA | CAG | | | | |
| Arginine | R | Arg | AGA | AGG | CGA | CGC | CGG | CGU |
| Serine | S | Ser | AGC | AGU | UCA | UCC | UCG | UCU |
| Threonine | T | Thr | ACA | ACC | ACG | ACU | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Valine | V | Val | GUA | GUC | GUG | GUU |
| Tryptophan | W | Trp | UGG | | | |
| Tyrosine | Y | Tyr | UAC | UAU | | |

Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well known in the art, such as DNASTAR$^{TM}$ software. Preferably, amino acid changes in the protein variants disclosed herein are conservative amino acid changes, *i.e.,* substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and generally can be made without altering a biological activity of a resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.,* Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p.224). Exemplary conservative substitutions are described in U.S. Provisional Patent Application No. 61/241,647 , the disclosure of which is herein incorporated by reference.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cysteine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.,* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions may be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like.

Polypeptide variants further include glycosylated forms, aggregative conjugates with other molecules, and covalent conjugates with unrelated chemical moieties (*e.g.,* pegylated molecules). Covalent variants can be prepared by linking functionalities to groups which are found in the amino acid chain or at the N- or C-terminal residue, as is known in the art. Variants also include allelic variants, species variants, and muteins. Truncations or deletions of regions which do not affect functional activity of the proteins are also variants.

In one embodiment, where expression of two or more polypeptides is desired, the polynucleotide sequences encoding them can be separated by and IRES sequence as discussed elsewhere herein. In another embodiment, two or more polypeptides can be expressed as a fusion protein that comprises one or more self-cleaving polypeptide sequences.

Polypeptides of the present invention include fusion polypeptides. In preferred embodiments, fusion polypeptides and polynucleotides encoding fusion polypeptides are provided, *e.g.,* CARs. Fusion polypeptides and fusion proteins refer to a polypeptide having at least two, three, four, five, six, seven, eight, nine, or ten or more polypeptide segments. Fusion polypeptides are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. The polypeptides of the fusion protein can be in any order or a specified order. Fusion polypeptides or fusion proteins can also include conservatively modified variants, polymorphic variants, alleles, mutants, subsequences, and interspecies homologs, so long as the desired transcriptional activity of the fusion polypeptide is preserved. Fusion polypeptides may be produced by chemical synthetic methods or by chemical linkage between the two moieties or may generally be prepared using other standard techniques. Ligated DNA sequences comprising the fusion polypeptide are operably linked to suitable transcriptional or translational control elements as discussed elsewhere herein.

In one embodiment, a fusion partner comprises a sequence that assists in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Other fusion partners may be selected so as to increase the solubility of the protein or to enable the protein to be targeted to desired intracellular compartments or to facilitate transport of the fusion protein through the cell membrane.

Fusion polypeptides may further comprise a polypeptide cleavage signal between each of the polypeptide domains described herein. In addition, polypeptide site can be put into any linker peptide sequence. Exemplary polypeptide cleavage signals include polypeptide cleavage recognition sites such as protease cleavage sites, nuclease cleavage sites (*e.g.,* rare restriction enzyme recognition sites, self-cleaving ribozyme recognition sites), and self-cleaving viral oligopeptides (see deFelipe and Ryan, 2004. *Traffic*, 5(8); 616-26).

Suitable protease cleavages sites and self-cleaving peptides are known to the skilled person (*see, e.g.,* in Ryan *et al.*, 1997. *J. Gener. Virol.* 78, 699-722; Scymczak *et al.* (2004) Nature Biotech. 5, 589-594). Exemplary protease cleavage sites include, but are not limited to the cleavage sites of potyvirus NIa proteases (*e.g.,* tobacco etch virus protease), potyvirus HC proteases, potyvirus P1 (P35) proteases, byovirus NIa proteases, byovirus RNA-2-encoded proteases, aphthovirus L proteases, enterovirus 2A proteases, rhinovirus 2A proteases, picorna 3C proteases, comovirus 24K proteases, nepovirus 24K proteases, RTSV (rice tungro spherical virus) 3C-like protease, PYVF (parsnip yellow fleck virus) 3C-like protease, heparin, thrombin, factor Xa and enterokinase. Due to its high cleavage stringency, TEV (tobacco etch virus) protease cleavage sites are preferred in one embodiment, *e.g.,* EXXYXQ(G/S) (SEQ ID NO: 57), for example, ENLYFQG (SEQ ID NO: 58) and ENLYFQS (SEQ ID NO: 59), wherein X represents any amino acid (cleavage by TEV occurs between Q and G or Q and S).

In a particular embodiment, self-cleaving peptides include those polypeptide sequences obtained from potyvirus and cardiovirus 2A peptides, FMDV (foot-and-mouth disease virus), equine rhinitis A virus, Thosea asigna virus and porcine teschovirus.

In certain embodiments, the self-cleaving polypeptide site comprises a 2A or 2A-like site, sequence or domain (Donnelly *et al.*, 2001. *J. Gen. Virol.* 82:1027-1041).

TABLE 2: Exemplary 2A sites include the following sequences:

| | |
|---|---|
| SEQ ID NO: 60 | LLNFDLLKLAGDVESNPGP |
| SEQ ID NO: 61 | TLNFDLLKLAGDVESNPGP |
| SEQ ID NO: 62 | LLKLAGDVESNPGP |
| SEQ ID NO: 63 | NFDLLKLAGDVESNPGP |
| SEQ ID NO: 64 | QLLNFDLLKLAGDVESNPGP |
| SEQ ID NO: 65 | APVKQTLNFDLLKLAGDVESNPGP |
| SEQ ID NO: 66 | VTELLYRMKRAETYCPRPLLAIHPTEARHKQKIVAPVKQT |
| SEQ ID NO: 67 | LNFDLLKLAGDVESNPGP |
| SEQ ID NO: 68 | LLAIHPTEARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP |
| SEQ ID NO: 69 | EARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP |

In preferred embodiments, a polypeptide contemplated herein comprises a CAR polypeptide.

## E. POLYNUCLEOTIDES

In preferred embodiments, a polynucleotide encoding one or more CAR polypeptides is provided, *e.g.,* SEQ ID NOs: 30-44, 70, and 72. As used herein, the terms "polynucleotide" or "nucleic acid" refers to messenger RNA (mRNA), RNA, genomic RNA (gRNA), plus strand RNA (RNA(+)), minus strand RNA (RNA(-)), genomic DNA (gDNA), complementary DNA (cDNA) or recombinant DNA. Polynucleotides include single and double stranded polynucleotides. Preferably, polynucleotides of the invention include polynucleotides or variants having at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any of the reference sequences described herein (*see, e.g.,* Sequence Listing), typically where the variant maintains at least one biological activity of the reference sequence. In various illustrative embodiments, the present invention contemplates, in part, polynucleotides comprising expression vectors, viral vectors, and transfer plasmids, and compositions, and cells comprising the same.

In particular embodiments, polynucleotides are provided by this invention that encode at least about 5, 10, 25, 50, 100, 150, 200, 250, 300, 350, 400, 500, 1000, 1250, 1500, 1750, or 2000 or more contiguous amino acid residues of a polypeptide of the invention, as well as all intermediate lengths. It will be readily understood that "intermediate lengths*,"* in this context, means any length between the quoted values, such as 6, 7, 8, 9, *etc.*, 101, 102, 103, *etc.*; 151, 152, 153, *etc.*; 201, 202, 203, *etc.*

As used herein, the terms "polynucleotide variant" and "variant" and the like refer to polynucleotides displaying substantial sequence identity with a reference polynucleotide sequence or polynucleotides that hybridize with a reference sequence under stringent conditions that are defined hereinafter. These terms include polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides compared to a reference polynucleotide. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide.

The recitations "sequence identity" or, for example, comprising a "sequence 50% identical to," as used herein, refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.,* A, T, C, G, I) or the identical amino acid residue (*e.g.,* Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Included are nucleotides and polypeptides having at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any of the reference sequences described herein, typically where the polypeptide variant maintains at least one biological activity of the reference polypeptide.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, Nucl. Acids Res. 25:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc, 1994-1998, Chapter 15.

As used herein, “isolated polynucleotide” refers to a polynucleotide that has been purified from the sequences which flank it in a naturally-occurring state, *e.g.*, a DNA fragment that has been removed from the sequences that are normally adjacent to the fragment. An “isolated polynucleotide” also refers to a complementary DNA (cDNA), a recombinant DNA, or other polynucleotide that does not exist in nature and that has been made by the hand of man.

Terms that describe the orientation of polynucleotides include: 5' (normally the end of the polynucleotide having a free phosphate group) and 3' (normally the end of the polynucleotide having a free hydroxyl (OH) group). Polynucleotide sequences can be annotated in the 5' to 3' orientation or the 3' to 5' orientation. For DNA and mRNA, the 5' to 3' strand is designated the “sense,” “plus,” or “coding” strand because its sequence is identical to the sequence of the premessenger (premRNA) [except for uracil (U) in RNA, instead of thymine (T) in DNA]. For DNA and mRNA, the complementary 3' to 5' strand which is the strand transcribed by the RNA polymerase is designated as “template,” “antisense,” “minus,” or “non-coding” strand. As used herein, the term “reverse orientation” refers to a 5' to 3' sequence written in the 3' to 5' orientation or a 3' to 5' sequence written in the 5' to 3' orientation.

The terms “complementary” and “complementarity” refer to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, the complementary strand of the DNA sequence 5' A G T C A T G 3' is 3' T C A G T A C 5'. The latter sequence is often written as the reverse complement with the 5' end on the left and the 3' end on the right, 5' C A T G A C T 3'. A sequence that is equal to its reverse complement is said to be a palindromic sequence. Complementarity can be “partial,” in which only some of the nucleic acids’ bases are matched according to the base pairing rules. Or, there can be "complete" or "total" complementarity between the nucleic acids.

Moreover, it will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide, or fragment of variant thereof, as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention, for example polynucleotides that are optimized for human and/or primate codon selection. Further, alleles of the genes comprising the polynucleotide sequences provided herein may also be used. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides.

The term "nucleic acid cassette" as used herein refers to genetic sequences within a vector which can express a RNA, and subsequently a protein. The nucleic acid cassette contains the gene of interest, *e.g.,* a CAR. The nucleic acid cassette is positionally and sequentially oriented within the vector such that the nucleic acid in the cassette can be transcribed into RNA, and when necessary, translated into a protein or a polypeptide, undergo appropriate post-translational modifications required for activity in the transformed cell, and be translocated to the appropriate compartment for biological activity by targeting to appropriate intracellular compartments or secretion into extracellular compartments. Preferably, the cassette has its 3' and 5' ends adapted for ready insertion into a vector, *e.g.,* it has restriction endonuclease sites at each end. In a preferred embodiment of the invention, the nucleic acid cassette contains the sequence of a chimeric antigen receptor used to treat a B cell malignancy. The cassette can be removed and inserted into a plasmid or viral vector as a single unit.

In particular embodiments, polynucleotides include at least one polynucleotide-of-interest. As used herein, the term "polynucleotide-of-interest" refers to a polynucleotide encoding a polypeptide (*i.e.,* a polypeptide-of-interest), inserted into an expression vector that is desired to be expressed. A vector may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 polynucleotides-of-interest. In certain embodiments, the polynucleotide-of-interest encodes a polypeptide that provides a therapeutic effect in the treatment or prevention of a disease or disorder. Polynucleotides-of-interest, and polypeptides encoded therefrom, include both polynucleotides that encode wild-type polypeptides, as well as functional variants and fragments thereof. In particular embodiments, a functional variant has at least 80%, at least 90%, at least 95%, or at least 99% identity to a corresponding wild-type reference polynucleotide or polypeptide sequence. In certain embodiments, a functional variant or fragment has at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of a biological activity of a corresponding wild-type polypeptide.

In one embodiment, the polynucleotide-of-interest does not encode a polypeptide but serves as a template to transcribe miRNA, siRNA, or shRNA, ribozyme, or other inhibitory RNA. In various other embodiments, a polynucleotide comprises a polynucleotide-of-interest encoding a CAR and one or more additional polynucleotides-of-interest including but not limited to an inhibitory nucleic acid sequence including, but not limited to: an siRNA, an miRNA, an shRNA, and a ribozyme.

As used herein, the terms “siRNA” or “short interfering RNA” refer to a short polynucleotide sequence that mediates a process of sequence-specific post-transcriptional gene silencing, translational inhibition, transcriptional inhibition, or epigenetic RNAi in animals (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Fire *et al.*, 1998, *Nature*, 391, 806; Hamilton *et al.*, 1999, *Science*, 286, 950-951; Lin *et al.*, 1999, *Nature*, 402, 128-129; Sharp, 1999, *Genes & Dev.*, 13, 139-141; and Strauss, 1999, *Science*, 286, 886). In certain embodiments, an siRNA comprises a first strand and a second strand that have the same number of nucleosides; however, the first and second strands are offset such that the two terminal nucleosides on the first and second strands are not paired with a residue on the complimentary strand. In certain instances, the two nucleosides that are not paired are thymidine resides. The siRNA should include a region of sufficient homology to the target gene, and be of sufficient length in terms of nucleotides, such that the siRNA, or a fragment thereof, can mediate down regulation of the target gene. Thus, an siRNA includes a region which is at least partially complementary to the target RNA. It is not necessary that there be perfect complementarity between the siRNA and the target, but the correspondence must be sufficient to enable the siRNA, or a cleavage product thereof, to direct sequence specific silencing, such as by RNAi cleavage of the target RNA. Complementarity, or degree of homology with the target strand, is most critical in the antisense strand. While perfect complementarity, particularly in the antisense strand, is often desired, some embodiments include one or more, but preferably 10, 8, 6, 5, 4, 3, 2, or fewer mismatches with respect to the target RNA. The mismatches are most tolerated in the terminal regions, and if present are preferably in a terminal region or regions, *e.g.,* within 6, 5, 4, or 3 nucleotides of the 5' and/or 3' terminus. The sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double-strand character of the molecule.

In addition, an siRNA may be modified or include nucleoside analogs. Single stranded regions of an siRNA may be modified or include nucleoside analogs, *e.g.,* the unpaired region or regions of a hairpin structure, *e.g.,* a region which links two complementary regions, can have modifications or nucleoside analogs. Modification to stabilize one or more 3'- or 5'-terminus of an siRNA, *e.g.,* against exonucleases, or to favor the antisense siRNA agent to enter into RISC are also useful. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis. Each strand of an siRNA can be equal to or less than 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. The strand is preferably at least 19 nucleotides in length. For example, each strand can be between 21 and 25 nucleotides in length. Preferred siRNAs have a duplex region of 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs, and one or more overhangs of 2-3 nucleotides, preferably one or two 3' overhangs, of 2-3 nucleotides.

As used herein, the terms “miRNA” or “microRNA” s refer to small non-coding RNAs of 20–22 nucleotides, typically excised from ~70 nucleotide foldback RNA precursor structures known as pre-miRNAs. miRNAs negatively regulate their targets in one of two ways depending on the degree of complementarity between the miRNA and the target. First, miRNAs that bind with perfect or nearly perfect complementarity to protein-coding mRNA sequences induce the RNA-mediated interference (RNAi) pathway. miRNAs that exert their regulatory effects by binding to imperfect complementary sites within the 3' untranslated regions (UTRs) of their mRNA targets, repress target-gene expression post-transcriptionally, apparently at the level of translation, through a RISC complex that is similar to, or possibly identical with, the one that is used for the RNAi pathway. Consistent with translational control, miRNAs that use this mechanism reduce the protein levels of their target genes, but the mRNA levels of these genes are only minimally affected. miRNAs encompass both naturally occurring miRNAs as well as artificially designed miRNAs that can specifically target any mRNA sequence. For example, in one embodiment, the skilled artisan can design short hairpin RNA constructs expressed as human miRNA (*e.g.,* miR-30 or miR-21) primary transcripts. This design adds a Drosha processing site to the hairpin construct and has been shown to greatly increase knockdown efficiency (Pusch *et al.*, 2004). The hairpin stem consists of 22-nt of dsRNA (*e.g.,* antisense has perfect complementarity to desired target) and a 15-19-nt loop from a human miR. Adding the miR loop and miR30 flanking sequences on either or both sides of the hairpin results in greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs without microRNA. Increased Drosha and Dicer processing translates into greater siRNA/miRNA production and greater potency for expressed hairpins.

As used herein, the terms “shRNA” or “short hairpin RNA” refer to double-stranded structure that is formed by a single self-complementary RNA strand. shRNA constructs containing a nucleotide sequence identical to a portion, of either coding or non-coding sequence, of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. In certain preferred embodiments, the length of the duplex-forming portion of an shRNA is at least 20, 21 or 22 nucleotides in length, *e.g.,* corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the shRNA construct is at least 25, 50, 100, 200, 300 or 400 bases in length. In certain embodiments, the shRNA construct is 400-800 bases in length. shRNA constructs are highly tolerant of variation in loop sequence and loop size.\

As used herein, the term “ribozyme” refers to a catalytically active RNA molecule capable of site-specific cleavage of target mRNA. Several subtypes have been described, *e.g.,* hammerhead and hairpin ribozymes. Ribozyme catalytic activity and stability can be improved by substituting deoxyribonucleotides for ribonucleotides at noncatalytic bases. While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy particular mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA has the following sequence of two bases: 5′-UG-3′. The construction and production of hammerhead ribozymes is well known in the art.

A preferred method of delivery of a polynucleotide-of-interest that comprises an siRNA, an miRNA, an shRNA, or a ribozyme comprises one or more regulatory sequences, such as, for example, a strong constitutive pol III, *e.g.,* human U6 snRNA promoter, the mouse U6 snRNA promoter, the human and mouse H1 RNA promoter and the human tRNA-val promoter, or a strong constitutive pol II promoter, as described elsewhere herein.

The polynucleotides of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters and/or enhancers, untranslated regions (UTRs), signal sequences, Kozak sequences, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, internal ribosomal entry sites (IRES), recombinase recognition sites (*e.g.,* LoxP, FRT, and Att sites), termination codons, transcriptional termination signals, and polynucleotides encoding self-cleaving polypeptides, epitope tags, as disclosed elsewhere herein or as known in the art, such that their overall length may vary considerably. It is therefore contemplated that a polynucleotide fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

Polynucleotides can be prepared, manipulated and/or expressed using any of a variety of well established techniques known and available in the art. In order to express a desired polypeptide, a nucleotide sequence encoding the polypeptide, can be inserted into appropriate vector. Examples of vectors are plasmid, autonomously replicating sequences, and transposable elements. Additional exemplary vectors include, without limitation, plasmids, phagemids, cosmids, artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses. Examples of categories of animal viruses useful as vectors include, without limitation, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.,* herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.,* SV40). Examples of expression vectors are pClneo vectors (Promega) for expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells. In particular embodiments, he coding sequences of the chimeric proteins disclosed herein can be ligated into such expression vectors for the expression of the chimeric protein in mammalian cells.

In particular embodiments, the vector is an episomal vector or a vector that is maintained extrachromosomally. As used herein, the term "episomal" refers to a vector that is able to replicate without integration into host's chromosomal DNA and without gradual loss from a dividing host cell also meaning that said vector replicates extrachromosomally or episomally. The vector is engineered to harbor the sequence coding for the origin of DNA replication or "ori" from a lymphotrophic herpes virus or a gamma herpesvirus, an adenovirus, SV40, a bovine papilloma virus, or a yeast, specifically a replication origin of a lymphotrophic herpes virus or a gamma herpesvirus corresponding to oriP of EBV. In a particular aspect, the lymphotrophic herpes virus may be Epstein Barr virus (EBV), Kaposi's sarcoma herpes virus (KSHV), Herpes virus saimiri (HS), or Marek's disease virus (MDV). Epstein Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV) are also examples of a gamma herpesvirus. Typically, the host cell comprises the viral replication transactivator protein that activates the replication.

The "control elements" or "regulatory sequences" present in an expression vector are those non-translated regions of the vector—origin of replication, selection cassettes, promoters, enhancers, translation initiation signals (Shine Dalgarno sequence or Kozak sequence) introns, a polyadenylation sequence, 5' and 3' untranslated regions—which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including ubiquitous promoters and inducible promoters may be used.

In particular embodiments, a vector for use in practicing the invention including, but not limited to expression vectors and viral vectors, will include exogenous, endogenous, or heterologous control sequences such as promoters and/or enhancers. An "endogenous" control sequence is one which is naturally linked with a given gene in the genome. An "exogenous" control sequence is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter. A "heterologous" control sequence is an exogenous sequence that is from a different species than the cell being genetically manipulated.

The term "promoter" as used herein refers to a recognition site of a polynucleotide (DNA or RNA) to which an RNA polymerase binds. An RNA polymerase initiates and transcribes polynucleotides operably linked to the promoter. In particular embodiments, promoters operative in mammalian cells comprise an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated and/or another sequence found 70 to 80 bases upstream from the start of transcription, a CNCAAT region where N may be any nucleotide.

The term "enhancer" refers to a segment of DNA which contains sequences capable of providing enhanced transcription and in some instances can function independent of their orientation relative to another control sequence. An enhancer can function cooperatively or additively with promoters and/or other enhancer elements. The term "promoter/enhancer" refers to a segment of DNA which contains sequences capable of providing both promoter and enhancer functions.

The term "operably linked", refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In one embodiment, the term refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, and/or enhancer) and a second polynucleotide sequence, *e.g.,* a polynucleotide-of-interest, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

As used herein, the term "constitutive expression control sequence" refers to a promoter, enhancer, or promoter/enhancer that continually or continuously allows for transcription of an operably linked sequence. A constitutive expression control sequence may be a "ubiquitous" promoter, enhancer, or promoter/enhancer that allows expression in a wide variety of cell and tissue types or a "cell specific," "cell type specific," "cell lineage specific," or "tissue specific" promoter, enhancer, or promoter/enhancer that allows expression in a restricted variety of cell and tissue types, respectively.

Illustrative ubiquitous expression control sequences suitable for use in particular embodiments of the invention include, but are not limited to, a cytomegalovirus (CMV) immediate early promoter, a viral simian virus 40 (SV40) (*e.g.,* early or late), a Moloney murine leukemia virus (MoMLV) LTR promoter, a Rous sarcoma virus (RSV) LTR, a herpes simplex virus (HSV) (thymidine kinase) promoter, H5, P7.5, and P11 promoters from vaccinia virus, an elongation factor 1-alpha (EF1a) promoter, early growth response 1 (EGR1), ferritin H (FerH), ferritin L (FerL), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), eukaryotic translation initiation factor 4A1 (EIF4A1), heat shock 70kDa protein 5 (HSPA5), heat shock protein 90kDa beta, member 1 (HSP90B1), heat shock protein 70kDa (HSP70), β-kinesin (β-KIN), the human ROSA 26 locus (Irions *et al.*, *Nature Biotechnology* 25, 1477 - 1482 (2007)), a Ubiquitin C promoter (UBC), a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β-actin (CAG) promoter, a β-actin promoter and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter (Challita *et al.*, *J Virol.* 69(2):748-55 (1995)).

In one embodiment, a vector of the invention comprises a MND promoter.

In one embodiment, a vector of the invention comprises an EF1a promoter comprising the first intron of the human EF1a gene.

In one embodiment, a vector of the invention comprises an EF1a promoter that lacks the first intron of the human EF1a gene.

In a particular embodiment, it may be desirable to express a polynucleotide comprising a CAR from a T cell specific promoter.

As used herein, "conditional expression" may refer to any type of conditional expression including, but not limited to, inducible expression; repressible expression; expression in cells or tissues having a particular physiological, biological, or disease state, *etc.* This definition is not intended to exclude cell type or tissue specific expression. Certain embodiments of the invention provide conditional expression of a polynucleotide-of-interest*, e.g.,* expression is controlled by subjecting a cell, tissue, organism, *etc.*, to a treatment or condition that causes the polynucleotide to be expressed or that causes an increase or decrease in expression of the polynucleotide encoded by the polynucleotide-of-interest.

Illustrative examples of inducible promoters/systems include, but are not limited to, steroid-inducible promoters such as promoters for genes encoding glucocorticoid or estrogen receptors (inducible by treatment with the corresponding hormone), metallothionine promoter (inducible by treatment with various heavy metals), MX-1 promoter (inducible by interferon), the "GeneSwitch" mifepristone-regulatable system (Sirin *et al.*, 2003, *Gene*, 323:67), the cumate inducible gene switch (WO 2002/088346), tetracycline-dependent regulatory systems, *etc.*

Conditional expression can also be achieved by using a site specific DNA recombinase. According to certain embodiments of the invention the vector comprises at least one (typically two) site(s) for recombination mediated by a site specific recombinase. As used herein, the terms "recombinase" or "site specific recombinase" include excisive or integrative proteins, enzymes, co-factors or associated proteins that are involved in recombination reactions involving one or more recombination sites (*e.g.,* two, three, four, five, seven, ten, twelve, fifteen, twenty, thirty, fifty, *etc.*), which may be wild-type proteins (see Landy, Current Opinion in Biotechnology 3:699-707 (1993)), or mutants, derivatives (*e.g.,* fusion proteins containing the recombination protein sequences or fragments thereof), fragments, and variants thereof. Illustrative examples of recombinases suitable for use in particular embodiments of the present invention include, but are not limited to: Cre, Int, IHF, Xis, Flp, Fis, Hin, Gin, ΦC31, Cin, Tn3 resolvase, TndX, XerC, XerD, TnpX, Hjc, Gin, SpCCE1, and ParA.

The vectors may comprise one or more recombination sites for any of a wide variety of site specific recombinases. It is to be understood that the target site for a site specific recombinase is in addition to any site(s) required for integration of a vector, *e.g.,* a retroviral vector or lentiviral vector. As used herein, the terms "recombination sequence," "recombination site," or "site specific recombination site" refer to a particular nucleic acid sequence to which a recombinase recognizes and binds.

For example, one recombination site for Cre recombinase is loxP which is a 34 base pair sequence comprising two 13 base pair inverted repeats (serving as the recombinase binding sites) flanking an 8 base pair core sequence (see FIG. 1 of Sauer, B., *Current Opinion in Biotechnology* 5:521-527 (1994)). Other exemplary loxP sites include, but are not limited to: lox511 (Hoess *et al.*, 1996; Bethke and Sauer, 1997), lox5171 (Lee and Saito, 1998), lox2272 (Lee and Saito, 1998), m2 (Langer *et al.*, 2002), lox71 (Albert *et al.*, 1995), and lox66 (Albert *et al.*, 1995).

Suitable recognition sites for the FLP recombinase include, but are not limited to: FRT (McLeod, *et al.*, 1996), $F_1$, $F_2$, $F_3$ (Schlake and Bode, 1994), $F_4$, $F_5$ (Schlake and Bode, 1994), FRT(LE) (Senecoff *et al.*, 1988), FRT(RE) (Senecoff *et al.*, 1988).

Other examples of recognition sequences are the attB, attP, attL, and attR sequences, which are recognized by the recombinase enzyme λ Integrase, *e.g.,* phi-c31. The φC31 SSR mediates recombination only between the heterotypic sites attB (34 bp in length) and attP (39 bp in length) (Groth *et al.*, 2000). attB and attP, named for the attachment sites for the phage integrase on the bacterial and phage genomes, respectively, both contain imperfect inverted repeats that are likely bound by φC31 homodimers (Groth *et al.*, 2000). The product sites, attL and attR, are effectively inert to further φC31-mediated recombination (Belteki *et al.*, 2003), making the reaction irreversible. For catalyzing insertions, it has been found that attB-bearing DNA inserts into a genomic attP site more readily than an attP site into a genomic attB site (Thyagarajan *et al.*, 2001; Belteki *et al.*, 2003). Thus, typical strategies position by homologous recombination an attP-bearing "docking site" into a defined locus, which is then partnered with an attB-bearing incoming sequence for insertion.

As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. *See, e.g.,* Jackson *et al.*, 1990. *Trends Biochem Sci* 15(12):477-83) and Jackson and Kaminski. 1995. *RNA* 1(10):985-1000. In particular embodiments, the vectors contemplated by the invention, include one or more polynucleotides-of-interest that encode one or more polypeptides. In particular embodiments, to achieve efficient translation of each of the plurality of polypeptides, the polynucleotide sequences can be separated by one or more IRES sequences or polynucleotide sequences encoding self-cleaving polypeptides.

As used herein, the term "Kozak sequence" refers to a short nucleotide sequence that greatly facilitates the initial binding of mRNA to the small subunit of the ribosome and increases translation. The consensus Kozak sequence is (GCC)RCCATGG, where R is a purine (A or G) (Kozak, 1986. *Cell.* 44(2):283-92, and Kozak, 1987. *Nucleic Acids Res.* 15(20):8125-48). In particular embodiments, the vectors contemplated by the invention, comprise polynucleotides that have a consensus Kozak sequence and that encode a desired polypeptide, *e.g.,* a CAR.

In some embodiments of the invention, a polynucleotide or cell harboring the polynucleotide utilizes a suicide gene, including an inducible suicide gene to reduce the risk of direct toxicity and/or uncontrolled proliferation. In specific aspects, the suicide gene is not immunogenic to the host harboring the polynucleotide or cell. A certain example of a suicide gene that may be used is caspase-9 or caspase-8 or cytosine deaminase. Caspase-9 can be activated using a specific chemical inducer of dimerization (CID).

In certain embodiments, vectors comprise gene segments that cause the immune effector cells of the invention, *e.g.,* T cells, to be susceptible to negative selection *in vivo*. By "negative selection" is meant that the infused cell can be eliminated as a result of a change in the *in vivo* condition of the individual. The negative selectable phenotype may result from the insertion of a gene that confers sensitivity to an administered agent, for example, a compound. Negative selectable genes are known in the art, and include, inter alia the following: the Herpes simplex virus type I thymidine kinase (HSV-I TK) gene (Wigler *et al.*, Cell 11:223, 1977) which confers ganciclovir sensitivity; the cellular hypoxanthine phosphribosyltransferase (HPRT) gene, the cellular adenine phosphoribosyltransferase (APRT) gene, and bacterial cytosine deaminase, (Mullen *et al.*, Proc. Natl. Acad. Sci. USA. 89:33 (1992)).

In some embodiments, genetically modified immune effector cells, such as T cells, comprise a polynucleotide further comprising a positive marker that enables the selection of cells of the negative selectable phenotype *in vitro*. The positive selectable marker may be a gene which, upon being introduced into the host cell expresses a dominant phenotype permitting positive selection of cells carrying the gene. Genes of this type are known in the art, and include, inter alia, hygromycin-B phosphotransferase gene (hph) which confers resistance to hygromycin B, the amino glycoside phosphotransferase gene (neo or aph) from Tn5 which codes for resistance to the antibiotic G418, the dihydrofolate reductase (DHFR) gene, the adenosine deaminase gene (ADA), and the multi-drug resistance (MDR) gene.

Preferably, the positive selectable marker and the negative selectable element are linked such that loss of the negative selectable element necessarily also is accompanied by loss of the positive selectable marker. Even more preferably, the positive and negative selectable markers are fused so that loss of one obligatorily leads to loss of the other. An example of a fused polynucleotide that yields as an expression product a polypeptide that confers both the desired positive and negative selection features described above is a hygromycin phosphotransferase thymidine kinase fusion gene (HyTK). Expression of this gene yields a polypeptide that confers hygromycin B resistance for positive selection *in vitro*, and ganciclovir sensitivity for negative selection *in vivo*. *See* Lupton S. D., et al, Mol. and Cell. Biology 1 1:3374- 3378, 1991. In addition, in preferred embodiments, the polynucleotides of the invention encoding the chimeric receptors are in retroviral vectors containing the fused gene, particularly those that confer hygromycin B resistance for positive selection *in vitro*, and ganciclovir sensitivity for negative selection *in vivo*, for example the HyTK retroviral vector described in Lupton, S. D. *et al.* (1991), supra. See also the publications of PCT US91/08442 and PCT/US94/05601, by S. D. Lupton, describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable markers with negative selectable markers.

Preferred positive selectable markers are derived from genes selected from the group consisting of hph, nco, and gpt, and preferred negative selectable markers are derived from genes selected from the group consisting of cytosine deaminase, HSV-I TK, VZV TK, HPRT, APRT and gpt. Especially preferred markers are bifunctional selectable fusion genes wherein the positive selectable marker is derived from hph or neo, and the negative selectable marker is derived from cytosine deaminase or a TK gene or selectable marker. Inducible Suicide Genes

## F. VIRAL VECTORS

In particular embodiments, a cell (*e.g.,* an immune effector cell) is transduced with a retroviral vector, *e.g.,* a lentiviral vector, encoding a CAR. For example, an immune effector cell is transduced with a vector encoding a CAR that comprises a humanized anti-BCMA antibody or antigen binding fragment thereof that binds a BCMA polypeptide, with an intracellular signaling domain of CD3ζ, CD28, 4-1BB, Ox40, or any combinations thereof. Thus, these transduced cells can elicit a CAR-mediated cytotoxic response.

Retroviruses are a common tool for gene delivery (Miller, 2000, *Nature*. 357: 455-460). In particular embodiments, a retrovirus is used to deliver a polynucleotide encoding a chimeric antigen receptor (CAR) to a cell. As used herein, the term "retrovirus" refers to an RNA virus that reverse transcribes its genomic RNA into a linear double-stranded DNA copy and subsequently covalently integrates its genomic DNA into a host genome. Once the virus is integrated into the host genome, it is referred to as a "provirus." The provirus serves as a template for RNA polymerase II and directs the expression of RNA molecules which encode the structural proteins and enzymes needed to produce new viral particles.

Illustrative retroviruses suitable for use in particular embodiments, include, but are not limited to: Moloney murine leukemia virus (M-MuLV), Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), spumavirus, Friend murine leukemia virus, Murine Stem Cell Virus (MSCV) and Rous Sarcoma Virus (RSV)) and lentivirus.

As used herein, the term “lentivirus” refers to a group (or genus) of complex retroviruses. Illustrative lentiviruses include, but are not limited to: HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2); visna-maedi virus (VMV) virus; the caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV). In one embodiment, HIV based vector backbones (*i.e.*, HIV cis-acting sequence elements) are preferred. In particular embodiments, a lentivirus is used to deliver a polynucleotide comprising a CAR to a cell.

Retroviral vectors and more particularly lentiviral vectors may be used in practicing particular embodiments of the present invention. Accordingly, the term “retrovirus” or “retroviral vector”, as used herein is meant to include “lentivirus” and “lentiviral vectors” respectively.

The term “vector” is used herein to refer to a nucleic acid molecule capable transferring or transporting another nucleic acid molecule. The transferred nucleic acid is generally linked to, *e.g.,* inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication in a cell, or may include sequences sufficient to allow integration into host cell DNA. Useful vectors include, for example, plasmids (*e.g.,* DNA plasmids or RNA plasmids), transposons, cosmids, bacterial artificial chromosomes, and viral vectors. Useful viral vectors include, *e.g.,* replication defective retroviruses and lentiviruses.

As will be evident to one of skill in the art, the term “viral vector” is widely used to refer either to a nucleic acid molecule (*e.g.,* a transfer plasmid) that includes virus-derived nucleic acid elements that typically facilitate transfer of the nucleic acid molecule or integration into the genome of a cell or to a viral particle that mediates nucleic acid transfer. Viral particles will typically include various viral components and sometimes also host cell components in addition to nucleic acid(s).

The term viral vector may refer either to a virus or viral particle capable of transferring a nucleic acid into a cell or to the transferred nucleic acid itself. Viral vectors and transfer plasmids contain structural and/or functional genetic elements that are primarily derived from a virus. The term “retroviral vector” refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, that are primarily derived from a retrovirus. The term “lentiviral vector” refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, including LTRs that are primarily derived from a lentivirus. The term “hybrid vector” refers to a vector, LTR or other nucleic acid containing both retroviral, *e.g.,* lentiviral, sequences and non-lentiviral viral sequences. In one embodiment, a hybrid vector refers to a vector or transfer plasmid comprising retroviral *e.g.,* lentiviral, sequences for reverse transcription, replication, integration and/or packaging.

In particular embodiments, the terms “lentiviral vector,” “lentiviral expression vector” may be used to refer to lentiviral transfer plasmids and/or infectious lentiviral particles. Where reference is made herein to elements such as cloning sites, promoters, regulatory elements, heterologous nucleic acids, *etc.*, it is to be understood that the sequences of these elements are present in RNA form in the lentiviral particles of the invention and are present in DNA form in the DNA plasmids of the invention.

At each end of the provirus are structures called “long terminal repeats” or “LTRs.” The term “long terminal repeat (LTR)” refers to domains of base pairs located at the ends of retroviral DNAs which, in their natural sequence context, are direct repeats and contain U3, R and U5 regions. LTRs generally provide functions fundamental to the expression of retroviral genes (*e.g.,* promotion, initiation and polyadenylation of gene transcripts) and to viral replication. The LTR contains numerous regulatory signals including transcriptional control elements, polyadenylation signals and sequences needed for replication and integration of the viral genome. The viral LTR is divided into three regions called U3, R and U5. The U3 region contains the enhancer and promoter elements. The U5 region is the sequence between the primer binding site and the R region and contains the polyadenylation sequence. The R (repeat) region is flanked by the U3 and U5 regions. The LTR composed of U3, R and U5 regions and appears at both the 5' and 3' ends of the viral genome. Adjacent to the

5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient packaging of viral RNA into particles (the Psi site).

As used herein, the term “packaging signal” or “packaging sequence” refers to sequences located within the retroviral genome which are required for insertion of the viral RNA into the viral capsid or particle, see *e.g.,* Clever *et al.,* 1995. *J. of Virology*, Vol. 69, No. 4; pp. 2101–2109. Several retroviral vectors use the minimal packaging signal (also referred to as the psi [Ψ] sequence) needed for encapsidation of the viral genome. Thus, as used herein, the terms “packaging sequence,” “packaging signal,” “psi” and the symbol “Ψ,” are used in reference to the non-coding sequence required for encapsidation of retroviral RNA strands during viral particle formation.

In various embodiments, vectors comprise modified 5' LTR and/or 3' LTRs. Either or both of the LTR may comprise one or more modifications including, but not limited to, one or more deletions, insertions, or substitutions. Modifications of the 3' LTR are often made to improve the safety of lentiviral or retroviral systems by rendering viruses replication-defective. As used herein, the term “replication-defective” refers to virus that is not capable of complete, effective replication such that infective virions are not produced (*e.g.,* replication-defective lentiviral progeny). The term “replication-competent” refers to wild-type virus or mutant virus that is capable of replication, such that viral replication of the virus is capable of producing infective virions (*e.g.,* replication-competent lentiviral progeny).

“Self-inactivating” (SIN) vectors refers to replication-defective vectors, *e.g.,* retroviral or lentiviral vectors, in which the right (3') LTR enhancer-promoter region, known as the U3 region, has been modified (*e.g.,* by deletion or substitution) to prevent viral transcription beyond the first round of viral replication. This is because the right (3') LTR U3 region is used as a template for the left (5') LTR U3 region during viral replication and, thus, the viral transcript cannot be made without the U3 enhancer-promoter. In a further embodiment of the invention, the 3' LTR is modified such that the U5 region is replaced, for example, with an ideal poly(A) sequence. It should be noted that modifications to the LTRs such as modifications to the 3' LTR, the 5' LTR, or both 3' and 5' LTRs, are also included in the invention.

An additional safety enhancement is provided by replacing the U3 region of the 5' LTR with a heterologous promoter to drive transcription of the viral genome during production of viral particles. Examples of heterologous promoters which can be used include, for example, viral simian virus 40 (SV40) (*e.g.,* early or late), cytomegalovirus (CMV) (*e.g.,* immediate early), Moloney murine leukemia virus (MoMLV), Rous sarcoma virus (RSV), and herpes simplex virus (HSV) (thymidine kinase) promoters. Typical promoters are able to drive high levels of transcription in a Tat-independent manner. This replacement reduces the possibility of recombination to generate replication-competent virus because there is no complete U3 sequence in the virus production system. In certain embodiments, the heterologous promoter has additional advantages in controlling the manner in which the viral genome is transcribed. For example, the heterologous promoter can be inducible, such that transcription of all or part of the viral genome will occur only when the induction factors are present. Induction factors include, but are not limited to, one or more chemical compounds or the physiological conditions such as temperature or pH, in which the host cells are cultured.

In some embodiments, viral vectors comprise a TAR element. The term “TAR” refers to the “trans-activation response” genetic element located in the R region of lentiviral (*e.g.,* HIV) LTRs. This element interacts with the lentiviral trans-activator (tat) genetic element to enhance viral replication. However, this element is not required in embodiments wherein the U3 region of the 5' LTR is replaced by a heterologous promoter.

The “R region” refers to the region within retroviral LTRs beginning at the start of the capping group (*i.e.*, the start of transcription) and ending immediately prior to the start of the poly A tract. The R region is also defined as being flanked by the U3 and U5 regions. The R region plays a role during reverse transcription in permitting the transfer of nascent DNA from one end of the genome to the other.

As used herein, the term “FLAP element” refers to a nucleic acid whose sequence includes the central polypurine tract and central termination sequences (cPPT and CTS) of a retrovirus, *e.g.,* HIV-1 or HIV-2. Suitable FLAP elements are described in U.S. Pat. No. 6,682,907 and in Zennou, *et al.*, 2000, *Cell*, 101:173. During HIV-1 reverse transcription, central initiation of the plus-strand DNA at the central polypurine tract (cPPT) and central termination at the central termination sequence (CTS) lead to the formation of a three-stranded DNA structure: the HIV-1 central DNA flap. While not wishing to be bound by any theory, the DNA flap may act as a cis-active determinant of lentiviral genome nuclear import and/or may increase the titer of the virus. In particular embodiments, the retroviral or lentiviral vector backbones comprise one or more FLAP elements upstream or downstream of the heterologous genes of interest in the vectors. For example, in particular embodiments a transfer plasmid includes a FLAP element. In one embodiment, a vector of the invention comprises a FLAP element isolated from HIV-1.

In one embodiment, retroviral or lentiviral transfer vectors comprise one or more export elements. The term "export element" refers to a cis-acting post-transcriptional regulatory element which regulates the transport of an RNA transcript from the nucleus to the cytoplasm of a cell. Examples of RNA export elements include, but are not limited to, the human immunodeficiency virus (HIV) rev response element (RRE) (*see e.g.,* Cullen *et al.*, 1991. *J. Virol.* 65: 1053; and Cullen *et al.*, 1991. *Cell* 58: 423), and the hepatitis B virus post-transcriptional regulatory element (HPRE). Generally, the RNA export element is placed within the 3' UTR of a gene, and can be inserted as one or multiple copies.

In particular embodiments, expression of heterologous sequences in viral vectors is increased by incorporating posttranscriptional regulatory elements, efficient polyadenylation sites, and optionally, transcription termination signals into the vectors. A variety of posttranscriptional regulatory elements can increase expression of a heterologous nucleic acid at the protein, *e.g.,* woodchuck hepatitis virus posttranscriptional regulatory element (WPRE; Zufferey *et al.*, 1999, *J. Virol.*, 73:2886); the posttranscriptional regulatory element present in hepatitis B virus (HPRE) (Huang *et al., Mol. Cell. Biol.*, 5:3864); and the like (Liu *et al.,* 1995, *Genes Dev.*, 9:1766). In particular embodiments, vectors of the invention comprise a posttranscriptional regulatory element such as a WPRE or HPRE

In particular embodiments, vectors of the invention lack or do not comprise a posttranscriptional regulatory element such as a WPRE or HPRE because in some instances these elements increase the risk of cellular transformation and/or do not substantially or significantly increase the amount of mRNA transcript or increase mRNA stability. Therefore, in some embodiments, vectors of the invention lack or do not comprise a WPRE or HPRE as an added safety measure.

Elements directing the efficient termination and polyadenylation of the heterologous nucleic acid transcripts increases heterologous gene expression.

Transcription termination signals are generally found downstream of the polyadenylation signal. In particular embodiments, vectors comprise a polyadenylation sequence 3′ of a polynucleotide encoding a polypeptide to be expressed. The term “polyA site” or “polyA sequence” as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript by RNA polymerase II. Polyadenylation sequences can promote mRNA stability by addition of a polyA tail to the 3′ end of the coding sequence and thus, contribute to increased translational efficiency. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. Illustrative examples of polyA signals that can be used in a vector of the invention, includes an ideal polyA sequence (*e.g.,* AATAAA, ATTAAA, AGTAAA), a bovine growth hormone polyA sequence (BGHpA), a rabbit β-globin polyA sequence (rβgpA), or another suitable heterologous or endogenous polyA sequence known in the art.

In certain embodiments, a retroviral or lentiviral vector further comprises one or more insulator elements. Insulators elements may contribute to protecting lentivirus-expressed sequences, *e.g.,* therapeutic polypeptides, from integration site effects, which may be mediated by cis-acting elements present in genomic DNA and lead to deregulated expression of transferred sequences (*i.e.,* position effect; *see, e.g.,* Burgess-Beusse *et al.*, 2002, *Proc. Natl. Acad. Sci., USA*, 99:16433; and Zhan *et al.*, 2001, *Hum. Genet.*, 109:471).. In some embodiments, transfer vectors comprise one or more insulator element the 3′ LTR and upon integration of the provirus into the host genome, the provirus comprises the one or more insulators at both the 5′ LTR or 3′ LTR, by virtue of duplicating the 3′ LTR. Suitable insulators for use in the invention include, but are not limited to, the chicken β-globin insulator (see Chung *et al.*, 1993. Cell 74:505; Chung *et al.*, 1997. PNAS 94:575; and Bell *et al.*, 1999. Cell 98:387, incorporated by reference herein). Examples of insulator elements include, but are not limited to, an insulator from an β-globin locus, such as chicken HS4.

According to certain specific embodiments of the invention, most or all of the viral vector backbone sequences are derived from a lentivirus, *e.g.*, HIV-1. However, it is to be understood that many different sources of retroviral and/or lentiviral sequences can be used, or combined and numerous substitutions and alterations in certain of the lentiviral sequences may be accommodated without impairing the ability of a transfer vector to perform the functions described herein. Moreover, a variety of lentiviral vectors are known in the art, *see* Naldini *et al.*, (1996a, 1996b, and 1998); Zufferey *et al.*, (1997); Dull *et al.*, 1998, U.S. Pat. Nos. 6,013,516; and 5,994,136, many of which may be adapted to produce a viral vector or transfer plasmid of the present invention.

In various embodiments, the vectors of the invention comprise a promoter operably linked to a polynucleotide encoding a CAR polypeptide. The vectors may have one or more LTRs, wherein either LTR comprises one or more modifications, such as one or more nucleotide substitutions, additions, or deletions. The vectors may further comprise one of more accessory elements to increase transduction efficiency (*e.g.,* a cPPT/FLAP), viral packaging (*e.g.,* a Psi (Ψ) packaging signal, RRE), and/or other elements that increase therapeutic gene expression (*e.g.,* poly (A) sequences), and may optionally comprise a WPRE or HPRE.

In a particular embodiment, the transfer vector of the invention comprises a left (5') retroviral LTR; a central polypurine tract/DNA flap (cPPT/FLAP); a retroviral export element; a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; and a right (3') retroviral LTR; and optionally a WPRE or HPRE.

In a particular embodiment, the transfer vector of the invention comprises a left (5') retroviral LTR; a retroviral export element; a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; a right (3') retroviral LTR; and a poly (A) sequence; and optionally a WPRE or HPRE. In another particular embodiment, the invention provides a lentiviral vector comprising: a left (5') LTR; a cPPT/FLAP; an RRE; a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; a right (3') LTR; and a polyadenylation sequence; and optionally a WPRE or HPRE.

In a certain embodiment, the invention provides a lentiviral vector comprising: a left (5') HIV-1 LTR; a Psi (Ψ) packaging signal; a cPPT/FLAP; an RRE; a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; a right (3') self-inactivating (SIN) HIV-1 LTR; and a rabbit β-globin polyadenylation sequence; and optionally a WPRE or HPRE.

In another embodiment, the invention provides a vector comprising: at least one LTR; a central polypurine tract/DNA flap (cPPT/FLAP); a retroviral export element; and a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; and optionally a WPRE or HPRE.

In particular embodiment, the present invention provides a vector comprising at least one LTR; a cPPT/FLAP; an RRE; a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; and a polyadenylation sequence; and optionally a WPRE or HPRE.

In a certain embodiment, the present invention provides at least one SIN HIV-1 LTR; a Psi (Ψ) packaging signal; a cPPT/FLAP; an RRE; a promoter active in a T cell, operably linked to a polynucleotide encoding CAR polypeptide contemplated herein; and a rabbit β-globin polyadenylation sequence; and optionally a WPRE or HPRE.

A "host cell" includes cells electroporated, transfected, infected, or transduced *in vivo, ex vivo,* or *in vitro* with a recombinant vector or a polynucleotide of the invention. Host cells may include packaging cells, producer cells, and cells infected with viral vectors. In particular embodiments, host cells infected with viral vector of the invention are administered to a subject in need of therapy. In certain embodiments, the term "target cell" is used interchangeably with host cell and refers to transfected, infected, or transduced cells of a desired cell type. In preferred embodiments, the target cell is a T cell.

Large scale viral particle production is often necessary to achieve a reasonable viral titer. Viral particles are produced by transfecting a transfer vector into a packaging cell line that comprises viral structural and/or accessory genes, *e.g.,* gag, pol, env, tat, rev, vif, vpr, vpu, vpx, or nef genes or other retroviral genes.

As used herein, the term "packaging vector" refers to an expression vector or viral vector that lacks a packaging signal and comprises a polynucleotide encoding one, two, three, four or more viral structural and/or accessory genes. Typically, the packaging vectors are included in a packaging cell, and are introduced into the cell via transfection, transduction or infection. Methods for transfection, transduction or infection are well known by those of skill in the art. A retroviral/lentiviral transfer vector of the present invention can be introduced into a packaging cell line, via transfection, transduction or infection, to generate a producer cell or cell line. The packaging vectors of the present invention can be introduced into human cells or cell lines by standard methods including, *e.g.,* calcium phosphate transfection, lipofection or electroporation. In some embodiments, the packaging vectors are introduced into the cells together with a dominant selectable marker, such as neomycin, hygromycin, puromycin, blastocidin, zeocin, thymidine kinase, DHFR, Gln synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. A selectable marker gene can be linked physically to genes encoding by the packaging vector, *e.g.,* by IRES or self cleaving viral peptides.

Viral envelope proteins (env) determine the range of host cells which can ultimately be infected and transformed by recombinant retroviruses generated from the cell lines. In the case of lentiviruses, such as HIV-1, HIV-2, SIV, FIV and EIV, the env proteins include gp41 and gp120. Preferably, the viral env proteins expressed by packaging cells of the invention are encoded on a separate vector from the viral gag and pol genes, as has been previously described.

Illustrative examples of retroviral-derived env genes which can be employed in the invention include, but are not limited to: MLV envelopes, 10A1 envelope, BAEV, FeLV-B, RD114, SSAV, Ebola, Sendai, FPV (Fowl plague virus), and influenza virus envelopes. Similarly, genes encoding envelopes from RNA viruses (*e.g.,* RNA virus families of Picornaviridae, Calciviridae, Astroviridae, Togaviridae, Flaviviridae, Coronaviridae, Paramyxoviridae, Rhabdoviridae, Filoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Reoviridae, Birnaviridae, Retroviridae) as well as from the DNA viruses (families of Hepadnaviridae, Circoviridae, Parvoviridae, Papovaviridae, Adenoviridae, Herpesviridae, Poxyiridae, and Iridoviridae) may be utilized. Representative examples include , FeLV, VEE, HFVW, WDSV, SFV, Rabies, ALV, BIV, BLV, EBV, CAEV, SNV, ChTLV, STLV, MPMV, SMRV, RAV, FuSV, MH2, AEV, AMV, CT10, and EIAV.

In other embodiments, envelope proteins for pseudotyping a virus of present invention include, but are not limited to any of the following virus: Influenza A such as H1N1, H1N2, H3N2 and H5N1 (bird flu), Influenza B, Influenza C virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, Rotavirus, any virus of the Norwalk virus group, enteric adenoviruses, parvovirus, Dengue fever virus, Monkey pox, Mononegavirales, Lyssavirus such as rabies virus, Lagos bat virus, Mokola virus, Duvenhage virus, European bat virus 1 & 2 and Australian bat virus, Ephemerovirus, Vesiculovirus, Vesicular Stomatitis Virus (VSV), Herpesviruses such as Herpes simplex virus types 1 and 2, varicella zoster, cytomegalovirus, Epstein-Bar virus (EBV), human herpesviruses (HHV), human herpesvirus type 6 and 8, Human immunodeficiency virus (HIV), papilloma virus, murine gammaherpesvirus, Arenaviruses such as Argentine hemorrhagic fever virus, Bolivian hemorrhagic fever virus, Sabia-associated hemorrhagic fever virus, Venezuelan hemorrhagic fever virus, Lassa fever virus, Machupo virus, Lymphocytic choriomeningitis virus (LCMV), Bunyaviridiae such as Crimean-Congo hemorrhagic fever virus, Hantavirus, hemorrhagic fever with renal syndrome causing virus, Rift Valley fever virus, Filoviridae (filovirus) including Ebola hemorrhagic fever and Marburg hemorrhagic fever, Flaviviridae including Kaysanur Forest disease virus, Omsk hemorrhagic fever virus, Tick-borne encephalitis causing virus and Paramyxoviridae such as Hendra virus and Nipah virus, variola major and variola minor (smallpox), alphaviruses such as Venezuelan equine encephalitis virus, eastern equine encephalitis virus, western equine encephalitis virus, SARS-associated coronavirus (SARS-CoV), West Nile virus, any encephaliltis causing virus.

In one embodiment, the invention provides packaging cells which produce recombinant retrovirus, *e.g.,* lentivirus, pseudotyped with the VSV-G glycoprotein.

The terms “pseudotype” or “pseudotyping” as used herein, refer to a virus whose viral envelope proteins have been substituted with those of another virus possessing preferable characteristics. For example, HIV can be pseudotyped with vesicular stomatitis virus G-protein (VSV-G) envelope proteins, which allows HIV to infect a wider range of cells because HIV envelope proteins (encoded by the env gene) normally target the virus to CD4+ presenting cells. In a preferred embodiment of the invention, lentiviral envelope proteins are pseudotyped with VSV-G. In one embodiment, the invention provides packaging cells which produce recombinant retrovirus, *e.g.,* lentivirus, pseudotyped with the VSV-G envelope glycoprotein.

As used herein, the term “packaging cell lines” is used in reference to cell lines that do not contain a packaging signal, but do stably or transiently express viral structural proteins and replication enzymes (*e.g.,* gag, pol and env) which are necessary for the correct packaging of viral particles. Any suitable cell line can be employed to prepare packaging cells of the invention. Generally, the cells are mammalian cells. In a particular embodiment, the cells used to produce the packaging cell line are human cells. Suitable cell lines which can be used include, for example, CHO cells, BHK cells, MDCK cells, C3H 10T1/2 cells, FLY cells, Psi-2 cells, BOSC 23 cells, PA317 cells, WEHI cells, COS cells, BSC 1 cells, BSC 40 cells, BMT 10 cells, VERO cells, W138 cells, MRC5 cells, A549 cells, HT1080 cells, 293 cells, 293T cells, B-50 cells, 3T3 cells, NIH3T3 cells, HepG2 cells, Saos-2 cells, Huh7 cells, HeLa cells, W163 cells, 211 cells, and 211A cells. In preferred embodiments, the packaging cells are 293 cells, 293T cells, or A549 cells. In another preferred embodiment, the cells are A549 cells.

As used herein, the term “producer cell line” refers to a cell line which is capable of producing recombinant retroviral particles, comprising a packaging cell line and a transfer vector construct comprising a packaging signal. The production of infectious viral particles and viral stock solutions may be carried out using conventional techniques. Methods of preparing viral stock solutions are known in the art and are illustrated by, *e.g.,* Y. Soneoka *et al.* (1995) *Nucl. Acids Res.* 23:628-633, and N. R. Landau *et al.* (1992) *J. Virol.* 66:5110-5113. Infectious virus particles may be collected from the packaging cells using conventional techniques. For example, the infectious particles can be collected by cell lysis, or collection of the supernatant of the cell culture, as is known in the art. Optionally, the collected virus particles may be purified if desired. Suitable purification techniques are well known to those skilled in the art.

The delivery of a gene(s) or other polynucleotide sequence using a retroviral or lentiviral vector by means of viral infection rather than by transfection is referred to as “transduction.” In one embodiment, retroviral vectors are transduced into a cell through infection and provirus integration. In certain embodiments, a target cell, *e.g.,* a T cell, is “transduced” if it comprises a gene or other polynucleotide sequence delivered to the cell by infection using a viral or retroviral vector. In particular embodiments, a transduced cell comprises one or more genes or other polynucleotide sequences delivered by a retroviral or lentiviral vector in its cellular genome.

In particular embodiments, host cells transduced with viral vector of the invention that expresses one or more polypeptides, are administered to a subject to treat and/or prevent a B cell malignancy. Other methods relating to the use of viral vectors in gene therapy, which may be utilized according to certain embodiments of the present invention, can be found in, *e.g.,* Kay, M. A. (1997) *Chest* 111(6 Supp.):138S-142S; Ferry, N. and Heard, J. M. (1998) *Hum. Gene Ther.* 9:1975-81; Shiratory, Y. *et al.* (1999) Liver 19:265-74; Oka, K. *et al.* (2000) *Curr. Opin. Lipidol.* 11:179-86; Thule, P. M. and Liu, J. M. (2000) *Gene Ther.* 7:1744-52; Yang, N. S. (1992) *Crit. Rev. Biotechnol.* 12:335-56; Alt, M. (1995) *J. Hepatol.* 23:746-58; Brody, S. L. and Crystal, R. G. (1994) *Ann. N.Y. Acad. Sci.* 716:90-101; Strayer, D. S. (1999) *Expert Opin. Investig. Drugs* 8:2159-2172; Smith-Arica, J. R. and Bartlett, J. S. (2001) *Curr. Cardiol. Rep.* 3:43-49; and Lee, H. C. *et al.* (2000) *Nature* 408:483-8.

### G. GENETICALLY MODIFIED CELLS

The present invention contemplates, in particular embodiments, cells genetically modified to express the CARs contemplated herein, for use in the treatment of B cell related conditions. As used herein, the term “genetically engineered” or “genetically modified” refers to the addition of extra genetic material in the form of DNA or RNA into the total genetic material in a cell. The terms, “genetically modified cells,” “modified cells,” and, “redirected cells,” are used interchangeably. As used herein, the term “gene therapy” refers to the introduction of extra genetic material in the form of DNA or RNA into the total genetic material in a cell that restores, corrects, or modifies expression of a gene, or for the purpose of expressing a therapeutic polypeptide, *e.g.,* a CAR.

In particular embodiments, the CARs contemplated herein are introduced and expressed in immune effector cells so as to redirect their specificity to a target antigen of interest, *e.g.,* a BCMA polypeptide. An “immune effector cell,” is any cell of the immune system that has one or more effector functions (*e.g.,* cytotoxic cell killing activity, secretion of cytokines, induction of ADCC and/or CDC).

Immune effector cells of the invention can be autologous/autogeneic (“self”) or non-autologous (“non-self,” *e.g.,* allogeneic, syngeneic or xenogeneic).

“Autologous,” as used herein, refers to cells from the same subject.

“Allogeneic,” as used herein, refers to cells of the same species that differ genetically to the cell in comparison.

“Syngeneic,” as used herein, refers to cells of a different subject that are genetically identical to the cell in comparison.

“Xenogeneic,” as used herein, refers to cells of a different species to the cell in comparison. In preferred embodiments, the cells of the invention are allogeneic.

Illustrative immune effector cells used with the CARs contemplated herein include T lymphocytes. The terms “T cell” or “T lymphocyte” are art-recognized and are intended to include thymocytes, immature T lymphocytes, mature T lymphocytes, resting T lymphocytes, or activated T lymphocytes. A T cell can be a T helper (Th) cell, for example a T helper 1 (Th1) or a T helper 2 (Th2) cell. The T cell can be a helper T cell (HTL; $CD4^+$ T cell) $CD4^+$ T cell, a cytotoxic T cell (CTL; $CD8^+$ T cell), $CD4^+CD8^+$ T cell, $CD4^-CD8^-$ T cell, or any other subset of T cells. Other illustrative populations of T cells suitable for use in particular embodiments include naïve T cells and memory T cells.

As would be understood by the skilled person, other cells may also be used as immune effector cells with the CARs as described herein. In particular, immune effector cells also include NK cells, NKT cells, neutrophils, and macrophages. Immune effector cells also include progenitors of effector cells wherein such progenitor cells can be induced to differentiate into an immune effector cells *in vivo* or *in vitro*. Thus, in particular embodiments, immune effector cell includes progenitors of immune effectors cells such as hematopoietic stem cells (HSCs) contained within the CD34$^+$ population of cells derived from cord blood, bone marrow or mobilized peripheral blood which upon administration in a subject differentiate into mature immune effector cells, or which can be induced *in vitro* to differentiate into mature immune effector cells.

As used herein, immune effector cells genetically engineered to contain BCMA-specific CAR may be referred to as, "BCMA-specific redirected immune effector cells."

The term, "CD34$^+$ cell," as used herein refers to a cell expressing the CD34 protein on its cell surface. "CD34," as used herein refers to a cell surface glycoprotein (*e.g.,* sialomucin protein) that often acts as a cell-cell adhesion factor and is involved in T cell entrance into lymph nodes. The CD34$^+$ cell population contains hematopoietic stem cells (HSC), which upon administration to a patient differentiate and contribute to all hematopoietic lineages, including T cells, NK cells, NKT cells, neutrophils and cells of the monocyte/macrophage lineage.

The present invention provides methods for making the immune effector cells which express the CAR contemplated herein. In one embodiment, the method comprises transfecting or transducing immune effector cells isolated from an individual such that the immune effector cells express one or more CAR as described herein. In certain embodiments, the immune effector cells are isolated from an individual and genetically modified without further manipulation *in vitro*. Such cells can then be directly re-administered into the individual. In further embodiments, the immune effector cells are first activated and stimulated to proliferate *in vitro* prior to being genetically modified to express a CAR. In this regard, the immune effector cells may be cultured before and/or after being genetically modified (*i.e.*, transduced or transfected to express a CAR contemplated herein).

In particular embodiments, prior to *in vitro* manipulation or genetic modification of the immune effector cells described herein, the source of cells is obtained from a subject. In particular embodiments, the CAR-modified immune effector cells comprise T cells. T cells can be obtained from a number of sources including, but not limited to, peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled person, such as sedimentation, *e.g.,* FICOLL™ separation. In one embodiment, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocyte, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing. The cells can be washed with PBS or with another suitable solution that lacks calcium, magnesium, and most, if not all other, divalent cations. As would be appreciated by those of ordinary skill in the art, a washing step may be accomplished by methods known to those in the art, such as by using a semiautomated flowthrough centrifuge. For example, the Cobe 2991 cell processor, the Baxter CytoMate, or the like. After washing, the cells may be resuspended in a variety of biocompatible buffers or other saline solution with or without buffer. In certain embodiments, the undesirable components of the apheresis sample may be removed in the cell directly resuspended culture media.

In certain embodiments, T cells are isolated from peripheral blood mononuclear cells (PBMCs) by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient. A specific subpopulation of T cells, expressing one or more of the following markers: CD3, CD28, CD4, CD8, CD45RA, and CD45RO, can be further isolated by positive or negative selection techniques. In one embodiment, a specific subpopulation of T cells, expressing CD3, CD28, CD4, CD8, CD45RA, and CD45RO is further isolated by positive or negative selection techniques. For example, enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method for use herein is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for $CD4^+$ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. Flow cytometry and cell sorting may also be used to isolate cell populations of interest for use in the present invention.

PBMC may be directly genetically modified to express CARs using methods contemplated herein. In certain embodiments, after isolation of PBMC, T lymphocytes are further isolated and in certain embodiments, both cytotoxic and helper T lymphocytes can be sorted into naïve, memory, and effector T cell subpopulations either before or after genetic modification and/or expansion.

$CD8^+$ cells can be obtained by using standard methods. In some embodiments, $CD8^+$ cells are further sorted into naive, central memory, and effector cells by identifying cell surface antigens that are associated with each of those types of $CD8^+$ cells.

In certain embodiments, naive $CD8^+$ T lymphocytes are characterized by the expression of phenotypic markers of naive T cells including CD62L, CCR7, CD28, CD3, CD 127, and CD45RA.

In particular embodiments, memory T cells are present in both $CD62L^+$ and $CD62L^-$ subsets of $CD8^+$ peripheral blood lymphocytes. PBMC are sorted into $CD62L^-CD8^+$ and $CD62L^+CD8^+$ fractions after staining with anti-CD8 and anti- CD62L antibodies. I n some embodiments, the expression of phenotypic markers of central memory T cells include CD45RO, CD62L, CCR7, CD28, CD3, and CD127 and are negative for granzyme B. In some embodiments, central memory T cells are $CD45RO^+$, $CD62L^+$, $CD8^+$ T cells.

In some embodiments, effector T cells are negative for CD62L, CCR7, CD28, and CD127, and positive for granzyme B and perforin.

In certain embodiments, $CD4^+$ T cells are further sorted into subpopulations. For example, $CD4^+$ T helper cells can be sorted into naive, central memory, and effector cells by identifying cell populations that have cell surface antigens. $CD4^+$ lymphocytes can be obtained by standard methods. In some embodiments, naïve $CD4^+$

T lymphocytes are CD45RO$^-$, CD45RA$^+$, CD62L$^+$ CD4$^+$ T cell. In some embodiments, central memory CD4$^+$ cells are CD62L positive and CD45RO positive. In some embodiments, effector CD4$^+$ cells are CD62L and CD45RO negative.

The immune effector cells, such as T cells, can be genetically modified following isolation using known methods, or the immune effector cells can be activated and expanded (or differentiated in the case of progenitors) *in vitro* prior to being genetically modified. In a particular embodiment, the immune effector cells, such as T cells, are genetically modified with the chimeric antigen receptors contemplated herein (*e.g.,* transduced with a viral vector comprising a nucleic acid encoding a CAR) and then are activated and expanded *in vitro*. In various embodiments, T cells can be activated and expanded before or after genetic modification to express a CAR, using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681 ; 7, 144,575; 7,067,318; 7, 172,869; 7,232,566; 7, 175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005.

Generally, the T cells are expanded by contact with a surface having attached thereto an agent that stimulates a CD3 TCR complex associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the T cells. T cell populations may be stimulated by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (*e.g.,* bryostatin) in conjunction with a calcium ionophore. Co-stimulation of accessory molecules on the surface of T cells, is also contemplated.

In particular embodiments, PBMCs or isolated T cells are contacted with a stimulatory agent and costimulatory agent, such as anti-CD3 and anti-CD28 antibodies, generally attached to a bead or other surface, in a culture medium with appropriate cytokines, such as IL-2, IL-7, and/or IL-15. To stimulate proliferation of either CD4$^+$ T cells or CD8$^+$ T cells, an anti-CD3 antibody and an anti-CD28 antibody. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diacione, Besancon, France) can be used as can other methods commonly known in the art (Berg *et al.*, Transplant Proc. 30(8):3975-3977, 1998; Haanen *et al.*, J. Exp. Med. 190(9): 13191328, 1999; Garland *et al.*, J. Immunol Meth. 227( 1 -2):53-63, 1999). Anti-CD3 and anti-CD28 antibodies attached to the same bead serve as a “surrogate” antigen presenting cell (APC). In other embodiments, the T cells may be activated and stimulated to proliferate with feeder cells and appropriate antibodies and cytokines using methods such as those described in US6040177; US5827642; and WO2012129514.

In other embodiments, artificial APC (aAPC) made by engineering K562, U937, 721.221, T2, and C1R cells to direct the stable expression and secretion, of a variety of co-stimulatory molecules and cytokines. In a particular embodiment K32 or U32 aAPCs are used to direct the display of one or more antibody-based stimulatory molecules on the AAPC cell surface. Expression of various combinations of genes on the aAPC enables the precise determination of human T-cell activation requirements, such that aAPCs can be tailored for the optimal propagation of T-cell subsets with specific growth requirements and distinct functions. The aAPCs support *ex vivo* growth and long-term expansion of functional human CD8 T cells without requiring the addition of exogenous cytokines, in contrast to the use of natural APCs. Populations of T cells can be expanded by aAPCs expressing a variety of costimulatory molecules including, but not limited to, CD137L (4-1BBL), CD134L (OX40L), and/or CD80 or CD86. Finally, the aAPCs provide an efficient platform to expand genetically modified T cells and to maintain CD28 expression on CD8 T cells. aAPCs provided in WO 03/057171 and US2003/0147869 are hereby incorporated by reference in their entirety.

In one embodiment, $CD34^+$ cells are transduced with a nucleic acid construct in accordance with the invention. In certain embodiments, the transduced $CD34^+$ cells differentiate into mature immune effector cells *in vivo* following administration into a subject, generally the subject from whom the cells were originally isolated. In another embodiment, $CD34^+$ cells may be stimulated *in vitro* prior to exposure to or after being genetically modified with a CAR as described herein, with one or more of the following cytokines: Flt-3 ligand (FLT3), stem cell factor (SCF), megakaryocyte growth and differentiation factor (TPO), IL-3 and IL-6 according to the methods described previously (Asheuer *et al.*, 2004; Imren, *et al.*, 2004).

The invention provides a population of modified immune effector cells for the treatment of cancer, the modified immune effector cells comprising a CAR as disclosed herein. For example, a population of modified immune effector cells are prepared from peripheral blood mononuclear cells (PBMCs) obtained from a patient diagnosed with B cell malignancy described herein (autologous donors). The PBMCs form a heterogeneous population of T lymphocytes that can be $CD4^+$, $CD8^+$, or $CD4^+$ and $CD8^+$.

The PBMCs also can include other cytotoxic lymphocytes such as NK cells or NKT cells. An expression vector carrying the coding sequence of a CAR contemplated herein can be introduced into a population of human donor T cells, NK cells or NKT cells. Successfully transduced T cells that carry the expression vector can be sorted using flow cytometry to isolate CD3 positive T cells and then further propagated to increase the number of these CAR protein expressing T cells in addition to cell activation using anti-CD3 antibodies and or anti-CD28 antibodies and IL-2 or any other methods known in the art as described elsewhere herein. Standard procedures are used for cryopreservation of T cells expressing the CAR protein T cells for storage and/or preparation for use in a human subject. In one embodiment, the *in vitro* transduction, culture and/or expansion of T cells are performed in the absence of non-human animal derived products such as fetal calf serum and fetal bovine serum. Since a heterogeneous population of PBMCs is genetically modified, the resultant transduced cells are a heterogeneous population of modified cells comprising a BCMA targeting CAR as contemplated herein.

In a further embodiment, a mixture of, *e.g.,* one, two, three, four, five or more, different expression vectors can be used in genetically modifying a donor population of immune effector cells wherein each vector encodes a different chimeric antigen receptor protein as contemplated herein. The resulting modified immune effector cells forms a mixed population of modified cells, with a proportion of the modified cells expressing more than one different CAR proteins.

In one embodiment, the invention provides a method of storing genetically modified murine, human or humanized CAR protein expressing immune effector cells which target a BCMA protein, comprising cryopreserving the immune effector cells such that the cells remain viable upon thawing. A fraction of the immune effector cells expressing the CAR proteins can be cryopreserved by methods known in the art to provide a permanent source of such cells for the future treatment of patients afflicted with the B cell related condition. When needed, the cryopreserved transformed immune effector cells can be thawed, grown and expanded for more such cells.

As used herein, “cryopreserving,” refers to the preservation of cells by cooling to sub-zero temperatures, such as (typically) 77 K or −196° C. (the boiling point of liquid nitrogen). Cryoprotective agents are often used at sub-zero temperatures to prevent the cells being preserved from damage due to freezing at low temperatures or warming to room temperature. Cryopreservative agents and optimal cooling rates can protect against cell injury. Cryoprotective agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock and Bishop, Nature, 1959; 183: 1394-1395; Ashwood-Smith, Nature, 1961; 190: 1204-1205), glycerol, polyvinylpyrrolidine (Rinfret, Ann. N.Y. Acad. Sci., 1960; 85: 576), and polyethylene glycol (Sloviter and Ravdin, Nature, 1962; 196: 48). The preferred cooling rate is 1° to 3° C/minute. After at least two hours, the T cells have reached a temperature of −80° C. and can be placed directly into liquid nitrogen (−196° C.) for permanent storage such as in a long-term cryogenic storage vessel.

## H. COMPOSITIONS AND FORMULATIONS

The compositions contemplated herein may comprise one or more polypeptides, polynucleotides, vectors comprising same, genetically modified immune effector cells, *etc.*, as contemplated herein. Compositions include, but are not limited to pharmaceutical compositions. A "pharmaceutical composition" refers to a composition formulated in pharmaceutically-acceptable or physiologically-acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. It will also be understood that, if desired, the compositions of the invention may be administered in combination with other agents as well, such as, *e.g.,* cytokines, growth factors, hormones, small molecules, chemotherapeutics, pro-drugs, drugs, antibodies, or other various pharmaceutically-active agents. There is virtually no limit to other components that may also be included in the compositions, provided that the additional agents do not adversely affect the ability of the composition to deliver the intended therapy.

The phrase "pharmaceutically acceptable" is employed herein to refer 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 problem or complication, commensurate with a reasonable benefit/risk ratio.

As used herein "pharmaceutically acceptable carrier, diluent or excipient" includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, surfactant, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals. Exemplary pharmaceutically acceptable carriers include, but are not limited to, to sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; tragacanth; malt; gelatin; talc; cocoa butter, waxes, animal and vegetable fats, paraffins, silicones, bentonites, silicic acid, zinc oxide; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and any other compatible substances employed in pharmaceutical formulations.

In particular embodiments, compositions of the present invention comprise an amount of CAR-expressing immune effector cells contemplated herein. As used herein, the term “amount” refers to “an amount effective” or “an effective amount” of a genetically modified therapeutic cell, *e.g.,* T cell, to achieve a beneficial or desired prophylactic or therapeutic result, including clinical results.

A “prophylactically effective amount” refers to an amount of a genetically modified therapeutic cell effective to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount is less than the therapeutically effective amount.

A “therapeutically effective amount” of a genetically modified therapeutic cell may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the stem and progenitor cells to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the virus or transduced therapeutic cells are outweighed by the therapeutically beneficial effects. The term “therapeutically effective amount” includes an amount that is effective to “treat” a subject (*e.g.,* a patient). When a therapeutic amount is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the T cells described herein may be administered at a dosage of $10^2$ to $10^{10}$ cells/kg body weight, preferably $10^5$ to $10^6$ cells/kg body weight, including all integer values within those ranges. The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 mLs or less, even 250 mLs or 100 mLs or less. Hence the density of the desired cells is typically greater than $10^6$ cells/ml and generally is greater than $10^7$ cells/ml, generally $10^8$ cells/ml or greater. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$, $10^{11}$, or $10^{12}$ cells. In some aspects of the present invention, particularly since all the infused cells will be redirected to a particular target antigen, lower numbers of cells, in the range of $10^6$/kilogram ($10^6$-$10^{11}$ per patient) may be administered. CAR expressing cell compositions may be administered multiple times at dosages within these ranges. The cells may be allogeneic, syngeneic, xenogeneic, or autologous to the patient undergoing therapy. If desired, the treatment may also include administration of mitogens (*e.g.,* PHA) or lymphokines, cytokines, and/or chemokines (*e.g.,* IFN-γ, IL-2, IL-12, TNF-alpha, IL-18, and TNF-beta, GM-CSF, IL-4, IL-13, Flt3-L, RANTES, MIP1α, *etc.*) as described herein to enhance induction of the immune response.

Generally, compositions comprising the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, compositions comprising the CAR-modified T cells contemplated herein are used in the treatment of B cell malignancies. The CAR-modified T cells of the present invention may be administered either alone, or as a pharmaceutical composition in combination with carriers, diluents, excipients, and/or with other components such as IL-2 or other cytokines or cell populations. In particular embodiments, pharmaceutical compositions contemplated herein comprise an amount of genetically modified T cells, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients.

Pharmaceutical compositions of the present invention comprising a CAR-expressing immune effector cell population, such as T cells, 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 of the present invention are preferably formulated for parenteral administration, *e.g.,* intravascular (intravenous or intraarterial), intraperitoneal or intramuscular administration.

The liquid pharmaceutical compositions, whether they be solutions, suspensions or other like form, may include one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. An injectable pharmaceutical composition is preferably sterile.

In a particular embodiment, compositions contemplated herein comprise an effective amount of CAR-expressing immune effector cells, alone or in combination with one or more therapeutic agents. Thus, the CAR-expressing immune effector cell compositions may be administered alone or in combination with other known cancer treatments, such as radiation therapy, chemotherapy, transplantation, immunotherapy, hormone therapy, photodynamic therapy, *etc.* The compositions may also be administered in combination with antibiotics. Such therapeutic agents may be accepted in the art as a standard treatment for a particular disease state as described herein, such as a particular cancer. Exemplary therapeutic agents contemplated include cytokines, growth factors, steroids, NSAIDs, DMARDs, anti-inflammatories, chemotherapeutics, radiotherapeutics, therapeutic antibodies, or other active and ancillary agents.

In certain embodiments, compositions comprising CAR-expressing immune effector cells disclosed herein may be administered in conjunction with any number of chemotherapeutic agents. Illustrative examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine resume; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2”-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (“Ara-C”); cyclophosphamide; thiotepa; taxoids, *e.g.* paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®., Rhne-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000;

difluoromethylomithine (DMFO); retinoic acid derivatives such as Targretin™ (bexarotene), Panretin™ (alitretinoin) ; ONTAK™ (denileukin diftitox) ; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on cancers such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A variety of other therapeutic agents may be used in conjunction with the compositions described herein. In one embodiment, the composition comprising CAR-expressing immune effector cells is administered with an anti-inflammatory agent. Anti-inflammatory agents or drugs include, but are not limited to, steroids and glucocorticoids (including betamethasone, budesonide, dexamethasone, hydrocortisone acetate, hydrocortisone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone), nonsteroidal anti-inflammatory drugs (NSAIDS) including aspirin, ibuprofen, naproxen, methotrexate, sulfasalazine, leflunomide, anti-TNF medications, cyclophosphamide and mycophenolate.

Other exemplary NSAIDs are chosen from the group consisting of ibuprofen, naproxen, naproxen sodium, Cox-2 inhibitors such as VIOXX® (rofecoxib) and CELEBREX® (celecoxib), and sialylates. Exemplary analgesics are chosen from the group consisting of acetaminophen, oxycodone, tramadol of proporxyphene hydrochloride. Exemplary glucocorticoids are chosen from the group consisting of cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, or prednisone. Exemplary biological response modifiers include molecules directed against cell surface markers (*e.g.,* CD4, CD5, *etc.*), cytokine inhibitors, such as the TNF antagonists (*e.g.,* etanercept (ENBREL®), adalimumab (HUMIRA®) and infliximab (REMICADE®), chemokine inhibitors and adhesion molecule inhibitors. The biological response modifiers include monoclonal antibodies as well as recombinant forms of molecules. Exemplary DMARDs include azathioprine, cyclophosphamide, cyclosporine, methotrexate, penicillamine, leflunomide, sulfasalazine, hydroxychloroquine, Gold (oral (auranofin) and intramuscular) and minocycline.

Illustrative examples of therapeutic antibodies suitable for combination with the CAR modified T cells contemplated herein, include but are not limited to, bavituximab, bevacizumab (avastin), bivatuzumab, blinatumomab, conatumumab, daratumumab, duligotumab, dacetuzumab, dalotuzumab, elotuzumab (HuLuc63), gemtuzumab, ibritumomab, indatuximab, inotuzumab, lorvotuzumab, lucatumumab, milatuzumab, moxetumomab, ocaratuzumab, ofatumumab, rituximab, siltuximab, teprotumumab, and ublituximab.

In certain embodiments, the compositions described herein are administered in conjunction with a cytokine. By “cytokine” as used herein is meant a generic term for proteins released by one cell population that act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-beta; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, beta, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-15, a tumor necrosis factor such as TNF-alpha or TNF-beta; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture, and biologically active equivalents of the native sequence cytokines.

## I. THERAPEUTIC METHODS

The genetically modified immune effector cells contemplated herein provide improved methods of adoptive immunotherapy for use in the treatment of B cell related conditions that include, but are not limited to immunoregulatory conditions and hematological malignancies.

In particular embodiments, the specificity of a primary immune effector cell is redirected to B cells by genetically modifying the primary immune effector cell with a CAR contemplated herein. In various embodiments, a viral vector is used to genetically modify an immune effector cell with a particular polynucleotide encoding a CAR comprising a humanized anti-BCMA antigen binding domain that binds a BCMA polypeptide; a hinge domain; a transmembrane (TM) domain, a short oligo- or polypeptide linker, that links the TM domain to the intracellular signaling domain of the CAR; and one or more intracellular co-stimulatory signaling domains; and a primary signaling domain.

In one embodiment, the present invention includes a type of cellular therapy where T cells are genetically modified to express a CAR that targets BCMA expressing B cells, and the CAR T cell is infused to a recipient in need thereof. The infused cell is able to kill disease causing B cells in the recipient. Unlike antibody therapies, CAR T cells are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained cancer therapy.

In one embodiment, the CAR T cells of the invention can undergo robust *in vivo* T cell expansion and can persist for an extended amount of time. In another embodiment, the CAR T cells of the invention evolve into specific memory T cells that can be reactivated to inhibit any additional tumor formation or growth.

In particular embodiments, compositions comprising immune effector cells comprising the CARs contemplated herein are used in the treatment of conditions associated with abnormal B cell activity.

Illustrative examples of conditions that can be treated, prevented or ameliorated using the immune effector cells comprising the CARs contemplated herein include, but are not limited to: systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, autoimmune hemolytic anemia, idiopathic thrombocytopenia purpura, anti-phospholipid syndrome, Chagas' disease, Grave's disease, Wegener's granulomatosis, poly-arteritis nodosa, Sjogren's syndrome, pemphigus vulgaris, scleroderma, multiple sclerosis, anti-phospholipid syndrome, ANCA associated vasculitis, Goodpasture's disease, Kawasaki disease, and rapidly progressive glomerulonephritis.

The modified immune effector cells may also have application in plasma cell disorders such as heavy-chain disease, primary or immunocyte-associated amyloidosis, and monoclonal gammopathy of undetermined significance (MGUS).

As use herein, "B cell malignancy" refers to a type of cancer that forms in B cells (a type of immune system cell) as discussed *infra*.

In particular embodiments, compositions comprising CAR-modified T cells contemplated herein are used in the treatment of hematologic malignancies, including but not limited to B cell malignancies such as, for example, multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL).

Multiple myeloma is a B cell malignancy of mature plasma cell morphology characterized by the neoplastic transformation of a single clone of these types of cells. These plasma cells proliferate in BM and may invade adjacent bone and sometimes the blood. Variant forms of multiple myeloma include overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma (see, for example, Braunwald, *et al.* (eds), *Harrison's Principles of Internal Medicine*, 15th Edition (McGraw-Hill 2001)).

Non-Hodgkin lymphoma encompasses a large group of cancers of lymphocytes (white blood cells). Non-Hodgkin lymphomas can occur at any age and are often marked by lymph nodes that are larger than normal, fever, and weight loss. There are many different types of non-Hodgkin lymphoma. For example, non-Hodgkin's lymphoma can be divided into aggressive (fast-growing) and indolent (slow-growing) types. Although non-Hodgkin lymphomas can be derived from B cells and T-cells, as used herein, the term "non-Hodgkin lymphoma" and "B cell non-Hodgkin lymphoma" are used interchangeably. B cell non-Hodgkin lymphomas (NHL) include Burkitt lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), diffuse large B cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma. Lymphomas that occur after bone marrow or stem cell transplantation are usually B cell non-Hodgkin lymphomas.

Chronic lymphocytic leukemia (CLL) is an indolent (slow-growing) cancer that causes a slow increase in immature white blood cells called B lymphocytes, or B cells. Cancer cells spread through the blood and bone marrow, and can also affect the lymph nodes or other organs such as the liver and spleen. CLL eventually causes the bone marrow to fail. Sometimes, in later stages of the disease, the disease is called small lymphocytic lymphoma.

In particular embodiments, methods comprising administering a therapeutically effective amount of CAR-expressing immune effector cells contemplated herein or a composition comprising the same, to a patient in need thereof, alone or in combination with one or more therapeutic agents, are provided. In certain embodiments, the cells of the invention are used in the treatment of patients at risk for developing a condition associated with abnormal B cell activity or a B cell malignancy. Thus, the present invention provides methods for the treatment or prevention of a condition associated with abnormal B cell activity or a B cell malignancy comprising administering to a subject in need thereof, a therapeutically effective amount of the CAR-modified cells contemplated herein.

As used herein, the terms “individual” and “subject” are often used interchangeably and refer to any animal that exhibits a symptom of a disease, disorder, or condition that can be treated with the gene therapy vectors, cell-based therapeutics, and methods disclosed elsewhere herein. In preferred embodiments, a subject includes any animal that exhibits symptoms of a disease, disorder, or condition of the hematopoietic system, *e.g.,* a B cell malignancy, that can be treated with the gene therapy vectors, cell-based therapeutics, and methods disclosed elsewhere herein. Suitable subjects (*e.g.,* patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included. Typical subjects include human patients that have a B cell malignancy, have been diagnosed with a B cell malignancy, or are at risk or having a B cell malignancy.

As used herein, the term “patient” refers to a subject that has been diagnosed with a particular disease, disorder, or condition that can be treated with the gene therapy vectors, cell-based therapeutics, and methods disclosed elsewhere herein.

As used herein “treatment” or “treating,” includes any beneficial or desirable effect on the symptoms or pathology of a disease or pathological condition, and may include even minimal reductions in one or more measurable markers of the disease or condition being treated. Treatment can involve optionally either the reduction or amelioration of symptoms of the disease or condition, or the delaying of the progression of the disease or condition. “Treatment” does not necessarily indicate complete eradication or cure of the disease or condition, or associated symptoms thereof.

As used herein, “prevent,” and similar words such as “prevented,” “preventing” *etc.*, indicate an approach for preventing, inhibiting, or reducing the likelihood of the occurrence or recurrence of, a disease or condition. It also refers to delaying the onset or recurrence of a disease or condition or delaying the occurrence or recurrence of the symptoms of a disease or condition. As used herein, “prevention” and similar words also includes reducing the intensity, effect, symptoms and/or burden of a disease or condition prior to onset or recurrence of the disease or condition.

By “enhance” or “promote,” or “increase” or “expand” refers generally to the ability of a composition contemplated herein, *e.g.,* a genetically modified T cell or vector encoding a CAR, to produce, elicit, or cause a greater physiological response (*i.e.*, downstream effects) compared to the response caused by either vehicle or a control molecule/composition. A measurable physiological response may include an increase in T cell expansion, activation, persistence, and/or an increase in cancer cell killing ability, among others apparent from the understanding in the art and the description herein. An “increased” or “enhanced” amount is typically a “statistically significant” amount, and may include an increase that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.,* 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.,* 1.5, 1.6, 1.7. 1.8, *etc.*) the response produced by vehicle or a control composition.

By “decrease” or “lower,” or “lessen,” or “reduce,” or “abate” refers generally to the ability of composition contemplated herein to produce, elicit, or cause a lesser physiological response (*i.e.*, downstream effects) compared to the response caused by either vehicle or a control molecule/composition. A “decrease” or “reduced” amount is typically a “statistically significant” amount, and may include an decrease that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.,* 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.,* 1.5, 1.6, 1.7. 1.8, *etc.*) the response (reference response) produced by vehicle, a control composition, or the response in a particular cell lineage.

By “maintain,” or “preserve,” or “maintenance,” or “no change,” or “no substantial change,” or “no substantial decrease” refers generally to the ability of a composition contemplated herein to produce, elicit, or cause a lesser physiological response (*i.e.*, downstream effects) in a cell, as compared to the response caused by either vehicle, a control molecule/composition, or the response in a particular cell lineage. A comparable response is one that is not significantly different or measurable different from the reference response.

In one embodiment, a method of treating a B cell related condition in a subject in need thereof comprises administering an effective amount, *e.g.,* therapeutically effective amount of a composition comprising genetically modified immune effector cells contemplated herein. The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

In certain embodiments, it may be desirable to administer activated immune effector cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate immune effector cells therefrom according to the present invention, and reinfuse the patient with these activated and expanded immune effector cells. This process can be carried out multiple times every few weeks. In certain embodiments, immune effector cells can be activated from blood draws of from 10cc to 400cc. In certain embodiments, immune effector cells are activated from blood draws of 20cc, 30cc, 40cc, 50cc, 60cc, 70cc, 80cc, 90cc, 100cc, 150cc, 200cc, 250cc, 300cc, 350cc, or 400cc or more. Not to be bound by theory, using this multiple blood draw/multiple reinfusion protocol may serve to select out certain populations of immune effector cells.

The administration of the compositions contemplated herein may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. In a preferred embodiment, compositions are administered parenterally. The phrases “parenteral administration” and “administered parenterally” as used herein refers to modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravascular, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intratumoral, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. In one embodiment, the compositions contemplated herein are administered to a subject by direct injection into a tumor, lymph node, or site of infection.

In one embodiment, a subject in need thereof is administered an effective amount of a composition to increase a cellular immune response to a B cell related condition in the subject. The immune response may include cellular immune responses mediated by cytotoxic T cells capable of killing infected cells, regulatory T cells, and helper T cell responses. Humoral immune responses, mediated primarily by helper T cells capable of activating B cells thus leading to antibody production, may also be induced. A variety of techniques may be used for analyzing the type of immune responses induced by the compositions of the present invention, which are well described in the art; *e.g.,* Current Protocols in Immunology, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001) John Wiley & Sons, NY, N.Y.

In the case of T cell-mediated killing, CAR-ligand binding initiates CAR signaling to the T cell, resulting in activation of a variety of T cell signaling pathways that induce the T cell to produce or release proteins capable of inducing target cell apoptosis by various mechanisms. These T cell-mediated mechanisms include (but are not limited to) the transfer of intracellular cytotoxic granules from the T cell into the target cell, T cell secretion of pro-inflammatory cytokines that can induce target cell killing directly (or indirectly via recruitment of other killer effector cells), and up regulation of death receptor ligands (*e.g.* FasL) on the T cell surface that induce target cell apoptosis following binding to their cognate death receptor (*e.g.* Fas) on the target cell.

In one embodiment, the invention provides a method of treating a subject diagnosed with a B cell related condition comprising removing immune effector cells from a subject diagnosed with a BCMA-expressing B cell related condition, genetically modifying said immune effector cells with a vector comprising a nucleic acid encoding a CAR as contemplated herein, thereby producing a population of modified immune effector cells, and administering the population of modified immune effector cells to the same subject. In a preferred embodiment, the immune effector cells comprise T cells.

In certain embodiments, the present invention also provides methods for stimulating an immune effector cell mediated immune modulator response to a target cell population in a subject comprising the steps of administering to the subject an immune effector cell population expressing a nucleic acid construct encoding a CAR molecule.

The methods for administering the cell compositions described herein includes any method which is effective to result in reintroduction of *ex vivo* genetically modified immune effector cells that either directly express a CAR of the invention in the subject or on reintroduction of the genetically modified progenitors of immune effector cells that on introduction into a subject differentiate into mature immune effector cells that express the CAR. One method comprises transducing peripheral blood T cells *ex vivo* with a nucleic acid construct in accordance with the invention and returning the transduced cells into the subject.

All publications, patent applications, and issued patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or issued patent were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

# EXAMPLES

## EXAMPLE 1

### Construction of anti-BCMA CARs

CARs containing humanized anti-BCMA scFv antibodies were designed to contain an MND promoter operably linked to anti-BCMA scFv, a hinge and transmembrane domain from CD8α and a CD137 co-stimulatory domains followed by the intracellular signaling domain of the CD3ζ chain. Figure 1. The anti-BCMA CARs comprise a CD8α signal peptide (SP) sequence for the surface expression on immune effector cells. The polynucleotide sequences of the anti-BCMA CARs are set forth in SEQ ID NOs: 30 to 44, 70, and 72; polypeptide sequences of the anti-BCMA CARs are set forth in SEQ ID NOs: 15 to 29, 71, and 73; and the vector maps is shown in Figure 1. Tables 3 to 5 show the Identity, Genbank Reference, Source Name and Citation for the various nucleotide segments of various exemplary anti-BCMA CAR lentiviral vectors.

**Table 3.**

| Nucleotides | Identity | GenBank Reference | Source Name | Citation |
|---|---|---|---|---|
| 1-185 | pUC19 plasmid backbone | Accession #L09137.2<br>nt 1 – 185 | pUC19 | New England Biolabs |
| 185-222 | Linker | Not applicable | Synthetic | Not applicable |
| 223-800 | CMV | Not Applicable | pHCMV | (1994) PNAS 91: 9564-68 |
| 801-1136 | R, U5, PBS, and packaging sequences | Accession #M19921.2<br>nt 454-789 | pNL4-3 | Maldarelli, et.al. (1991)<br>J Virol: 65(11):5732-43 |
| 1137-1139 | Gag start codon (ATG) changed to stop codon (TAG) | Not Applicable | Synthetic | Not applicable |
| 1140-1240 | HIV-1 gag sequence | Accession #M19921.2<br>nt 793-893 | pNL4-3 | Maldarelli, et.al. (1991)<br>J Virol: 65(11):5732-43 |
| 1241-1243 | HIV-1 gag sequence changed to a second stop codon | Not Applicable | Synthetic | Not applicable |
| 1244-1595 | HIV-1 gag sequence | Accession #M19921.2 | pNL4-3 | Maldarelli, et.al. |

| Nucleotides | Identity | GenBank Reference | Source Name | Citation |
|---|---|---|---|---|
| | | nt 897-1248 | | (1991)<br>J Virol: 65(11):5732-43 |
| 1596-1992 | HIV-1 pol<br>cPPT/CTS | Accession #M19921.2<br>nt 4745-5125 | pNL4-3 | Maldarelli, et.al. (1991)<br>J Virol: 65(11):5732-43 |
| 1993-2517 | HIV-1, isolate HXB3 env region (RRE) | Accession #M14100.1<br>nt 1875-2399 | PgTAT-CMV | Malim, M. H.<br>Nature (1988) 335:181-183 |
| 2518-2693 | HIV-1 env sequences S/A | Accession #M19921.2<br>nt 8290-8470 | pNL4-3 | Maldarelli, et.al. (1991)<br>J Virol: 65(11):5732-43 |
| 2694-2708 | Linker | Not applicable | Synthetic | Not applicable |
| 2709-3096 | MND | Not applicable | rSPA.mPro.MND | Challita *et al.* (1995)<br>J.Virol. 69: 748-755 |
| 3097-3125 | Linker | Not applicable | Synthetic | Not applicable |
| 3126-3188 | Signal peptide | | Synthetic | Not applicable |
| 3189-3926 | Anti-BCMA scFv | Not applicable | Synthetic | Not applicable |
| 3927-3935 | Linker | Not applicable | Synthetic | Not applicable |
| 3936-4142 | CD8a hinge and TM | Accession # NM_001768 | Synthetic | Milone et al (2009)<br>Mol Ther 17(8):1453-64 |
| 4143-4268 | CD137 (4-1BB) signaling domain | Accession # NM_001561 | Synthetic | Milone et al (2009)<br>Mol Ther 17(8):1453-64 |
| 4269-4607 | CD3-ζ signaling domain | Accession # NM_000734 | Synthetic | Milone et al (2009)<br>Mol Ther 17(8):1453-64 |
| 4608-4718 | HIV-1 ppt and part of U3 | Accession #M19921.2<br>nt 9005-9110 | pNL4-3 | Maldarelli, et.al. (1991)<br>J Virol: 65(11):5732-43 |
| 4719-4835 | HIV-1 part of U3 (399bp deletion) and R | Accession #M19921.2<br>nt 9511-9627 | pNL4-3 | Maldarelli, et.al. (1991) |

| Nucleotides | Identity | GenBank Reference | Source Name | Citation |
|---|---|---|---|---|
| | | | | J Virol: 65(11):5732-43 |
| 4836-4859 | Synthetic polyA | Not applicable[2] | Synthetic | Levitt, N. Genes & Dev (1989) 3:1019-1025 |
| 4860-4878 | Linker | Not applicable[1] | Synthetic | Not Applicable[1] |
| 4879-7351 | pUC19 backbone | Accession #L09137.2<br>nt 2636-2686 | pUC19 | New England Biolabs (Attached) |

**Table 4.**

| Nucleotides | Identity | GenBank Reference | Source Name | Citation |
|---|---|---|---|---|
| 1-185 | pUC19 plasmid backbone | Accession #L09137.2 nt 1 – 185 | pUC19 | New England Biolabs (attached) |
| 185-222 | Linker | Not applicable[1] | Synthetic | Not applicable[1] |
| 223-800 | CMV | Not Applicable[2] | pHCMV | (1994) PNAS 91: 9564-68 |
| 801-1136 | R, U5, PBS, and packaging sequences | Accession #M19921.2 nt 454-789 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1137-1139 | Gag start codon (ATG) changed to stop codon (TAG) | Not Applicable[1] | Synthetic | Not applicable[1] |
| 1140-1240 | HIV-1 gag sequence | Accession #M19921.2 nt 793-893 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1241-1243 | HIV-1 gag sequence changed to a second stop codon | Not Applicable[1] | Synthetic | Not applicable[1] |
| 1244-1595 | HIV-1 gag sequence | Accession #M19921.2 nt 897-1248 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1596-1992 | HIV-1 pol cPPT/CTS | Accession #M19921.2 nt 4745-5125 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1993-2517 | HIV-1, isolate HXB3 env region (RRE) | Accession #M14100.1 nt 1875-2399 | PgTAT-CMV | Malim, M. H. Nature (1988) 335:181-183 |
| 2518-2693 | HIV-1 env sequences S/A | Accession #M19921.2 nt 8290-8470 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 2694-2708 | Linker | Not applicable | Synthetic | Not applicable[1] |

| | | | | |
|---|---|---|---|---|
| 2709-3096 | MND | Not applicable[2] | pccl-c-MNDU3c-x2 | Challita *et al.* (1995) J.Virol. 69: 748-755 |
| 3097-3125 | Linker | Not applicable | Synthetic | Not applicable[1] |
| 3126-3188 | Signal peptide | Accession # NM_001768 | CD8a signal peptide | Not applicable[3] |
| 3189-3926 | Humanized BCMA10.2 scFv (VL-linker-VH) | Not applicable | Synthetic | Not applicable[1] |
| 3927-3935 | Linker | Not applicable | Synthetic | Not applicable |
| 3936-4124 | CTLA4 hinge and TM | Accession # NM_001037631 | cytotoxic T-lymphocyte-associated protein 4 (CTLA4) | Not applicable[1] |
| 4125-4250 | CD137 (4-1BB) signaling domain | Accession # NM_001561 | CD137 signaling domain | Milone et al (2009) Mol Ther 17(8):1453-64 |
| 4251-4589 | CD3-ζ signaling domain | Accession # NM_000734 | CD3-ζ signaling domain | Milone et al (2009) Mol Ther 17(8):1453-64 |
| 4590-4700 | HIV-1 ppt and part of U3 | Accession #M19921.2 nt 9005-9110 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 4701-4817 | HIV-1 part of U3 (399bp deletion) and R | Accession #M19921.2 nt 9511-9627 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 4818-4841 | Synthetic polyA | Not applicable | Synthetic | Levitt, N. Genes & Dev (1989) 3:1019-1025 |
| 4842-4860 | Linker | Not applicable | Synthetic | Not Applicable[1] |
| 4861-7333 | pUC19 backbone | Accession #L09137.2 nt 2636-2686 | pUC19 | New England Biolabs (Attached) |

**Table 5.**

| Nucleotides | Identity | GenBank Reference | Source Name | Citation |
|---|---|---|---|---|
| 1-185 | pUC19 plasmid backbone | Accession #L09137.2 nt 1 – 185 | pUC19 | New England Biolabs (attached) |
| 185-222 | Linker | Not applicable | Synthetic | Not applicable[1] |
| 223-800 | CMV | Not Applicable | pHCMV | (1994) PNAS 91: 9564-68 |

| | | | | |
|---|---|---|---|---|
| 801-1136 | R, U5, PBS, and packaging sequences | Accession #M19921.2 nt 454-789 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1137-1139 | Gag start codon (ATG) changed to stop codon (TAG) | Not Applicable | Synthetic | Not applicable[1] |
| 1140-1240 | HIV-1 gag sequence | Accession #M19921.2 nt 793-893 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1241-1243 | HIV-1 gag sequence changed to a second stop codon | Not Applicable | Synthetic | Not applicable[1] |
| 1244-1595 | HIV-1 gag sequence | Accession #M19921.2 nt 897-1248 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1596-1992 | HIV-1 pol cPPT/CTS | Accession #M19921.2 nt 4745-5125 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 1993-2517 | HIV-1, isolate HXB3 env region (RRE) | Accession #M14100.1 nt 1875-2399 | PgTAT-CMV | Malim, M. H. Nature (1988) 335:181-183 |
| 2518-2693 | HIV-1 env sequences S/A | Accession #M19921.2 nt 8290-8470 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 2694-2708 | Linker | Not applicable | Synthetic | Not applicable[1] |
| 2709-3096 | MND | Not applicable | pccl-c-MNDU3c-x2 | Challita *et al.* (1995) J.Virol. 69: 748-755 |
| 3097-3125 | Linker | Not applicable | Synthetic | Not applicable[1] |
| 3126-3188 | Signal peptide | Accession # NM_001768 | CD8a signal peptide | Not applicable[3] |
| 3189-3926 | Humanized BCMA10.5 scFv (VL-linker-VH) | Not applicable | Synthetic | Not applicable[1] |
| 3927-3935 | Linker | Not applicable | Synthetic | Not applicable |
| 3936-4118 | PD-1 hinge and TM | Accession # NM_005018 | Programmed cell death 1 (PDCD1) | Not applicable[1] |
| 4119-4244 | CD137 (4-1BB) signaling domain | Accession # NM_001561 | CD137 signaling domain | Milone et al (2009) Mol Ther 17(8):1453-64 |
| 4245-4583 | CD3-ζ signaling domain | Accession # NM_000734 | CD3-ζ signaling domain | Milone et al (2009) Mol Ther 17(8):1453-64 |

| | | | | |
|---|---|---|---|---|
| 4584-4694 | HIV-1 ppt and part of U3 | Accession #M19921.2 nt 9005-9110 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 4695-4811 | HIV-1 part of U3 (399bp deletion) and R | Accession #M19921.2 nt 9511-9627 | pNL4-3 | Maldarelli, et.al. (1991) J Virol: 65(11):5732-43 |
| 4818-4841 | Synthetic polyA | Not applicable | Synthetic | Levitt, N. Genes & Dev (1989) 3:1019-1025 |
| 4836-4854 | Linker | Not applicable | Synthetic | Not Applicable[1] |
| 4855-7327 | pUC19 backbone | Accession #L09137.2 nt 2636-2686 | pUC19 | New England Biolabs (Attached) |

## EXAMPLE 2

### HIGH THROUGHPUT SCREEN OF HUMANIZED ANTI-BCMA CAR

A microculture assay was developed to rapidly screen anti-BCMA CARs. The microculture assay can compare 15-20 CAR modified T cells (CAR T cells). Peripheral blood mononuclear cells (PBMC) were cultured in 24-well plates in media containing IL-2 (CellGenix) and antibodies specific for CD3 and CD28 (Miltenyi Biotec) to initiate outgrowth of T cells. The expanded T cells were >99% CD3 positive T cells after 7 days of culture.

T cells expanded from PBMC were modified by lentiviruses to express the anti-BCMA CARs. Transient lentiviral supernatants were added to the PBMC culture at 1:1 ratio (volume:volume) one day after culture initiation. Cultures were split as needed with fresh media containing IL-2 after becoming optically confluent using an inverted light microscope. After twelve days of culture, CAR T cells were harvested and evaluated for CAR expression and function.

Expression of CARs on the T cell surface was assayed by flow cytometry using an antibody reactive to the single chain variable fragment portion of the CARs (goat anti-mouse Ig (GAM), Life Technologies). The average expression of each of the 15 CARs in three separate normal donors is shown in Table 6. Expression of CARs was comparable, ranging from 47% to 63% CAR-positive T cells. Transduction efficiency (represented as VCN in Table 6) among the various CAR constructs tested. VCN was assayed by PCR using primers that amplify integrated virus.

**Table 6.**

| Construct | Percent sequence change | Ave CAR expression | Ave VCN |
|---|---|---|---|
| anti-BCMA-20 | 19.6 | 47.0 | 5.7 |
| anti-BCMA-10 | 19.15 | 49.1 | 6.6 |
| anti-BCMA-21 | 18.5 | 55.4 | 6.1 |
| anti-BCMA-30 | 18.25 | 48.5 | 6.3 |
| anti-BCMA-11 | 18.05 | 50.1 | 5.6 |
| anti-BCMA-22 | 17.25 | 57.6 | 6.7 |
| anti-BCMA-31 | 17.15 | 55.0 | 5.6 |
| anti-BCMA-12 | 16.8 | 53.8 | 5.5 |
| anti-BCMA-23 | 16.4 | 62.3 | 6.1 |
| anti-BCMA-24 | 15.95 | 56.1 | 6.6 |
| anti-BCMA-13 | 15.95 | 54.0 | 5.7 |
| anti-BCMA-32 | 15.9 | 59.0 | 7.8 |
| anti-BCMA-14 | 15.5 | 52.9 | 6.8 |
| anti-BCMA-33 | 15.05 | 59.2 | 6.2 |
| anti-BCMA-34 | 14.6 | 56.0 | 6.2 |

## EXAMPLE 3

## ANTIGEN SPECIFIC REACTIVITY OF CAR T CELLS

Antigen-specific reactivity was examined after co-culture with BCMA-positive cell lines. Anti-BCMA CAR T cells were co-cultured with BCMA-engineered K562 (K562-BCMA) cells for 24 hours. Reactivity of the anti-BCMA CAR T cells to K562-BCMA was assayed by IFN-gamma (IFNγ) release into the supernatant by ELISA. All humanized anti-BCMA CAR T cells tested released similar amounts of IFNγ and the amounts were comparable the parental mouse anti-BCMA CAR. Figure 2. In contrast, IFNγ was not detected in cultures containing either untransduced or CD19-specific CAR T cells confirming that BCMA-specificity of the CAR T cells was required for reactivity to K562-BCMA cells.

In another set of experiments, tumor cytolytic activity of T cells expressing one of five humanized anti-BCMA CARs was examined. anti-BCMA CAR T cells were co-cultured with K562-BCMA cells for four hours. The five humanized anti-BCMA CAR T cells exhibited similar cytolytic activity to K562-BCMA across three T cell:tumor ratios. Figure 3. Surprisingly, all humanized anti-BCMA CAR T cells tested showed greater cytolytic activity than the parental mouse anti-BCMA CAR T cell.

## EXAMPLE 4

### PHENOTYPE AND FUNCTION OF HUMANIZED ANTI-BCMA CAR T CELLS

The phenotype and function of three humanized anti-BCMA constructs (anti-BCMA-10, anti-BCMA-11, anti-BCMA-31) were evaluated in a culture system directly scalable to large clinical manufacturing processes. Briefly, peripheral blood mononuclear cells (PBMC) were cultured in static flasks in media containing IL-2 (CellGenix) and antibodies specific for CD3 and CD28 (Miltenyi Biotec). $2x10^8$ transducing units of lentivirus encoding anti-BCMA CARs were added one day after culture initiation. Anti-BCMA CAR T cells were maintained in log-phase by adding fresh media containing IL-2 for a total of ten days of culture. T cells transduced with mouse anti- BCMA CAR (BCMA-02), the three humanized anti-BCMA CARs, or an anti-CD19 CAR lacking signaling capacity (CAR19Δ) were expanded from three normal donors (800290, 800309, 801269). CAR T cells were assessed for CAR expression and antigen and tumor cell reactivity at the end of culture.

Expression of anti- BCMA CARs on the T cell surface was assayed by flow cytometry using an antibody reactive to the single chain variable fragment portion of the CAR (goat anti-mouse Ig (GAM), Life Technologies). Figure 4. Comparable CAR expression was observed for both mouse (msBCMA-02) or humanized sequences (anti-BCMA-10, anti-BCMA-11, anti-BCMA-31). No significant differences in mouse or humanized anti-BCMA CAR expression was observed between all three donors (Table7).

**Table 7.**

| | CAR Expression (GaM) | | | | | |
|---|---|---|---|---|---|---|
| | **UnTD** | **CAR19Δ** | **BCMA-02** | **BCMA-10** | **BCMA-11** | **BCMA-31** |
| **800290** | 2.22 | 19.1 | 60.9 | 85.2 | 64.0 | 68.4 |
| **800309** | 2.37 | 13.9 | 56.6 | 77.9 | 54.4 | 57.9 |
| **801269** | 2.16 | 19.1 | 52.2 | 64.1 | 57.2 | 57.6 |
| **Average (±SE)** | 2.25 (±0.1) | 17.4 (±1.7) | 56.6 (±2.5) | 75.7 (±6.2) | 58.5 (±2.8) | 61.3 (±3.6) |

There was no significant difference in transduction efficiency among the anti-BCMA CAR T cells. Vector copy number (VCN), assayed by PCR using primers that amplify integrated virus, was comparable in all anti-BCMA CAR T cells (Table 8).

**Table 8.**

| | Vector Copy Number (VCN) | | | | | |
|---|---|---|---|---|---|---|
| | **UnTD** | **CAR19D** | **BCMA-02** | **BCMA-10** | **BCMA-11** | **BCMA-31** |
| **800290** | 0 | 1.4 | 4.3 | 3.9 | 3.1 | 3.6 |
| **800309** | 0 | 1.1 | 3.9 | 3.1 | 2.3 | 2.8 |
| **801269** | 0 | 1.1 | 3.9 | 2.6 | 2.5 | 2.7 |
| **Average (±SE)** | 0 | 1.2 (±0.2) | 4.0 (±0.2) | 3.2 (±0.7) | 2.6 (±0.4) | 3.0 (±0.5) |

The reactivity of the anti-BCMA CAR T cells to BCMA positive cell lines and tumors was evaluated. Comparable IFNγ release was observed after co-culture of anti-BCMA CAR T cells with K562-BCMA (expressing low or high amounts of BCMA) cells or multiple myeloma cell lines (RPMI-8226, NCI-H929) but not to BCMA-negative cell lines (HDLM-2, K562). Figure 5.

Cytolytic activity of T cells expressing anti-BCMA CARs was examined. Anti-BCMA CAR T cells were co-cultured with K562-BCMA cells for four hours. All anti-BCMA CAR T cells tested caused comparable K562-BCMA cell cytotoxicity. Figure 6.

## EXAMPLE 5

### TONIC CYTOKINE RELEASE IN HUMANIZED ANTI-BCMA CAR T CELLS

In particular embodiments, anti-BCMA CAR molecules may release cytokines in the absence of stimulation. This antigen-independent ("tonic") cytokine release can be observed after *in vitro* culture in conditions which lack the presence of its antigen.

Tonic cytokine release from T cells engineered to express a humanized anti-BCMA-10 CAR was observed. The humanized anti-BCMA10 was derived from mouse anti-BCMA-02 to reduce potential immunogenicity caused be immune reactions to mouse sequences. In total, less than 20% sequence difference exists between anti-BCMA-02 and anti-BCMA-10 CAR molecules. Despite these changes, no difference in CAR expression or reactivity to BCMA antigens were observed between mouse anti-BCMA-02 and humanized anti-BCMA-10 (Figure 5 and Table 7). Yet in the absence of BCMA, humanized anti-BCMA-10 CAR T cells released significantly more inflammatory cytokines. Figure 7.

Mouse anti-BCMA-02 or humanized anti-BCMA-10 CAR T cells were cultured overnight in media containing human serum but lacking any BCMA. Levels of 12 cytokines released into the supernatant were determined using a multiplex bead approach (Luminex assay). $5x10^4$ humanized anti-BCMA-10 CAR T cells released up to 5ng/ml of inflammatory cytokines including MIP1α, MIP1β, IFNγ, GMCSF, IL-8, and TNFα. The same amount of mouse anti-BCMA-02 CAR T cells released less than 200pg/ml. Neither CAR modification impacted the release of anti-inflammatory cytokines including IL-10 and IL-4.

Tonic cytokine release raised concerns of known toxicity caused by excessive cytokine release in patients treated with anti-CD19 CAR T cells. The high antigen-independent, tonic cytokine release observed from humanized anti-BCMA-10 CAR T cells could cause toxic levels of cytokines in patients even in the absence of BCMA antigen. The sequence changes made to mouse anti-BCMA-02 to generate humanized anti-BCMA-10 could introduce nascent reactivity to proteins in human serum not seen in mouse anti-BCMA-02 T cells. Reactivity to human serum proteins could explain "tonic" cytokine release seen in humanized anti-BCMA-10.

Mouse anti-BCMA-02 and humanized anti-BCMA-10 CAR T cells were rested for 48 hours in media containing fetal calf serum instead of human serum. The CAR T cells were switched to media containing human serum and the amount of IFNγ released was compared to cultures kept in fetal calf serum. Culture media did not impact IFN-γ release from either CAR T cell. No IFNγ was detected at the end of culture of mouse anti-BCMA-02 CAR T cells while humanized anti-BCMA10 cultures contained about 4ng/ml IFNγ regardless of the culture media (Figure 8). Lack of reactivity in mouse anti-BCMA-02 CAR T cells was not due to T cell dysfunction: Both anti-BCMA CAR T cells reacted to plate-immobilized BCMA (data not shown).

## EXAMPLE 6

### TUNING CYTOKINE RELEASE IN HUMANIZED ANTI-BCMA CAR T CELLS

CARs containing mouse sequences have the potential to cause undesirable immune responses. Anecdotal evidence in CAR clinical trials have suggested these immune responses can limit the efficacy of CAR T cells in some patients. Humanization of mouse sequences is a strategy to reduce the immune responses to mouse sequences. Yet humanized anti-BCMA-10 CAR introduced tonic inflammatory release. The antigen recognition domain of anti-BCMA-10 CAR T cells is tethered to the T cell surface using a CD8α transmembrane domain. The identity of the transmembrane domain and its impact the dimerization of CAR molecules and the subsequent activation in the absence of antigen was examined.

Anti-BCMA-10 CAR molecules were constructed to replace the CD8α transmembrane domain with either a CTLA-4 (anti-BCMA-10.2) or a PD-1 (anti-BCMA-10.5) transmembrane domain (Figure 9). Expression of anti-BCMA-10.2 and anti-BCMA-10.5 was compared to anti-BCMA-10 by staining recombinant human BCMA protein conjugated to IgG1 Fc-PE and assaying using flow cytometry. Table 9 shows the results from two representative normal donor T cells which robust surface expression of all three CAR molecules was observed.

**Table 9**

| Construct | Donor 1 | Donor2 |
|---|---|---|
| anti-BCMA-10 | 90.4 | 87.7 |
| anti-BCMA-10.2 | 86.2 | 86.7 |
| anti-BCMA-10.5 | 76.2 | 78.0 |

The reactivity of the anti-BCMA CAR T cells to BCMA positive K562 cell lines was evaluated. Comparable IFNγ release was observed after co-culture of anti-BCMA-10, anti-BCMA-10.2, or anti-BCMA-10.5 with K562-BCMA cells (Figure 10). These data show that changing the transmembrane domain to sequences from either CTLA-4 or PD-1 did not impact reactivity of the anti-BCMA-10 CAR T cell.

Tonic cytokine release was assessed by measuring IFNγ in the supernatants of overnight cultures with the mouse anti-BCMA-02, or humanized anti-BCMA-10, anti-BCMA-10.2 or anti-BCMA-10.5. Anti-BCMA-02 cultures contained low amounts of IFNγ as observed previously. Supernatants from anti-BCMA-10 cultures contained higher amounts of IFNγ. Anti-BCMA-10.2 and anti-BCMA-10.5 cultures contained IFNγ levels comparable to anti-BCMA-02 and lower levels than anti-BCMA-10 CAR T cells (Figure 11). These data suggest that changing the transmembrane domain can reduce the tonic cytokine release without affecting the antigen reactivity of CAR T cells.

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

# CLAIMS

1. A chimeric antigen receptor (CAR) comprising: an extracellular domain that comprises a humanized anti-BCMA (B cell maturation antigen) antibody or antigen binding fragment thereof that binds one or more epitopes of a human BCMA polypeptide; a transmembrane domain, one or more intracellular co-stimulatory signaling domains, and a primary signaling domain.

2. The CAR of claim 1, wherein the humanized anti-BCMA antibody or antigen binding fragment that binds the human BCMA polypeptide is selected from the group consisting of: a Camel Ig, Ig NAR, Fab fragments, Fab' fragments, F(ab)'2 fragments, F(ab)'3 fragments, Fv, single chain Fv antibody ("scFv"), bis-scFv, (scFv)2, minibody, diabody, triabody, tetrabody, disulfide stabilized Fv protein ("dsFv"), and single-domain antibody (sdAb, Nanobody).

3. The CAR of claim 2, wherein the humanized anti-BCMA antibody or antigen binding fragment that binds the human BCMA polypeptide is an scFv.

4. The CAR of any one of claims 1 to 3, wherein the humanized anti-BCMA antibody or antigen binding fragment thereof comprises one or more CDRs as set forth in any one of SEQ ID NOs: 1-3.

5. The CAR of any one of claims 1 to 4, wherein the humanized anti-BCMA antibody or antigen binding fragment thereof comprises one or more CDRs as set forth in any one of SEQ ID NOs: 4-6.

6. The CAR of any one of claims 1 to 5, wherein the humanized anti-BCMA antibody or antigen binding fragment thereof comprises a variable light chain sequence as set forth in any one of SEQ ID NOs: 7-9.

7. The CAR of claim 6, wherein the variable light chain sequence comprises the CDR sequences set forth in SEQ ID NOs: 1-3.

8. The CAR of any one of claims 1 to 7, wherein the humanized anti-BCMA antibody or antigen binding fragment thereof comprises a variable heavy chain sequence as set forth in any one of SEQ ID NOs: 10-14.

9. The CAR of claim 8, wherein the variable heavy chain sequence comprises the CDR sequences set forth in SEQ ID NOs: 4-6

10. The CAR of any one of claims 1 to 9, wherein the transmembrane domain is from a polypeptide selected from the group consisting of: alpha, beta or zeta chain of the T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD 154, and PD1.

11. The CAR of any one of claims 1 to 9, wherein the transmembrane domain is from a polypeptide selected from the group consisting of: CD8α; CD4, CD45, PD1, and CD152.

12. The CAR of any one of claims 1 to 11, wherein the transmembrane domain is from CD8α.

13. The CAR of any one of claims 1 to 11, wherein the transmembrane domain is from PD1.

14. The CAR of any one of claims 1 to 11, wherein the transmembrane domain is from CD152.

15. The CAR of any one of claims 1 to 14, wherein the one or more co-stimulatory signaling domains are from a co-stimulatory molecule selected from the group consisting of: CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83,

CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70.

16. The CAR of any one of claims 1 to 14, wherein the one or more co-stimulatory signaling domains are from a co-stimulatory molecule selected from the group consisting of: CD28, CD134, and CD137.

17. The CAR of any one of claims 1 to 14, wherein the one or more co-stimulatory signaling domains are from a co-stimulatory molecule selected from the group consisting of: CD28, CD134, and CD137.

18. The CAR of any one of claims 1 to 14, wherein the one or more co-stimulatory signaling domains is from CD28.

19. The CAR of any one of claims 1 to 14, wherein the one or more co-stimulatory signaling domains is from CD134.

20. The CAR of any one of claims 1 to 14, wherein the one or more co-stimulatory signaling domains is from CD137.

21. The CAR of any one of claims 1 to 20, further comprising a hinge region polypeptide.

22. The CAR of claim 21, wherein the hinge region polypeptide comprises a hinge region of CD8α.

23. The CAR of claim 21, wherein the hinge region polypeptide comprises a hinge region of PD1.

24. The CAR of claim 21, wherein the hinge region polypeptide comprises a hinge region of CD152.

25. The CAR of any one of claims 1 to 24, further comprising a spacer region.

26. The CAR of claim 25, wherein the spacer region polypeptide comprises CH2 and CH3 regions of IgG1 or IgG4.

27. The CAR of any one of claims 1 to 26, further comprising a signal peptide.

28. The CAR of claim 27, wherein the signal peptide comprises an IgG1 heavy chain signal polypeptide, a CD8α signal polypeptide, or a human GM-CSF receptor alpha signal polypeptide.

29. The CAR of any one of claims 1 to 28, comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 15-29, 71, and 73.

30. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 15.

31. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 16.

32. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 17.

33. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 18.

34. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 19.

35. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 20.

36. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 21.

37. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 22.

38. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 23.

39. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 24.

40. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 25.

41. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 26.

42. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 27.

43. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 28.

44. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 29.

45. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 71.

46. The CAR of any one of claims 1 to 29, comprising an amino acid sequence as set forth in SEQ ID NO: 73.

47. A polynucleotide encoding a CAR of any one of claims 1 to 46.

48. A polynucleotide encoding a CAR, wherein the polynucleotide sequence is set forth in any one of SEQ ID NOs: 30-44, 70, and 72.

49. A vector comprising the polynucleotide of claim 47 or claim 48.

50. The vector of claim 49, wherein the vector is an expression vector.

51. The vector of claim 49, wherein the vector is an episomal vector.

52. The vector of claim 49, wherein the vector is a viral vector.

53. The vector of claim 49, wherein the vector is a retroviral vector.

54. The vector of claim 49, wherein the vector is a lentiviral vector.

55. The vector of claim 49, wherein the lentiviral vector is selected from the group consisting essentially of: human immunodeficiency virus 1 (HIV-1); human immunodeficiency virus 2 (HIV-2), visna-maedi virus (VMV) virus; caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

56. The vector according to any one of claims 52 to 55, comprising a left (5') retroviral LTR, a Psi (Ψ) packaging signal, a central polypurine tract/DNA flap (cPPT/FLAP), a retroviral export element; a promoter operably linked to the polynucleotide of claim 26 or claim 27; and a right (3') retroviral LTR.

57. The vector of claim 56, further comprising a heterologous polyadenylation sequence.

58. The vector of claim 56 or claim 57, further comprising a hepatitis B virus posttranscriptional regulatory element (HPRE) or woodchuck post-transcriptional regulatory element (WPRE).

59. The vector of any one of claims 56 to 58, wherein the promoter of the 5' LTR is replaced with a heterologous promoter.

60. The vector of claim 59, wherein the heterologous promoter is a cytomegalovirus (CMV) promoter, a Rous Sarcoma Virus (RSV)promoter, or an Simian Virus 40 (SV40) promoter.

61. The vector of any one of claims 56 to 60, wherein the 5' LTR or 3' LTR is a lentivirus LTR.

62. The vector of any one of claims 56 to 61, wherein the 3' LTR comprises one or more modifications.

63. The vector of any one of claims 56 to 62, wherein the 3' LTR comprises one or more deletions.

64. The vector of any one of claims 56 to 63, wherein the 3' LTR is a self-inactivating (SIN) LTR.

65. The vector of any one of claims 56 to 64, wherein the polyadenylation sequence is a bovine growth hormone polyadenylation or signal rabbit β-globin polyadenylation sequence.

66. The vector of any one of claims 59 to 65, wherein the polynucleotide of claim 57 or claim 58 comprises an optimized Kozak sequence.

67. The vector of any one of claims 56 to 66, wherein the promoter operably linked to the polynucleotide of claim 57 or claim 58 is selected from the group consisting of: a cytomegalovirus immediate early gene promoter (CMV), an elongation factor 1 alpha promoter (EF1-α), a phosphoglycerate kinase-1 promoter (PGK), a ubiquitin-C promoter (UBQ-C), a cytomegalovirus enhancer/chicken beta-actin promoter (CAG), polyoma enhancer/herpes simplex thymidine kinase promoter (MC1), a beta actin promoter (β-ACT), a simian virus 40 promoter (SV40), and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter.

68. An immune effector cell comprising the vector of any one of claims 49 to 67.

69. The immune effector cell of claim 68, wherein the immune effector cell is selected from the group consisting of: a T lymphocyte and a natural killer (NK) cell.

70. A composition comprising the immune effector cell of claim 68 or claim 69 and a physiologically acceptable excipient.

71. A method of generating an immune effector cell comprising a CAR according to any one of claims 1 to 46 comprising introducing into an immune effector cell the vector of an one of claims 49 to 67.

72. The method of claim 71, further comprising stimulating the immune effector cell and inducing the cell to proliferate by contacting the cell with antibodies that bind CD3 and antibodies that bind to CD28; thereby generating a population of immune effector cells.

73. The method of claim 72, wherein the immune effector cell is stimulated and induced to proliferate before introducing the vector.

74. The method of claim 72, wherein the immune effector cells comprise T lymphocytes.

75. The method of claim 72, wherein the immune effector cells comprise NK cells.

76. A method of treating a B cell related condition in a subject in need thereof, comprising administering to the subject a therapeutically effect amount of the composition of claim 70.

77. The method of claim 76, wherein the B cell related condition is multiple myeloma, non-Hodgkin’s lymphoma, B cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, post-transplant lymphoproliferative disorder, an immunoregulatory disorder, rheumatoid arthritis, myasthenia gravis, idiopathic thrombocytopenia purpura, anti-phospholipid syndrome, Chagas' disease, Grave's disease, Wegener's granulomatosis, poly-arteritis nodosa, Sjogren's syndrome, pemphigus vulgaris, scleroderma, multiple sclerosis, anti-phospholipid syndrome, ANCA associated vasculitis, Goodpasture's disease, Kawasaki disease, autoimmune hemolytic anemia, and rapidly progressive glomerulonephritis, heavy-chain disease, primary or immunocyte-associated amyloidosis, or monoclonal gammopathy of undetermined significance.

78. The method of claim 76, wherein the B cell related condition is a B cell malignancy.

79. The method of claim 78, wherein the B cell malignancy is multiple myeloma (MM) or non-Hodgkin’s lymphoma (NHL).

80. The method of claim 79, wherein the MM is selected from the group consisting of: overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

81. The method of claim 79, wherein the NHL is selected from the group consisting of: Burkitt lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma.

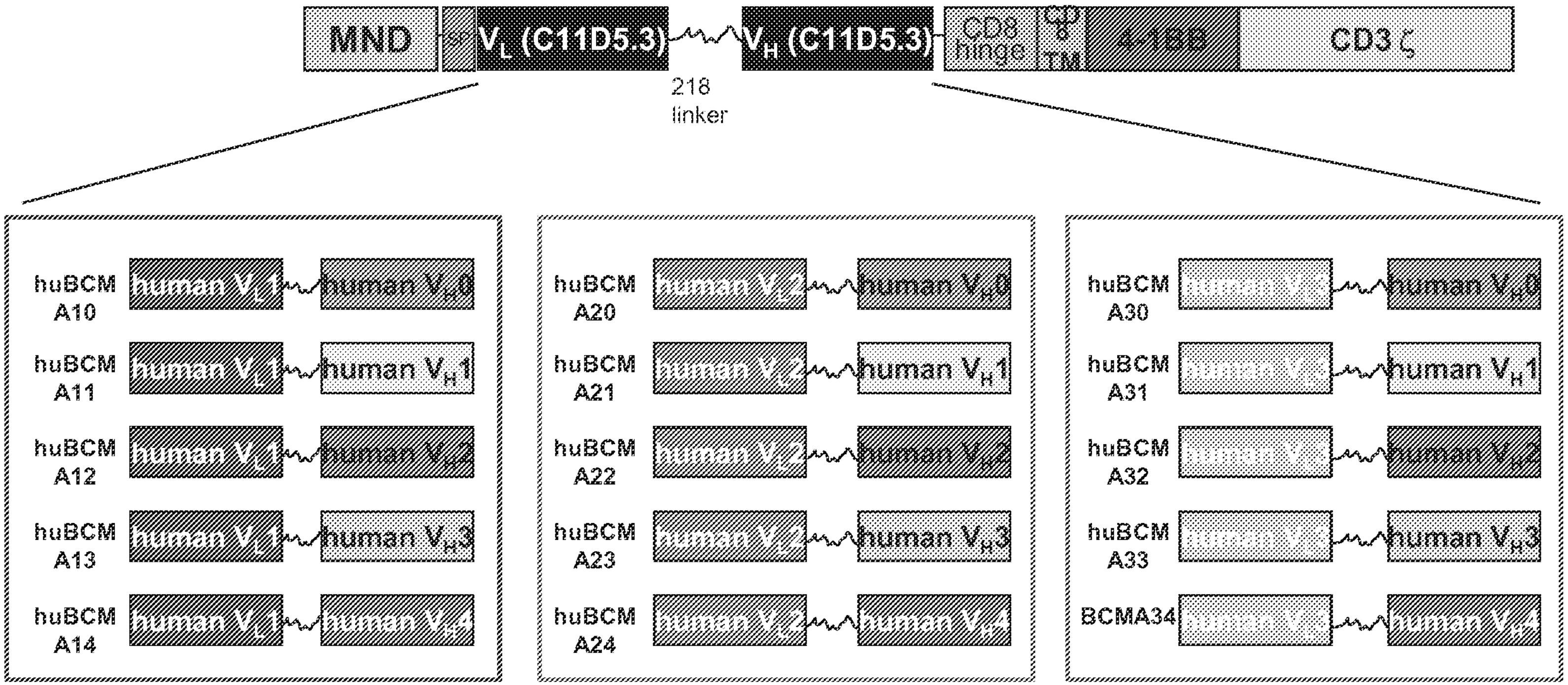
82. The method of claim 76, wherein the B cell related condition is a plasma cell malignancy.

83. The method of claim 76, wherein the B cell related condition is an autoimmune disease.

84. The method of claim 83, wherein the autoimmune disease is systemic lupus erythematosus.

85. The method of claim 83, wherein the B cell related condition is rheumatoid arthritis.

86. The method of claim 83, wherein the B cell related condition is idiopathic thrombocytopenia purpura, or myasthenia gravis, or autoimmune hemolytic anemia.


MND
SP
$V_L$ (C11D5.3)
218 linker
$V_H$ (C11D5.3)
CD8 hinge
CD8 TM
4-1BB
CD3 ζ
huBCM A10
human $V_L$1
human $V_H$0
huBCM A11
human $V_L$1
human $V_H$1
huBCM A12
human $V_L$1
human $V_H$2
huBCM A13
human $V_L$1
human $V_H$3
huBCM A14
human $V_L$1
human $V_H$4
huBCM A20
human $V_L$2
human $V_H$0
huBCM A21
human $V_L$2
human $V_H$1
huBCM A22
human $V_L$2
human $V_H$2
huBCM A23
human $V_L$2
human $V_H$3
huBCM A24
human $V_L$2
human $V_H$4
huBCM A30
human $V_H$0
huBCM A31
human $V_H$1
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huBCM A33
human $V_H$3
BCMA34
human $V_H$4

FIG. 1

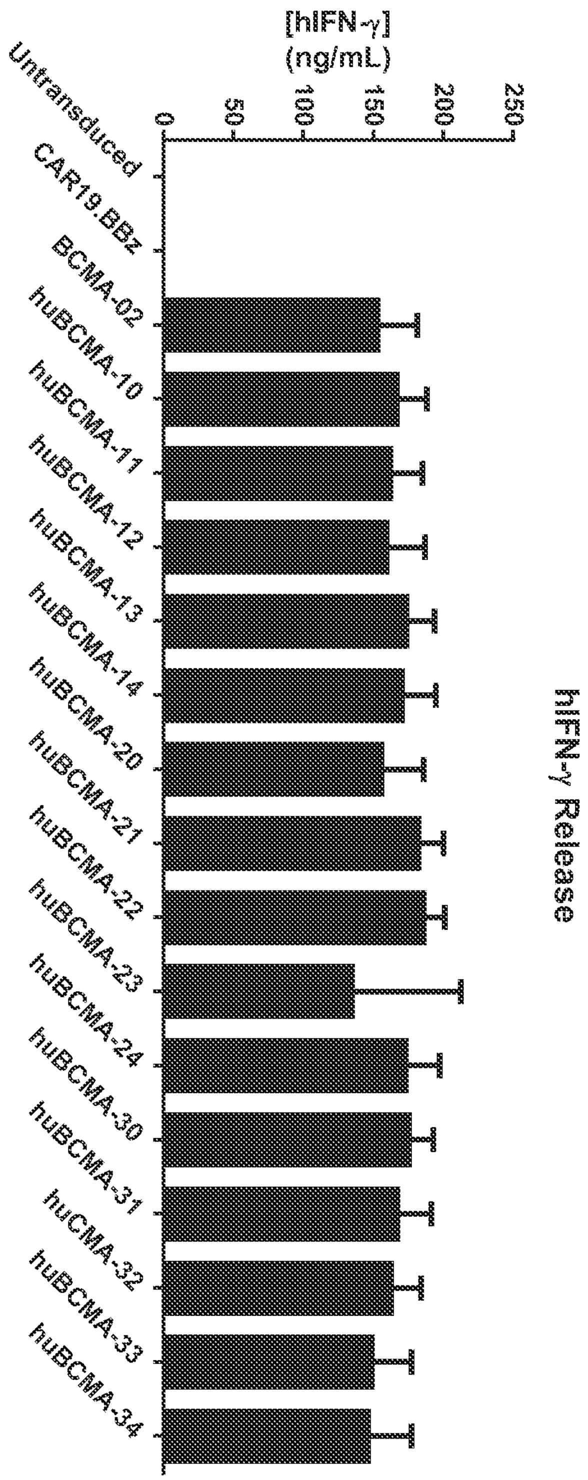

hIFN-γ Release
[hIFN-γ]
(ng/mL)
0
50
100
150
200
250
Untransduced
CAR19.BBz
BCMA-02
huBCMA-10
huBCMA-11
huBCMA-12
huBCMA-13
huBCMA-14
huBCMA-20
huBCMA-21
huBCMA-22
huBCMA-23
huBCMA-24
huBCMA-30
huBCMA-31
huCMA-32
huBCMA-33
huBCMA-34

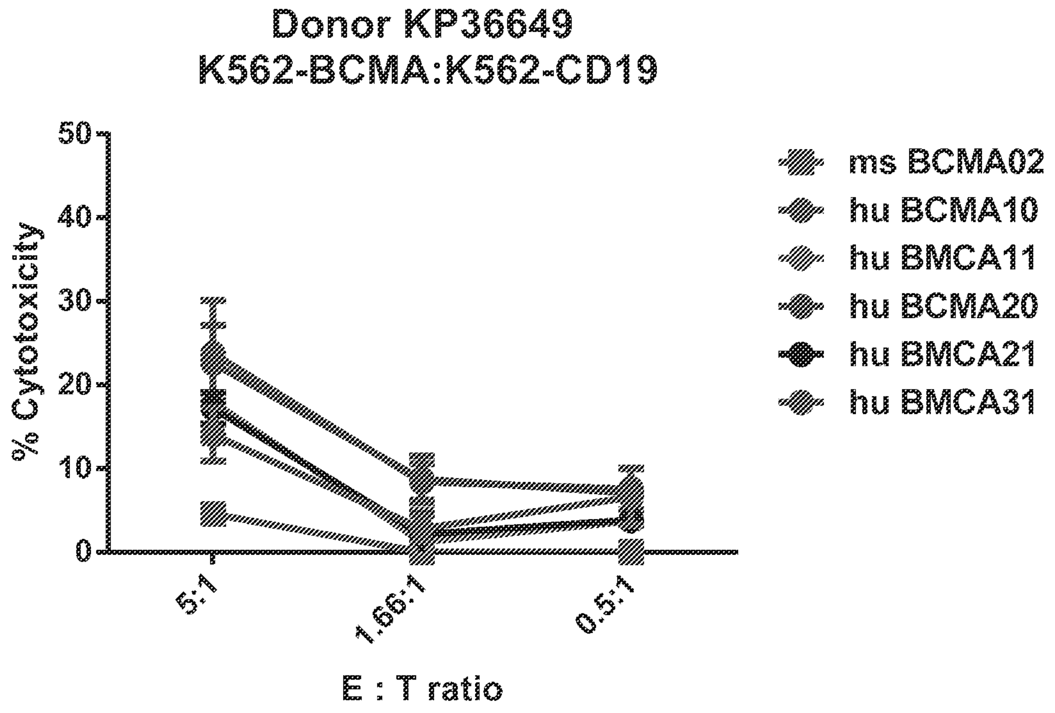
FIG. 2

FIG. 3

**Day 18**

**BCMA expression established with flow cytometry**

| BCMA$^{pos}$ tumor | BCMA$^{neg}$ tumor |
|---|---|
| K562-BCMA (bulk) | K562-CD19 |



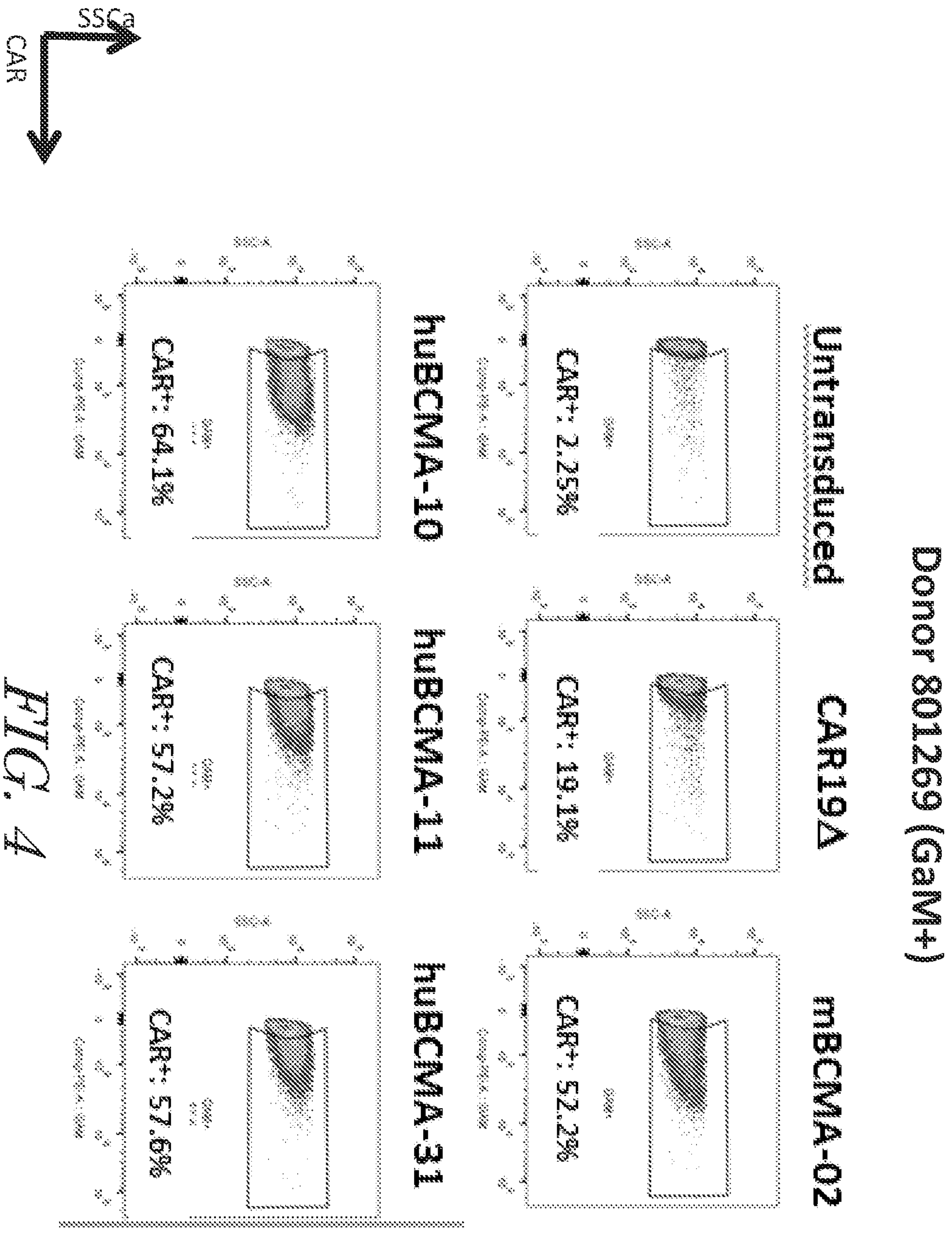
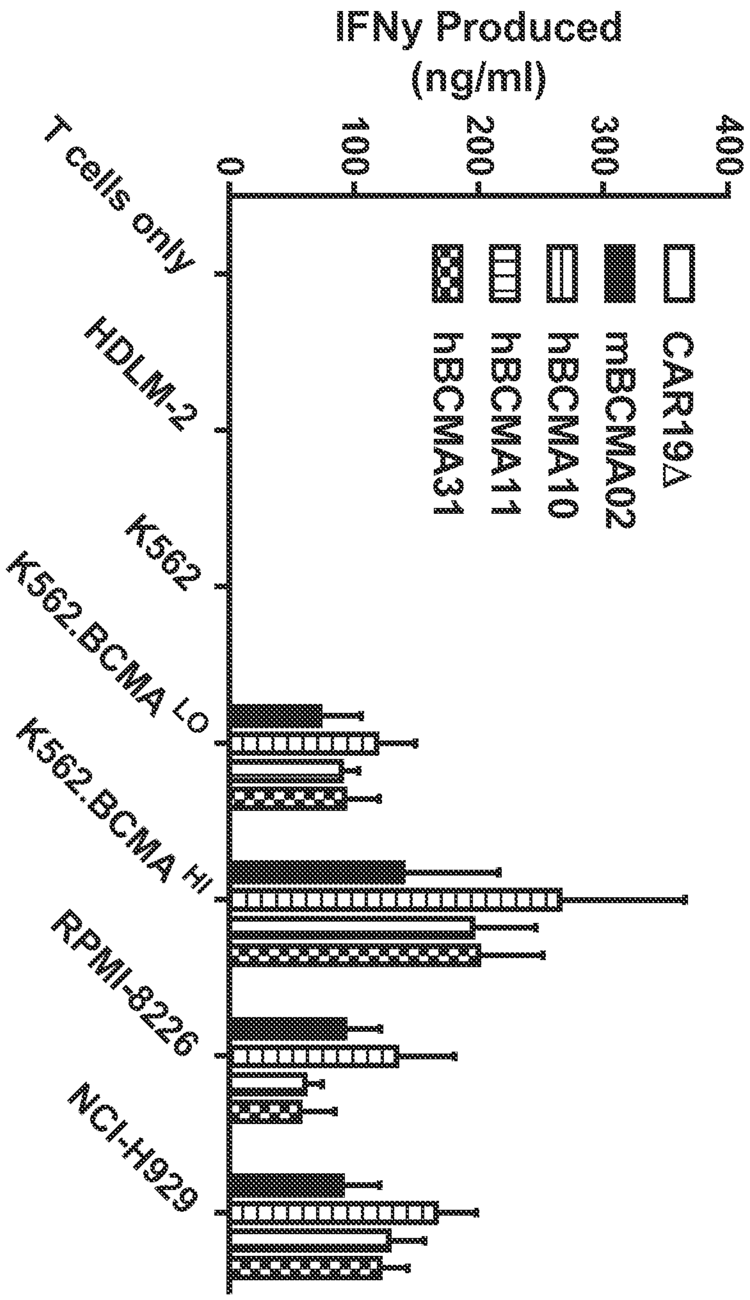

SSCa
CAR
Donor 801269 (GaM+)
Untransduced
CAR+: 2.25%
CAR19Δ
CAR+: 19.1%
mBCMA-02
CAR+: 52.2%
huBCMA-10
CAR+: 64.1%
huBCMA-11
CAR+: 57.2%
huBCMA-31
CAR+: 57.6%

FIG. 4


IFNy Produced (ng/ml)
0
100
200
300
400
CAR19Δ
mBCMA02
hBCMA10
hBCMA11
hBCMA31
T cells only
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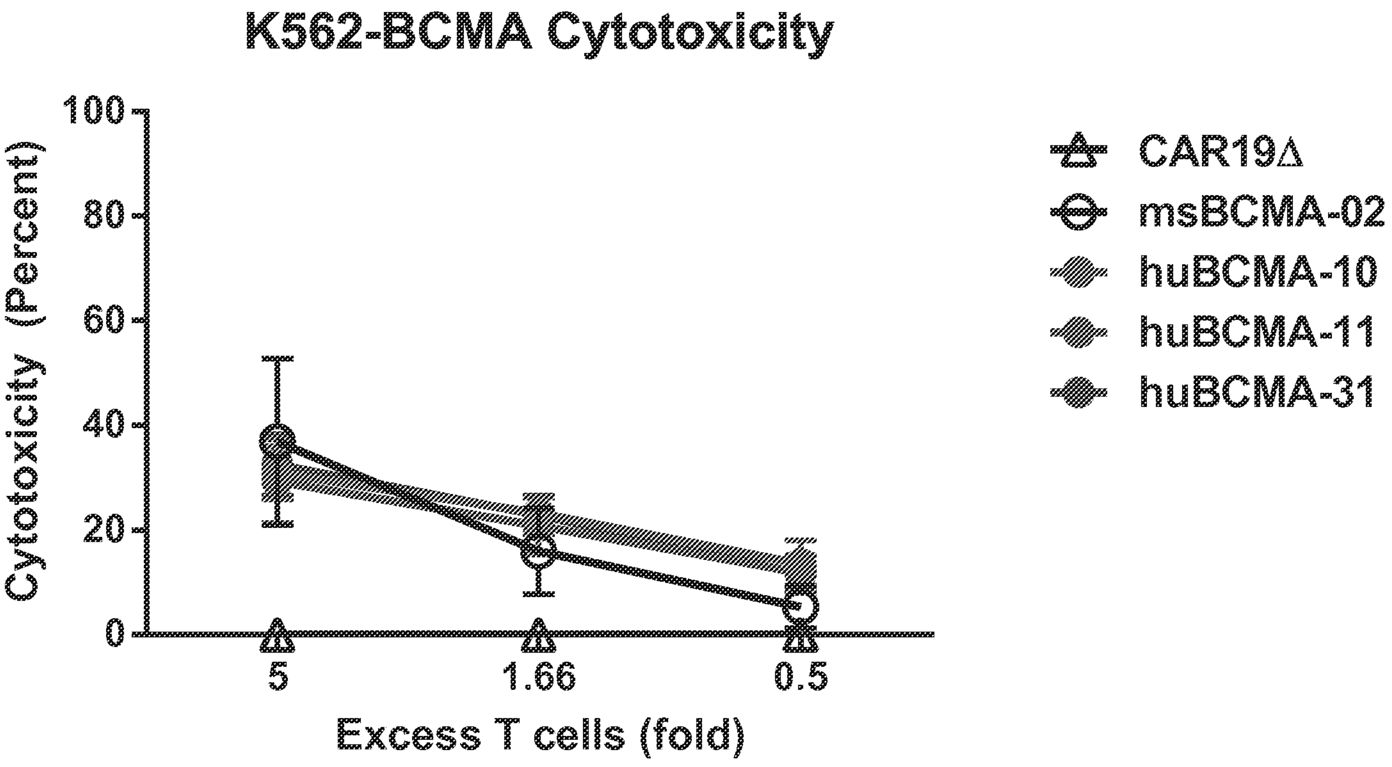
NCI-H929

*FIG. 5*



*Donors 800290, 800309, 801269

*FIG. 6*

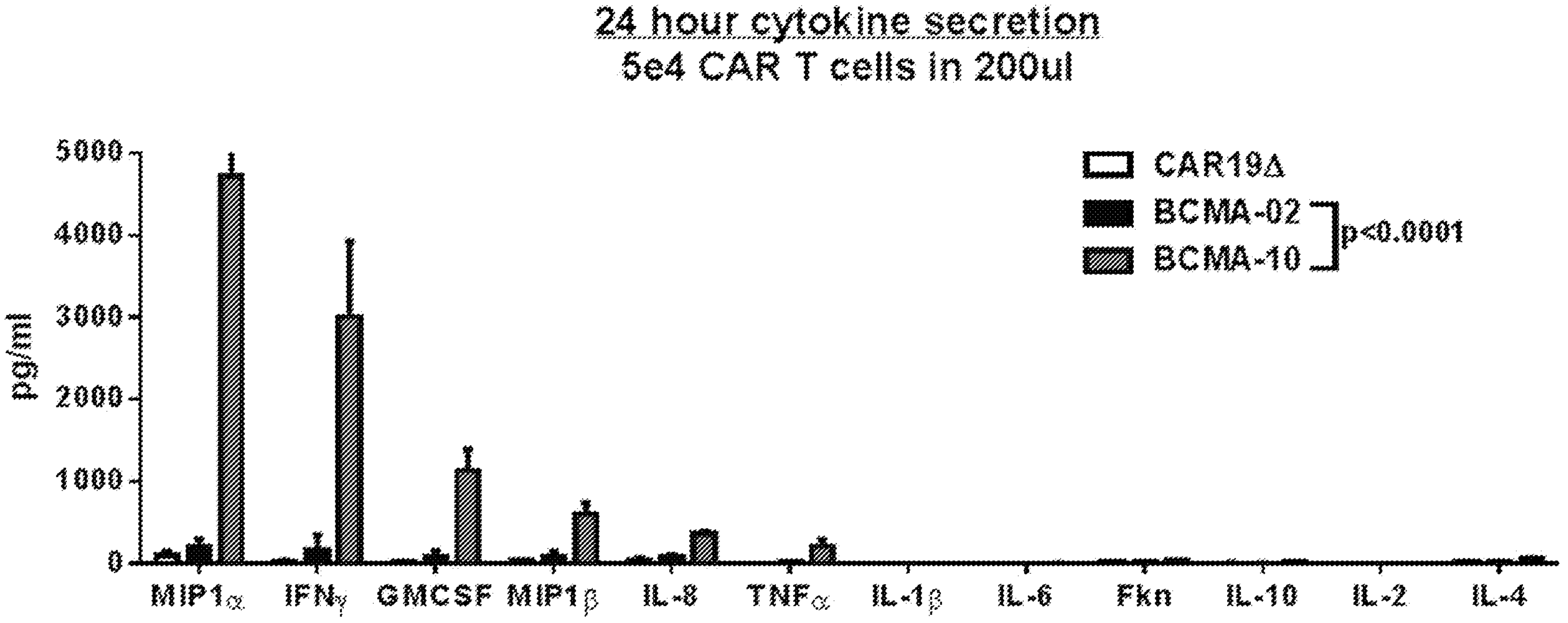

24 hour cytokine secretion
5e4 CAR T cells in 200ul
pg/ml
0
1000
2000
3000
4000
5000
CAR19Δ
BCMA-02
BCMA-10
p<0.0001
MIP1α
IFNγ
GMCSF
MIP1β
IL-8
TNFα
IL-1β
IL-6
Fkn
IL-10
IL-2
IL-4

FIG. 7

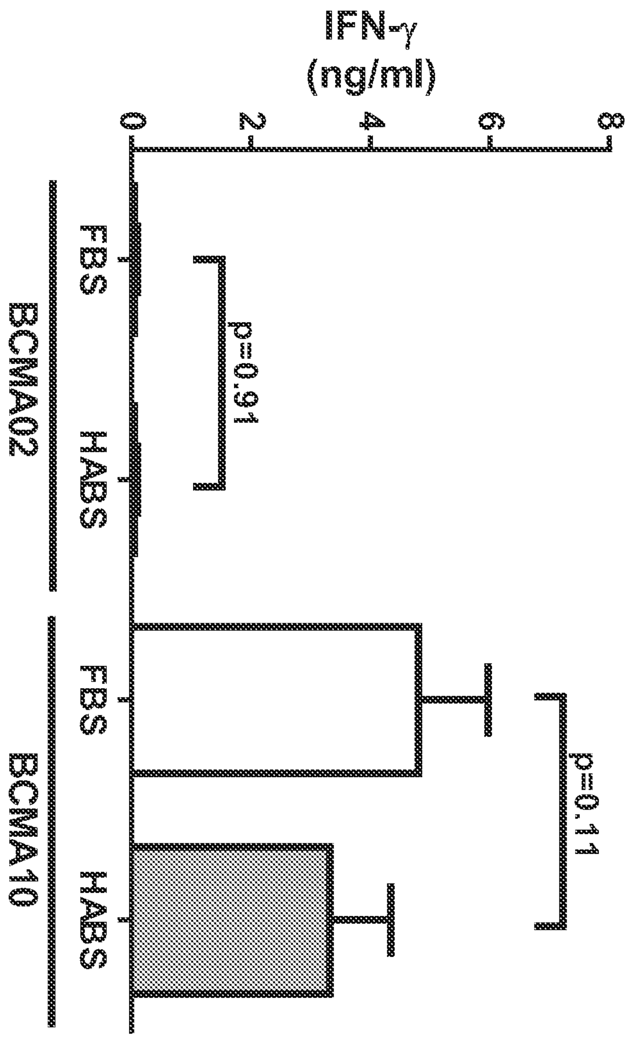
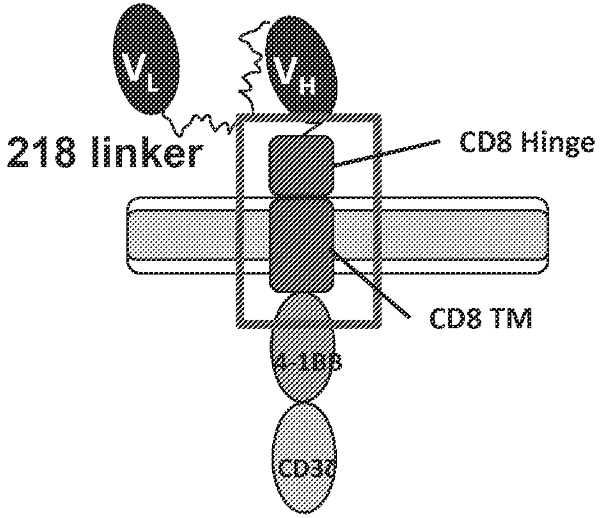
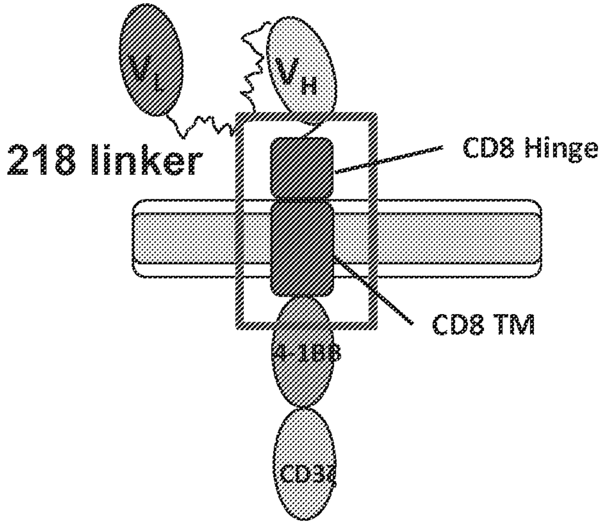


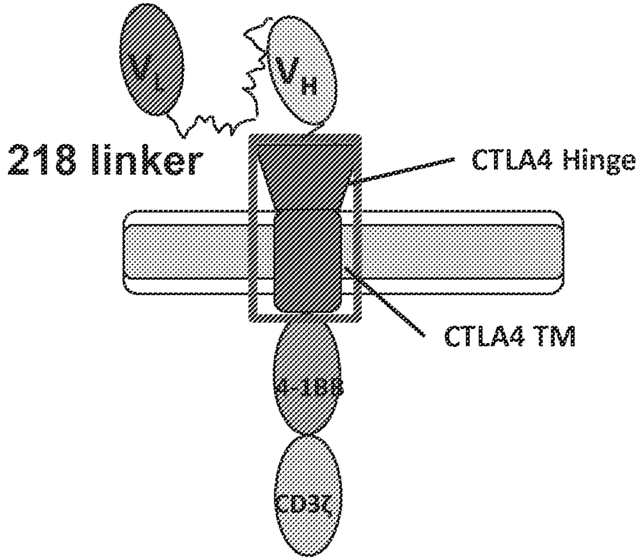
FIG. 8



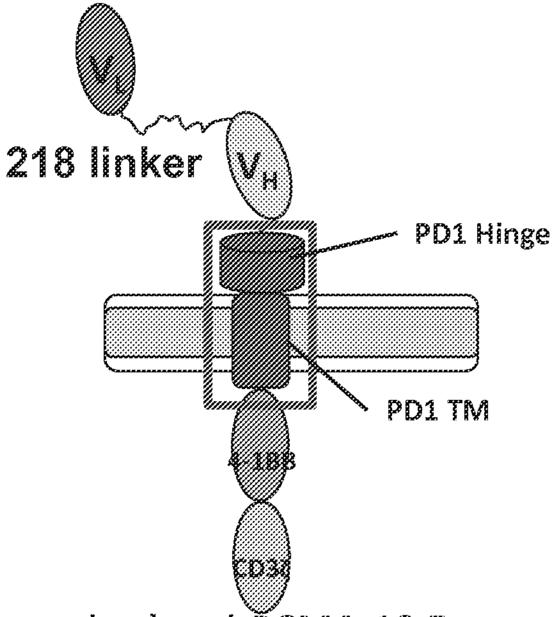
Mouse anti-BCMA-02



Humanized anti-BCMA-10



Humanized anti-BCMA-10.2



Humanized anti-BCMA-10.5

*FIG. 9*

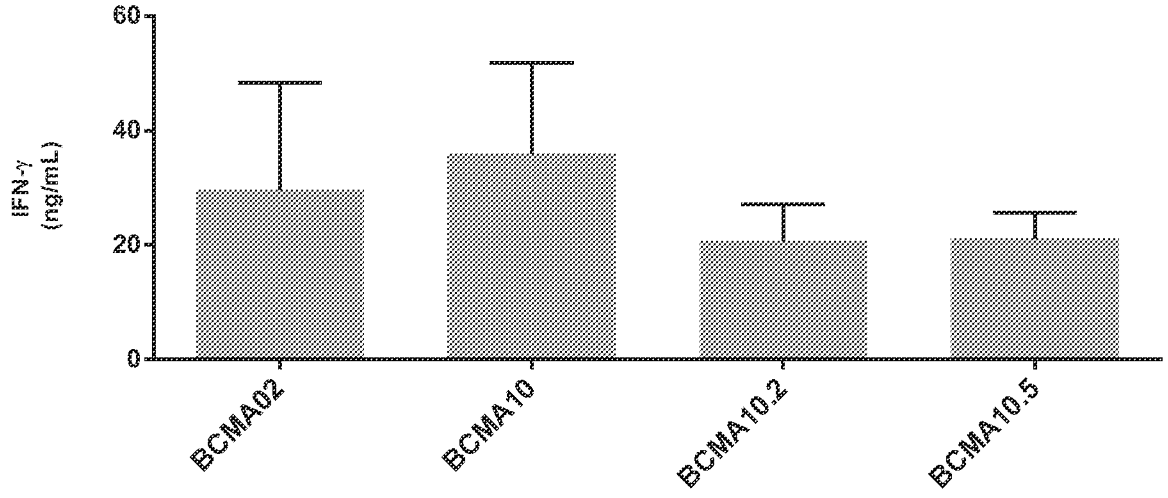


FIG. 10

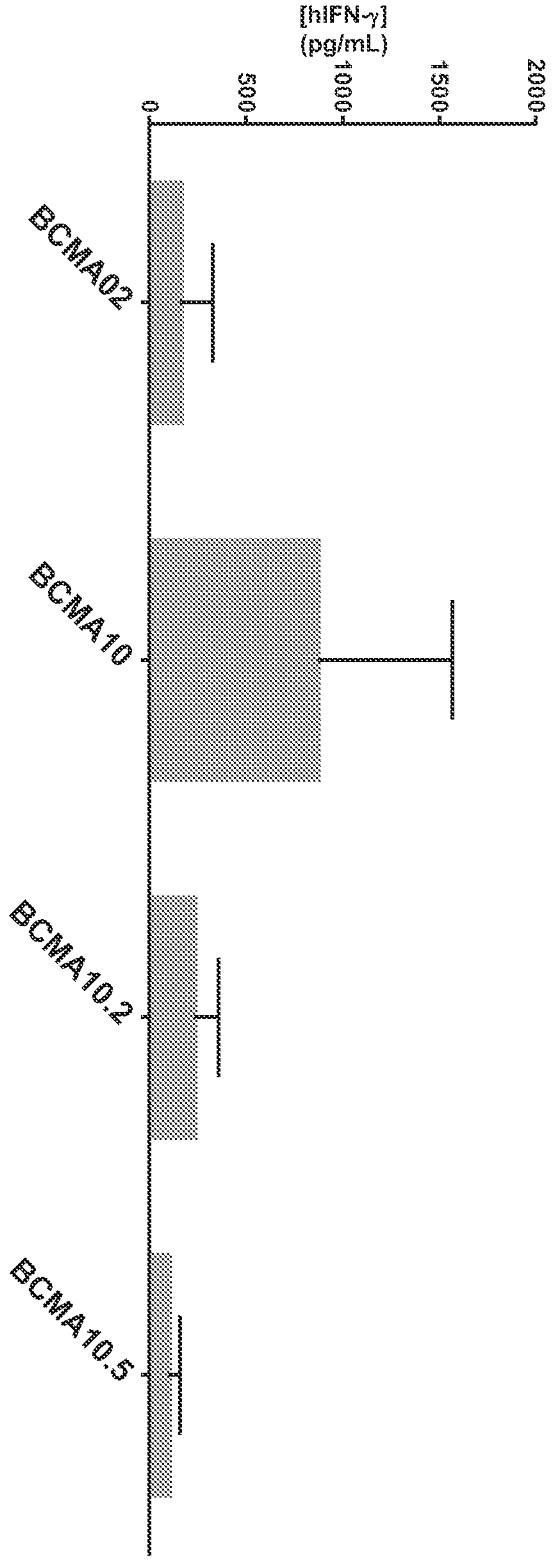

[hIFN-γ]
(pg/mL)
0
500
1000
1500
2000
BCMA02
BCMA10
BCMA10.2
BCMA10.5

FIG. 11