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(54) **SMART CAR DEVICES AND DE CAR
POLYPEPTIDES FOR TREATING DISEASE
AND METHODS FOR ENHANCING IMMUNE
RESPONSES**

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(57) **ABSTRACT**

In an aspect, the present invention relates generally to the field of treating disease with CAR devices, Smart CAR devices, DE CAR devices, and/or Smart-DE CAR devices. The present invention also relates generally to the genetic modification of cytotoxic T-lymphocytes to reduce target cell killing by apoptosis and/or increase production of lytic proteins at desired times. In an aspect, the invention relates to the use of these genetically modified T-lymphocytes and/or natural killer cells with CAR devices, Smart CAR devices, DE CAR devices, and/or Smart-DE CAR devices to enhance the immune response against a disease.

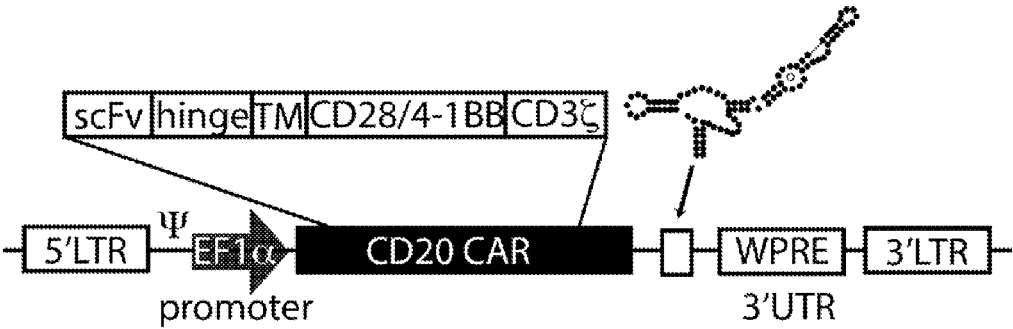


FIG. 1



FIG. 2

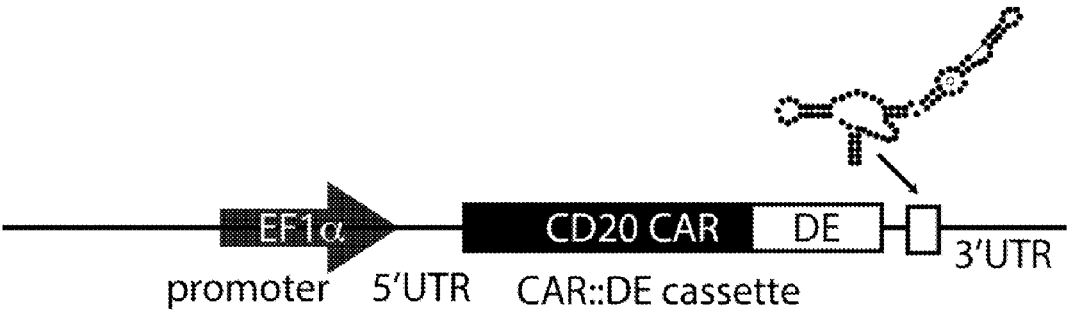


FIG. 3

**SMART CAR DEVICES AND DE CAR
POLYPEPTIDES FOR TREATING DISEASE
AND METHODS FOR ENHANCING IMMUNE
RESPONSES**

CROSS REFERENCE TO RELATED
APPLICATIONS

[0001] This application claims priority to provisional application Ser. No. 62/264,771 filed on Dec. 8, 2015, and 62/276,449 filed on Jan. 8, 2016.

REFERENCE TO SEQUENCE LISTING, TABLE
OR COMPUTER PROGRAM

[0002] The official copy of the Sequence Listing is submitted concurrently with the specification as an ASCII formatted text file via EFS-Web, with a file name of "CBI00016_ST25.txt", a creation date of Nov. 28, 2016, and a size of 40 kilobytes. The Sequence Listing filed via EFS-Web is part of the specification and is incorporated in its entirety by reference herein.

BACKGROUND OF THE INVENTION

[0003] CAR T cell therapy targets B-cell specific antigens and has been shown to be effective at inducing complete responses for acute lymphoblastic leukemia and other B-cell-related malignancies and has been shown to be effective at achieving and sustaining remissions for refractory/relapsed acute lymphoblastic leukemia (Maude et al., NEJM, 371:1507, 2014). However, dangerous side effects related to cytokine release syndrome (CRS), tumor lysis syndrome (TLS), B-cell aplasia and on-tumor, off-target toxicities have been seen in some patients.

[0004] There are currently two extant strategies to control CAR technology. The first is an inducible "kill switch." In this approach, one or more "suicide" genes that initiate apoptotic pathways are incorporated into the CAR construct (Budde et al. PLoS1, 2013 doi:10.1371/journal.pone.0082742). Activation of these suicide genes is initiated by the addition of AP1903 (also known as rimiducid), a lipid-permeable tachrolimus analog that initiates homodimerization of the human protein FKBP12 (Fv), to which the apoptosis-inducing proteins are translationally fused. In the ideal scenario, these kill switches endeavor to sacrifice the long-term surveillance benefit of CAR technology to safeguard against toxicity. However, in vivo, these suicide switches are not likely to realize this goal, as they are operating against powerful selection pressures for CAR T-cells that do not respond to AP1903, a situation worsened by the inimical error-prone retroviral copying associated with the insertion of stable transgenes into patient T-cells. In this scenario, non-responsive CAR T-cell clones will continue to proliferate and kill target cells in an antigen-dependent manner. Thus, kill switch technology is unlikely to provide an adequate safeguard against toxicity.

[0005] The second CAR regulatory approach is transient CAR expression, which can be achieved in several ways. In one approach, T-cells are harvested from unrelated donors, the HLA genes are deleted by genome-editing technology and CAR-encoding transgenes are inserted into the genome of these cells. Upon adoptive transfer, these CAR T-cells will be recognized by the recipient's immune system as being foreign and destroyed, thus the CAR exposure in this system is transient. In another transient CAR exposure

approach, mRNA of a CAR-encoding gene is introduced into harvested patient T-cells (Beatty, G L 2014. Cancer Immunology Research 2 (2): 112-20. doi:10.1158/2326-6066.CIR-13-0170). As mRNA has a short half-life and is not replicated in the cell or stably maintained, there is no permanent alteration of the CAR-expressing T-cell, thus the CAR expression and activity will be for a short period of time. However, as with the kill-switch approach, these transient CAR exposure approaches sacrifice the surveillance benefit of CARs. Additionally, with these transient systems acute toxicity can be difficult to control.

[0006] It is an object of the invention to use CAR, DE-CAR, Smart-CAR, and/or Smart-DE-CAR with immune cells (e.g., T-cells, natural killer cells, macrophages, etc.) to treat disease in a patient. It is a further object of the invention to enhance a patient's immune response to a disease by using CARs, DE-CARs, Smart-CARs, and/or Smart-DE-CARs with cytotoxic T-cells that have been genetically modified to reduce target cell killing by apoptosis.

SUMMARY OF THE INVENTION

[0007] The invention relates to CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR constructs for use in the treatment of disease. In some embodiments, the host cell for the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is modified to enhance the immune response of the patient when treated with the host cell containing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR. In some embodiments, a host cell is used with the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR because the host cell kills by cytolysis of target cells. In some embodiments, the host cell is a natural killer cell. In some embodiments, the host cell is a T-lymphocyte modified to reduce apoptosis killing via FasL when the T-lymphocyte is activated by a CAR or DE-CAR polypeptide. In some embodiments, the T-lymphocyte is genetically modified to reduce presentation of FasL. In some embodiments, the FasL locus of the host T-lymphocyte is modified to reduce or eliminate expression of functional FasL. In some embodiments, the FasL is mutated to a less active or inactive form of FasL. In some embodiments, the FasL locus of the host T-lymphocyte is knocked out using genome editing tools. In some embodiments, the FasL locus is knocked out using CRISPR/Cas9, TALEN, Zinc-Finger Nuclease, or equivalent systems.

[0008] In some embodiments, the host cell is a T-lymphocyte or a natural killer cell modified to increase killing by lytic proteins (perforin, granzymes) when the T-lymphocyte or natural killer cell is activated by a CAR or DE-CAR polypeptide. In some embodiments, the T-cell is genetically modified so that lytic proteins can be expressed at desired times. In some embodiments, nucleic acids encoding lytic proteins are operably linked to an inducible control region, an RNA control device, and/or a degron. Using this inducible control the T-lymphocytes and/or natural killer cell can be armed with granules containing the lytic proteins at a desired time. In some embodiments, the inducible control is used to increase the number of granules containing lytic proteins in the T-lymphocyte, thereby increasing target cell killing by the lytic proteins. In some embodiments, the T-lymphocytes containing the CAR or DE-CAR polypeptide are activated with cytokines (e.g., IL-2, IL-12, IL-15) that induce production of lytic proteins and formation of granules with these proteins. In some embodiments, transcription factors that upregulate expression of lytic proteins are

expressed or activated. In some embodiments, the transcription factor is T-bet and/or Eomesodermin.

[0009] In some embodiments, the T-lymphocyte is obtained from a syngeneic, allogeneic, or other nonself donor. In some embodiments, the syngeneic, allogeneic or other nonself T-lymphocyte is genetically modified by knocking out the alpha chain of the T-cell receptor whereby graft versus host reactions are reduced.

[0010] In some embodiments, the host cell is a T-lymphocyte, a natural killer cell, or a B-lymphocyte that has been genetically modified to express a polypeptide regulating proliferation and/or activation of T-lymphocytes, natural killer cells, and/or B-lymphocytes when desired. In some embodiments, the polypeptide regulating proliferation and/or activation is a cytokine. In some embodiments, the polypeptide regulating proliferation and/or activation is IL-2, IL-7 and/or IL-15. In some embodiments, polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is expressed and expands the number of T-lymphocytes, natural killer cells, or B-lymphocyte with or without a CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR, or maintains or extends the life of T-lymphocytes, natural killer cells, or B-lymphocytes with or without a CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR. In some embodiments, the T-lymphocyte, natural killer cell, or B-lymphocyte is modified so that the nucleic acids encoding the endogenous polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are under the control of a heterologous control region that is inducible. In some embodiments, a transgene is cloned into the T-lymphocyte, natural killer cell, or B-lymphocyte wherein the transgene encodes the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) under the control of an inducible control region, a RNA control device, and/or a degron.

[0011] In some embodiments, the disease is a malignancy. In some embodiments, the malignancy is a sarcoma, carcinoma, melanoma, chordoma, malignant histiocytoma, mesothelioma, glioblastoma, neuroblastoma, medulloblastoma, malignant meningioma, malignant schwannoma, leukemia, lymphoma, myeloma, myelodysplastic syndrome, and/or myeloproliferative disease. In some embodiments, the malignancy is multiple myeloma. In some embodiments, the malignancy is a CD19 and/or CD20 positive B-cell lymphoma.

[0012] In some embodiments, the disease is an autoimmune disease, such as, for example a neurological disorder (e.g., multiple sclerosis), a rheumatological disorder (e.g., rheumatoid arthritis, systemic sclerosis, systemic lupus), a hematological immunocytopenia (pure red cell aplasia, immune thrombopenia, pure white cell aplasia), or a gastrointestinal disorder (inflammatory bowel disease).

[0013] In some embodiments, the disease is an infectious disease. In some embodiments, the infectious disease is a virus, bacteria or eukaryotic pathogen or parasite. In some embodiments, the virus is a Retroviridae (e.g. human immunodeficiency viruses such as HIV-1 and HIV-LP), Picornaviridae (e.g. poliovirus, hepatitis A virus, enterovirus, human coxsackievirus, rhinovirus, and echovirus), rubella virus, coronavirus, vesicular stomatitis virus, rabies virus, ebola virus, parainfluenza virus, mumps virus, measles virus, respiratory syncytial virus, influenza virus, hepatitis B virus, parvovirus, Adenoviridae, Herpesviridae [e.g. type 1 and type 2 herpes simplex virus (HSV), varicella-zoster virus,

cytomegalovirus (CMV), and herpes virus], Poxviridae (e.g. smallpox virus, vaccinia virus, and pox virus), or hepatitis C virus. In some embodiments, the bacteria is a *Chlamydia* (*Chlamydia*), *Ehrlichia*, *Rickettsia*, *Salmonella*, *Neisseria*, *Brucella*, *Mycobacterium*, *Listeria*, *Francisella*, *Legionella*, *Yersinia*, *Nocardia*, *Rhodococcus*, and/or *Coxiella*. In some embodiments, there is provided a CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR capable of binding to an antigen found on host cells infected with an infectious pathogen (e.g., a virus, a bacteria, a protozoan, or a fungus). Examples of bacterial pathogens that may infect host cells include, *Helicobacter pylori*, *Legionella pneumophila*, a bacterial strain of *Mycobacteria* sps. (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, or *M. goodii*), *Neisseria meningitidis*, *Listeria monocytogenes*, *R. rickettsia*, *Salmonella* spp., *Brucella* spp., *Shigella* spp., or certain *E. coli* strains or other bacteria that have acquired genes encoding invasive factors. In some embodiments, the eukaryotic pathogen is a *Histoplasma*, *Cryptococcus*, *Trypanosoma*, *Apicomplexans* (e.g., *Plasmodium*), and/or *Pneumocystis*.

[0014] In some embodiments, the CAR or DE-CAR is specific for a hematopoietic antigen and its construct comprises a nucleic acid encoding a CAR (chimeric antigen receptor), optionally a nucleic acid encoding a Destabilizing Element, and/or optionally a nucleic acid encoding a RNA control device. In some embodiments, the hematopoietic antigen is found on cells of a hematopoietic malignancy, for example, a leukemia stem cell or an acute myeloid leukemia (AML) cell. In some embodiments, the leukemia stem cell antigen is, for example, CD 13, CD 25, CD 32, CD 33, CD 34, CD 38, CD 44, CD 45RA, CD 47, CD 90, CD 123, CLL-1, and/or TIM3. In some embodiments, the AML antigen is, for example, CD 33, CD 34, CD 38, CD 44, CD 45, CD 45RA, CD 47, CD 64, CD 66, CD 123, CD 133, CD 157, CLL-1, CXCR4, LeY, PR1, RHAMM (CD 168), TIM-3, and/or WT1. In some embodiments, the hematopoietic antigen is a B-cell antigen, for example, CD19 and/or CD20. In some embodiments, the hematopoietic antigen is a memory B-cell antigen, for example, CD 19, CD 21, CD 27, CD 40, and/or CD84. In some embodiments, the hematopoietic antigen is a T-cell antigen, for example, CD3 and/or CD4. In some embodiments, the hematopoietic antigen is a memory T-cell antigen, for example, CCR5, CCR7, CD11a, CD27, CD28, CD45RA, CD45RO, CD57, and/or CD62L. In some embodiments, the hematopoietic antigen is a hematopoietic stem cell antigen. For example, CD 34, CD 41, CD 45, CD 90, CD 117, CD 123, and/or CD 133. Other hematopoietic stem cell antigens include, for example, CD13, CD33, CD 44, CD 47, CD 96, Mpl, Flt3, Esam1, Robo4, and TIM3.

[0015] When the treatment utilizes a CAR and/or DE-CAR that is specific for a hematopoietic stem cell antigen, the treatment will optionally involve a transplant of hematopoietic stem cells and/or a transplant of bone marrow. In some embodiments, the transplanted hematopoietic stem cells and/or bone marrow are obtained from an autologous source (the patient), a syngeneic source (an identical twin), or an allogeneic source (a sibling, parent, or third person with similar or identical HLA markers). In these embodiments, the T-lymphocytes and/or natural killer cells expressing CAR and/or DE-CARs are used to reduce the population of hematopoietic stem cells in the patient in preparation for the transplant with hematopoietic stem cells and/or bone

marrow. Prior to or at the same time as this treatment to reduce the patients HSCs, the patient may receive a treatment for the hematopoietic disorder. For example, the patient could be treated with chemotherapy and/or radiation, or T-lymphocytes and/or natural killer cells with CARs and/or DE-CARs targeted at certain hematopoietic cells (e.g., malignant cells in a cancer, or memory cells in autoimmune diseases).

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 provides a schematic diagram of a chimeric antigen receptor-RNA control device (Smart CAR).

[0017] FIG. 2 provides a schematic diagram of a chimeric antigen receptor-Destabilizing Element (DE-CAR).

[0018] FIG. 3 provides a schematic diagram of a chimeric antigen receptor-Destabilizing Element—RNA control device (Smart-DE-CAR).

DETAILED DESCRIPTION OF THE INVENTION

[0019] Before the various embodiments are described, it is to be understood that the teachings of this disclosure are not limited to the particular embodiments described, and as such can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present teachings will be limited only by the appended claims.

[0020] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present teachings, some exemplary methods and materials are now described.

[0021] It must be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims can be drafted to exclude any optional element. As such, this statement is intended to serve as an antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation. Numerical limitations given with respect to concentrations or levels of a substance are intended to be approximate, unless the context clearly dictates otherwise. Thus, where a concentration is indicated to be (for example) 10 g, it is intended that the concentration be understood to be at least approximately or about 10 g.

[0022] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which can be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present teachings. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

DEFINITIONS

[0023] In reference to the present disclosure, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill

in the art, unless specifically defined otherwise. Accordingly, the following terms are intended to have the following meanings.

[0024] As used herein, an “antibody” is defined to be a protein functionally defined as a ligand-binding protein and structurally defined as comprising an amino acid sequence that is recognized by one of skill as being derived from the variable region of an immunoglobulin. An antibody can consist of one or more polypeptides substantially encoded by immunoglobulin genes, fragments of immunoglobulin genes, hybrid immunoglobulin genes (made by combining the genetic information from different animals), or synthetic immunoglobulin genes. The recognized, native, immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes and multiple D-segments and J-segments. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Antibodies exist as intact immunoglobulins, as a number of well characterized fragments produced by digestion with various peptidases, or as a variety of fragments made by recombinant DNA technology. Antibodies can derive from many different species (e.g., rabbit, sheep, camel, human, or rodent, such as mouse or rat), or can be synthetic. Antibodies can be chimeric, humanized, CDR grafted, or humanized. Antibodies can be monoclonal or polyclonal, multiple or single chained, fragments or intact immunoglobulins.

[0025] As used herein, an “antibody fragment” is defined to be at least one portion of an intact antibody, or recombinant variants thereof, and refers to the antigen binding domain, e.g., an antigenic determining variable region of an intact antibody, that is sufficient to confer recognition and specific binding of the antibody fragment to a target, such as an antigen. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')₂, and Fv fragments, scFv antibody fragments, linear antibodies, single domain antibodies such as sdAb (either V_L or V_H), camelid VHH domains, and multispecific antibodies formed from antibody fragments. The term “scFv” is defined to be a fusion protein comprising at least one antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked via a short flexible polypeptide linker, and capable of being expressed as a single chain polypeptide, and wherein the scFv retains the specificity of the intact antibody from which it is derived. Unless specified, as used herein an scFv may have the V_L and V_H variable regions in either order, e.g., with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise V_L-linker-V_H or may comprise V_H-linker-V_L.

[0026] As used herein, an “antigen” is defined to be a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including, but not limited to, virtually all proteins or peptides, including glycosylated polypeptides, phosphorylated polypeptides, and other post-translation modified polypeptides including polypeptides modified with lipids, can serve as an antigen. Furthermore, antigens can be

derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an “antigen” as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to encode polypeptides that elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a “gene” at all. It is readily apparent that an antigen can be synthesized or can be derived from a biological sample, or can be a macromolecule besides a polypeptide. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a fluid with other biological components.

[0027] As used herein, “antigen spreading” or “epitope spreading” refers to the development of an immune response to epitopes and/or antigens distinct from, and noncross-reactive with, the epitope and/or antigen targeted by a CAR. Diversification, or the ability of the immune system to attack multiple targets on a pathogen or diseased cell enhances the immune response to the diseased cell or pathogen.

[0028] As used herein, an “aptamer” is defined to be a nucleic acid sequence that interacts with a ligand under normal physiological conditions.

[0029] As used herein, “bone marrow transplant” is defined to be the replacement of a patient’s hematopoietic stem cells in the bone marrow with bone marrow from a donor. Bone marrow transplants may be autologous, syngeneic or allogeneic. In an autologous transplant, the patient receives their own bone marrow/stem cells. In a syngeneic transplant, the patient receives bone marrow/stem cells from an identical twin. In an allogeneic transplant, the patient receives bone marrow/stem cells from a sibling, parent, other related person, or an unrelated person.

[0030] As used herein, the terms “Chimeric Antigen Receptor” and the term “CAR” are used interchangeably. As used herein, a “CAR” is defined to be a fusion protein comprising antigen recognition moieties and cell-activation elements.

[0031] As used herein, a “CAR T-cell” or “CAR T-lymphocyte” are used interchangeably, and are defined to be a T-cell containing the capability of producing CAR polypeptide, regardless of actual expression level. For example a T-cell that is capable of expressing CARs and contains nucleic acid sequences for the expression of a CAR is a CAR T-cell.

[0032] As used herein, a “costimulatory element” or “costimulatory signaling domain” or “costimulatory polypeptide” are defined to be the intracellular portion of a costimulatory polypeptide. A costimulatory polypeptide can be represented in the following protein families: TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), and activating natural killer cell receptors. Examples of such polypeptides include CD27, CD28, 4-1BB (CD137), OX40, GITR, CD30, CD40, ICOS, BAFRR, HVEM, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3, MyD88, and the like.

[0033] As used herein, a “destabilizing element” or a “DE” or a “Degron” are used interchangeably, and are defined to be a polypeptide sequence that is inducibly resistant or susceptible to degradation in the cellular context by the addition or subtraction of a ligand, and which confers this stability modulation to a co-translated polypeptide to which it is fused in cis.

[0034] As used herein, an “effective amount” or “therapeutically effective amount” are used interchangeably, and defined to be an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result.

[0035] As used herein, an “epitope” is defined to be the portion of an antigen capable of eliciting an immune response, or the portion of an antigen that binds to an antibody. Epitopes can be a protein sequence or subsequence that is recognized by an antibody.

[0036] As used herein, an “expression vector” and an “expression construct” are used interchangeably, and are both defined to be a plasmid, virus, or other nucleic acid designed for protein expression in a cell. The vector or construct is used to introduce a gene into a host cell whereby the vector will interact with polymerases in the cell to express the protein encoded in the vector/construct. The expression vector and/or expression construct may exist in the cell extrachromosomally or integrated into the chromosome. When integrated into the chromosome the nucleic acids comprising the expression vector or expression construct will be an expression vector or expression construct.

[0037] As used herein, an “extracellular element” is defined as the antigen binding or recognition element of a Chimeric Antigen Receptor.

[0038] As used herein, a “hematopoietic cell” is defined to be a cell that arises from a pluripotent, hematopoietic stem cell. “Hematopoietic cells” include hematopoietic stem cells. This includes but is not limited to myeloid progenitor cells, lymphoid progenitor cells, megakaryocytes, erythrocytes, mast cells, myeloblasts, basophils, neutrophils, eosinophils, macrophages, thrombocytes, monocytes, natural killer cells, T lymphocytes, B lymphocytes and plasma cells.

[0039] As used herein, a “hematopoietic stem cell” is defined to be a cell capable of self-renewal, and capable of differentiation into some or all of the cell types found in the blood (e.g., myeloid cells or lymphoid cells). As used herein, hematopoietic stem cells may have one or more of the following cell surface antigens: CD 34, CD 38, CD 41, CD 45, CD 90, CD 105, CD 117, CD 123, and/or CD 133. Other hematopoietic stem cell surface antigens may include, for example, CD13, CD33, CD 44, CD 47, CD 96, and TIM3.

[0040] As used herein, a “hematopoietic stem cell transplant” is defined to be the replacement of a patient’s hematopoietic stem cells with hematopoietic stem cells from a donor. Hematopoietic stem cell transplants may be autologous, syngeneic or allogeneic. In an autologous transplant, the patient receives their own hematopoietic stem cells. In a syngeneic transplant, the patient receives hematopoietic stem cells from an identical twin. In an allogeneic transplant, the patient receives hematopoietic stem cells from a sibling, parent, other related person, or an unrelated person.

[0041] As used herein, an “information transmission element” refers to a nucleic acid that transmits information between the sensor element and the regulatory element.

[0042] As used herein, an “intracellular element” is defined as the portion of a Chimeric Antigen Receptor that resides on the cytoplasmic side of the eukaryotic cell’s cytoplasmic membrane, and transmits a signal into the eukaryotic cell. The “intracellular signaling element” is that portion of the intracellular element which transduces the effector function signal which directs the eukaryotic cell to perform a specialized function.

[0043] As used herein, a “regulatory element” is defined to be a nucleic acid that encodes the system control function of the RNA control device. The regulatory element has an RNA sequence that produces a distinct behavior in an RNA control device in response to ligand binding. Examples of regulatory elements include ribozymes, antisense RNA, RNAi, siRNA, shRNA, RNase III substrates, splicing elements, ribosome binding sites, IRES sequences, transcription terminators, attenuators, and other RNA secondary structures that can be used to regulate polypeptide expression.

[0044] As used herein, a “RNA control device” is defined to be an RNA molecule that can adopt different structures and behaviors that correspond to different gene regulatory activities.

[0045] As used herein, a “RNA virus (+) strand” is defined to be a polynucleotide of the sense strand encoding an element of an RNA virus.

[0046] As used herein, a “sensor element” is defined to be a nucleic acid that can bind a ligand. Ligand binding by the sensor element changes the activity of the regulatory element of the RNA control device.

[0047] As used herein, a “T-lymphocyte” or T-cell” is defined to be a hematopoietic cell that normally develops in the thymus. T-lymphocytes or T-cells include, but are not limited to, natural killer T cells, regulatory T cells, helper T cells, cytotoxic T cells, memory T cells, gamma delta T cells and mucosal invariant T cells.

[0048] As used herein, “transfected” or “transformed” or “transduced” are defined to be a process by which exogenous nucleic acid is transferred or introduced into a host cell. A “transfected” or “transformed” or “transduced” cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

[0049] As used herein, a “transmembrane element” is defined as the element between the extracellular element and the intracellular element. A portion of the transmembrane element exists within the cell membrane.

Destabilizing Elements

[0050] Destabilizing elements (DE) are stability-affecting polypeptides capable of interacting with a small-molecule ligand, the presence, absence, or amount of which ligand is used to modulate the stability of the DE-polypeptide of interest. In some embodiments, the polypeptide of interest is an immunomodulatory polypeptide. In some embodiments, the polypeptide of interest is a CAR. In some embodiments, binding of ligand by a DE-CAR reduces the degradation rate of the DE-CAR polypeptide in the eukaryotic cell. In some embodiments, binding of ligand by the DE-CAR increases the degradation rate of the DE-CAR in the eukaryotic cell.

[0051] In some embodiments, the DE is derived from a naturally-occurring ligand binding protein. In some embodiments, the ligand binding protein is a variant of the FKBP protein. In some embodiments, the variant FKBP polypep-

tide has one or more of the following substitutions: F15S, V24A, H25R, F36V, E60G, M66T, R71G, D100G, D100N, E102G, K105I, and L106P from SEQ ID NO: 1. In some embodiments, the variant FKBP has the polypeptide sequence of SEQ ID NOS: 2 or 3. In some embodiments, the variant FKBP polypeptide has the polypeptide TRGVE-EVAEGVLLRRRGN (SEQ ID NO: 4) fused to the C-terminus of the FKBP. In some embodiments, the variant FKBP ligand is Shield 1, or a small molecule structurally related to rapamycin. In some embodiments, binding of ligand to the variant FKBP polypeptide in the DE-CAR stabilizes the DE-CAR and reduces the degradation rate of the DE-CAR in the eukaryotic cell. In some embodiments binding of ligand to the variant FKBP polypeptide in the DE-CAR destabilizes the DE-CAR and increases the degradation rate of the DE-CAR in the eukaryotic cell. Examples of variant FKBP nucleic acids and polypeptides are described in US published patent application 20120178168 A1 published on Jul. 12, 2012, which is hereby incorporated by reference in its entirety for all purposes.

[0052] In some embodiments, the ligand binding protein is a variant of the DHFR protein. In some embodiments, the variant DHFR polypeptide has one or more of the following substitutions: H12L, H12Y, N18T, A19V, M42T, I61F, T68S, R98H, Y100I, F103L, F103S, H114R, and G121V from SEQ ID NO: 5. In some embodiments, the variant DHFR has the polypeptide sequence of SEQ ID NOS: 6 or 7. In some embodiments, the variant DHFR polypeptide has one or more of the following groups of substitutions: H12L/Y100I, H12Y/Y100I, N18T/A19V, M42T/H114R, I61F/T68S, and R98H/F103S from SEQ ID NO: 5. In some embodiments, the variant DHFR ligand is trimethoprim, or a structurally related variant of trimethoprim. In some embodiments, binding of ligand to the variant DHFR polypeptide in the DE-CAR reduces the degradation rate of the DE-CAR in the eukaryotic cell. In some embodiments binding of ligand to the variant DHFR polypeptide in the DE-CAR increases the degradation rate of the DE-CAR in the eukaryotic cell. Examples of variant DHFR nucleic acids and polypeptides are described in US published patent application 20120178168 A1 published on Jul. 12, 2012, which is hereby incorporated by reference in its entirety for all purposes.

[0053] In some embodiments, the ligand binding protein is an estrogen receptor binding domain (ERBD) with a degron fused to the C-terminus of the ERBD (a variant ERBD). In some embodiments, the ERBD is derived from SEQ ID NO: 8 or 9. In some embodiments, a “degron” is an amino acid sequence that interacts with the cellular protein degradation machinery and specifies degradation of itself and any fusion protein of which it is a part. In some embodiments, the degron may be, for example, RRRG; (SEQ ID NO: 10), the bacterial YALAA peptide (SEQ ID NO: 11), the yeast CL1 degron, RRRGN (SEQ ID NO: 12), where N is an amino acid. In some embodiments, a peptide RRRG (SEQ ID NO: 10), optionally having a fifth residue is contemplated. In some embodiments, the ligand for the variant ERBD is CMP8 (9a-(4-Chlorobenzyl)-7-hydroxy-4-[4-(2-piperidin-1-ylethoxy)phenyl]-1,2,9-,9a-tetrahydro-3H-fluoren-3-one), 4-hydroxytamoxifen, fulvestrant or raloxifene. In some embodiments, the variant ERBD has a spacer peptide between the estrogen receptor binding domain and the degron. In some embodiments, the spacer is between 2-20,

4-20, 4-18, 4-16, 4-14, 4-12, 4-10, 4-8, 6-8 or 8 amino acid residues. In some embodiments, the variant ERBD has a polypeptide having at least about 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to KHKILHRLLDSS (SEQ ID NO: 13), wherein this polypeptide is located between the spacer and the degron, or between the ERBD and the degron. Examples of variant ERBD nucleic acids, polypeptides, and ligands are described in published US patent application 20140255361, which is hereby incorporated by reference in its entirety for all purposes.

[0054] In some embodiments, the DE is derived from phototropin 1 of *Avena sativa* (AsLOV2). In some embodiments, the DE is an AsLOV2 (SEQ ID NO: 14) that has a degron attached to the C-terminus of phototropin 1 (a variant AsLOV2). In some embodiments, the degron fused to the C-terminus of phototropin 1 is RRRG (SEQ ID NO: 9) or RRRGN (SEQ ID NO: 11). In some embodiments, the variant AsLOV2 includes one or more of the amino acid substitutions: V416A, V416I, N482K, N482R, D522E, D522A, G528A, V529N, I532A, and N538E (positions 14, 80, 120, 126, 127, 130, and 136, respectively, of SEQ ID NO: 14). In some embodiments, the variant AsLOV2 is SEQ ID NOS: 15 or 16 and the variant AsLOV2 is fused to a degron at the C-terminus. When these DE's are exposed to blue light the degron at the C-terminus is exposed and degradation of the DE-polypeptide of interest is increased. In some embodiments, a variant AsLOV2 is fused with a CAR to make a DE-CAR that is regulated by blue light. Examples of variant AsLOV2 DEs are described in Bongers et al., ACS Chem. Biol. 2014, vol. 9, pp. 111-115, and Usherenko et al., BMC Systems Biology 2014, vol. 8, pp. 128-143, which are incorporated by reference in their entirety for all purposes.

[0055] Other DEs can be derived from other ligand binding polypeptides by fusing in frame a nucleic acid encoding the ligand binding polypeptide with a nucleic acid encoding a reporter. This construct is mutagenized by well-known methods, and then mutants with increased or decreased reporter activity in response to ligand binding are identified by a selection or screening. In some embodiments, variants obtained in a first round of mutagenesis and selection/screening are further mutagenized using random mutagenesis and/or creation of combinatorial libraries of the amino acid substitutions obtained in the first round of mutagenesis and/or substitution of other amino acids at the positions identified in the first round of mutagenesis. In some embodiments, the reporter polypeptide is a light emitting polypeptide such as green fluorescent polypeptide (GFP). In some embodiments, the reporter polypeptide can be used in a selection such as, for example, a reporter polypeptide that provides a cell with antibiotic resistance or the ability to grow in a certain nutrient environment or the ability to make a certain essential nutrient (e.g., the enzyme DHFR can be used in selection schemes with certain mammalian cell lines).

[0056] Other DEs can be derived from other ligand binding polypeptides using a degron as described above for ERBD. In some embodiments, a degron is fused to the C-terminus of the ligand binding polypeptide. In some embodiments, the degron is fused to the N-terminus of the ligand binding polypeptide. In some embodiments, the ligand binding polypeptide is a ligand binding domain derived from the ligand binding polypeptide, or is some other truncated form of the ligand binding polypeptide that

has the ligand binding property. In some embodiments, a nucleic acid encoding the ligand binding domain fused to a degron is fused in frame with a nucleic acid encoding a reporter. This construct is mutagenized by well-known methods, and then mutants with increased or decreased reporter activity in response to ligand binding are identified by a selection or screening. In some embodiments, variants obtained in a first round of mutagenesis and selection/screening are further mutagenized using random mutagenesis and/or creation of combinatorial libraries of the amino acid substitutions obtained in the first round of mutagenesis and/or substitution of other amino acids at the positions identified in the first round of mutagenesis.

[0057] Other ligand binding polypeptides from which variants can be made for use as DEs, include for example, enzymes, antibodies or antibody fragments or antibody fragments engineered by recombinant DNA methods with the variable domain, ligand binding receptors, or other proteins. Examples of enzymes include bromodomain-containing proteins, FKBP variants, or prokaryotic DHFR variants. Examples of receptor elements useful in making DEs include: variant ERBD, or other receptors that have ligands which are nontoxic to mammals, especially humans.

[0058] In some embodiments, the ligand(s) for the DE are selected for optimization of certain attributes for therapeutic attractiveness. These attributes include, specificity to the target DE, affinity to the DE, bioavailability, stability, commercial availability, cost, available related chemical, bio-orthogonality, or combinations thereof. In some embodiments, the ligands are permeable to the plasma membrane, or are transported across the plasma membrane of a eukaryotic cell. In some embodiments, the ligand is orally dosable to a subject. In some embodiments, the ligand is inert (a pro-ligand) and is metabolized by normal flora or the subject to produce the active ligand. In some embodiments, the ligand has a serum half-life greater than 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, 24 hours, 48 hours, 96 hours or more. In some embodiments, the ligand has a serum half-life less than 96 hours, 48 hours, 24 hours, 18 hours, 12 hours, 10 hours, 8 hours, 6 hours, 4 hours, 2 hours or 1 hour or less. In some embodiments the ligand has a serum half-life between 1 and 96 hours, between 2 and 48 hours, between 8 and 36 hours, between 10 and 28 hours, between 12 and 24 hours, between 12 and 48 hours, between 8 and 48 hours or between 16 and 18 hours. In some embodiments, the ligand can cross the blood-brain barrier. In some embodiments, the ligand is small and lipophilic. In some embodiments, the ligand cannot normally exist in human bodies or be introduced by normal diet. In some embodiments, the affinity, as measured by K_d, of the ligands to the target RNA control device is less than 500 nM, 100 nM, 50 nM, 20 nM, 10 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.5 nM, 0.1 nM or less. In some embodiments the ligand is a protein. In some embodiments, the ligand is a small molecule. In some embodiments, the ligand is a nucleic acid.

RNA Control Devices

[0059] In some embodiments, the Ribonucleic acid (RNA) control devices of the invention exhibit tunable regulation of gene expression, design modularity, and target specificity. The RNA control devices of the invention can act to rewire information flow through cellular networks and reprogram cellular behavior in response to changes in the cellular environment. In regulating polypeptide expression, the RNA

control devices of the invention can serve as synthetic cellular sensors to monitor temporal and spatial fluctuations in the levels of diverse input molecules. RNA control devices represent powerful tools for constructing ligand-controlled gene regulatory systems tailored to modulate the expression of the CAR, DE-CAR, and/or other polypeptides of the invention in response to specific effector molecules enabling RNA regulation of target CAR, DE-CAR, and/or other constructs in various living systems.

[0060] The RNA control devices of the invention may be either trans-acting or cis-acting. By trans-acting, it is meant that the RNA control device exerts its ligand-dependent activity on a molecule, e.g. another nucleic acid, that is different from the RNA control device, e.g. not linked through a phosphodiester (or equivalent) backbone linker, and even more preferably not covalently linked to the RNA control device at all. By cis-acting, it is meant that the RNA control device exerts its ligand-dependent activity on the same contiguous nucleic acid, i.e., a nucleic acid that is covalently linked to the RNA control device, e.g., through a phosphodiester (or equivalent) backbone linker.

[0061] In some embodiments, the RNA control devices of the invention comprise a regulatory element, a sensor element, and an information transmission element (ITE) that functionally couples the regulatory element and the sensor element. In some embodiments, the ITE of the subject invention is based on the strand-displacement mechanism. Such a strand-displacement mechanism uses competitive binding of two nucleic acid sequences (e.g., the competing strand and the RNA control device strand) to a general transmission region of the RNA control device (e.g., the base stem of the aptamer) to result in disruption or restoration of the regulatory element in response to ligand binding to the sensor element.

[0062] In some embodiments, the aptamer-regulated nucleic acid platform is fully modular, enabling ligand response and regulatory function (e.g., transcript targeting) to be engineered by swapping elements within the subject regulated nucleic acid. This provides a platform for the construction of tailor-made sensor element regulated nucleic acids for a variety of different ligands. Ligand binding of the sensor element in sensor-regulated nucleic acids is designed separately from the targeting capability of the regulatory element by swapping only the sensor element. Likewise, the targeting capability of the regulatory element can be designed separately from the ligand binding of the sensor element by swapping the regulatory element so that a different gene or molecule is targeted without affecting the sensor element. Thus, the subject sensor element-regulated nucleic acids present a powerful, flexible method of tailoring spatial and temporal gene expression in both natural and engineered contexts.

[0063] In some embodiments, the RNA control devices are cis-acting RNA sequences that regulate the production of cognate protein encoded by a messenger RNA (mRNA). In some embodiments RNA control devices comprise RNA with sequences that enable direct or indirect binding of a ligand. In some embodiments, binding of a ligand to the RNA control device results in the level of protein product derived from the mRNA is augmented or diminished. In some embodiments, RNA control devices comprise ribo-switches which are segments of mRNA that binds a small molecule.

[0064] An example of an RNA control device is the theophylline responsive switch, comprising an aptamer (a ligand binding component) and hammerhead ribozyme (gene regulating component) (Win and Smolke 2007 PNAS 104 (36): 14283-88, which is hereby incorporated by reference in its entirety for all purposes). Upon aptamer binding of theophylline, the ribozyme becomes inactive and enables the expression of the desired transgene. In the absence of binding of theophylline, the ribozyme self cleaves, leading to nuclease driven degradation of mRNA, inhibiting expression.

[0065] In some embodiments, the RNA control device comprises a sensor element and a regulatory element. In some embodiments the sensor element is an RNA aptamer. In some embodiments, the RNA control device comprises more than one sensor element. In some embodiments the regulatory element is a ribozyme. In some embodiments the ribozyme is a hammerhead ribozyme. In some embodiments, the ribozyme is a hairpin ribozyme, or a hepatitis delta virus (HDV) ribozyme, or a Varkud Satellite (VS) ribozyme, or a glmS ribozyme. In other embodiments the ribozyme is a ribozymes known in the art.

[0066] In some embodiments, the RNA control device is embedded within a nucleic acid that encodes a transgene. In some embodiments the transgene of interest encodes a chimeric antigen receptor or a DE-chimeric antigen receptor.

[0067] In some embodiments an RNA control device or devices are embedded within a DNA sequence. In some embodiments, the RNA control device is encoded for in messenger RNA. In some embodiments multiple RNA control devices are encoded in cis with a transgene-encoding mRNA. In some embodiments, the RNA control device is repeated. In some embodiments the nucleic acid that is used to encode the RNA control device is repeated. By including multiple RNA control devices, sensitivity and dose response may be tailored or optimized. In some embodiments multiple RNA control devices are included, with each RNA control device being specific for a different ligand. This embodiment can mitigate unintentional expression due to endogenously produced ligands that interact with the sensor element.

RNA Control Devices: Sensor Elements

[0068] In some embodiments, the sensor-regulated polynucleotides may further comprise a functional group or a functional agent, e.g., an intercalator or an alkylating agent. In some embodiments, the sensor-regulated polynucleotides may comprise synthetic or non-natural nucleotides and analogs (e.g., 6-mercaptopurine, 5-fluorouracil, 5-iodo-2'-deoxyuridine and 6-thioguanine) or may include modified nucleic acids. Exemplary modifications include cytosine exocyclic amines, substitution of 5-bromouracil, backbone modifications, methylations, and unusual base-pairing combinations. Additional analogs include at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxytriethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanaine, 1-methylinosine, 2,2-dimethylguanaine, 2-methyladenine, 2-methylguanaine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanaine, 5-methylaminomethyluracil,

5-methoxyaminomethyl-2-thiouracil; beta-D-mannosylqueosine, 5-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

[0069] In some embodiments, the sensor element comprises an aptamer that responds to ligand binding to favor an allosteric change in the regulatory element that alters the ability of the regulatory element to interact with its target molecule. Ligand binding in this embodiment switches the regulatory element from “off” to “on,” or vice versa. The sensor-element, therefore, acts as a switch that turns the activity of the RNA control device “off” and/or “on” in response to ligand binding. In some embodiments, the response of the sensor (aptamer) element to the ligand may also depend on the ligand identity and/or the amount or concentration of ligand exposed to the sensor (aptamer) element. In some embodiments, an aptamer may bind small molecules, such as drugs, metabolites, intermediates, cofactors, transition state analogs, ions, metals, nucleic acids, and toxins. Alternatively, an aptamer may bind natural and synthetic polymers, including proteins, peptides, nucleic acids, polysaccharides, glycoproteins, hormones, receptors and cell surfaces such as cell walls and cell membranes.

[0070] In some embodiments, an “aptamer” is a nucleic acid molecule, such as RNA or DNA that is capable of binding to a specific molecule with high affinity and specificity (Ellington et al., *Nature* 346, 818-22 (1990); and Tuerk et al., *Science* 249, 505-10 (1990), which are hereby incorporated by reference in their entirety for all purposes). Exemplary ligands that bind to an aptamer include, without limitation, small molecules, such as drugs, metabolites, intermediates, cofactors, transition state analogs, ions, metals, nucleic acids, and toxins. In some embodiments, aptamers may also bind natural and synthetic polymers, including proteins, peptides, nucleic acids, polysaccharides, glycoproteins, hormones, receptors and cell surfaces such as cell walls and cell membranes. In some embodiments, the binding of a ligand to an aptamer, which is typically RNA, causes or favors a conformational change in the regulatory element and alters its ability to interact with its target molecule. In some embodiments, ligand binding affects the regulatory element’s ability to mediate gene inactivation, transcription, translation, or otherwise interfere with the normal activity of the target gene or mRNA, for example.

[0071] Aptamers can be made that bind to a wide variety of molecules. Each of these aptamer molecules can be used as a modulator of gene expression using the methods of the invention. In some embodiments, organic molecules, nucleotides, amino acids, polypeptides, target features on cell surfaces, ions, metals, salts, saccharides, are used as ligands for making an aptamer that can specifically bind to the respective ligand. In some embodiments, organic dyes such as Hoechst 33258 are used as target ligands for in vitro aptamer selection (Werstuck and Green, *Science* 282:296-298 (1998), which is hereby incorporated by reference in its entirety for all purposes). In some embodiments, small organic molecules like dopamine, theophylline, sulforhodamine B, and cellobiose are used as ligands in the isolation of aptamers. In some embodiments, aptamers re isolated that

bind to antibiotics such as kanamycin A, lividomycin, tobramycin, neomycin B, viomycin, chloramphenicol and streptomycin. For a review of aptamers that recognize small molecules, see Famulok, *Science* 9:324-9 (1999), which is hereby incorporated by reference in its entirety for all purposes.

[0072] In some embodiments, the RNA control device of the invention is comprised of RNA. In other embodiments of the invention, the RNA control device can instead be composed entirely of DNA, or partially of DNA, or partially of other nucleotide analogs. In some embodiments, translation is inhibited in vivo with an RNA control device comprised of RNA. Such aptamer-regulated RNAs are preferably introduced into a cell as a DNA that encodes the RNA control device such that transcription results in the RNA control device. In some embodiments, the RNA control device itself can be introduced into a cell.

[0073] In some embodiments, the binding affinity of the aptamer for its ligand must be sufficiently strong and the structure formed by the aptamer when bound to its ligand must be significant enough so as to switch an RNA control device of the invention between “on” and “off” states. In some embodiments, the association constant for the aptamer and associated ligand is preferably such that the ligand functions to bind to the aptamer and have the desired effect at the concentration of ligand obtained upon administration of the ligand to a subject. For in vivo use, for example, the association constant should be such that binding occurs well below the concentration of ligand that can be achieved in the serum or other tissue, preferably well below the concentration of ligand that can be achieved intracellularly since cellular membranes may not be sufficiently permeable to allow the intracellular ligand concentration to approach the level in the serum or extracellular environment. In some embodiments, the required ligand concentration for in vivo use is also below that which could have undesired effects on the organism.

Ligands for RNA Control Devices

[0074] RNA control devices can be controlled via the addition of exogenous or endogenous ligands. In some embodiments, the ligands are selected for optimization of certain attributes for therapeutic attractiveness. These attributes include, specificity to the target RNA control device, affinity to the RNA control device, bioavailability, stability, commercial availability, cost, available related chemical, bio-orthogonality, or combinations thereof. In some embodiments, the ligands are permeable to the plasma membrane, or are transported across the plasma membrane of a eukaryotic cell. In some embodiments, the ligand is orally dosable to a subject. In some embodiments, the ligand is inert (a pro-ligand) and is metabolized by normal flora or the subject to produce the active ligand. In some embodiments, the ligand has a serum half-life greater than 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, 24 hours, 48 hours, 96 hours or more. In some embodiments, the ligand has a serum half-life less than 96 hours, 48 hours, 24 hours, 18 hours, 12 hours, 10 hours, 8 hours, 6 hours, 4 hours, 2 hours or 1 hour or less. In some embodiments the ligand has a serum half-life between 1 and 96 hours, between 2 and 48 hours, between 8 and 36 hours, between 10 and 28 hours, between 12 and 24 hours, between 12 and 48 hours, between 8 and 48 hours or between 16 and 18 hours. In some embodiments, the ligand can cross the blood-brain barrier. In some embodi-

ments, the ligand is small and lipophilic. In some embodiments, the ligand cannot normally exist in human bodies or be introduced by normal diet. In some embodiments, the affinity, as measured by K_d, of the ligands to the target RNA control device is less than 500 nM, 100 nM, 50 nM, 20 nM, 10 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.5 nM, 0.1 nM or less. In some embodiments the ligand is a protein. In some embodiments, the ligand is a small molecule. In some embodiments, the ligand is a nucleic acid.

[0075] In some embodiments, the ligand is a naturally occurring, secreted metabolite. For example, a ligand that is uniquely produced by a tumor, or present in the tumor microenvironment is the ligand for the sensor element and binding of this ligand to the sensor element changes the activity of the RNA control device. Thus the control device is responsive and controlled through chemical signaling or proximity to a tumor.

[0076] In some embodiments, the ligand is selected for its pharmacodynamic or ADME behavior. For example ligands may be preferentially localized to specific portions of the human anatomy and physiology. For example certain molecules are preferentially absorbed or metabolized in the gut, the liver, the kidney etc. In some embodiments the ligand is selected to demonstrate preferential pharmacodynamic behavior in a particular organ. For example, it would be useful to have a ligand that preferentially localizes to the colon for a colorectal carcinoma so that the peak concentration of the ligand is at the required site, whereas the concentrations in the rest of the body is minimized, preventing undesired, nonspecific toxicity. In some embodiments the ligand is selected to demonstrate non preferential pharmacodynamic behavior. For example, for disseminated tumors like hematological malignancies, it would be useful to have non variant concentration of the ligand throughout the body.

RNA Control Devices: Regulatory Elements

[0077] In some embodiments, the regulatory element comprises a ribozyme, or an antisense nucleic acid, or an RNAi sequence or precursor that gives rise to a siRNA or miRNA, or an shRNA or precursor thereof, or an RNase III substrate, or an alternative splicing element, or a transcription terminator, or a ribosome binding site, or an IRES, or a polyA site.

[0078] In some embodiments, the regulatory element of an RNA control device comprises an antisense sequence and acts through an antisense mechanism in modulating expression of a target gene. For instance, an RNA control device may comprise a regulatory element that comprises an antisense sequence for inhibiting expression of a target gene and an aptamer element that binds to a ligand. The binding of the ligand to the aptamer element causes a conformational change in the RNA control device that alters the ability of the antisense sequence of the regulatory element to inhibit expression of the target sequence.

[0079] In some embodiments, an RNA control device, for example, can be a component of an expression plasmid which, when transcribed in the eukaryotic cell, modulates expression of a target through the regulatory element. Alternatively, the RNA control device can be generated outside of the target cell, subsequently introduced into the target cell to modulate expression of the target. RNA control devices may be modified so that they are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for

use in RNA control devices are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Pat. Nos. 5,176,996; 5,264,564; and 5,256,775, which are hereby incorporated by reference in their entirety for all purposes). General approaches to constructing oligomers useful in antisense technology have been reviewed, for example, by van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668, which are hereby incorporated by reference in their entirety for all purposes.

[0080] In some embodiments, the regulatory element of an RNA control device comprises a regulatory element that comprises an RNAi sequence and acts through an RNAi or miRNA mechanism in modulating expression of a target gene. For instance, an RNA control device may comprise a regulatory element that comprises a miRNA or siRNA sequence for inhibiting expression of a target gene and an aptamer element that binds to a ligand. The binding of the ligand to the aptamer element causes a conformational change in the aptamer-regulated nucleic acid that alters the ability of the miRNA or siRNA sequence of the regulatory element to inhibit expression of the target sequence. In some embodiments, a regulatory element comprises a miRNA or siRNA sequence that is between about 19 nucleotides and about 35 nucleotides in length, or preferably between about 25 nucleotides and about 35 nucleotides. In some embodiments, the regulatory element is a hairpin loop that may be processed by RNase III enzymes. As used herein, the term "RNAi" means an RNA-mediated mechanism for attenuating gene expression and includes small RNA-mediated silencing mechanisms. RNA-mediated silencing mechanisms include inhibition of mRNA translation and directed cleavage of targeted mRNAs. The sequence targeted by the regulatory element can be selected from untranscribed sequences that regulate transcription of a target gene at the genomic level.

[0081] In some embodiments, an RNAi construct contains a nucleotide sequence that hybridizes under the physiologic conditions of the cell to the nucleotide sequence of at least a portion of the mRNA transcript for the gene to be inhibited (i.e., the "target" gene). The double-stranded RNA only needs to be sufficiently similar to natural RNA for its ability to mediate RNAi. Thus, the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism or evolutionary divergence. The number of tolerated nucleotide mismatches between the target sequence and the RNAi construct sequence is no more than 1 in 5 basepairs, or 1 in 10 basepairs, or 1 in 20 basepairs, or 1 in 50 basepairs. Mismatches in the center of the siRNA duplex are most critical and may abolish cleavage of the target RNA. In contrast, nucleotides at the 3' end of the siRNA strand that is complementary to the target RNA do not significantly contribute to specificity of target recognition. However, certain miRNA designs, such as the mir-30 based miRNA designs, may feature a bulge of about a few nucleotides in the middle of the guide sequence.

[0082] In some embodiments, the subject RNAi constructs are "siRNAs." These nucleic acids are between about 19-35 nucleotides in length, and even more preferably 21-23 nucleotides in length, e.g., corresponding in length to the fragments generated by nuclease "dicing" of longer double-stranded RNAs. The siRNAs are understood to recruit nuclease complexes and guide the complexes to the target

mRNA by pairing to the specific sequences. As a result, the target mRNA is degraded by the nucleases in the protein complex or translation is inhibited. In a particular embodiment, the 21-23 nucleotides siRNA molecules comprise a 3' hydroxyl group.

[0083] In some embodiments, the subject RNAi constructs are “miRNAs.” microRNAs (miRNAs) are small non-coding RNAs that direct post transcriptional regulation of gene expression through interaction with homologous mRNAs. miRNAs control the expression of genes by binding to complementary sites in target mRNAs. miRNAs are processed by nucleolytic cleavage from larger double-stranded precursor molecules. These precursor molecules are often hairpin structures of about 70 nucleotides in length, with 25 or more nucleotides that are base-paired in the hairpin. The RNase III-like enzymes Droscha and Dicer (which may also be used in siRNA processing) cleave the miRNA precursor to produce an miRNA. The processed miRNA is single-stranded and incorporates into a protein complex, termed RISC or miRNP. This RNA-protein complex targets a complementary mRNA. miRNAs inhibit translation or direct cleavage of target mRNAs. (Brennecke et al., *Genome Biology* 4:228 (2003); Kim et al., *Mol. Cells*. 19:1-15 (2005), which are hereby incorporated by reference in their entirety for all purposes).

[0084] In some embodiments, the regulatory element is a ribozyme. In some embodiments, the ribozyme self-cleaves its phosphate backbone in the presence of appropriate cofactors, such as divalent metals. The products of this intramolecular RNA cleavage yield a 2',3'-cyclic phosphate on the upstream cleavage fragment, and a 5'OH on the downstream cleavage fragment. An example of a self-cleaving ribozyme is a hammerhead ribozyme that cleaves the 3' untranslated region of the gene product mRNA. The ribozyme can be coupled to a sensor element that binds a ligand so that the ribozyme cleaves the mRNA either in the presence or absence of the ligand. Binding of the ligand can either induce ribozyme cleavage or inhibit ribozyme cleavage depending on the design of the RNA control device.

[0085] In some embodiments, the RNA control devices have multiple regulatory elements, and/or multiple sensor elements. In some embodiments, the multiple sensor elements recognize different ligands. In some embodiments, the multiple sensor elements have different effects on the regulatory element.

Chimeric Antigen Receptors

[0086] In some embodiments, chimeric antigen receptors (CARs) are fused proteins comprising an extracellular antigen-binding/recognition element, a transmembrane element that anchors the receptor to the cell membrane and at least one intracellular element. These CAR elements are known in the art, for example as described in patent application US20140242701, which is incorporated by reference in its entirety for all purposes herein. In some embodiments, the CAR of the invention is a recombinant polypeptide construct comprising at least an extracellular antigen binding element, a transmembrane element and an intracellular signaling element comprising a functional signaling element derived from a stimulatory molecule. In some embodiments, the stimulatory molecule is the zeta chain associated with the T cell receptor complex. In some embodiments, the cytoplasmic signaling element further comprises one or more functional signaling elements derived from at least one costimulatory

molecule. In some embodiments, the costimulatory molecule is chosen from 4-1BB (i.e., CD137), CD27 and/or CD28. In some embodiments, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition element, a transmembrane element and an intracellular signaling element comprising a functional signaling element derived from a stimulatory molecule. In some embodiments, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition element, a transmembrane element and an intracellular signaling element comprising a functional signaling element derived from a co-stimulatory molecule and a functional signaling element derived from a stimulatory molecule. In some embodiments, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition element, a transmembrane element and an intracellular signaling element comprising two functional signaling elements derived from one or more co-stimulatory molecule(s) and a functional signaling element derived from a stimulatory molecule. In some embodiments, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition element, a transmembrane element and an intracellular signaling element comprising at least two functional signaling elements derived from one or more co-stimulatory molecule(s) and a functional signaling element derived from a stimulatory molecule. In some embodiments, the CAR comprises an optional leader sequence at the amino-terminus (N-ter) of the CAR fusion protein. In some embodiments, the CAR further comprises a leader sequence at the N-terminus of the extracellular antigen recognition element, wherein the leader sequence is optionally cleaved from the antigen recognition element (e.g., a scFv) during cellular processing and localization of the CAR to the cellular membrane.

Chimeric Antigen Receptor—Extracellular Element

[0087] In some embodiments, the “extracellular element capable of binding to an antigen” used for the CAR of the present invention is an element comprising an oligopeptide or polypeptide that can bind to a target antigen, and includes, for example, an antigen-binding domain of an antibody and a ligand-binding domain of a receptor. In some embodiments, this element binds to and interacts with an antigen, for example, an antigen present on a cell surface of a target cell, and thereby imparts specificity to a cell expressing a CAR. Particularly useful examples of the extracellular element in the present invention include extracellular elements derived from antibodies (H chain and L chain) and variable regions of a TCR (TCR α , TCR β , TCR γ , TCR δ), CD8 α , CD8 β , CD11A, CD11B, CD11C, CD18, CD29, CD49A, CD49B, CD49D, CD49E, CD49F, CD61, CD41, and CD51. In some embodiments, the entire protein may be used effectively. In some embodiments, a domain capable of binding to an antigen or a ligand, for example, an extracellular domain of an antibody Fab fragment, an antibody variable region [V region of H chain (VH) and V region of L chain (VL)] or a receptor can be used. In some embodiments, a scFv can be used. In some embodiments, the extracellular element is selected to be from a polypeptide that is native to the species of the subject which will be administered the eukaryotic cell with the DE-CAR and/or Smart-DE-CAR. In some embodiments, a portion of a domain can be used as long as it retains the ability to bind antigen.

[0088] As described in U.S. Pat. Nos. 5,359,046, 5,686,281 and 6,103,521 (which are hereby incorporated by reference in their entirety for all purposes), the extracellular element may be obtained from any of the wide variety of extracellular elements or secreted proteins associated with ligand binding and/or signal transduction. In some embodiments, the extracellular element is part of a protein which is monomeric, homodimeric, heterodimeric, or associated with a larger number of proteins in a non-covalent complex. In some embodiments, the extracellular element may consist of an Ig heavy chain which may in turn be covalently associated with Ig light chain by virtue of the presence of CH1 and hinge regions, or may become covalently associated with other Ig heavy/light chain complexes by virtue of the presence of hinge, CH2 and CH3 domains. In the latter case, the heavy/light chain complex that becomes joined to the chimeric construct may constitute an antibody with a specificity distinct from the antibody specificity of the chimeric construct. Depending on the function of the antibody, the desired structure and the signal transduction, the entire chain may be used or a truncated chain may be used, where all or a part of the CH1, CH2, or CH3 domains may be removed or all or part of the hinge region may be removed.

[0089] In some embodiments, the extracellular element for the CAR of the present invention may be an extracellular element that binds to only one antigen or ligand, or an extracellular element that binds to two or more antigens or ligands. In some embodiments, CARs, Smart CARs, DE-CARs, and Smart-DE-CARs of the invention comprise one extracellular element or two or more extracellular elements. In some embodiments, the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR is a bispecific CAR and targets two different antigens. In some embodiments, the bispecific CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR controls an inhibitory CAR and an amplifying CAR, each addressable by different ligands. This embodiment provides positive and negative control over the activity of the eukaryotic cell. In some embodiments, the antigen-specific targeting regions of the CAR, Smart CAR, DE-CAR, and Smart-DE-CAR may be arranged in tandem and may be separated by linker peptides. In some embodiments, the antigens targeted by CAR, Smart CAR, DE-CAR, and Smart-DE-CAR may be antigens on a diseased cell (such as a cancerous B-cell) or antigens that are expressed on separate cells that each contribute to a disease. In some embodiments, the antigens targeted by the CAR, Smart CAR, DE-CAR, and Smart-DE-CAR are antigens which are either directly or indirectly involved in the disease.

[0090] In some embodiments, the extracellular element can be selected from antibodies recognizing a target antigen or molecules interacting with the antigen. Examples of antigens include a viral antigen, a bacterial (particularly, infectious bacterial) antigen, a parasite antigen, a cell surface marker on a target cell related to a certain condition (e.g. a tumor antigen), and a surface molecule of an immunity-related cell.

[0091] The extracellular element can be any polypeptide that binds to the antigen including but not limited to a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody, single chain antibodies, diabodies, and a functional fragment thereof, including but not limited to a single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (VHH) of

a camelid derived nanobody, and to an alternative scaffold known in the art to function as antigen binding domain, such as a recombinant fibronectin domain, and the like. In some embodiments, it is beneficial for the antigen binding element to be derived from the same species in which the CAR will ultimately be used. For example, for use in humans, it may be beneficial for the antigen binding element of the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR to comprise human or humanized or humanized or human chimeric residues for the antigen binding element of an antibody or antibody fragment.

[0092] A humanized antibody can be produced using a variety of techniques known in the art, including but not limited to, CDR-grafting (see, e.g., European Patent No. EP 239,400; International Publication No. WO 91/09967; and U.S. Pat. Nos. 5,225,539, 5,530,101, and 5,585,089, each of which is incorporated herein in its entirety by reference), veneering or resurfacing (see, e.g., European Patent Nos. EP 592,106 and EP 519,596; Padlan, 1991, *Molecular Immunology*, 28(4/5):489-498; Studnicka et al., 1994, *Protein Engineering*, 7(6):805-814; and Roguska et al., 1994, *PNAS*, 91:969-973, each of which is incorporated herein by its entirety by reference), chain shuffling (see, e.g., U.S. Pat. No. 5,565,332, which is incorporated herein in its entirety by reference), and techniques disclosed in, e.g., U.S. Patent Application Publication No. US2005/0042664, U.S. Patent Application Publication No. US2005/0048617, U.S. Pat. No. 6,407,213, U.S. Pat. No. 5,766,886, International Publication No. WO 9317105, Tan et al., *J. Immunol.*, 169:1119-25 (2002), Caldas et al., *Protein Eng.*, 13(5):353-60 (2000), Morea et al., *Methods*, 20(3):267-79 (2000), Baca et al., *J. Biol. Chem.*, 272(16):10678-84 (1997), Roguska et al., *Protein Eng.*, 9(10):895-904 (1996), Couto et al., *Cancer Res.*, 55 (23 Supp):5973s-5977s (1995), Couto et al., *Cancer Res.*, 55(8):1717-22 (1995), Sandhu J S, *Gene*, 150(2):409-10 (1994), and Pedersen et al., *J. Mol. Biol.*, 235(3):959-73 (1994), each of which is incorporated herein in its entirety by reference. Often, framework residues in the framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, for example improve, antigen binding. These framework substitutions are identified by methods well-known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Pat. No. 5,585,089; and Riechmann et al., 1988, *Nature*, 332:323, which are incorporated herein by reference in their entireties.)

[0093] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987), which are incorporated by reference in their entirety for all purposes). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (see, e.g., Nicholson et al.

Mol. Immun. 34 (16-17): 1157-1165 (1997); Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993), which are incorporated herein by reference in their entirety for all purposes). In some embodiments, the framework region, e.g., all four framework regions, of the heavy chain variable region are derived from a VH4₄₋₅₉ germline sequence. In one embodiment, the framework region can comprise, one, two, three, four or five modifications, e.g., substitutions, e.g., from the amino acid at the corresponding murine sequence. In one embodiment, the framework region, e.g., all four framework regions of the light chain variable region are derived from a VK3_{1.25} germline sequence. In one embodiment, the framework region can comprise, one, two, three, four or five modifications, e.g., substitutions, e.g., from the amino acid at the corresponding murine sequence.

[0094] A “humaneered” antibody refers to an engineered human antibody having a binding specificity of a reference antibody. A “humaneered” antibody for use in this invention has an immunoglobulin molecule that contains minimal sequence derived from a donor immunoglobulin. Typically, an antibody is “humaneered” by joining a DNA sequence encoding a binding specificity determinant (BSD) from the CDR3 region of the heavy chain of the reference antibody to human V_H segment sequence and a light chain CDR3BSD from the reference antibody to a human V_L segment sequence. A “BSD” refers to a CDR3-FR4 region, or a portion of this region that mediates binding specificity. A binding specificity determinant therefore can be a CDR3-FR4, a CDR3, a minimal essential binding specificity determinant of a CDR3 (which refers to any region smaller than the CDR3 that confers binding specificity when present in the V region of an antibody), the D segment (with regard to a heavy chain region), or other regions of CDR3-FR4 that confer the binding specificity of a reference antibody. Methods for humaneering are provided in US patent application publication no. 20050255552 and US patent application publication no. 20060134098 (which are incorporated by reference in their entirety for all purposes).

[0095] In some embodiments, there is provided a Smart CAR capable of binding to an antigen derived from Retroviridae (e.g. human immunodeficiency viruses such as HIV-1 and HIV-LP), Picornaviridae (e.g. poliovirus, hepatitis A virus, enterovirus, human coxsackievirus, rhinovirus, and echovirus), rubella virus, coronavirus, vesicular stomatitis virus, rabies virus, ebola virus, parainfluenza virus, mumps virus, measles virus, respiratory syncytial virus, influenza virus, hepatitis B virus, parvovirus, Adenoviridae, Herpesviridae [e.g. type 1 and type 2 herpes simplex virus (HSV), varicella-zoster virus, cytomegalovirus (CMV), and herpes virus], Poxviridae (e.g. smallpox virus, vaccinia virus, and pox virus), or hepatitis C virus.

[0096] In some embodiments, antigens specific for infectious diseases targeted by the Smart CARs of the invention include but are not limited to any one or more of anthrax toxin, CCR5, CD4, clumping factor A, cytomegalovirus, cytomegalovirus glycoprotein B, endotoxin, *Escherichia coli*, hepatitis B surface antigen, hepatitis B virus, HIV-1, Hsp90, Influenza A hemagglutinin, lipoteichoic acid, *Pseudomonas aeruginosa*, rabies virus glycoprotein, respiratory syncytial virus and TNF- α . Other antigens specific for infectious diseases will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

[0097] In some embodiments, there is provided a Smart CAR capable of binding to an antigen derived from a bacterial strain of Staphylococci, *Streptococcus*, *Escherichia coli*, *Pseudomonas*, or *Salmonella*. In some embodiments, a phagocytic immune cell is engineered with a Smart CAR specific for these or other pathogenic bacteria. Such Smart CAR engineered immune cells are useful in treating septicemia. Examples of bacterial pathogens that can be targeted by such Smart CARs include, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, Group A *Streptococcus*, Group B *Streptococcus* (*Streptococcus agalactiae*), *Streptococcus pneumoniae*, and *Clostridium tetani*. In some embodiments, there is provided a Smart CAR capable of binding to an antigen found on host cells infected with an infectious pathogen (e.g., a virus, a bacteria, a protozoan, or a fungus). Examples of bacterial pathogens that may infect host cells include, *Helicobacter pylori*, *Legionella pneumophila*, a bacterial strain of *Mycobacteria* sps. (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, or *M. goodii*), *Neisseria meningitidis*, *Listeria monocytogenes*, *R. rickettsia*, *Salmonella* spp., *Brucella* spp., *Shigella* spp., or certain *E. coli* strains or other bacteria that have acquired genes with invasive factors. Examples of viral pathogens that may infect host cells include, Retroviridae (e.g. human immunodeficiency viruses such as HIV-1 and HIV-LP), Picornaviridae (e.g. poliovirus, hepatitis A virus, enterovirus, human coxsackievirus, rhinovirus, and echovirus), rubella virus, coronavirus, vesicular stomatitis virus, rabies virus, ebola virus, parainfluenza virus, mumps virus, measles virus, respiratory syncytial virus, influenza virus, hepatitis B virus, parvovirus, Adenoviridae, Herpesviridae [e.g. type 1 and type 2 herpes simplex virus (HSV), varicella-zoster virus, cytomegalovirus (CMV), and herpes virus], Poxviridae (e.g. smallpox virus, vaccinia virus, and pox virus), or hepatitis C virus.

[0098] In some embodiments, there is provided a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR capable of binding to a tumor antigen such as any one or more of 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD21, CD22, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNTO888, CTLA-4, DR5, EGFR, EpCAM, EphA3, CD3, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HER2/neu, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-1, IgG1, L1-CAM, IL-13, IL-6, insulin-like growth factor I receptor, alpha 5 β 1-integrin, integrin α v β 3, MORAb-009, MS4A1, MUC1, mucin CanAg, N-glycolylneuraminic acid, NPC-1C, PDGF-R α , PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF β 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, 707-AP, ART-4, B7H4, BAGE, β -catenin/m, Bcr-abl, MN/C IX antibody, CAMEL, CAP-1, CASP-8, CD25, CDC27/m, CDK4/m, CT, Cyp-B, DAM, ErbB3, ELF2M, EMMPRIN, ETV6-AML1, G250, GAGE, GnT-V, Gp100, HAGE, HLA-A*0201-R170I, HPV-E7, HSP70-2M, HST-2, hTERT (or hTRT), iCE, IL-2R, IL-5, KIAA0205, LAGE, LDLR/FUT, MAGE, MART-1/melan-A, MART-2/Ski, MC1R, myosin/m, MUM-1, MUM-2, MUM-3, NA88-A, PAP, proteinase-3, p190 minor bcr-abl, Pml/RAR α ,

PRAME, PSA, PSM, PSMA, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, survivin, TPI/m, TRP-1, TRP-2, TRP-2/INT2, WT1, NY-Eso-1 or NY-Eso-B or vimentin. Other antigens specific for cancer will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

[0099] In some embodiments, antigens specific for hematopoietic stem cells are targeted by the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the invention including but not limited to any one or more of CD 34, CD 41, CD 45, CD 90, CD 117, CD 123, and/or CD 133. Other hematopoietic stem cell surface antigens that may be targeted include, for example, CD13, CD33, CD 44, CD 47, CD 96, Mpl, Flt3, Esam1, Robo4, and TIM3. Other antigens specific for hematopoietic stem cells will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

[0100] In some embodiments, antigens for acute myeloid leukemia (AML) are targeted by the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the invention including but not limited to any one or more of CD 33, CD 34, CD 38, CD 44, CD 45, CD 45RA, CD 47, CD 64, CD 66, CD 123, CD 133, CD 157, CLL-1, CXCR4, LeY, PR1, RHAMM (CD 168), TIM-3, and/or WT1. In some embodiments, the monoclonal antibody 293C3-SDIE is used for the extracellular element. (Rothfelder et al., 2015, <https://ash.confex.com/ash/2015/webprogram/Paper81121.html>) Other antigens specific for AML will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

[0101] In some embodiments, antigens for leukemia stem cells (LSC) are targeted by the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the invention including but not limited to any one or more of CD 13, CD 25, CD 32, CD 33, CD 34, CD 38, CD 44, CD 45RA, CD 47, CD 90, CD 123, CLL-1, and/or TIM3. Other antigens specific for LSC will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

[0102] In some embodiments, antigens specific for memory B-cells are targeted by the CAR, Smart CAR, DE-CAR and/or Smart-DE-CARs of the invention include but are not limited to any one or more of CD 19, CD 21, CD 27, CD 40, and/or CD84. Other antigens specific for memory B-cells will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

[0103] In some embodiments, antigens specific for memory T-cells are targeted by the CAR, Smart CAR, DE-CAR and/or Smart-DE-CARs of the invention include but are not limited to any one or more of CCR5, CCR7, CD11a, CD27, CD28, CD45RA, CD45RO, CD57, and/or CD62L. Other antigens specific for memory T-cells will be apparent to those of skill in the art and may be used in connection with alternate embodiments of the invention.

Intracellular Element

[0104] In some embodiments, the intracellular element is a molecule that can transmit a signal into a cell when the extracellular element of CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR binds to (interacts with) an antigen. In some embodiments, the intracellular signaling element is generally responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR has been

introduced. The term “effector function” refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term “intracellular signaling element” refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While the entire intracellular signaling domain can be employed, in many cases the intracellular element or intracellular signaling element need not consist of the entire domain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used as long as it transduces the effector function signal. The term intracellular signaling element is thus also meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal. Examples of intracellular signaling elements for use in the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the invention include the cytoplasmic sequences of the T cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any recombinant sequence that has the same functional capability.

[0105] In some embodiments, the intracellular signaling element of the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR can comprise the CD3-zeta signaling domain by itself or it can be combined with any other desired intracellular signaling element(s) useful in the context of a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the invention. For example, the intracellular signaling element of the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR can comprise a CD3 zeta chain portion and a costimulatory signaling element. The costimulatory signaling element refers to a portion of the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising the intracellular element of a costimulatory molecule. In some embodiments, a costimulatory molecule is a cell surface molecule other than an antigen receptor or its ligands that enhances response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. For example, CD27 costimulation has been demonstrated to enhance expansion, effector function, and survival of human CAR T-lymphocytes in vitro and augments human T-lymphocyte persistence and antitumor activity in vivo (Song et al. Blood. 2012; 119(3):696-706, which is incorporated by reference in its entirety for all purposes). In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention comprises the primary cytoplasmic signaling sequence and/or the secondary cytoplasmic signaling sequence as the intracellular element.

[0106] In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention comprises an intracellular element of a GITR as the intracellular element. The intracellular element of a GITR includes variants having the same function. The term “variant” means any variant comprising substitution, deletion or addition of one or a few to plural amino acids, provided that the variant substantially retains the same function as the original sequence possesses. An example of the intracellular element of a GITR used in the present invention includes an

intracellular domain comprising amino acid numbers 193 to 241 of a GITR (NCBI RefSeq: NP_004186.1, SEQ ID NO: 43).

[0107] For the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention, in addition to the intracellular element of a GITR, an intracellular element derived from other polypeptides can be used. Examples of other intracellular elements include cytoplasmic sequences derived from a TCR complex and a costimulatory molecule, and any variant having the same function as those sequences.

[0108] The primary cytoplasmic signaling sequence regulates primary activation of a TCR complex. The primary cytoplasmic signaling sequence that stimulates the activation may comprise a signal transduction motif known as an immunoreceptor tyrosine-based activation motif (ITAM) [Nature, vol. 338, pp. 383-384 (1989)]. On the other hand, the primary cytoplasmic signaling sequence that acts in an inhibitory way comprises a signal transduction motif known as an immunoreceptor tyrosine-based inhibition motif (ITIM) [J Immunol., vol. 162, No. 2, pp. 897-902 (1999)]. In the present invention, an intracellular element having an ITAM or an ITIM can be used.

[0109] In some embodiments, the intracellular element having an ITAM includes intracellular elements having ITAM derived from CD3 ζ , FcR γ , FcRP β , CD3 γ , CD3 δ , CD3 ϵ , CD5, CD22, CD79a, CD79b, and CD66d. Examples of the ITAM include peptides having sequences of amino acid numbers 51 to 164 of CD3 ζ (NCBI RefSeq: NP_932170.1, SEQ ID NO: 17), amino acid numbers 45 to 86 of Fc ϵ RI γ (NCBI RefSeq: NP_004097.1, SEQ ID NO: 18), amino acid numbers 201 to 244 of Fc ϵ RI β (NCBI RefSeq: NP_000130.1, SEQ ID NO: 19), amino acid numbers 139 to 182 of CD3 γ (NCBI RefSeq: NP_000064.1, SEQ ID NO: 20), amino acid numbers 128 to 171 of CD3 δ (NCBI RefSeq: NP_000723.1, SEQ ID NO: 21), amino acid numbers 153 to 207 of CD3 ϵ (NCBI RefSeq: NP_000724.1, SEQ ID NO: 22), amino acid numbers 402 to 495 of CD5 (NCBI RefSeq: NP_055022.2, SEQ ID NO: 23), amino acid numbers 707 to 847 of CD22 (NCBI RefSeq: NP_001762.2, SEQ ID NO: 24), amino acid numbers 166 to 226 of CD79a (NCBI RefSeq: NP_001774.1, SEQ ID NO: 25), amino acid numbers 182 to 229 of CD79b (NCBI RefSeq: NP_000617.1, SEQ ID NO: 26), and amino acid numbers 177 to 252 of CD66d (NCBI RefSeq: NP_001806.2, SEQ ID NO: 27), and their variants having the same function as these peptides have. The amino acid number based on amino acid sequence information of NCBI RefSeq ID or GenBank described herein is numbered based on the full length of the precursor (comprising a signal peptide sequence etc.) of each protein.

[0110] Examples of the intracellular element comprising a secondary cytoplasmic signaling sequence that can be used in the present invention include sequences derived from CD2, CD4, CD5, CD8a, CD80, CD28, CD134, CD137, ICOS, and CD154. Specific examples thereof include peptides having sequences of amino acid numbers 236 to 351 of CD2 (NCBI RefSeq: NP_001758.2, SEQ ID NO: 28), amino acid numbers 421 to 458 of CD4 (NCBI RefSeq: NP_000607.1, SEQ ID NO: 29), amino acid numbers 402 to 495 of CD5 (NCBI RefSeq: NP_055022.2, SEQ ID NO: 30), amino acid numbers 207 to 235 of CD8 α (NCBI RefSeq: NP_001759.3, SEQ ID NO: 31), amino acid numbers 196 to 210 of CD8 β (GenBank: AAA35664.1, SEQ ID NO: 32), amino acid numbers 181 to 220 of CD28 (NCBI RefSeq:

NP_006130.1, SEQ ID NO: 33), amino acid numbers 214 to 255 of CD137 (4-1BB, NCBI RefSeq: NP_001552.2, SEQ ID NO: 34), amino acid numbers 241 to 277 of CD134 (OX40, NCBI RefSeq: NP_003318.1, SEQ ID NO: 35), and amino acid numbers 166 to 199 of ICOS (NCBI RefSeq: NP_036224.1, SEQ ID NO: 36), and their variants having the same function as these peptides have.

[0111] The present invention includes a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising only an intracellular element of a GITR as the intracellular element, and a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising one or more, for example, 2 or more intracellular elements in addition to the intracellular element of a GITR. Examples include a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising an intracellular element of a GITR and an intracellular element of CD3 as the intracellular elements, and a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising an intracellular element of a GITR, an intracellular element of CD3 and an intracellular element of CD28 as the intracellular elements. In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprises two or more copies of the same intracellular element which are linked in tandem. In some embodiments, the present invention provides a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR in which an intracellular element of a GITR is arranged on a C-terminal side relative to an intracellular element of CD3 ζ , that is, a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising an intracellular element of CD3 ζ and an intracellular element of a GITR which are linked in this order from the N-terminal side. In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR are obtained by further adding an intracellular element of CD28 to the aforementioned CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR, that is, a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising an intracellular element of CD28, an intracellular element of CD3 ζ , and an intracellular element of a GITR which are linked in this order from the N-terminal side, and a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising an intracellular element of CD3 ζ , an intracellular element of a GITR, and an intracellular element of CD28 which are linked in this order from the N-terminal side. In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR has an intracellular element of a GITR arranged on a C-terminal side.

[0112] In some embodiments, a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR comprising a plurality of intracellular elements has an oligopeptide linker or a polypeptide linker inserted between the intracellular elements to link the elements. In some embodiments, the linker has a length of 2 to 10 amino acids. In some embodiments, the linker has a glycine-serine continuous sequence.

[0113] In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR expressing cell described herein can further express another agent, e.g., an agent which enhances the activity of a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR expressing cell. For example, an agent which inhibits an inhibitory molecule. Inhibitory molecules, e.g., PD1, can, in some embodiments, decrease the ability of a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, IDO, NDO, and TGFR beta. In one

embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling element described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PD1, LAG3, CTLA4, CD160, BTLA, LAIR1, TIM3, 2B4 and TIGIT, or a fragment of any of these (e.g., at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling element described herein (e.g., comprising a costimulatory element (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling element (e.g., a CD3 zeta signaling element described herein). In some embodiments, the agent comprises a first polypeptide of PD1 or a fragment thereof (e.g., at least a portion of an extracellular element of PD1), and a second polypeptide of an intracellular signaling element described herein (e.g., a CD28 signaling element described herein and/or a CD3 zeta signaling element described herein). PD1 is an inhibitory member of the CD28 family of receptors that also includes CD28, CTLA-4, ICOS, and BTLA. PD-1 is expressed on activated B cells, T cells and myeloid cells (Agata et al. 1996 *Int. Immunol* 8:765-75, which is incorporated by reference in its entirety for all purposes). Two ligands for PD1, PD-L1 and PD-L2 have been shown to downregulate T cell activation upon binding to PD1 (Freeman et al. 2000 *J Exp Med* 192:1027-34; Latchman et al. 2001 *Nat Immunol* 2:261-8; Carter et al. 2002 *Eur J Immunol* 32:634-43, which are incorporated by reference in their entirety for all purposes). PD-L1 is abundant in human cancers (Dong et al. 2003 *J Mol Med* 81:281-7; Blank et al. 2005 *Cancer Immunol. Immunother* 54:307-314; Konishi et al. 2004 *Clin Cancer Res* 10:5094, which are incorporated by reference in their entirety for all purposes). Immune suppression can be reversed by inhibiting the local interaction of PD1 with PD-L1.

Transmembrane Element and Spacer Element

[0114] The CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention comprises a transmembrane element. The transmembrane element is attached to the extracellular element of the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR. In some embodiments, a transmembrane element includes one or more additional amino acids adjacent to the transmembrane region, e.g., one or more amino acid associated with the extracellular region of the protein from which the transmembrane was derived (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the extracellular region) and/or one or more additional amino acids associated with the intracellular region of the protein from which the transmembrane protein is derived (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the intracellular region). In some embodiments, the transmembrane element is associated with one of the other elements used in the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR. In some embodiments, the transmembrane element is selected or modified by amino acid substitution to avoid binding of such elements to the transmembrane elements of the same or different surface membrane proteins, e.g., to minimize interactions with other members of the receptor complex. In some embodiments, the transmembrane element is capable of homodimerization with another CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR on the cell surface. In some embodiments, the amino acid sequence of the transmem-

brane element may be modified or substituted so as to minimize interactions with the binding elements of the native binding partner present in the same cell.

[0115] The transmembrane element may be contributed by the protein contributing the multispecific extracellular inducer clustering element, the protein contributing the effector function signaling element, the protein contributing the proliferation signaling portion, or by a totally different protein. For the most part it will be convenient to have the transmembrane element naturally associated with one of the elements. In some cases it will be desirable to employ the transmembrane element of the ζ , η or Fc ϵ R1 γ chains which contain a cysteine residue capable of disulfide bonding, so that the resulting chimeric protein will be able to form disulfide linked dimers with itself, or with unmodified versions of the ζ , η or Fc ϵ R1 γ chains or related proteins. In some embodiments, the transmembrane element will be selected or modified by amino acid substitution to avoid binding of such elements to the transmembrane elements of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. In some embodiments it will be desirable to employ the transmembrane element of ζ , η , Fc ϵ R1- γ and - β , MBI (Ig α), B29 or CD3- γ , ζ , or ϵ , in order to retain physical association with other members of the receptor complex.

[0116] In some embodiments, the transmembrane element is derived from a natural polypeptide, or may be artificially designed. Transmembrane elements derived from a natural polypeptide can be obtained from any membrane-binding or transmembrane protein. For example, a transmembrane element of a T cell receptor α or β chain, a CD3 ζ chain, CD28, CD3 ϵ , CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, or a GITR can be used. An artificially designed transmembrane element n is a polypeptide mainly comprising hydrophobic residues such as leucine and valine. In some embodiments, a triplet of phenylalanine, tryptophan and valine is found at each end of the synthetic transmembrane element. In some embodiments, a short oligopeptide linker or a polypeptide linker, for example, a linker having a length of 2 to 10 amino acids can be arranged between the transmembrane element and the intracellular element. In some embodiments, a linker sequence having a glycine-serine continuous sequence can be used.

[0117] In some embodiments, a transmembrane element having a sequence of amino acid numbers 153 to 180 of CD28 (NCBI RefSeq: NP_006130.1, SEQ ID NO: 37) can be used as the transmembrane element. In some embodiments, a transmembrane element having a sequence of amino acid numbers 162 to 183 of a GITR (NCBI RefSeq: NP_004186.1, SEQ ID NO: 38) can be used.

[0118] In some embodiments, a spacer element can be arranged between the extracellular element and the transmembrane element, or between the intracellular element and the transmembrane element. In some embodiments, a spacer element is an oligopeptide or polypeptide that serves to link the transmembrane element with the extracellular element and/or the transmembrane element with the intracellular element. In some embodiments, the spacer element comprises up to 300 amino acids, or 10 to 100 amino acids, or 25 to 50 amino acids.

[0119] In some embodiments, the spacer element has a sequence that promotes binding of a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR with an antigen and/or

enhances signaling into a cell. Examples of such amino acids include cysteine, a charged amino acid, and serine and threonine in a potential glycosylation site, and these amino acids can be used as an amino acid constituting the spacer element.

[0120] In some embodiments, the spacer element comprises amino acid numbers 118 to 178 which is a hinge region of CD8 α (NCBI RefSeq: NP_001759.3, SEQ ID NO: 39), amino acid numbers 135 to 195 of CD8 β (GenBank: AAA35664.1, SEQ ID NO: 40), amino acid numbers 315 to 396 of CD4 (NCBI RefSeq: NP_000607.1, SEQ ID NO: 41), or amino acid numbers 137 to 152 of CD28 (NCBI RefSeq: NP_006130.1, SEQ ID NO: 42). In some embodiments, the spacer element is a part of a constant region of an antibody H chain or L chain (CH1 region or CL region). In some embodiments, the spacer element may be an artificially synthesized sequence.

[0121] In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention forms a polymer, particularly, a dimer. For example, cysteine is inserted into the spacer element and/or the transmembrane element to polymerize (dimerize) the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR.

[0122] In some embodiments, a signal peptide sequence can be linked to the N-terminus of a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR nucleic acid or polypeptide. The signal peptide sequence exists at the N-terminus of many secretory proteins and membrane proteins, and has a length of 15 to 30 amino acids. Since many of the protein molecules mentioned above as the intracellular element have signal peptide sequences, the signal peptides can be used as a signal peptide for the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention.

Chimeric Antigen Receptors Coupled with Destabilizing Elements (DE-CAR)

[0123] In some embodiments of the present invention, destabilizing elements, as described above, are combined in cis with a CAR, as described above, so that the amount of the CAR polypeptide in the eukaryotic cell is under the control of the DE. This is one embodiment of the DE-CAR of the invention.

[0124] In some embodiments, destabilizing elements are selected for use with a CAR depending upon the eukaryotic cell that will host the DE-CAR, or the target of the DE-CAR, or the subject to be administered the eukaryotic cell with a DE-CAR, or a combination of the foregoing.

[0125] In some embodiments, the DE-CAR will have DE(s) that can be induced to increase polypeptide stability in the eukaryotic cell and/or the DE-CAR will have DE(s) that can be induced to increase the degradation rate of the DE-CAR polypeptide in the eukaryotic cell. In some embodiments, the DE(s) of the DE-CAR can be induced to decrease the degradation rate of DE-CAR polypeptide in the eukaryotic cell.

[0126] In some embodiments, the amount of DE-CAR in the host cell is modulated by the presence, absence and/or amount of ligand for the DE. The presence, absence and/or amount of ligand alters the degradation rate of the DE-CAR in the host cell and results in a gain, loss or maintenance of the amount of DE-CAR in the host cell. In this manner, the presence, absence and/or amount of is used to change (or maintain) the amount of DE-CAR in the cell and thus change (or maintain) the reactivity of the host cell towards the target of the DE-CAR.

Chimeric Antigen Receptors: Side-CARs

[0127] In some embodiments, the CARs, Smart CARs, DE-CAR, and/or Smart-DE-CARs of the invention are comprised of at least two parts which associate to form a functional CAR or DE-CAR. In some embodiments, the extracellular antigen binding element is expressed as a separate part from the transmembrane element, optional spacer, and the intracellular element of a CAR. In some embodiments, the separate extracellular binding element is associated with the host cell membrane (through a means other than a transmembrane polypeptide). In some embodiments, the intracellular element is expressed as a separate part from the extracellular element, transmembrane element, and optionally the spacer. In some embodiments the extracellular element and intracellular element are expressed separately and each has a transmembrane element, and optionally a spacer. In some embodiments, each part of the CAR or DE-CAR has an association element ("side-CAR") for bringing the two parts together to form a functional CAR or DE-CAR.

[0128] In some embodiments, host cells make both parts of the CAR or DE-CAR. In some embodiments, different host cells make one part of the CAR or DE-CAR. In some embodiments, one part is made ex vivo, and a host cell makes the other part of the CAR or DE-CAR. In this embodiment, and the host cell expressing one part and the ex vivo part may be administered together or separately to the subject. In some embodiments, the nucleic acids encoding one or both parts of the CAR or DE-CAR are under the control of an inducible control region, an RNA control device, and/or a degron, providing control at transcription, mRNA stability, translation, and polypeptide stability stages.

[0129] In some embodiments, the side-CAR acts in response to a small molecule, polypeptide, or other stimulus (e.g., light, heat, etc.) Upon binding with the small molecule, polypeptide, or interacting with the other stimulus, the side-CAR is able to associate with the other side-CAR element bringing together the two parts of the CAR or DE-CAR. In some embodiments, one part of the CAR or DE-CAR is membrane bound through a transmembrane polypeptide segment and the other part is not. In this embodiment, a side-CAR is attached to transmembrane portion of membrane bound part on the opposite site of the membrane from the CAR part attached to the transmembrane element. In some embodiments, the side-CAR is attached to the transmembrane element through a spacer polypeptide.

[0130] In some embodiments, the side-CAR is, for example, FK506 binding protein (FKBP), calcineurin subunit A, cyclophilin, FKBP-rapamycin associated protein, Gyrase B (gyrB), DHFR, DmrB, PYL, ABI, Cry2, CIB1, GAI and/or GID1. In some embodiments, the small molecule that interacts with the side-CAR is, for example, rapamycin, rapamycin analog, coumermycin, methotrexate, AP20187, abscisic acid, and/or gibberellin. In some embodiments, the stimulus is binding to the small molecule, or adsorption of light, for example, blue light. In some embodiments, the side-CAR is activated to react chemically with the other side-CAR.

[0131] In some embodiments, an antibody is used to associate the two side-CARs and form an active CAR and/or DE-CAR. In some embodiments, the two side-CARs shares epitopes that are bound by the antibody so that the antibody can crosslink the side-CARs together. In some embodiments, the side-CARs have different epitopes and the antibody is a bispecific antibody with one arm of the antibody binding to one of the epitopes on one of the side-CARs and

the other arm of the antibody binding to a different epitope on the other side-CAR. In some embodiments, the extracellular element does not have a side-CAR and the bispecific antibody recognizes an epitope on the extracellular element. In some embodiments, no side-CAR is used and the bispecific antibody recognizes an epitope on the extracellular element and an epitope on the extracellular side of the transmembrane element. In some embodiments, the antibody for crosslinking the side-CARs has the same specie origin as the patient. In some embodiments, the antibody is a chimeric antibody, humanized antibody, humanized antibody, or other combination antibody formed from framework regions taken from an antibody of one specie combined with all or part of the CDRs of another antibody from a different species. In some embodiments, the antibody is a human, mouse, rat, or other mammalian antibody. In some embodiments, the side-CAR and its epitope are recognized as self. In some embodiments, the bispecific antibody is made of framework regions from one species. In some embodiments, the framework regions of the bispecific antibody are human. In some embodiments, the bispecific antibody is made from two fully human antibodies. In some embodiments, the bispecific antibody is made from two antibodies from the same species.

[0132] In some embodiments, the extracellular element is not membrane associated and is free in solution. In this embodiment, the extracellular element and the host cell with the transmembrane element-intracellular element must find each other before they associate through the side-CARs and/or via an antibody. This search phase adds another level of post-translational control over CAR and/or DE-CAR activity. In some embodiments, the extracellular element is made *ex vivo* and the amount of extracellular element administered to the patient is used to control the activity of the CAR and/or DE-CAR. The half-life of the extracellular element can also be controlled by selecting a full length, or fragment of the extracellular element with the desired half-life. The extracellular element can also be modified to raise or lower its half-life. For example, the extracellular element could be glycosylated or PEGylated to increase its half-life. Controlling the half-life of the extracellular element will impact the level of control achieved by administering different doses of extracellular element during treatment. In some embodiments, the extracellular element has an Fc portion that is bound by the host-cell side-CAR when the extracellular element is bound to its epitope. In some embodiments, the host cell side-CAR is CD 16, which binds to Fc regions of the extracellular element when the extracellular element is bound to epitope.

[0133] In some embodiments, the extracellular element is associated with the host cell membrane through a tether. In some embodiments, the tether is through a glycosphosphatidylinositol (GPI) which binds to the phosphate groups of the membrane bilayer. In this embodiment, the extracellular element includes a GPI signal sequence on its C-terminal end. In some embodiments, the human GPI signal sequence is, for example:

SEQ ID NO: 44
TNATTKAAGGALQSTASLFVVSLSLLHLYS (CD24)

SEQ ID NO: 45
VSQVKISGAPTLSPSLLGLLLPAGFILVYLEF (CNTN1)

-continued

SEQ ID NO: 46
PEVRVLHSHGHSAPRLFPPLAWTVLLPLLLQLTP (EPNA1)

SEQ ID NO: 47
EAPEPIFTSNNSCSSPGGCRFLSTIPVLWRLGGS (EPNA2)

SEQ ID NO: 48
QVPKLEKSI SGTSPKREHLPLAVGIAFFLMTFLAS (EPNA3)

SEQ ID NO: 49
ESAEPSRGENAAQTTPRIPSRLLAILLFLMLLTL (EPNA5)

SEQ ID NO: 50
YAAAMSGAGPWAAWPFLSLALMLLWLLS (FOLI)

SEQ ID NO: 51
SVRGINGSISLAVPLWLLAASLLCLLSKC (LSAMP)

SEQ ID NO: 52
TTDAHPGRSVVPALLPLLAGTLLLETTATAP (PPB1)

SEQ ID NO: 53
DSEGGALPSLTCSLTPLGLALVLTWVGLGPC (RTN4R)

In this embodiment, the extracellular element also has an N-terminal signal sequence that directs the extracellular element into the endoplasmic reticulum during translation. In this embodiment, the extracellular element is tethered to the cell membrane through GPI and so the side-CAR of the extracellular element and the side-CAR of the transmembrane element—intracellular element can associate upon interaction with the appropriate small molecule, polypeptide (e.g., antibody), or physical stimuli. In addition, in this embodiment, activity of the CAR or DE-CAR can be controlled by reducing the amount of membrane tethered extracellular element using an enzyme, for example, phospholipase C which can cleave the GPI link liberating the extracellular element from the host cell. This cleavage of the GPI link reduces CAR or DE-CAR activity because the interaction between the two parts of the CAR or DE-CAR is reduced (the extracellular element is diluted by this cleavage). In some embodiments, the activity of the enzyme, e.g., phospholipase C, is regulated. In some embodiments, the enzyme activity is regulated by a competitive inhibitor, e.g., inhibitors of phospholipase c are commercially available from Sigma-Aldrich and Santa Cruz Biotechnology.

Lymphocyte Expansion Molecule

[0134] In some embodiments, a lymphocyte expansion molecule (“LEM”) is used in the invention. Examples of LEMs include, but are not limited to mouse LEM or the BC05111 gene as found at Uniprot or NCBI, human LEM or the C1ORF 177 gene as found at Uniprot or NCBI, and other polypeptides having homology to human or mouse LEM and the activity of LEM. Nucleic acids encoding these LEMs are also part of this aspect of the invention.

[0135] In some embodiments, the LEM is combined with a DE to regulate the expression of the LEM. When a DE is used, it is operably placed in *cis* to the LEM, so the amount of LEM polypeptide in the eukaryotic cell is under the control of the DE.

[0136] In some embodiments, destabilizing elements are selected for use with a LEM depending upon the eukaryotic cell that will host the DE-LEM, or the target cell of the DE-LEM, or the subject to be administered the eukaryotic cell with a DE-LEM, or a combination of the foregoing.

[0137] In some embodiments, one or more DEs with the same or different ligands are placed in cis with the LEM. In some embodiments, some or all of the DEs can be induced to increase the amount of DE-LEM polypeptide in the eukaryotic cell and/or some or all of the DE(s) can be induced to decrease the amount of DE-LEM polypeptide in the eukaryotic cell. In some embodiments, the ligands for the different DEs can be added in a coordinated fashion to produce different amounts of DE-LEM polypeptide in the eukaryotic cell over time. In some embodiments, the ligands for the different DEs can be added in a coordinated fashion to produce a constant or stable amount of DE-LEM polypeptide in the eukaryotic cell over a period of time.

[0138] In some embodiments, binding of ligand to the DE of the DE-LEM induces a change in the conformation of the DE-LEM that increases or decreases the degradation rate of the DE-LEM polypeptide in the eukaryotic cell.

[0139] In some embodiments of the present invention, RNA control devices, as described above, are combined in cis with a LEM, as described above, so that the expression level of the LEM is under the control of the RNA control device. In some embodiments of the present invention, the RNA control device operates in trans to the LEM so that the expression level of the LEM is under the control of the RNA control device.

[0140] In some embodiments, RNA control devices are selected for use with a LEM depending upon the eukaryotic cell that will host the Smart LEM, or the target cell of the Smart LEM, or the subject to be administered the eukaryotic cell with a Smart LEM, or a combination of the foregoing.

[0141] In some embodiments, the Smart LEM will have regulatory element(s) that can be induced to increase polypeptide expression in the eukaryotic cell and/or the Smart LEM will have regulatory element(s) that can be induced to decrease polypeptide expression in the eukaryotic cell. In some embodiments, the ligands for the different RNA control devices can be added in a coordinated fashion to produce different amounts of LEM polypeptide in the eukaryotic cell over time. In some embodiments, the ligands for the different RNA control devices can be added in a coordinated fashion to maintain a constant or stable amount of LEM polypeptide in the eukaryotic cell over a period of time.

[0142] The regulatory element of the Smart LEM may operate through any of the means described above (e.g., ribozyme activity, antisense, RNAi, secondary structure sequestration of mRNA structures, etc.). Binding of ligand to the sensor element of the Smart LEM RNA induces a change in the conformational equilibria of the Smart LEM which increase or decrease translation of the LEM RNA into LEM polypeptide.

[0143] In some embodiments, the LEM is under the control of both DE(s) and RNA control device(s). In some embodiments, some or all of the DEs and some or all of the RNA control devices will have regulatory element(s) that can be induced to increase the amount of LEM polypeptide in the eukaryotic cell and/or some or all of the DEs and some or all of the RNA control devices will have regulatory element(s) that can be induced to decrease the amount of LEM polypeptide in the eukaryotic cell. In some embodiments, the ligands for the different DEs and RNA control devices can be added in a coordinated fashion to produce different amounts of DE-LEM polypeptide in the eukaryotic cell over time. In some embodiments, the ligands for the different DEs and RNA control devices can be added in a

coordinated fashion to maintain a constant or stable amount of DE-LEM polypeptide in the eukaryotic cell over a period of time.

Eukaryotic Cells

[0144] In the present invention, various eukaryotic cells can be used as the eukaryotic cell of the invention. In some embodiments, the eukaryotic cells of the invention are animal cells. In some embodiments, the eukaryotic cells are mammalian cells, such as mouse, rat, rabbit, hamster, porcine, bovine, feline, or canine. In some embodiments, the mammalian cells are cells of primates, including but not limited to, monkeys, chimpanzees, gorillas, and humans. In some embodiments, the mammalian cells are mouse cells, as mice routinely function as a model for other mammals, most particularly for humans (see, e.g., Hanna, J. et al., *Science* 318:1920-23, 2007; Holtzman, D. M. et al., *J Clin Invest.* 103(6):R15-R21, 1999; Warren, R. S. et al., *J Clin Invest.* 95: 1789-1797, 1995; each publication is incorporated by reference in its entirety for all purposes). Animal cells include, for example, fibroblasts, epithelial cells (e.g., renal, mammary, prostate, lung), keratinocytes, hepatocytes, adipocytes, endothelial cells, and hematopoietic cells. In some embodiments, the animal cells are adult cells (e.g., terminally differentiated, dividing or non-dividing) or embryonic cells (e.g., blastocyst cells, etc.) or stem cells. In some embodiments, the eukaryotic cell is a cell line derived from an animal or other source.

[0145] In some embodiments, the eukaryotic cell is a cell found in the circulatory system of a mammal, including humans. Exemplary circulatory system cells include, among others, T-lymphocytes, natural killer cells, B-cells, macrophages, neutrophils, or the like, and precursor cells of the same. As a group, these cells are defined to be circulating eukaryotic cells of the invention. In some embodiments, the eukaryotic cells are derived from any of these circulating eukaryotic cells. The present invention may be used with any of these circulating cells or eukaryotic cells derived from the circulating cells. In some embodiments, the eukaryotic cell is a T-lymphocyte or T-lymphocyte precursor or progenitor cell. In some embodiments, the eukaryotic cell is a helper T-lymphocyte, a cytotoxic T-lymphocyte, a memory T-lymphocyte, a regulatory T-lymphocyte, a natural killer T-lymphocyte, a mucosal associated invariant T-lymphocyte, a gamma delta T lymphocyte, or a precursor or progenitor cell to the aforementioned. In some embodiments, the eukaryotic cell is a natural killer cell, or a precursor or progenitor cell to the natural killer cell. In some embodiments, the eukaryotic cell is a B-cell, or a B-cell precursor or progenitor cell.

[0146] In some embodiments, a source of cells is obtained from a subject. The subject may be any living organisms. In some embodiments, the cells are derived from cells obtained from a subject. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. In some embodiments, T-lymphocytes can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In some embodiments, any number of T-lymphocyte lines available in the art, may be used. In some embodiments, T-lymphocytes can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as

Ficoll separation. In some embodiments, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T-lymphocytes, monocytes, granulocytes, B-cells, other nucleated white blood cells, red blood cells, and platelets. In some embodiments, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In an alternative aspect, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Initial activation steps in the absence of calcium can lead to magnified activation.

[0147] In some embodiments, the host T-lymphocytes are modified to reduce apoptosis mediated killing of target cells by CAR or DE-CAR T-cells. For example, the host T-cells can be genetically modified to knock-out FasL which will prevent the CAR or DE-CAR T-cell from killing target cells by FasL mediated apoptosis. In some embodiments, FasL is knocked out using the CRISPR/Cas9, TALEN, Zinc-Finger Nuclease, or equivalent systems (e.g., Cong et al. *Science* 339.6121 (2013): 819-823, Li et al., *Nucl. Acids Res* (2011): gkr188, Gaj et al. *Trends in Biotechnology* 31.7 (2013): 397-405, all of which are incorporated by reference in their entirety for all purposes). In some embodiments, double allele knockouts of the FasL gene can be obtained using a dual antibiotic resistant selection with Cas9 as described in Park et al., *PLoS One* 9:e95101 (2014), which is incorporated by reference in its entirety for all purposes, or the Cas9 gRNA approach as described in Zhang et al., *Methods* 69:171-178 (2014), which is incorporated by reference in its entirety for all purposes. In some embodiments, double allele knock outs are obtained using a Cas9 system with multiple gRNAs targeted to the FasL gene of the host T-cell. These host T-lymphocytes with a double knockout of FasL are then used as host cells for the CAR, Smart-CAR, DE-CAR, and/or Smart-DE-CAR constructs of the invention.

[0148] In some embodiments, the T-lymphocytes or natural killer cells are engineered to express lytic proteins at desired times. In some embodiments, the lytic proteins are perforin, granzyme A, granzyme B and granzyme K, or one or more of these lytic proteins. In some embodiments, the lytic protein is perforin. In some embodiments, the nucleic acid encoding the lytic protein is operably linked to an inducible control region, an RNA control device, and/or a degen. In these embodiments, the transcription control, RNA processing control, or polypeptide stability control is used to provide expression of the lytic protein, and its subsequent inclusion in granules at a desired time. For example, the lytic proteins can be expressed ex vivo in preparation for administration to a patient so that the administered T-lymphocytes and/or natural killer cells have sufficient granules of lytic proteins to kill target cells. In another example, the lytic proteins can be expressed when the T-lymphocytes and/or natural killer cells are at the disease site so as to recharge the cells granules for a subsequent round of target cell killing. In some embodiments, the T-lymphocyte and/or natural killer cell is engineered to express cytokines that induce lytic protein expression at a desired time. In this embodiment, the T-lymphocyte and/or natural killer cell is engineered as described below to express cytokines such as, for example, IL-2, IL-12 and/or

IL-15 at a desired time. In some embodiments, the T-lymphocyte and/or natural killer cell is engineered to express a transcription factor that induces lytic protein expression at a desired time. In some embodiments, the transcription factor is T-bet and/or Eomesodermin.

[0149] In some embodiments, a source of cells is obtained from an allogeneic or other nonself donor. In some embodiments, allogeneic or nonself T-lymphocytes are obtained from the donor using any of the methods described above. In some embodiments, the allogeneic or nonself T-lymphocytes can be used directly as host cells for the CAR, Smart-CAR, DE-CAR, and/or Smart-DE-CAR constructs of the invention. In some embodiments, the allogeneic or nonself T-lymphocytes are modified to reduce graft versus host reactions. For example, the allogeneic or nonself T-lymphocytes can be genetically modified to knock-out the alpha chain of the T-lymphocyte receptor. Knock out of the alpha chain of the T-lymphocyte receptor inhibits the ability of the modified T-lymphocyte to recognize nonself and so can reduce graft versus host reactions from the allogeneic or nonself CAR, Smart-CAR, DE-CAR, and/or Smart-DE-CAR T-cells. In some embodiments, the alpha chain is knocked out using the CRISPR/Cas9, TALEN, Zinc-Finger Nuclease, or equivalent systems (e.g., Cong et al. *Science* 339.6121 (2013): 819-823, Li et al., *Nucl. Acids Res* (2011): gkr188, Gaj et al. *Trends in Biotechnology* 31.7 (2013): 397-405, all of which are incorporated by reference in their entirety for all purposes). In some embodiments, double allele knockouts of the alpha gene can be obtained using a dual antibiotic resistant selection with Cas9 as described in Park et al., *PLoS One* 9:e95101 (2014), which is incorporated by reference in its entirety for all purposes, or the Cas9 gRNA approach as described in Zhang et al., *Methods* 69:171-178 (2014), which is incorporated by reference in its entirety for all purposes. In some embodiments, double allele knock outs are obtained using a Cas9 system with multiple gRNAs targeted to the alpha chain gene of the allogeneic or nonself T-lymphocyte. These allogeneic or nonself T-lymphocytes with a double knockout of the alpha chain of the T-lymphocyte receptor are then used as host cells for the CAR, Smart-CAR, DE-CAR, and/or Smart-DE-CAR constructs of the invention.

[0150] In some embodiments, the host T-lymphocyte, host natural killer cell, or host B-lymphocyte for the CAR, Smart-CAR, DE-CAR, and/or Smart-DE-CAR is engineered to express a polypeptide regulating proliferation and/or activation of T-lymphocytes, natural killer cells, and/or B-lymphocytes when desired. In some embodiments, the polypeptide regulating proliferation and/or activation is a cytokine. In some embodiments, the polypeptide regulating proliferation and/or activation is IL-2, IL-7 and/or IL-15. In some embodiments, the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are heterologous to the T-lymphocyte, B-lymphocyte, and/or natural killer cell. In some embodiments, the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are from the same species as the T-lymphocyte, B-lymphocyte, and/or natural killer cell. In some embodiments the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are mammalian. In some embodiments, the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are human, mouse, rat, dog, or cat. In some embodiments, the polypeptide regulating proliferation and/or activation (e.g.,

IL-2, IL-7, and/or IL-15) is/are from different sources. In some embodiments, the endogenous genes in the T-lymphocyte, B-lymphocyte, or natural killer cell encoding the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7 and/or IL-15) are placed under the control of an inducible control region by using CRISPR/Cas9, TALEN, Zinc-Finger Nuclease, or equivalent systems with appropriate guide sequences and the desired control region. In this embodiment, the gene editing system inserts nucleic acids encoding the desired control region upstream of the endogenous nucleic acids encoding the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15). In some embodiments, a transgene encoding the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7 and/or IL-15) is/are placed under the control of an inducible control region, and/or a degron is fused at an appropriate location to the coding sequence of the polypeptide regulating proliferation and/or activation, and/or a RNA control device is fused at an appropriate location to the coding sequence of the polypeptide regulating proliferation and/or activation. In some embodiments, the transgene, DE-transgene, Smart-transgene, and/or Smart-DE-transgene is engineered into the host T-lymphocyte, B-lymphocyte, or natural killer cell. This controllable endogenous gene, transgene, DE-transgene, Smart-transgene, and/or Smart-DE-transgene provides for expression of the polypeptide, (e.g., IL-2, IL-7 and/or IL-15) at times when it is desired, for example, to maintain or extend the life of the host cell with the CAR, Smart-CAR, DE-CAR, and/or Smart-DE-CAR. In some embodiments, the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are expressed and the number of T-lymphocytes, B-lymphocytes, and/or natural killer cells is increased.

[0151] Enrichment of a T-lymphocyte population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. In some embodiments, cells are enriched by cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry using a cocktail of monoclonal antibodies directed to cell surface markers present on the cells. For example, to enrich for CD4+ cells, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD1 b, CD16, HLA-DR, and CD8. In some embodiments, it may be desirable to enrich for regulatory T-lymphocytes which typically express CD4+, CD25+, CD62Lhi, GITR+, and FoxP3+. Alternatively, in certain aspects, T regulatory cells are depleted by anti-C25 conjugated beads or other similar method of selection.

[0152] T-lymphocytes may be activated and expanded generally using methods as described, for example, in U.S. Pat. Nos. 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005, each of which is incorporated by reference in its entirety for all purposes.

[0153] In some embodiments, natural killer cells may be expanded in the presence of a myeloid cell line that has been genetically modified to express membrane bound IL-15 and 4-1BB ligand (CD137L). A cell line modified in this way which does not have MHC class I and II molecules is highly susceptible to natural killer cell lysis and activates natural killer cells. For example, K562 myeloid cells can be transduced with a chimeric protein construct consisting of human

IL-15 mature peptide fused to the signal peptide and transmembrane domain of human CD8 α and GFP. Transduced cells can then be single-cell cloned by limiting dilution and a clone with the highest GFP expression and surface IL-15 selected. This clone can then be transduced with human CD137L, creating a K562-mb 15-137L cell line. To preferentially expand natural killer cells, peripheral blood mononuclear cell cultures containing natural killer cells are cultured with a K562-mb 15-137L cell line in the presence of 10 IU/mL of IL-2 for a period of time sufficient to activate and enrich for a population of natural killer cells. This period can range from 2 to 20 days, preferably about 5 days. Expanded natural killer cells may then be transduced with the anti-CD 19-BB-t chimeric receptor.

Nucleic Acids

[0154] In some embodiments, the present invention relates to the nucleic acids that encode, at least in part, the individual peptides, polypeptides, proteins, and RNA control devices of the present invention. In some embodiments, the nucleic acids may be natural, synthetic or a combination thereof. The nucleic acids of the invention may be RNA, mRNA, DNA or cDNA.

[0155] In some embodiments, the nucleic acids of the invention also include expression vectors, such as plasmids, or viral vectors, or linear vectors, or vectors that integrate into chromosomal DNA. Expression vectors can contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of cells. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria. In eukaryotic host cells, e.g., mammalian cells, the expression vector can be integrated into the host cell chromosome and then replicate with the host chromosome. Similarly, vectors can be integrated into the chromosome of prokaryotic cells.

[0156] Expression vectors also generally contain a selection gene, also termed a selectable marker. Selectable markers are well-known in the art for prokaryotic and eukaryotic cells, including host cells of the invention. Generally, the selection gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. In some embodiments, an exemplary selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Other selectable markers for use in bacterial or eukaryotic (including mammalian) systems are well-known in the art.

[0157] An example of a promoter that is capable of expressing a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR transgene in a mammalian T cell is the EF1 α promoter. The native EF1 α promoter drives expression of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. The EF1 α promoter has been extensively used in

mammalian expression plasmids and has been shown to be effective in driving CAR expression from transgenes cloned into a lentiviral vector. See, e.g., Milone et al., *Mol. Ther.* 17(8): 1453-1464 (2009), which is incorporated by reference in its entirety for all purposes. Another example of a promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the elongation factor-1a promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention is not limited to the use of constitutive promoters.

[0158] Inducible promoters are also contemplated as part of the invention. Examples of inducible promoters include, but are not limited to a metallothionein promoter, a glucocorticoid promoter, a progesterone promoter, a tetracycline promoter, a c-fos promoter, the T-REx system of ThermoFisher which places expression from the human cytomegalovirus immediate-early promoter under the control of tetracycline operator(s), and RheoSwitch promoters of Intrexon. Karzenowski, D. et al., *BioTechniques* 39:191-196 (2005); Dai, X. et al., *Protein Expr. Purif* 42:236-245 (2005); Palli, S. R. et al., *Eur. J. Biochem.* 270:1308-1515 (2003); Dhadialla, T. S. et al., *Annual Rev. Entomol.* 43:545-569 (1998); Kumar, M. B. et al., *J. Biol. Chem.* 279:27211-27218 (2004); Verhaegent, M. et al., *Annal. Chem.* 74:4378-4385 (2002); Katalam, A. K., et al., *Molecular Therapy* 13:S103 (2006); and Karzenowski, D. et al., *Molecular Therapy* 13:S194 (2006), U.S. Pat. Nos. 8,895,306, 8,822,754, 8,748,125, 8,536,354, all of which are incorporated by reference in their entirety for all purposes.

[0159] Expression vectors of the invention typically have promoter elements, e.g., enhancers, to regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

[0160] In some embodiments, it may be desirable to modify the polypeptides of the present invention. One of skill will recognize many ways of generating alterations in a given nucleic acid construct to generate variant polypeptides. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (e.g., in conjunction with ligation and/or cloning to generate large nucleic acids) and other

well-known techniques (see, e.g., Gillam and Smith, *Gene* 8:81-97, 1979; Roberts et al., *Nature* 328:731-734, 1987, which is incorporated by reference in its entirety for all purposes). In some embodiments, the recombinant nucleic acids encoding the polypeptides of the invention are modified to provide preferred codons which enhance translation of the nucleic acid in a selected organism.

[0161] The polynucleotides of the invention also include polynucleotides including nucleotide sequences that are substantially equivalent to the polynucleotides of the invention. Polynucleotides according to the invention can have at least about 80%, more typically at least about 90%, and even more typically at least about 95%, sequence identity to a polynucleotide of the invention. The invention also provides the complement of the polynucleotides including a nucleotide sequence that has at least about 80%, more typically at least about 90%, and even more typically at least about 95%, sequence identity to a polynucleotide encoding a polypeptide recited above. The polynucleotide can be DNA (genomic, cDNA, amplified, or synthetic) or RNA. Methods and algorithms for obtaining such polynucleotides are well known to those of skill in the art and can include, for example, methods for determining hybridization conditions which can routinely isolate polynucleotides of the desired sequence identities.

[0162] Nucleic acids which encode protein analogs or variants in accordance with this invention (i.e., wherein one or more amino acids are designed to differ from the wild type polypeptide) may be produced using site directed mutagenesis or PCR amplification in which the primer(s) have the desired point mutations. For a detailed description of suitable mutagenesis techniques, see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) and/or *Current Protocols in Molecular Biology*, Ausubel et al., eds, Green Publishers Inc. and Wiley and Sons, N.Y. (1994), each of which is incorporated by reference in its entirety for all purposes. Chemical synthesis using methods well known in the art, such as that described by Engels et al., *Angew Chem Intl Ed.* 28:716-34, 1989 (which is incorporated by reference in its entirety for all purposes), may also be used to prepare such nucleic acids.

[0163] In some embodiments, amino acid "substitutions" for creating variants are preferably the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

[0164] The nucleic acid of the present invention can be linked to another nucleic acid so as to be expressed under control of a suitable promoter. The nucleic acid of the present invention can be also linked to, in order to attain efficient transcription of the nucleic acid, other regulatory elements that cooperate with a promoter or a transcription initiation site, for example, a nucleic acid comprising an

enhancer sequence, a polyA site, or a terminator sequence. In addition to the nucleic acid of the present invention, a gene that can be a marker for confirming expression of the nucleic acid (e.g. a drug resistance gene, a gene encoding a reporter enzyme, or a gene encoding a fluorescent protein) may be incorporated.

[0165] When the nucleic acid of the present invention is introduced into a cell *ex vivo*, the nucleic acid of the present invention may be combined with a substance that promotes transference of a nucleic acid into a cell, for example, a reagent for introducing a nucleic acid such as a liposome or a cationic lipid, in addition to the aforementioned excipients. Alternatively, a vector carrying the nucleic acid of the present invention is also useful. Particularly, a composition in a form suitable for administration to a living body which contains the nucleic acid of the present invention carried by a suitable vector is suitable for *in vivo* gene therapy.

Process for Producing Eukaryotic Cells Expressing CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs

[0166] A process for producing a cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention includes a step of introducing the nucleic acid encoding a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR described above into a eukaryotic cell. In some embodiments, this step is carried out *ex vivo*. For example, a cell can be transformed *ex vivo* with a virus vector or a non-virus vector carrying the nucleic acid of the present invention to produce a cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention.

[0167] In the process of the present invention, a eukaryotic cell as describe above is used. In some embodiments, a eukaryotic cell derived from a mammal, for example, a human cell, or a cell derived from a non-human mammal such as a monkey, a mouse, a rat, a pig, a horse, or a dog can be used. The cell used in the process of the present invention is not particularly limited, and any cell can be used. For example, a cell collected, isolated, purified or induced from a body fluid, a tissue or an organ such as blood (peripheral blood, umbilical cord blood etc.) or bone marrow can be used. Examples of such cells include, a B cell, a natural killer cell or a T-cell. In the present invention, particularly, use of a T cell, a precursor cell of a T cell (a hematopoietic stem cell, a lymphocyte precursor cell etc.) or a cell population containing them is preferable. In some embodiments, the T-cell is from an allogeneic or nonself donor. In some embodiments, the allogeneic or nonself T-cell is genetically modified to knockout of the alpha chain of the T-cell receptor to reduce graft versus host reactions. Examples of the T cell include a CD8-positive T cell, a CD4-positive T cell, a regulatory T cell, a cytotoxic T cell, and a tumor infiltrating lymphocyte. The cell population containing a T cell and a precursor cell of a T cell includes a PBMC. The aforementioned cells may be collected from a living body, obtained by expansion culture of a cell collected from a living body, or established as a cell strain. When transplantation of the produced CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR-expressing cell or a cell differentiated from the produced CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR expressing cell into a living body is desired, it is preferable to introduce the nucleic acid into a cell collected from the living body itself.

[0168] In some embodiments, the nucleic acid encoding the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention is inserted into a vector, and the vector

is introduced into a cell. In some embodiments, the nucleic acid encoding the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is introduced to the eukaryotic cell by transfection (e.g., Gorman, et al. *Proc. Natl. Acad. Sci.* 7922 (1982): 6777-6781. which is incorporated by reference in its entirety for all purposes), transduction (e.g., Cepko and Pear (2001) *Current Protocols in Molecular Biology* unit 9.9; DOI: 10.1002/0471142727.mb0909s36, which is incorporated by reference in its entirety for all purposes), calcium phosphate transformation (e.g., Kingston, Chen and Okayama (2001) *Current Protocols in Molecular Biology* Appendix 1C; DOI: 10.1002/0471142301.nsa01cs01, which is incorporated by reference in its entirety for all purposes), cell-penetrating peptides (e.g., Copolovici, Langel, Eriste, and Langel (2014) *ACS Nano* 2014 8 (3), 1972-1994; DOI: 10.1021/nn4057269, which is incorporated by reference in its entirety for all purposes), electroporation (e.g Potter (2001) *Current Protocols in Molecular Biology* unit 10.15; DOI: 10.1002/0471142735.im1015 s03 and Kim et al (2014) *Genome* 1012-19. doi:10.1101/gr.171322.113, Kim et al. 2014 describe the Amaza Nucleofector, an optimized electroporation system, both of these references are incorporated by reference in their entirety for all purposes), microinjection (e.g., McNeil (2001) *Current Protocols in Cell Biology* unit 20.1; DOI: 10.1002/0471143030.cb2001s18, which is incorporated by reference in its entirety for all purposes), liposome or cell fusion (e.g., Hawley-Nelson and Ciccarone (2001) *Current Protocols in Neuroscience* Appendix 1F; DOI: 10.1002/0471142301.nsa01fs10, which is incorporated by reference in its entirety for all purposes), mechanical manipulation (e.g. Sharon et al. (2013) *PNAS* 2013 110(6); DOI: 10.1073/pnas.1218705110, which is incorporated by reference in its entirety for all purposes) or other well-known technique for delivery of nucleic acids to eukaryotic cells. Once introduced, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR nucleic acid can be transiently expressed episomally, or can be integrated into the genome of the eukaryotic cell using well known techniques such as recombination (e.g., Lisby and Rothstein (2015) *Cold Spring Harb Perspect Biol.* March 2; 7(3). pii: a016535. doi: 10.1101/cshperspect.a016535, which is incorporated by reference in its entirety for all purposes), or non-homologous integration (e.g., Deyle and Russell (2009) *Curr Opin Mol Ther.* 2009 August; 11(4):442-7, which is incorporated by reference in its entirety for all purposes). The efficiency of homologous and non-homologous recombination can be facilitated by genome editing technologies that introduce targeted double-stranded breaks (DSB). Examples of DSB-generating technologies are CRISPR/Cas9, TALEN, Zinc-Finger Nuclease, or equivalent systems (e.g., Cong et al. *Science* 339.6121 (2013): 819-823, Li et al. *Nuc. Acids Res* (2011): gkr188, Gaj et al. *Trends in Biotechnology* 31.7 (2013): 397-405, all of which are incorporated by reference in their entirety for all purposes), transposons such as Sleeping Beauty (e.g., Singh et al (2014) *Immunol Rev.* 2014 January; 257(1):181-90. doi: 10.1111/imr.12137, which is incorporated by reference in its entirety for all purposes), targeted recombination using, for example, FLP recombinase (e.g., O'Gorman, Fox and Wahl *Science* (1991) 15:251(4999):1351-1355, which is incorporated by reference in its entirety for all purposes), CRE-LOX (e.g., Sauer and Henderson *PNAS* (1988): 85; 5166-5170), or equivalent systems, or other techniques known in the art for

integrating the nucleic acid encoding the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR into the eukaryotic cell genome.

[0169] In an embodiment, the nucleic acid encoding the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is integrated into the eukaryotic cell chromosome at a genomic safe harbor site, such as, for example, the CCR5, AAVS1, human ROSA26, or PSIP1 loci. (Sadelain et al., *Nature Rev.* 12:51-58 (2012); Fadel et al., *J. Virol.* 88(17):9704-9717 (2014); Ye et al., *PNAS* 111(26):9591-9596 (2014), all of which are incorporated by reference in their entirety for all purposes.) In an embodiment, the integration of the nucleic acid encoding the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR at the CCR5 or PSIP1 locus is done using a gene editing system, such as, for example, CRISPR, TALEN, or Zinc-Finger nuclease systems. In an embodiment, the eukaryotic cell is a human, T-lymphocyte and a CRISPR system is used to integrate the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR at the CCR5 or PSIP1 locus. In an embodiment, integration of the nucleic acid at CCR5 or PSIP1 using the CRISPR system also deletes a portion, or all, of the CCR5 gene or PSIP1 gene. In an embodiment, Cas9 in the eukaryotic cell may be derived from a plasmid encoding Cas9, an exogenous mRNA encoding Cas9, or recombinant Cas9 polypeptide alone or in a ribonucleoprotein complex. (Kim et al (2014) *Genome* 1012-19. doi:10.1101/gr.171322.113.; Wang et al (2013) *Cell* 153 (4). Elsevier Inc.: 910-18. doi:10.1016/j.cell.2013.04.025, both of which are incorporated by reference in their entirety for all purposes.)

[0170] Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (e.g., an artificial membrane vesicle). Other methods of state-of-the-art targeted delivery of nucleic acids are available, such as delivery of polynucleotides with targeted nanoparticles or other suitable sub-micron sized delivery system.

[0171] In some embodiments, transduction can be done with a virus vector such as a retrovirus vector (including an oncoretrovirus vector, a lentivirus vector, and a pseudo type vector), an adenovirus vector, an adeno-associated virus (AAV) vector, a simian virus vector, a vaccinia virus vector or a sendai virus vector, an Epstein-Barr virus (EBV) vector, and a HSV vector can be used. As the virus vector, a virus vector lacking the replicating ability so as not to self-replicate in an infected cell is preferably used.

[0172] In some embodiments, when a retrovirus vector is used, the process of the present invention can be carried out by selecting a suitable packaging cell based on a LTR sequence and a packaging signal sequence possessed by the vector and preparing a retrovirus particle using the packaging cell. Examples of the packaging cell include PG13 (ATCC CRL-10686), PA317 (ATCC CRL-9078), GP+E-86 and GP+envAm-12 (U.S. Pat. No. 5,278,056, which is incorporated by reference in its entirety for all purposes), and Psi-Crip (Proceedings of the National Academy of Sciences of the United States of America, vol. 85, pp. 6460-6464 (1988), which is incorporated by reference in its entirety for all purposes). A retrovirus particle can also be prepared using a 293 cell or a T cell having high transfection

efficiency. Many kinds of retrovirus vectors produced based on retroviruses and packaging cells that can be used for packaging of the retrovirus vectors are widely commercially available from many companies.

[0173] In some embodiments, a viral vector derived from a RNA virus is used to introduce the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR encoding polynucleotides. In some embodiments, the RNA virus vector encodes the reverse complement or antisense strand of the polynucleotide encoding the RNA control device and Smart CAR and/or Smart-DE-CAR construct (the complementary strand encodes the sense strand for the RNA control device, CAR construct, and optionally the DE). In this embodiment, the RNA control device is not active in the single stranded, RNA virus vector. In some embodiments, the sense strand of the RNA control device, CAR and optionally the DE construct is encoded in the RNA virus vector, and the viral vector with the RNA control device, CAR, and optionally the DE construct is maintained and replicated in the presence of ligand for the sensor element of the RNA control device. In some embodiments, the viral vector encoding the sense strand of the RNA control device, CAR, and optionally the DE construct in the viral vector is maintained and replicated without ligand for the sensor element.

[0174] In some embodiments, a non-virus vector is used in combination with a liposome and a condensing agent such as a cationic lipid as described in WO 96/10038, WO 97/18185, WO 97/25329, WO 97/30170 and WO 97/31934 (which are incorporated herein by reference in their entirety for all purposes). The nucleic acid of the present invention can be introduced into a cell by calcium phosphate transduction, DEAE-dextran, electroporation, or particle bombardment.

[0175] In some embodiments, chemical structures with the ability to promote stability and/or translation efficiency are used. The RNA preferably has 5' and 3' UTRs. In one embodiment, the 5' UTR is between one and 3000 nucleotides in length.

[0176] In some embodiments, the mRNA has both a cap on the 5' end and a 3' poly(A) tail which determine ribosome binding, initiation of translation and stability mRNA in the cell. On a circular DNA template, for instance, plasmid DNA, RNA polymerase produces a long concatameric product which is not suitable for expression in eukaryotic cells. The transcription of plasmid DNA linearized at the end of the 3' UTR results in normal sized mRNA which is not effective in eukaryotic transfection even if it is polyadenylated after transcription.

[0177] In the step of introducing a nucleic acid into a cell, a functional substance for improving the introduction efficiency can also be used (e.g. WO 95/26200 and WO 00/01836, which are incorporated herein by reference in their entirety for all purposes). Examples of the substance for improving the introduction efficiency include a substance having ability to bind to a virus vector, for example, fibronectin and a fibronectin fragment. In some embodiments, a fibronectin fragment having a heparin binding site, for example, a fragment commercially available as RetroNectin (registered trademark, CH-296, manufactured by TAKARA BIC INC.) can be used. Also, polybrene which is a synthetic polycation having an effect of improving the efficiency of infection of a retrovirus into a cell, a fibroblast growth factor, V type collagen, polylysine or DEAE-dextran can be used.

[0178] In a preferable aspect of the present invention, the functional substance can be used in a state of being immobilized on a suitable solid phase, for example, a container used for cell culture (plate, petri dish, flask or bag) or a carrier (microbeads etc.).

Eukaryotic Cells Expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR

[0179] The cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR of the present invention is a cell in which a nucleic acid encoding a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is introduced and expressed.

[0180] In some embodiments, a eukaryotic cell of the present invention binds to a specific antigen via the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR, causing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR to transmit a signal into the eukaryotic cell, and as a result, the eukaryotic cell is activated. The activation of the eukaryotic cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is varied depending on the kind of a eukaryotic cell and the intracellular element of the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR, and can be confirmed based on, for example, release of a cytokine, improvement of a cell proliferation rate, change in a cell surface molecule, or the like as an index. For example, release of a cytotoxic cytokine (a tumor necrosis factor, lymphotoxin, etc.) from the activated cell causes destruction of a target cell expressing an antigen. In addition, release of a cytokine or change in a cell surface molecule stimulates other immune cells, for example, a B cell, a dendritic cell, a natural killer cell, and/or a macrophage.

[0181] In some embodiments, eukaryotic cells expressing CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR constructs are detected using Protein L (a bacterial surface protein isolated from *Peptostreptococcus magnus* that selectively binds to variable light chains (kappa chain) of immunoglobulins. In some embodiments, Protein L is directly labeled with a reporter (e.g., a light emitting or absorbing moiety) or is labeled with an agent such as biotin. When biotin or related molecule is used to label the Protein L, binding of Protein L to eukaryotic cells displaying CAR or DE-CAR polypeptide is detected by adding a streptavidin (or similar paired molecule) labeled with reporter (e.g., phycoerythrin). Zheng et al., *J. Translational Med.*, 10:29 (2012), which is incorporated by reference in its entirety for all purposes. Protein L binding to eukaryotic cells containing CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR constructs demonstrates the presence of antibody light chain, the extracellular domain of a CAR, on the eukaryotic cell. This method of detecting CAR expression on the eukaryotic cell can also be used to quantitate the amount of CAR or DE-CAR polypeptide on the surface of the eukaryotic cell. In some embodiments, Protein L is used in QC and QA methodologies for making eukaryotic cells with the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR constructs of the invention.

[0182] In some embodiments, a eukaryotic cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is used as a therapeutic agent to treat a disease. The therapeutic agent comprises the eukaryotic cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR as an active ingredient, and may further comprise a suitable excipient. Examples of the excipient include pharmaceutically acceptable excipients for the composition. The disease against

which the eukaryotic cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is administered is not particularly limited as long as the disease shows sensitivity to the eukaryotic cell. Examples of diseases of the invention include a hematopoietic cell cancers and an autoimmune disease. The eukaryotic cell expressing the DE-CAR and/or Smart-DE-CAR of the present invention is administered for treatment of these diseases. The therapeutic agent comprising the eukaryotic cell expressing the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR as an active ingredient can be administered intradermally, intramuscularly, subcutaneously, intraperitoneally, intranasally, intraarterially, intravenously, intratumorally, or into an afferent lymph vessel, by parenteral administration, for example, by injection or infusion, although the administration route is not limited.

[0183] In some embodiments, the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR is used with a T-lymphocyte that has aggressive anti-tumor properties, such as those described in Pegram et al, CD28z CARs and armored CARs, 2014, *Cancer J.* 20(2):127-133, which is incorporated by reference in its entirety for all purposes. In some embodiments, the RNA control device of the invention is used with an armored DE-CAR in a T-lymphocyte.

[0184] In some embodiments, any of the above CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR embodiments can also include a DE-LEM, Smart LEM or Smart-DE-LEM to provide controlled expression of LEM or DE-LEM. In these embodiments, the amount of LEM or DE-LEM is controlled so that its expansion signal is provided at a desired time. This control of the expansion signal is achieved by altering the amount of ligand (s) for the DE(s) and/or RNA control devices associated with the LEM or DE-LEM whereby the amount of LEM or DE-LEM is altered. In some embodiments, control of the LEM expansion signal is achieved by adding exogenous LEM to the eukaryotic cells at a desired time.

Pharmaceutical Compositions

[0185] Pharmaceutical compositions of the present invention may comprise a CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR expressing cell, e.g., a plurality of CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR expressing cells, as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present invention are in one aspect formulated for intravenous administration.

[0186] Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). 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.

[0187] Suitable pharmaceutically acceptable excipients are well known to a person skilled in the art. Examples of the pharmaceutically acceptable excipients include phosphate buffered saline (e.g. 0.01 M phosphate, 0.138 M NaCl,

0.0027 M KCl, pH 7.4), an aqueous solution containing a mineral acid salt such as a hydrochloride, a hydrobromide, a phosphate, or a sulfate, saline, a solution of glycol or ethanol, and a salt of an organic acid such as an acetate, a propionate, a malonate or a benzoate. In some embodiments, an adjuvant such as a wetting agent or an emulsifier, and a pH buffering agent can also be used. In some embodiments, the pharmaceutically acceptable excipients described in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991) (which is incorporated herein by reference in its entirety for all purposes) can be appropriately used. The composition of the present invention can be formulated into a known form suitable for parenteral administration, for example, injection or infusion. In some embodiments, the composition of the present invention may comprise formulation additives such as a suspending agent, a preservative, a stabilizer and/or a dispersant, and a preservation agent for extending a validity term during storage.

[0188] A composition comprising the eukaryotic cells of the present invention as an active ingredient can be administered for treatment of, for example, a hematopoietic cancer or an autoimmune disease.

[0189] The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient trans arterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, intranasally, intraarterially, intratumorally, into an afferent lymph vessel, by intravenous (i.v.) injection, or intraperitoneally. In one aspect, the T cell compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In one aspect, the T-lymphocyte compositions of the present invention are administered by i.v. injection. The compositions of T-lymphocytes may be injected directly into a tumor, lymph node, or site of infection. In some embodiments, the administration is adoptive transfer.

[0190] When "an immunologically effective amount," "an anti-tumor effective amount," "a tumor-inhibiting effective amount," or "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). In some embodiments, a pharmaceutical composition comprising the eukaryotic cells described herein may be administered at a dosage of 10^4 to 10^9 cells/kg body weight, in some instances 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. In some embodiments, a eukaryotic cell composition may also be administered multiple times at these dosages. In some embodiments, eukaryotic cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, e.g., Rosenberg et al., *New Eng. J. of Med.* 319:1676, 1988, which is incorporated by reference in its entirety for all purposes).

Uses of Eukaryotic Cells with CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR

[0191] In some embodiments, nucleic acids encoding the CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention are used to express CAR and/or DE-CAR polypeptides in eukaryotic cells. In some embodiments, nucleic acids encoding CAR, Smart CAR, DE-CAR and/or

Smart-DE-CAR of the invention are used to express CAR and/or DE-CAR polypeptides in mammalian cells. In some embodiments, nucleic acids encoding CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention are used to express CAR and/or DE-CAR polypeptides in human cells or murine cells. In some embodiments, nucleic acids encoding CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention are used to express CAR and/or DE-CAR polypeptide in hematopoietic cells. In some embodiments, nucleic acids encoding CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention are used to express CAR and/or DE-CAR polypeptides in T-lymphocytes, natural killer cells, or B-cells. In some embodiments, nucleic acids encoding CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention are used to express CAR and/or DE-CAR polypeptides in T-lymphocytes or natural killer cells.

[0192] In some embodiments, the nucleic acids encoding the CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention are used to express a desired level of CAR and/or DE-CAR polypeptide on the surface of the eukaryotic cell. In this embodiment, the DE, RNA control device, and/or inducible promoter (e.g., the RheoSwitch promoter and control region) control the level of CAR and/or DE-CAR polypeptide expression, at least in part, and by modulating the level of activity of the DE, RNA control device, and/or the inducible promoter so a desired amount of CAR and/or DE-CAR polypeptide is expressed and displayed on the surface of the eukaryotic cell. In some embodiments, the DE increases the degradation rate of DE-CAR polypeptide in the eukaryotic cell and when ligand is bound by the DE, the rate of degradation decreases. In some embodiments, the DE increases degradation of the DE-CAR polypeptide when ligand is bound by the DE. In some embodiments, the RNA control device inhibits translation of the CAR and/or DE-CAR mRNA and when ligand binds to the sensor element of the RNA control device this inhibition of translation is reduced so that CAR and/or DE-CAR polypeptide expression is increased. In some embodiments, ligand for the DE, ligand for the RNA control device sensor, and/or ligand for the repressor or activator that acts at the inducible promoter (or control region) is added in increasing (or decreasing) amounts to the eukaryotic cells with the CARs, DE-CARs and/or Smart-DE-CARs until a desired level of CAR and/or DE-CAR polypeptide is made in the eukaryotic cell. In some embodiments, the amount of CAR and/or DE-CAR polypeptide is measured using antibodies specific for the CAR and/or DE-CAR polypeptide. In some embodiments, the amount of CAR and/or DE-CAR polypeptide is measured using the antigen recognized by the extracellular element. In some embodiments, the amount of CAR and/or DE-CAR polypeptide is measured in a functional assay of target cell killing. In some embodiments, the amount of CAR and/or DE-CAR polypeptide is measured in a functional assay for eukaryotic cell proliferation (induced by the CAR and/or DE-CAR polypeptide). In some embodiments, the above eukaryotic cell is a T-lymphocyte or a natural killer cell.

[0193] In some embodiments, the ligand for the DE, the ligand for the RNA control device sensor, and/or the ligand for the repressor or activator that acts at the inducible promoter (or control region) is added in increasing or decreasing amounts until a desired level of activity is obtained. In some embodiments, the desired activity is killing of a target cell. In some embodiments, target cell

killing occurs over a desired time period, e.g., the killing of a certain number of target cells in 12 hours, or 24 hours, or 36 hours, or two days, or 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 days, or two months, or 3, 4, 5, or 6 months. In some embodiments, target cell killing is expressed as a half-life for a standardized number of target cells. In this embodiment, the half-life of target cell killing can be 12 hours, 24 hours, 36 hours, or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 days, or two months, or 3, 4, 5, or 6 months. In some embodiments, the desired activity is proliferation. In some embodiments, the cell proliferation occurs with a doubling time of 12 hours, 24 hours, 36 hours, two days, or 3, 4, 5, 6, or 7 days. In some embodiments, the above eukaryotic cell is a T-lymphocyte or a natural killer cell.

[0194] In some embodiments, a regime of different amounts of ligand (for the sensor, DE, and/or repressor or activator that acts at the inducible promoter (or control region)) is added over time so that different desired levels of CAR and/or DE-CAR polypeptide are present on the eukaryotic cell at different times. For example, in the treatment of cancer in patients with CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR T-lymphocytes or CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR natural killer cells, the amount of CAR and/or DE-CAR polypeptide expression may be reduced initially to reduce toxicity from tumor lysis, and as tumor mass is cleared, the amount of CAR and/or DE-CAR polypeptide expression can be increased to kill the remaining tumor cells as these become more rare within the body. In some embodiments, the CAR and/or DE-CAR polypeptide expression may be increased initially, and as tumor mass is reduced the CAR and/or DE-CAR expression level is reduced to reduce killing of healthy tissue that also may express target antigen. In some embodiments, the reactivity towards tumor cells can be modulated so that the ratio of tumor cell killing to killing of normal tissue is maintained within a desired range. In some embodiments, the amount of CAR and/or DE-CAR polypeptide expressed on the surface of the eukaryotic cell is reduced by eukaryotic cell proliferation. As the eukaryotic cells proliferate, CAR and/or DE-CAR polypeptide will be diluted if the expression level from the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR nucleic acid is insufficient to keep the CAR and/or DE-CAR polypeptide copy number at the level found in the parent eukaryotic cell (i.e., if the parent cell does not double its amount of CAR and/or DE-CAR polypeptide then each daughter cell will have a decreased amount of CAR and/or DE-CAR polypeptide compared to the parent cell). In some embodiments, the CAR and/or DE-CAR polypeptide is designed to have a short half-life, in comparison to the doubling time for the eukaryotic cell in the subject. In some embodiments, the ligand(s) has a short half-life in the subject when compared to the doubling time of the eukaryotic cell in the subject. In some embodiments, an anti-ligand antibody or a different ligand binding molecule is administered to the subject or given to the eukaryotic cells (in vitro) so that the ligand binds to the antibody or ligand binding molecule and cannot react with the DE, sensor, repressor, and/or activator. In some embodiments, the above eukaryotic cell is a T-lymphocyte or a natural killer cell.

[0195] In some embodiments, eukaryotic cells with CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention expressed a desired amount of CAR and/or DE-

CAR polypeptide so that a subject containing the eukaryotic cells with the CAR and/or DE-CAR polypeptide produce a therapeutic level of target cell killing while keeping toxicity and adverse events at acceptable levels. In some embodiments, the above eukaryotic cell is a T-lymphocyte or a natural killer cell. For example, CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention can be used to reduce tumor lysis syndrome, cytokine storms, or healthy tissue killing by T-lymphocytes with the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR. In some embodiments, the amount of CAR and/or DE-CAR polypeptide expressed on the T-lymphocyte or the natural killer cell is individualized such that severe adverse events are prevented.

[0196] In some embodiments, the amount of CAR and/or DE-CAR polypeptide expressed by the eukaryotic cell is individualized for the subject by measuring the amount of target antigen on the subjects diseased tissue (or cells) and/or the amount of target antigen expressed on the subjects healthy tissue (or cells). In some embodiments, a biopsy of diseased and healthy tissue (or cells) is taken from the subject, and the amount of target antigen is measured using routine methods, e.g., antibodies to the target can be used to quantify target antigen expression. Based on the relative amount of target antigen expressed on diseased and healthy tissues (or cells) of the subject a desired level of CAR and/or DE-CAR activity for the eukaryotic cell can be determined. Eukaryotic cells with the CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention can then be programmed to express the desired amount of CAR and/or DE-CAR to give the desired level of CAR and/or DE-CAR activity. In some embodiments, the above eukaryotic cell is a T-lymphocyte or a natural killer cell.

[0197] In some embodiments, the Smart CAR and/or Smart-DE-CAR of the invention is associated with two or more RNA control devices. In some embodiments, different amounts of the two or more ligands for the DE and/or two or more RNA control devices and/or inducible promoter (repressor and/or activator) are added to the eukaryotic cells to produce a desired amount of CAR and/or DE-CAR polypeptide in the eukaryotic cell. In some embodiments, different regimes of combinations of the ligands are applied to the eukaryotic cells to produce a desired profile over time of the amount of CAR and/or DE-CAR polypeptide on the surface of the eukaryotic cell. In some embodiments, some of the RNA control devices increase expression of CAR and/or DE-CAR polypeptide when ligand for the sensor element is bound, and some of the RNA control devices decrease CAR and/or DE-CAR polypeptide expression when ligand for the sensor element is bound. In some embodiments, the above eukaryotic cell is a T-lymphocyte or a natural killer cell.

[0198] In some embodiments, CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs are used for genetically engineering T-lymphocytes for cancer immunotherapy. When used for immunotherapy applications, T-lymphocytes may be removed from a patient or donor (syngeneic or allogeneic) through leukopheresis and T-lymphocytes are preferentially sorted and saved. Optionally, T-lymphocytes from an allogeneic donor are genetically modified to knock-out the alpha chain of the T-lymphocyte receptor. T lymphocytes are subjected to lentiviral or retroviral introduction (or other means of nucleic acid introduction) of the transgene that encodes the CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR, expanded to target therapeutic cell concen-

trations and infused into the patient. Although CARs have been shown to be very effective at achieving and sustaining remissions for refractory/relapsed acute lymphoblastic leukemia (Maude et al., *NEJM*, 371:1507, 2014, which is incorporated by reference in its entirety for all purposes), dangerous side effects related to cytokine release syndrome (CRS), tumor lysis syndrome (TLS), B cell aplasia or “on-tumor, off-target” toxicities occur. Modulating CAR expression via the incorporation of DEs and/or RNA control devices in the Smart CAR, DE-CARs and/or Smart-DE-CARs of the invention can control these toxicities.

[0199] In some embodiments the nucleic acid sequences encoding a cognate RNA control device or devices are present in a nucleic acid locus encoding a chimeric antigen receptor transgene. In some embodiments, RNA control devices are encoded for as nucleic acid sequence in the vector proximal, distal, or within the ORF encoding a CAR or DE-CAR polypeptide. An example of a schematic of a vector is included in FIG. 1, adapted from (Budde et al., *PLoS1*, 2013, doi:10.1371/journal.pone.0082742, which is incorporated by reference in its entirety for all purposes). In some embodiments nucleic acid sequences encoding an RNA control device or devices are located within the 3' UTR region of the transgene. In some embodiments nucleic acid sequences encoding an RNA control device or devices are located in the 5' UTR region of the CAR or DE-CAR transgene. In some embodiments nucleic acid sequences encoding an RNA control device or devices are located within synthetic or natural introns flanked by coding or noncoding exons within the CAR transgene, or at intron/exon boundaries.

[0200] In some embodiments RNA control devices are present in cis within an mRNA encoding a CAR or a DE-CAR. In some embodiments, the CAR or DE-CAR is encoded by an mRNA. The Smart CAR, DE-CAR and/or Smart-DE-CAR mRNA may be delivered to T-lymphocytes via electroporation, transfection, or other methods known to those skilled in the art to create a transiently translated Smart CAR, DE-CAR and/or Smart-DE-CAR T-lymphocyte. The RNA control device may act to modulate cognate mRNA stability or translation. The DE with its ligand may modulate the amount of DE-CAR polypeptide in the eukaryotic cell. In some embodiments, these CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR mRNAs may be derived from in vitro transcription with a prokaryotic or bacteriophage RNA polymerase. In some embodiments, these CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR mRNAs may be derived from chemical synthesis of RNAs, enzymatic manipulation of chemically synthesized RNAs, or a combination of chemical synthesis and in vitro transcription of mRNAs. In some embodiments, CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR mRNAs may contain chemical modifications to base, sugar or backbone moieties, which may increase or decrease mRNA stability, alter the base-pairing characteristics of canonical RNA bases, or alter the translated polypeptide sequence by facilitating interactions between modified mRNA codons and charged non-cognate tRNAs.

[0201] In some embodiments, T-lymphocytes comprise CARs and/or DE-CARs with integrated RNA control devices. In some embodiments, combinational Smart DE-CAR T-lymphocytes are used, wherein independent T-lymphocytes express orthogonal DE-CARs, and/or Smart DE-CARs, and/or Smart-CARs that target distinct tumor-

associated antigens (TAAs). Targeting multiple TAAs simultaneously can direct a greater CTL response against the primary tumor or metastases and prevent relapse. A potential disadvantage of using DE-CAR T-lymphocytes and/or CAR T-cells is that there may be a higher probability of eliciting on-target, off-tumor effects, leading to toxicity. The coupling of DEs and/or RNA control devices to CARs in DE-CAR, and/or Smart-DE-CAR, and/or Smart CAR T-lymphocytes mitigates the toxicity concern while enabling a stronger response and relapse prevention of DE-CAR, and/or Smart-DE-CAR, and/or Smart-CAR T-lymphocytes. In some embodiments, Smart-DE-CAR T-lymphocytes are controlled by multiple ligands. In some embodiments, the DE and RNA control devices used in combinational DE-CAR, and/or Smart-DE-CAR, and/or Smart-CAR T-lymphocytes are specific for different ligands, or combinations of ligands, such that expression cross-talk is minimized or eliminated. In some embodiments, the DE-CAR and/or Smart-DE-CAR and/or Smart-CAR T-lymphocytes are used against a single tumor by targeting different tumor-associated surface antigens. In some embodiments, these DE-CAR and/or Smart-DE-CAR and/or Smart-CAR T-lymphocytes are used against a single tumor by targeting the same tumor-associated surface antigen, with different transmembrane, hinge, receptor, costimulatory elements, other aspects of the DE-CAR or combinations thereof. In some embodiments the DE-CAR and/or Smart-DE-CAR and/or Smart-CAR T-lymphocytes are used against clonally heterogeneous tumor types, wherein each population of DE-CAR and/or Smart-DE-CAR and/or Smart-CAR T-lymphocyte is specific for a particular TAA. In some embodiments, the relative populations of DE-CAR and/or Smart-DE-CAR and/or Smart-CAR T-lymphocytes is controlled. In some embodiments, combinations of ligands are dosed to induce expression of a specific population of DE-CAR and/or Smart-DE-CAR and/or Smart-CAR T-lymphocytes. In some embodiments, universal DE-CAR (uDE-CAR) T-lymphocytes are used. uDE-CAR T-lymphocytes are single T-lymphocytes that comprise more than one Car, DE-CAR, Smart-DE-CAR, and/or Smart-CAR or more than one means for CAR, DE-CAR, Smart-DE-CAR, and/or Smart-CAR expression. In some embodiments, DE-CARs in uDE-CAR T-lymphocytes are controlled by RNA control devices. In some embodiments uDE-CAR T-lymphocytes contain more than one, two, three or more DE-CARs and/or Smart-DE-CARs. In some embodiments, each DE-CAR in the uDE-CAR T-lymphocyte are controlled by RNA control devices and DEs. In some embodiments, only a subset of DE-CARs are controlled by RNA control devices and DEs. In some embodiments, the RNA control devices in an uDE-CAR T-lymphocyte are specific for different ligands, or combinations of ligands, such that expression cross-talk is minimized or eliminated.

[0202] In some embodiments, multiple orthogonally targeted CAR, DE-CAR, Smart-DE-CAR, and/or Smart-CAR T-lymphocytes are used, where different DE-CARs and/or CARs target separate antigens, or combinatorial DE-CAR T-lymphocytes where a single DE-CAR T-lymphocyte targets multiple antigens, whereby tumor cell killing is increased, and complete responses and sustained remissions are more likely. This combinatorial DE-CAR and/or CAR approach will be enhanced by RNA control devices which respond to bio-orthogonal ligands to control individual

DE-CAR and/or CAR expression within a single or population of modified T-lymphocytes.

[0203] In some embodiments, the T-lymphocyte, B-lymphocyte, and/or natural killer cell has been engineered to have inducible expression of a polypeptide regulating proliferation and/or activation of the T-lymphocyte, B-lymphocyte, and/or natural killer cell. In some embodiments, the polypeptide regulating proliferation and/or activation is a cytokine. In some embodiments, the polypeptide regulating proliferation and/or activation is IL-2, IL-7 and/or IL-15. These T-lymphocytes, B-lymphocytes, and/or natural killer cells are used with the CARs, Smart CARs, DE-CARs and/or Smart-DE-CARs of the invention and during treatment, the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are induced at desired times. In some embodiments, the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) is/are induced at times when antigen stimulation of the CAR or DE-CAR polypeptide on the T-lymphocyte, B-lymphocyte, or natural killer cell is low because the cells have down regulated the amount of displayed CAR or DE-CAR polypeptide or because the amount of target antigen has decreased, or both. The induced expression of the polypeptide regulating proliferation and/or activation (e.g., IL-2, IL-7, and/or IL-15) at these times of low antigen stimulation can maintain or extend the life of the CAR or DE-CAR T-lymphocyte, B-lymphocyte, or natural killer cell.

[0204] In some embodiments, the T-lymphocyte has been engineered to reduce function of or eliminate FasL on the T-lymphocyte. In some embodiments, the T-lymphocyte and/or natural killer cell has been engineered to express lytic proteins (or cytokines that induce lytic protein expression) at a desired time. The T-lymphocytes that are FasL modified, or the T-lymphocytes and/or natural killer cells that are modified to express lytic proteins at desired times are used as host cells for the CARs, Smart CARs, DE-CARs, and/or Smart-DE-CARs of the invention. In these embodiments, the host cell has been modified increase target cell killing by the lytic proteins of the T-lymphocyte or natural killer cell. Target cell killing by the lytic proteins is often pro-inflammatory and can result in antigen spreading, wherein other T-lymphocytes and/or natural killer cells are activated to attack the target cells through other antigens found on the target cells (the diseased or disease causing cells). In some embodiments, the target cells are cancer cells. In some embodiments, the target cells are patient cells infected by viral, bacterial, fungi, or other pathogen cells. In some embodiments, the target cells are bacterial, fungal or other pathogen cells.

[0205] In some embodiments, any of the above DE-CAR or Smart-DE-CAR embodiments can also include a LEM (inducible promoter), DE-LEM, Smart LEM or Smart-DE-LEM to provide controlled expression of LEM or DE-LEM. In these embodiments, the amount of LEM or DE-LEM is controlled so that its expansion signal is provided at a desired time. This control of the expansion signal is achieved by altering the amount of ligand (s) for the DE(s) and/or RNA control devices associated with the LEM or DE-LEM whereby the amount of LEM or DE-LEM is altered. In some embodiments, control of the LEM expansion signal is achieved by adding exogenous LEM to the eukaryotic cells at a desired time.

Treatment of Disease

[0206] In some embodiments, the invention relates to CAR, Smart CAR, DE-CAR and/or Smart-DE-CAR constructs for use in the treatment of certain hematopoietic disorders. In some embodiments, the hematopoietic disorder is a malignancy or an autoimmune disease. In some embodiments, the malignancy is a leukemia, lymphoma, myeloma, or sarcoma. In some embodiments, the malignancy is multiple myeloma. In some embodiments, the malignancy is a CD19 and/or CD20 positive B-cell lymphoma. In some embodiments, leukemias include, for example, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute monocytic leukemia (AMoL), B-cell prolymphocytic leukemia, Hairy cell leukemia, T-cell prolymphocytic leukemia, T-cell large granular lymphocytic leukemia, and NK-cell leukemia. In some embodiments, lymphomas include, for example, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, lymphoplasmacytic lymphoma, Splenic marginal zone lymphoma, small B-cell lymphoma, Waldenstrom macroglobulinemia, MALT lymphoma, Nodal marginal zone lymphoma, Pediatric follicular lymphoma, Mantle cell lymphoma, Diffuse large B-cell lymphoma (DLBCL), large B-cell lymphoma, Plasmablastic lymphoma, Burkitt lymphoma, and T-cell lymphoma. In some embodiments, myelomas include, for example, multiple myeloma, and plasma cell myeloma. In some embodiments, sarcomas include, for example, Histiocytic sarcoma, dendritic cell sarcoma, and Langerhans cell sarcoma.

[0207] In some embodiments, antigens for acute myeloid leukemia (AML) including but not limited to any one or more of CD 33, CD 34, CD 38, CD 44, CD 45, CD 45RA, CD 47, CD 64, CD 66, CD 123, CD 133, CD 157, CLL-1, CXCR4, LeY, PR1, RHAMM (CD 168), TIM-3, and/or WT1. In some embodiments, the monoclonal antibody 293C3-SDIE is used for the extracellular element. (Rothfelder et al., 2015, ash.confex.com/ash/2015/webprogram/Paper81121.html which is incorporated by reference in its entirety for all purposes) In some embodiments, potential antigens for B-cell malignancies include, for example, CD5, CD 10, CD 19, CD 20, CD 21, CD 22, CD 23, CD 43, and CD79a. In some embodiments, potential antigens for T-cell malignancies include, for example, CD2, CD3, CD4, CD5, CD7, and CD8. In some embodiments, potential antigens for NK cell malignancies include, for example, CD 16 and CD 56. In some embodiments, potential antigens for other myeloid malignancies include, for example, CD13, CD33, CD 38, and CD117. In some embodiments, potential antigens for dendritic cell malignancies include, for example, CD 11c and CD123. In some embodiments, potential antigens for monocyte malignancies include, for example, CD 14 and CD 33.

[0208] In some embodiments, potential antigens for hairy cell leukemias include, for example, CD 11, CD 19, CD 22, CD 25, and CD 103. In some embodiments, potential antigens for splenic marginal zone lymphoma include, for example, CD 19, CD22, and FMC7. In some embodiments, potential antigens for lymphoplasmacytic lymphoma include, for example, B19, FMC7, and CD38. In some embodiments, potential antigens for follicular lymphoma include, for example, CD19, CD22, CD23, and CD10.

[0209] In some embodiments, the extracellular antigen binding domain of a CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR binds to one of the above enumerated

antigens to target the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR to the hematopoietic malignancy. In some embodiments, the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR are placed in a T-lymphocyte. In some embodiments, the T-lymphocyte is a CD 4+ T-cell. In some embodiments, the T-lymphocyte is a CD 8+ T-cell. In some embodiments, the CAR, Smart CAR, DE-CAR, or Smart-DE-CAR is placed in a natural killer cell. In some embodiments, the T-lymphocyte or natural killer cell is obtained from the patient. In some embodiments, the T-lymphocyte or natural killer cell is obtained from an autologous, syngeneic, or allogeneic donor. Optionally, when the T-lymphocyte is obtained from an allogeneic source, the T-lymphocyte is genetically modified to knock-out the alpha chain of the T-lymphocyte receptor. In some embodiments, the T-lymphocytes and/or natural killer cells with the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR are administered to a patient with a hematologic malignancy as a treatment.

[0210] In some embodiments, T-lymphocytes and/or natural killer cells with the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR targeted to hematopoietic malignancies are co-administered with T-lymphocytes or natural killer cells with CARs, Smart CARs, DE-CARs, and/or Smart-DE-CARs targeted at hematopoietic stem cells. In some embodiments, potential antigens for hematopoietic stem cells include, for example, CD 34, CD 41, CD 45, CD 90, CD 117, CD 123, and CD 133. Other antigens found on hematopoietic stem cells are well known in the art and may also be targeted by the CARs, Smart CARs, DE-CARs, and/or Smart-DE-CARs of the invention. In this embodiment, the extracellular antigen binding domain binds to a hematopoietic stem cell antigen (e.g., CD 123) and targets a cell containing the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR to hematopoietic stem cells in a patient. In some embodiments, the CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR are placed in a T-lymphocyte. In some embodiments, the T-lymphocyte is a CD 4+ T-cell. In some embodiments, the T-lymphocyte is a CD 8+ T-cell. In some embodiments, the CAR, Smart CAR, DE-CAR, or Smart-DE-CAR is placed in a natural killer cell. In some embodiments, the T-lymphocyte or natural killer cell is obtained from a donor. In some embodiments, the donor is the patient (autologous). In some embodiments, the donor is a twin (syngeneic). In some embodiments, the donor is a sibling, parent or other third person (allogeneic). In some embodiments, the allogeneic, syngeneic or nonself T-lymphocyte is genetically modified to knockout the alpha chain of the T-lymphocyte receptor so that graft versus host reactions are reduced. In some embodiments, the patient is given a hematopoietic cell transplant or a bone marrow transplant, from an autologous, syngeneic, or allogeneic source, after the treatment with the T-lymphocyte and/or natural killer cell with CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR that target hematopoietic stem cells. In this embodiment, the T-lymphocyte and/or natural killer cell with CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR that target hematopoietic stem cells deplete the patient's hematopoietic stem cells, which are then repopulated by the stem cell or bone marrow graft. This embodiment allows the patient to receive a bone marrow or stem cell graft without having to undergo toxic chemotherapy or toxic radiation treatment.

[0211] In some embodiments, the patient with the hematopoietic malignancy receives a treatment with chemotherapy and/or radiation prior to or at the same time as

treatment with T-lymphocytes or natural killer cells with CARs, Smart CARs, DE-CARs, and/or Smart-DE-CARs targeted at hematopoietic stem cells.

[0212] In some embodiments, the hematopoietic disorder is an autoimmune disease, such as, for example neurological disorders, rheumatological disorders, hematological immunocytopenias, and gastrointestinal disorders (e.g., inflammatory bowel disease). In some embodiments, the neurological disorders include, for example, multiple sclerosis, myasthenia gravis, polyneuropathy, cerebellar degeneration, Guillain Barré syndrome, and amyotrophic lateral sclerosis. In some embodiments, rheumatological disorders include, for example, rheumatoid arthritis, systemic sclerosis, juvenile idiopathic arthritis, systemic lupus, erythematosus, dermatomyositis, mixed connective tissue disease, Bechet's disease, psoriatic arthritis, Ankylosing Spondylitis, Wegner's granulomatosis, and Cryoglobulinemia. In some embodiments, hematological immunocytopenias include, for example, immune thrombocytopenia, pure red cell aplasia, autoimmune hemolytic anemia, thrombotic thrombocytopenic purpura, Evan's syndrome, pancytopenia, and pure white cell aplasia.

[0213] In some embodiments, the extracellular antigen binding domain of a CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR binds to antigens found on memory T-lymphocytes, memory B-cells, and hematopoietic stem cells. Potential antigens for memory T-lymphocytes include, for example, CCR5, CCR7, CD11a, CD27, CD28, CD45RA, CD45RO, CD57, and/or CD62L. Potential antigens for memory B-cells include, for example, CD 19, CD 21, CD 27, CD 40, and/or CD84. Potential antigens for hematopoietic stem cells include, for example, CD 34, CD 41, CD 45, CD 90, CD 117, CD 123, and CD 133. In some embodiments, treatment of the patient with T-lymphocytes and/or natural killer cells with CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR that bind to memory cell antigens removes the memory cells that are renewing the B-cells and/or T-lymphocytes that are playing a role in the autoimmune disease. In some embodiments, treatment of the patient with T-lymphocytes and/or natural killer cells with CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR that bind to hematopoietic stem cells removes the patient's hematopoietic stem cells in preparation for a stem cell or bone marrow transplant. In this embodiment, the T-lymphocyte and/or natural killer cell with CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR that target hematopoietic stem cells deplete the patient's hematopoietic stem cells, which are then repopulated by the hematopoietic stem cell or bone marrow transplant. This embodiment allows the patient to receive a bone marrow or hematopoietic stem cell transplant without having to undergo toxic chemotherapy or toxic radiation treatment.

[0214] In some embodiments, the patient with the autoimmune disorder receives a treatment with chemotherapy and/or radiation prior to or at the same time as treatment with T-lymphocytes or natural killer cells with CARs, Smart CARs, DE-CARs, and/or Smart-DE-CARs targeted at hematopoietic stem cells.

Hematopoietic Stem Cell (or Bone Marrow) Transplant

[0215] Hematopoietic stem cell transplantation (HSCT) or bone marrow transplantation is the transplantation of blood stem cells derived from the bone marrow or blood. In some embodiments, hematopoietic stem cell transplantation procedures are performed using stem cells collected from the

peripheral blood. In some embodiments, collecting peripheral blood stem cells provides a larger number of hematopoietic stem cells, does not require that the donor be subjected to general anesthesia to collect the graft, and results in a shorter time to engraftment.

[0216] Autologous hematopoietic stem cell transplant involves isolation of hematopoietic stem cells from the patient and storage of the harvested hematopoietic stem cells until after an ablative treatment of the patient to deplete the hematopoietic stem cells in the patient's body. The patient's hematopoietic disorder is treated (e.g., CARs targeting a malignancy), and as a result of this treatment or as the result of an additional treatment, the patient's bone marrow stem cells are ablated in whole or in part, and then the patient's harvested stem cells are returned to their body. In some embodiments, a CAR, Smart CAR, DE-CAR, and/or Smart-DE-CAR is targeted to the patient's hematopoietic stem cells through an appropriate CD target. Such a CAR treatment primes the patient for receiving the hematopoietic stem cell/bone marrow transplant. Autologous transplants have the advantage of a lower risk of graft rejection and infection, since the recovery of immune function is rapid. Also, the incidence of a patient experiencing graft-versus-host disease is close to none as the donor and recipient are the same individual.

[0217] "Allogeneic hematopoietic stem cell transplantation" refers herein to a method of reconstituting hematopoiesis by transfusing hematopoietic stem cells from a related donor or an unrelated donor having an HLA type that is identical or similar to the patient. In order to collect or acquire the hematopoietic stem cells, it is necessary first to screen potential donors for their HLA types, e.g., donors may be relatives or found in bone marrow banks (e.g., the Japan Marrow Donor Program) or umbilical cord blood banks (e.g., the Japanese Cord Blood Bank Network). The donor is selected to have a HLA type which is identical or similar to that of the patient. Allogeneic hematopoietic stem cell transplantation may have a graft versus tumor effect, but there is a risk of graft versus host disease onset.

[0218] Given that three HLA types (HLA-A, -B and -DR) are inherited from each parent, in principle, the number of HLA types which should be considered in allogeneic hematopoietic stem cell transplantation is six. A certain degree of incompatibility between the HLA loci may strengthen the GVT effect. Hence, it is preferable to select a suitable donor according to the type of hematopoietic disorder, the age and health status of the patient, and the type of hematopoietic stem cell to be transplanted. Donor selection is carried out based on, in principle, the following classification. Because the HLA type is inherited, there is a $\frac{1}{4}$ probability of compatibility among siblings. For this reason, the frequency of GVHD and transfusion-related complications is generally low. In allogeneic hematopoietic cell transplantation, it is desirable first to seek a compatible donor from among relatives. In cases where an HLA-matched relative for all the HLA types has not been found, an HLA-matched person (HLA-matched unrelated donor) can be sought from a bone marrow bank. Given that the success rate for allogeneic hematopoietic stem cell transplantation from related donors in which five of the six HLA types match is comparable to that from HLA-matched non-related donors, even in cases where a relative that matches for all HLA types has not been found, a related donor with a partial mismatch may be selected. In cases

where a suitable donor cannot be found from among HLA-matched individuals or HLA-mismatched relatives, an HLA-mismatched unrelated person may be selected as the donor.

[0219] In addition, in selecting the donor, it is preferable to make a judgment which is also based on, for example, respiratory function, circulatory function, liver function, medical history for various diseases, and the presence or absence of infections and allergies. For more detailed selection criteria, reference may be made to the Manual of hematopoietic stem cell transplantation and diagnosis, first edition (published by Nihon Igakukan), or Manual of hematopoietic cell transplantation, revised third edition (published by Nihon Igakukan), both of which are incorporated by reference in their entirety for all purposes.

[0220] In some embodiments, the hematopoietic stem cells may, for example, be bone marrow cells supplemented with about 1-2% of T-lymphocytes to enhance the graft rate without eliciting the onset of GVHD. In some embodiments, hematopoietic stem cells are prepared by adding anti-T-lymphocyte antibody (e.g. a mixture of anti-CD3 antibody or anti-CD4 antibody with anti-CD8 antibody) to a cell population and then adding complement to kill the T-lymphocytes thereby removing the T-lymphocytes from the population or by adding anti-T-lymphocyte antibody and removing the cells coupled to the anti-T cell antibody selectively by physical means (e.g., a column that binds the anti-T-lymphocyte antibody). The purification (isolation) of T-lymphocytes can be made by removing erythrocytes from peripheral blood to provide mononuclear cells in the routine manner, adding said anti-T-lymphocyte antibody to this cell population and selectively recovering the cells coupled to the anti-T-lymphocyte antibody by the magnetic bead method or by adding the anti-T-lymphocyte antibody conjugated with a fluorescent dye to the mononuclear cell population and recovering the T-lymphocytes with an automatic fluorescent separation hardware. Other methods of preparing hematopoietic stem cells are found in the Manual of hematopoietic stem cell transplantation and diagnosis, first edition (published by Nihon Igakukan), or Manual of hematopoietic cell transplantation, revised third edition (published by Nihon Igakukan) both of which are incorporated by reference in their entirety for all purposes.

[0221] Hematopoietic stem cell transplantation also falls into three categories, depending on the type of cell used in the transplantation: bone marrow transplantation, peripheral blood stem cell transplantation, and umbilical cord blood transplantation. Bone marrow transplantation is a method of transplanting hematopoietic stem cells by transplanting bone marrow fluid. Bone marrow fluid can be obtained by placing the donor under general anesthesia and using a bone marrow needle to collect about 15 to 20 mL of fluid per body weight from three to five places on the left and right sides of the dorsum of the pelvis. Peripheral blood stem cell transplantation is a method wherein peripheral blood stem cells which have been mobilized in a large quantity from the bone marrow into the blood by G-CSF administration are transplanted. Peripheral blood stem cells can be obtained by subcutaneously injecting about 10 $\mu\text{g}/\text{kg}/\text{day}$ of G-CSF for 4 to 6 days, and using a blood component collection system to collect the cells on days 4 to 6 following injection. The timing of cell collection can be set by measuring the number of cells positive for the CD34 antigen, which is a hematopoietic stem cell marker present in the blood. Umbilical cord

blood transplantation is a method of transplanting hematopoietic stem cells present in umbilical cord blood. Cord blood which matches the patient can be sought from a cord blood bank by means of a HLA type test. In cord blood transplantation, although the number of stem cells that can be collected from umbilical cord blood is limited, compared with the other types of transplantation, GVHD does not readily arise. As a result, even if two out of six HLA type are incompatible, transplantation is possible. Methods of transplantation and methods of collecting, preparing or screening for bone marrow, peripheral blood stem cells or umbilical cord blood can be carried out based on the Manual of hematopoietic stem cell transplantation and diagnosis, first edition (published by Nihon Igakukan), or Manual of hematopoietic cell transplantation, revised third edition (published by Nihon Igakukan) both of which are incorporated by reference in their entirety for all purposes.

[0222] After the treatment to ablate or remove many or most of the patient's hematopoietic stem cells, the transplant hematopoietic stem cells (or bone marrow) are provided to the patient intravenously, similar to a blood transfusion. In some embodiments, the patient receives additional medication prior to, at the same time as, or after the transfusion of hematopoietic stem cells. The additional medication may include, for example, anti-inflammatories, immune suppressive drugs, and/or anti-allergy drugs (e.g., anti-histamines), G-CSF, GM-CSF, or other such immune stimulatory molecules, erythropoietin, thrombopoietin, etc.

[0223] The inventions disclosed herein will be better understood from the experimental details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the inventions as described more fully in the claims which follow thereafter. Unless otherwise indicated, the disclosure is not limited to specific procedures, materials, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Examples

Example 1. A Chimeric Antigen Receptor Targeted to CD123

[0224] A CAR is made using the anti-CD20 CAR cassette described in Budde 2013 (Budde et al. PLoS1, 2013 doi: 10.1371/journal.pone.0082742, which is hereby incorporated-by-reference in its entirety for all purposes), with an anti-CD123 mAb scFv (WO 2014/144622 and US20140271582, which are incorporated by reference in their entirety for all purposes) replacing the anti-CD20 extracellular domain. In an embodiment, the anti-CD 123 CAR also encodes the RNA control device, 3XL2bulge9 (Win and Smolke 2007 Proc. Natl Acad. Sci. 104 (36): 14283-88, which is hereby incorporated by reference in its entirety for all purposes), and/or the destabilizing element (DE) ecDHFR described in Iwamoto 2010 (Iwamoto et al. Chemistry and Biology, 2010 doi:10.1016/j.chembiol.2010.07.009, which is hereby incorporated by reference in its entirety for all purposes). A nucleic acid encoding the anti-CD20 CAR cassette is engineered to replace the anti-CD20 extracellular domain with the anti-CD 123 element, and optionally the RNA control device and/or the destabilizing element are also engineered into the cassette. The

anti-CD123 CAR with or without the RNA control device and/or the DE are cloned into appropriate expression vectors.

[0225] This anti-CD123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD123 DE-Smart CAR are transfected by routine methods into T-lymphocytes (Jurkat cells and/or primary human T-lymphocytes), and stable populations of T-lymphocytes are selected using appropriate antibiotics (or other selection schemes). T-cell populations with anti-anti-CD123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD123 DE-Smart CAR (CD20⁻/CD22⁻/CD3⁺) are activated by co-incubation with anti-CD3/CD28 beads.

[0226] Activated anti-CD123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD123 DE-Smart CAR T-lymphocytes are co-cultured with CD123⁺/CD3⁻ HSC target cells (e.g., human hematopoietic stem cells at ATCC PCS-800-012) at anti-CD123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD123 DE-Smart CAR T-lymphocyte:HSC target ratios of 2:1, 5:1, and 10:1. Ligand for the DE, trimethoprim, and/or ligand for the RNA control device, theophylline, is added to the culture medium at concentrations in the range of 500 LM to 1 mM (lower or greater concentrations can be used to titrate Smart-CAR activity to the desired level). The anti-CD123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD123 DE-Smart CAR T-lymphocytes and the HSC cells are grown together for 48 hours. Cultures are washed, and then stained with anti-CD123 and anti-CD3 reagents, followed by counting of CD123⁺ (HSC target cells) and CD3⁺ cells (anti-CD 123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD123 DE-Smart CAR T-lymphocytes). These measurements will identify the target cell killing rate (e.g., half-life) and the proliferation rate of the anti-CD123 CAR, anti-CD123 DE-CAR, anti-CD123 Smart CAR, and/or the anti-CD 123 DE-Smart CAR T-cells at different levels of CAR and/or DE-CAR expression.

Example 2. A Chimeric Antigen Receptor Targeted to CD133 (AML)

[0227] A CAR is made using the anti-CD20 CAR cassette described in Budde 2013 (Budde et al. PLoS1, 2013 doi: 10.1371/journal.pone.0082742, which is hereby incorporated-by-reference in its entirety for all purposes), with the anti-CD133 mAb 293C3-SDIE is used for the extracellular element (Rothfelder et al., 2015, ash.confex.com/ash/2015/webprogram/Paper81121.html, which is incorporated by reference in its entirety for all purposes) replacing the anti-CD20 extracellular domain. In an embodiment, the anti-CD133 CAR also encodes the RNA control device, 3XL2bulge9 (Win and Smolke 2007 Proc. Natl Acad. Sci. 104 (36): 14283-88, which is hereby incorporated by reference in its entirety for all purposes), and/or the destabilizing element (DE) ecDHFR described in Iwamoto 2010 (Iwamoto et al. Chemistry and Biology, 2010 doi: 10.1016/j.chembiol.2010.07.009, which is hereby incorporated by reference in its entirety for all purposes). A nucleic acid encoding the anti-CD20 CAR cassette is engineered to replace the anti-CD20 extracellular domain with the anti-CD133 element, and optionally the RNA control device and/or the destabilizing element are also engineered into the

cassette. The anti-CD133 CAR with or without the RNA control device and/or the DE are cloned into appropriate expression vectors.

[0228] This anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR are transfected by routine methods into T-lymphocytes (Jurkat cells and/or primary human T-lymphocytes), and stable populations of T-lymphocytes are selected using appropriate antibiotics (or other selection schemes). T-lymphocyte populations with anti-anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR (CD20⁻/CD22⁻/CD3⁺) are activated by co-incubation with anti-CD3/CD28 beads.

[0229] Activated anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD 133 DE-Smart CAR T-lymphocytes are co-cultured with CD133⁺/CD3⁻ AML target cells (e.g., U937, MV4-11, MOLM-14, HL-60 and/or KG1a) at anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-lymphocyte:AML target ratios of 2:1, 5:1, and 10:1. Ligand for the DE, trimethoprim, and/or ligand for the RNA control device, theophylline, is added to the culture medium at concentrations in the range of 500 LM to 1 mM (lower or greater concentrations can be used to titrate Smart-CAR activity to the desired level). The anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-lymphocytes and the AML cells are grown together for 48 hours. Cultures are washed, and then stained with anti-CD133 and anti-CD3 reagents, followed by counting of CD133⁺ (AML target cells) and CD3⁺ cells (anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-lymphocytes). These measurements will identify the target cell killing rate (e.g., half-life) and the proliferation rate of the anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-lymphocytes at different levels of CAR and/or DE-CAR expression.

Example 3. A Chimeric Antigen Receptor Targeted to CD133 (AML) in a FasL Knock Out T-Lymphocyte

[0230] A CAR is made using the anti-CD20 CAR cassette described in Budde 2013 (Budde et al. PLoS1, 2013 doi: 10.1371/journal.pone.0082742, which is hereby incorporated-by-reference in its entirety for all purposes), with the anti-CD133 mAb 293C3-SDIE is used for the extracellular element (Rothfelder et al., 2015, ash.confex.com/ash/2015/webprogram/Paper81121.html, which is incorporated by reference in its entirety for all purposes) replacing the anti-CD20 extracellular domain. In an embodiment, the anti-CD133 CAR also encodes the RNA control device, 3XL2bulge9 (Win and Smolke 2007 Proc. Natl Acad. Sci.

104 (36): 14283-88, which is hereby incorporated by reference in its entirety for all purposes), and/or the destabilizing element (DE) ecDHFR described in Iwamoto 2010 (Iwamoto et al. Chemistry and Biology, 2010 doi: 10.1016/j.chembiol.2010.07.009, which is hereby incorporated by reference in its entirety for all purposes). A nucleic acid encoding the anti-CD20 CAR cassette is engineered to replace the anti-CD20 extracellular domain with the anti-CD133 element, and optionally the RNA control device and/or the destabilizing element are also engineered into the cassette. The anti-CD133 CAR with or without the RNA control device and/or the DE are cloned into appropriate expression vectors.

[0231] T-lymphocytes (Jurkat cells and/or primary human T-cells), or stable populations of T-cells are genetically modified using CRISPR/cas9 to make a double-allele knockout of FasL. Multiple guide RNAs specific for FasL are designed and then together with the cas9 enzyme are introduced into the T-cells. Double allele FasL knockouts are identified by T-lymphocyte clones that do not stain with anti-FasL antibody.

[0232] This anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR are transfected by routine methods into the double-allele knockout FasL T-lymphocytes. T-cell populations with anti-anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR (CD20⁻/CD22⁻/CD3⁺) are activated by co-incubation with anti-CD3/CD28 beads.

[0233] Activated anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD 133 DE-Smart CAR T-lymphocytes are co-cultured with CD133⁺/CD3⁻ AML target cells (e.g., U937, MV4-11, MOLM-14, HL-60 and/or KG1a) at anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-cell:AML target ratios of 2:1, 5:1, and 10:1. Ligand for the DE, trimethoprim, and/or ligand for the RNA control device, theophylline, is added to the culture medium at concentrations in the range of 500 μM to 1 mM (lower or greater concentrations can be used to titrate Smart-CAR activity to the desired level). The anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-cells and the AML cells are grown together for 48 hours. Cultures are washed, and then stained with anti-CD133 and anti-CD3 reagents, followed by counting of CD133⁺ (AML target cells) and CD3⁺ cells (anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-lymphocytes). These measurements will identify the target cell killing rate (e.g., half-life) and the proliferation rate of the anti-CD133 CAR, anti-CD133 DE-CAR, anti-CD133 Smart CAR, and/or the anti-CD133 DE-Smart CAR T-cells at different levels of CAR and/or DE-CAR expression.

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Met Ile Ser Leu Ile Ala Ala Leu Ala Val Asp His Val Ile Gly Met
1           5           10           15

Glu Asn Ala Met Pro Trp Asn Leu Pro Ala Asp Leu Ala Trp Phe Lys
           20           25           30

Arg Asn Thr Leu Asn Lys Pro Val Ile Met Gly Arg His Thr Trp Glu
           35           40           45

Ser Ile Gly Arg Pro Leu Pro Gly Arg Lys Asn Ile Ile Leu Ser Ser
           50           55           60

Gln Pro Ser Thr Asp Asp Arg Val Thr Trp Val Lys Ser Val Asp Glu
           65           70           75           80

Ala Ile Ala Ala Cys Gly Asp Val Pro Glu Ile Met Val Ile Gly Gly
           85           90           95

Gly Arg Val Tyr Glu Gln Phe Leu Pro Lys Ala Gln Lys Leu Tyr Leu
           100          105          110

Thr His Ile Asp Ala Glu Val Glu Gly Asp Thr His Phe Pro Asp Tyr
           115          120          125

Glu Pro Asp Asp Trp Glu Ser Val Phe Ser Glu Phe His Asp Ala Asp
           130          135          140

Ala Gln Asn Ser His Ser Tyr Cys Phe Glu Ile Leu Glu Arg Arg
           145          150          155

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<210> SEQ ID NO 6
<211> LENGTH: 159
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: DHFR variant peptide
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (12)..(12)
<223> OTHER INFORMATION: Xaa = His, Leu, Tyr
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (18)..(18)
<223> OTHER INFORMATION: Xaa = Asn, Thr
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa = Ala, Val
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (42)..(42)
<223> OTHER INFORMATION: Xaa = Met, Thr
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (61)..(61)
<223> OTHER INFORMATION: Xaa = Ile, Phe
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (68)..(68)
<223> OTHER INFORMATION: Xaa = Thr, Ser
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (98)..(98)
<223> OTHER INFORMATION: Xaa = Arg, His
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (100)..(100)

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<223> OTHER INFORMATION: Xaa = Tyr, Ile
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (103)..(103)
<223> OTHER INFORMATION: Xaa = Phe, Leu, Ser
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (114)..(114)
<223> OTHER INFORMATION: Xaa = His, Arg
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (121)..(121)
<223> OTHER INFORMATION: Xaa = Gly, Val

<400> SEQUENCE: 6

Met Ile Ser Leu Ile Ala Ala Leu Ala Val Asp Xaa Val Ile Gly Met
 1             5             10             15

Glu Xaa Xaa Met Pro Trp Asn Leu Pro Ala Asp Leu Ala Trp Phe Lys
 20             25             30

Arg Asn Thr Leu Asn Lys Pro Val Ile Xaa Gly Arg His Thr Trp Glu
 35             40             45

Ser Ile Gly Arg Pro Leu Pro Gly Arg Lys Asn Ile Xaa Leu Ser Ser
 50             55             60

Gln Pro Ser Xaa Asp Asp Arg Val Thr Trp Val Lys Ser Val Asp Glu
 65             70             75             80

Ala Ile Ala Ala Cys Gly Asp Val Pro Glu Ile Met Val Ile Gly Gly
 85             90             95

Gly Xaa Val Xaa Glu Gln Xaa Leu Pro Lys Ala Gln Lys Leu Tyr Leu
 100            105            110

Thr Xaa Ile Asp Ala Glu Val Glu Xaa Asp Thr His Phe Pro Asp Phe
 115            120            125

Glu Pro Asp Asp Trp Glu Ser Val Phe Ser Glu Phe His Asp Ala Asp
 130            135            140

Ala Gln Asn Ser His Ser Tyr Cys Phe Glu Ile Leu Glu Arg Arg
 145            150            155

<210> SEQ ID NO 7
<211> LENGTH: 159
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: DHFR variant peptide
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (1)..(159)
<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 7

Met Ile Ser Leu Ile Ala Ala Leu Ala Val Asp Xaa Val Ile Gly Met
 1             5             10             15

Glu Xaa Xaa Met Pro Trp Asn Leu Pro Ala Asp Leu Ala Trp Phe Lys
 20             25             30

Arg Asn Thr Leu Asn Lys Pro Val Ile Xaa Gly Arg His Thr Trp Glu
 35             40             45

Ser Ile Gly Arg Pro Leu Pro Gly Arg Lys Asn Ile Xaa Leu Ser Ser
 50             55             60

Gln Pro Ser Xaa Asp Asp Arg Val Thr Trp Val Lys Ser Val Asp Glu
 65             70             75             80

Ala Ile Ala Ala Cys Gly Asp Val Pro Glu Ile Met Val Ile Gly Gly

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	85		90		95										
Gly	Xaa	Val	Xaa	Glu	Gln	Xaa	Leu	Pro	Lys	Ala	Gln	Lys	Leu	Tyr	Leu
			100					105							110
Thr	Xaa	Ile	Asp	Ala	Glu	Val	Glu	Xaa	Asp	Thr	His	Phe	Pro	Asp	Phe
			115					120						125	
Glu	Pro	Asp	Asp	Trp	Glu	Ser	Val	Phe	Ser	Glu	Phe	His	Asp	Ala	Asp
		130					135						140		
Ala	Gln	Asn	Ser	His	Ser	Tyr	Cys	Phe	Glu	Ile	Leu	Glu	Arg	Arg	
		145				150					155				

<210> SEQ ID NO 8
 <211> LENGTH: 273
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: ERBD Peptide

<400> SEQUENCE: 8

Ser	Leu	Ala	Leu	Ser	Leu	Thr	Ala	Asp	Gln	Met	Val	Ser	Ala	Leu	Leu
1				5					10						15
Asp	Ala	Glu	Pro	Pro	Ile	Leu	Tyr	Ser	Glu	Tyr	Asp	Pro	Thr	Arg	Pro
			20					25					30		
Phe	Ser	Glu	Ala	Ser	Met	Met	Gly	Leu	Leu	Thr	Asn	Leu	Ala	Asp	Arg
			35				40					45			
Glu	Leu	Val	His	Met	Ile	Asn	Trp	Ala	Lys	Arg	Val	Pro	Gly	Phe	Val
			50			55					60				
Asp	Leu	Thr	Leu	His	Asp	Gln	Val	His	Leu	Leu	Glu	Cys	Ala	Trp	Leu
			65		70					75					80
Glu	Ile	Leu	Met	Ile	Gly	Leu	Val	Trp	Arg	Ser	Met	Glu	His	Pro	Gly
				85					90					95	
Lys	Leu	Leu	Phe	Ala	Pro	Asn	Leu	Leu	Leu	Asp	Arg	Asn	Gln	Gly	Lys
			100					105						110	
Cys	Val	Glu	Gly	Met	Val	Glu	Ile	Phe	Asp	Met	Leu	Leu	Ala	Thr	Ser
			115					120						125	
Ser	Arg	Phe	Arg	Met	Met	Asn	Leu	Gln	Gly	Glu	Glu	Phe	Val	Cys	Leu
			130			135							140		
Lys	Ser	Ile	Ile	Leu	Leu	Asn	Ser	Gly	Val	Tyr	Thr	Phe	Leu	Ser	Ser
					150					155					160
Thr	Leu	Lys	Ser	Leu	Glu	Glu	Lys	Asp	His	Ile	His	Arg	Val	Leu	Asp
				165					170						175
Lys	Ile	Thr	Asp	Thr	Leu	Ile	His	Leu	Met	Ala	Lys	Ala	Gly	Leu	Thr
			180						185					190	
Leu	Gln	Gln	Gln	His	Gln	Arg	Leu	Ala	Gln	Leu	Leu	Leu	Ile	Leu	Ser
			195				200						205		
His	Ile	Arg	His	Met	Ser	Asn	Lys	Gly	Met	Glu	His	Leu	Tyr	Ser	Met
			210			215							220		
Lys	Cys	Lys	Asn	Val	Val	Pro	Leu	Tyr	Asp	Leu	Leu	Leu	Glu	Met	Leu
					230					235					240
Asp	Ala	His	Arg	Leu	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser	Thr	Arg	Lys
				245					250					255	
His	Lys	Ile	Leu	His	Arg	Leu	Leu	Gln	Asp	Ser	Ser	Arg	Arg	Arg	Gly
			260					265						270	

Asn

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<210> SEQ ID NO 9
<211> LENGTH: 245
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ERBD peptide

<400> SEQUENCE: 9

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
1           5           10          15
Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
20          25          30
Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
35          40          45
Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
50          55          60
Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
65          70          75          80
Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
85          90          95
Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
100         105        110
Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
115        120        125
Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
130        135        140
Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
145        150        155        160
Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
165        170        175
Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
180        185        190
Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
195        200        205
His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
210        215        220
Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
225        230        235        240
Asp Ala His Arg Leu
245

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<210> SEQ ID NO 10
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: degron peptide

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<400> SEQUENCE: 10

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Arg Arg Arg Gly
1

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<210> SEQ ID NO 11
<211> LENGTH: 5
<212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: bacterial degron peptide

<400> SEQUENCE: 11

Tyr Ala Leu Ala Ala
1 5

<210> SEQ ID NO 12

<211> LENGTH: 5

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: degron peptide

<400> SEQUENCE: 12

Arg Arg Arg Gly Asn
1 5

<210> SEQ ID NO 13

<211> LENGTH: 13

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: peptide

<400> SEQUENCE: 13

Lys His Lys Ile Leu His Arg Leu Leu Gln Asp Ser Ser
1 5 10

<210> SEQ ID NO 14

<211> LENGTH: 144

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: AsLOV2 peptide

<400> SEQUENCE: 14

Phe Leu Ala Thr Thr Leu Glu Arg Ile Glu Lys Asn Phe Val Ile Thr
1 5 10 15

Asp Pro Arg Leu Pro Asp Asn Pro Ile Ile Phe Ala Ser Asp Ser Phe
20 25 30

Leu Gln Leu Thr Glu Tyr Ser Arg Glu Glu Ile Leu Gly Arg Asn Cys
35 40 45

Arg Phe Leu Gln Gly Pro Glu Thr Asp Arg Ala Thr Val Arg Lys Ile
50 55 60

Arg Asp Ala Ile Asp Asn Gln Thr Glu Val Thr Val Gln Leu Ile Asn
65 70 75 80

Tyr Thr Lys Ser Gly Lys Lys Phe Trp Asn Leu Phe His Leu Gln Pro
85 90 95

Met Arg Asp Gln Lys Gly Asp Val Gln Tyr Phe Ile Gly Val Gln Leu
100 105 110

Asp Gly Thr Glu His Val Arg Asp Ala Ala Glu Arg Glu Gly Val Met
115 120 125

Leu Ile Lys Lys Thr Ala Glu Asn Ile Asp Glu Ala Ala Lys Glu Leu
130 135 140

<210> SEQ ID NO 15

<211> LENGTH: 144

<212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AsLOV2 variant peptide
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: Xaa = Val, Ala, Ile
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (80)..(80)
<223> OTHER INFORMATION: Xaa = Asn, Lys, Arg
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (120)..(120)
<223> OTHER INFORMATION: Xaa = Asp, Glu, Ala
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (126)..(126)
<223> OTHER INFORMATION: Xaa = Gly, Ala
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (127)..(127)
<223> OTHER INFORMATION: Xaa = Val, Asn
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (130)..(130)
<223> OTHER INFORMATION: Xaa = Ile, Ala
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (136)..(136)
<223> OTHER INFORMATION: Xaa = Asn, Glu, Asp
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (142)..(142)
<223> OTHER INFORMATION: Xaa = Lys or No Amino Acid
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (143)..(143)
<223> OTHER INFORMATION: Xaa = Glu or No Amino Acid
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (144)..(144)
<223> OTHER INFORMATION: Xaa = Leu or No Amino Acid

<400> SEQUENCE: 15

Phe Leu Ala Thr Thr Leu Glu Arg Ile Glu Lys Asn Phe Xaa Ile Thr
1           5           10           15

Asp Pro Arg Leu Pro Asp Asn Pro Ile Ile Phe Ala Ser Asp Ser Phe
                20           25           30

Leu Gln Leu Thr Glu Tyr Ser Arg Glu Glu Ile Leu Gly Arg Asn Cys
35           40           45

Arg Phe Leu Gln Gly Pro Glu Thr Asp Arg Ala Thr Val Arg Lys Ile
50           55           60

Arg Asp Ala Ile Asp Asn Gln Thr Glu Val Thr Val Gln Leu Ile Xaa
65           70           75           80

Tyr Thr Lys Ser Gly Lys Lys Phe Trp Asn Leu Phe His Leu Gln Pro
85           90           95

Met Arg Asp Gln Lys Gly Asp Val Gln Tyr Phe Ile Gly Val Gln Leu
100          105          110

Asp Gly Thr Glu His Val Arg Xaa Ala Ala Glu Arg Glu Xaa Xaa Met
115          120          125

Leu Xaa Lys Lys Thr Ala Glu Xaa Ile Asp Glu Ala Ala Xaa Xaa Xaa
130          135          140

<210> SEQ ID NO 16
<211> LENGTH: 144

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AsLOV2 variant peptide
<220> FEATURE:
<221> NAME/KEY: Xaa
<222> LOCATION: (1)..(144)
<223> OTHER INFORMATION: Xaa = any amino acid

<400> SEQUENCE: 16

Phe Leu Ala Thr Thr Leu Glu Arg Ile Glu Lys Asn Phe Xaa Ile Thr
1           5           10          15
Asp Pro Arg Leu Pro Asp Asn Pro Ile Ile Phe Ala Ser Asp Ser Phe
20          25          30
Leu Gln Leu Thr Glu Tyr Ser Arg Glu Glu Ile Leu Gly Arg Asn Cys
35          40          45
Arg Phe Leu Gln Gly Pro Glu Thr Asp Arg Ala Thr Val Arg Lys Ile
50          55          60
Arg Asp Ala Ile Asp Asn Gln Thr Glu Val Thr Val Gln Leu Ile Xaa
65          70          75          80
Tyr Thr Lys Ser Gly Lys Lys Phe Trp Asn Leu Phe His Leu Gln Pro
85          90          95
Met Arg Asp Gln Lys Gly Asp Val Gln Tyr Phe Ile Gly Val Gln Leu
100         105         110
Asp Gly Thr Glu His Val Arg Xaa Ala Ala Glu Arg Glu Xaa Xaa Met
115        120        125
Leu Xaa Lys Lys Thr Ala Glu Xaa Ile Asp Glu Ala Ala Xaa Xaa Xaa
130        135        140

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<210> SEQ ID NO 17
<211> LENGTH: 114
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CD3 zeta peptide

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<400> SEQUENCE: 17

Leu Arg Val Lys Phe Ser Arg Ser Ala Asp Ala Pro Ala Tyr Gln Gln
1           5           10          15
Gly Gln Asn Gln Leu Tyr Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu
20          25          30
Tyr Asp Val Leu Asp Lys Arg Arg Gly Arg Asp Pro Glu Met Gly Gly
35          40          45
Lys Pro Gln Arg Arg Lys Asn Pro Gln Glu Gly Leu Tyr Asn Glu Leu
50          55          60
Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser Glu Ile Gly Met Lys Gly
65          70          75          80
Glu Arg Arg Arg Gly Lys Gly His Asp Gly Leu Tyr Gln Gly Leu Ser
85          90          95
Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu His Met Gln Ala Leu Pro
100         105         110

Pro Arg

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<210> SEQ ID NO 18
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
 <223> OTHER INFORMATION: Fc epsilon receptor subunit gamma peptide

<400> SEQUENCE: 18

Arg Leu Lys Ile Gln Val Arg Lys Ala Ala Ile Thr Ser Tyr Glu Lys
 1 5 10 15

Ser Asp Gly Val Tyr Thr Gly Leu Ser Thr Arg Asn Gln Glu Thr Tyr
 20 25 30

Glu Thr Leu Lys His Glu Lys Pro Pro Gln
 35 40

<210> SEQ ID NO 19
 <211> LENGTH: 44
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Fc epsilon receptor subunit beta peptide

<400> SEQUENCE: 19

Ile Cys Gly Ala Gly Glu Glu Leu Lys Gly Asn Lys Val Pro Glu Asp
 1 5 10 15

Arg Val Tyr Glu Glu Leu Asn Ile Tyr Ser Ala Thr Tyr Ser Glu Leu
 20 25 30

Glu Asp Pro Gly Glu Met Ser Pro Pro Ile Asp Leu
 35 40

<210> SEQ ID NO 20
 <211> LENGTH: 44
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD3 gamma peptide

<400> SEQUENCE: 20

Gln Asp Gly Val Arg Gln Ser Arg Ala Ser Asp Lys Gln Thr Leu Leu
 1 5 10 15

Pro Asn Asp Gln Leu Tyr Gln Pro Leu Lys Asp Arg Glu Asp Asp Gln
 20 25 30

Tyr Ser His Leu Gln Gly Asn Gln Leu Arg Arg Asn
 35 40

<210> SEQ ID NO 21
 <211> LENGTH: 44
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD3 delta peptide

<400> SEQUENCE: 21

His Glu Thr Gly Arg Leu Ser Gly Ala Ala Asp Thr Gln Ala Leu Leu
 1 5 10 15

Arg Asn Asp Gln Val Tyr Gln Pro Leu Arg Asp Arg Asp Ala Gln
 20 25 30

Tyr Ser His Leu Gly Gly Asn Trp Ala Arg Asn Lys
 35 40

<210> SEQ ID NO 22
 <211> LENGTH: 55
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:

<223> OTHER INFORMATION: CD3 epsilon peptide

<400> SEQUENCE: 22

Lys Asn Arg Lys Ala Lys Ala Lys Pro Val Thr Arg Gly Ala Gly Ala
 1 5 10 15
 Gly Gly Arg Gln Arg Gly Gln Asn Lys Glu Arg Pro Pro Pro Val Pro
 20 25 30
 Asn Pro Asp Tyr Glu Pro Ile Arg Lys Gly Gln Arg Asp Leu Tyr Ser
 35 40 45
 Gly Leu Asn Gln Arg Arg Ile
 50 55

<210> SEQ ID NO 23

<211> LENGTH: 94

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: CD5 peptide

<400> SEQUENCE: 23

Tyr Lys Lys Leu Val Lys Lys Phe Arg Gln Lys Lys Gln Arg Gln Trp
 1 5 10 15
 Ile Gly Pro Thr Gly Met Asn Gln Asn Met Ser Phe His Arg Asn His
 20 25 30
 Thr Ala Thr Val Arg Ser His Ala Glu Asn Pro Thr Ala Ser His Val
 35 40 45
 Asp Asn Glu Tyr Ser Gln Pro Pro Arg Asn Ser His Leu Ser Ala Tyr
 50 55 60
 Pro Ala Leu Glu Gly Ala Leu His Arg Ser Ser Met Gln Pro Asp Asn
 65 70 75 80
 Ser Ser Asp Ser Asp Tyr Asp Leu His Gly Ala Gln Arg Leu
 85 90

<210> SEQ ID NO 24

<211> LENGTH: 141

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: CD22 peptide

<400> SEQUENCE: 24

Lys Leu Gln Arg Arg Trp Lys Arg Thr Gln Ser Gln Gln Gly Leu Gln
 1 5 10 15
 Glu Asn Ser Ser Gly Gln Ser Phe Phe Val Arg Asn Lys Lys Val Arg
 20 25 30
 Arg Ala Pro Leu Ser Glu Gly Pro His Ser Leu Gly Cys Tyr Asn Pro
 35 40 45
 Met Met Glu Asp Gly Ile Ser Tyr Thr Thr Leu Arg Phe Pro Glu Met
 50 55 60
 Asn Ile Pro Arg Thr Gly Asp Ala Glu Ser Ser Glu Met Gln Arg Pro
 65 70 75 80
 Pro Pro Asp Cys Asp Asp Thr Val Thr Tyr Ser Ala Leu His Lys Arg
 85 90 95
 Gln Val Gly Asp Tyr Glu Asn Val Ile Pro Asp Phe Pro Glu Asp Glu
 100 105 110

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Gly Ile His Tyr Ser Glu Leu Ile Gln Phe Gly Val Gly Glu Arg Pro
 115 120 125

Gln Ala Gln Glu Asn Val Asp Tyr Val Ile Leu Lys His
 130 135 140

<210> SEQ ID NO 25
 <211> LENGTH: 61
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD79a peptide

<400> SEQUENCE: 25

Arg Lys Arg Trp Gln Asn Glu Lys Leu Gly Leu Asp Ala Gly Asp Glu
 1 5 10 15

Tyr Glu Asp Glu Asn Leu Tyr Glu Gly Leu Asn Leu Asp Asp Cys Ser
 20 25 30

Met Tyr Glu Asp Ile Ser Arg Gly Leu Gln Gly Thr Tyr Gln Asp Val
 35 40 45

Gly Ser Leu Asn Ile Gly Asp Val Gln Leu Glu Lys Pro
 50 55 60

<210> SEQ ID NO 26
 <211> LENGTH: 64
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD79b peptide

<400> SEQUENCE: 26

Thr Leu Leu Ile Ile Leu Phe Ile Ile Val Pro Ile Phe Leu Leu Leu
 1 5 10 15

Asp Lys Asp Asp Ser Lys Ala Gly Met Glu Glu Asp His Thr Tyr Glu
 20 25 30

Gly Leu Asp Ile Asp Gln Thr Ala Thr Tyr Glu Asp Ile Val Thr Leu
 35 40 45

Arg Thr Gly Glu Val Lys Trp Ser Val Gly Glu His Pro Gly Pro Glu
 50 55 60

<210> SEQ ID NO 27
 <211> LENGTH: 76
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD66d peptide

<400> SEQUENCE: 27

Ala Lys Thr Gly Arg Thr Ser Ile Gln Arg Asp Leu Lys Glu Gln Gln
 1 5 10 15

Pro Gln Ala Leu Ala Pro Gly Arg Gly Pro Ser His Ser Ser Ala Phe
 20 25 30

Ser Met Ser Pro Leu Ser Thr Ala Gln Ala Pro Leu Pro Asn Pro Arg
 35 40 45

Thr Ala Ala Ser Ile Tyr Glu Leu Leu Lys His Asp Thr Asn Ile
 50 55 60

Tyr Cys Arg Met Asp His Lys Ala Glu Val Ala Ser
 65 70 75

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<210> SEQ ID NO 28
 <211> LENGTH: 116
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD2 peptide

 <400> SEQUENCE: 28

 Lys Arg Lys Lys Gln Arg Ser Arg Arg Asn Asp Glu Glu Leu Glu Thr
 1 5 10 15

 Arg Ala His Arg Val Ala Thr Glu Glu Arg Gly Arg Lys Pro His Gln
 20 25 30

 Ile Pro Ala Ser Thr Pro Gln Asn Pro Ala Thr Ser Gln His Pro Pro
 35 40 45

 Pro Pro Pro Gly His Arg Ser Gln Ala Pro Ser His Arg Pro Pro Pro
 50 55 60

 Pro Gly His Arg Val Gln His Gln Pro Gln Lys Arg Pro Pro Ala Pro
 65 70 75 80

 Ser Gly Thr Gln Val His Gln Gln Lys Gly Pro Pro Leu Pro Arg Pro
 85 90 95

 Arg Val Gln Pro Lys Pro Pro His Gly Ala Ala Glu Asn Ser Leu Ser
 100 105 110

 Pro Ser Ser Asn
 115

<210> SEQ ID NO 29
 <211> LENGTH: 38
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD4 peptide

 <400> SEQUENCE: 29

 Arg Cys Arg His Arg Arg Arg Gln Ala Glu Arg Met Ser Gln Ile Lys
 1 5 10 15

 Arg Leu Leu Ser Glu Lys Lys Thr Cys Gln Cys Pro His Arg Phe Gln
 20 25 30

 Lys Thr Cys Ser Pro Ile
 35

<210> SEQ ID NO 30
 <211> LENGTH: 94
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD5 peptide

 <400> SEQUENCE: 30

 Tyr Lys Lys Leu Val Lys Lys Phe Arg Gln Lys Lys Gln Arg Gln Trp
 1 5 10 15

 Ile Gly Pro Thr Gly Met Asn Gln Asn Met Ser Phe His Arg Asn His
 20 25 30

 Thr Ala Thr Val Arg Ser His Ala Glu Asn Pro Thr Ala Ser His Val
 35 40 45

 Asp Asn Glu Tyr Ser Gln Pro Pro Arg Asn Ser His Leu Ser Ala Tyr
 50 55 60

 Pro Ala Leu Glu Gly Ala Leu His Arg Ser Ser Met Gln Pro Asp Asn
 65 70 75 80

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Ser Ser Asp Ser Asp Tyr Asp Leu His Gly Ala Gln Arg Leu
 85 90

<210> SEQ ID NO 31
 <211> LENGTH: 29
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD8 alpha peptide

<400> SEQUENCE: 31

Asn His Arg Asn Arg Arg Arg Val Cys Lys Cys Pro Arg Pro Val Val
 1 5 10 15

Lys Ser Gly Asp Lys Pro Ser Leu Ser Ala Arg Tyr Val
 20 25

<210> SEQ ID NO 32
 <211> LENGTH: 15
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD8 beta peptide

<400> SEQUENCE: 32

Arg Arg Arg Arg Ala Arg Leu Arg Phe Met Lys Gln Phe Tyr Lys
 1 5 10 15

<210> SEQ ID NO 33
 <211> LENGTH: 40
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD28 peptide

<400> SEQUENCE: 33

Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
 1 5 10 15

Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
 20 25 30

Arg Asp Phe Ala Ala Tyr Arg Ser
 35 40

<210> SEQ ID NO 34
 <211> LENGTH: 42
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: CD137 TNF receptor member 9 peptide

<400> SEQUENCE: 34

Lys Arg Gly Arg Lys Lys Leu Leu Tyr Ile Phe Lys Gln Pro Phe Met
 1 5 10 15

Arg Pro Val Gln Thr Thr Gln Glu Glu Asp Gly Cys Ser Cys Arg Phe
 20 25 30

Pro Glu Glu Glu Glu Gly Gly Cys Glu Leu
 35 40

<210> SEQ ID NO 35
 <211> LENGTH: 37
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:

<223> OTHER INFORMATION: CD134 TNF receptor member 4

<400> SEQUENCE: 35

Arg Arg Asp Gln Arg Leu Pro Pro Asp Ala His Lys Pro Pro Gly Gly
 1 5 10 15

Gly Ser Phe Arg Thr Pro Ile Gln Glu Glu Gln Ala Asp Ala His Ser
 20 25 30

Thr Leu Ala Lys Ile
 35

<210> SEQ ID NO 36

<211> LENGTH: 34

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: T-cell costimulator (ICOS) peptide

<400> SEQUENCE: 36

Lys Lys Lys Tyr Ser Ser Ser Val His Asp Pro Asn Gly Glu Tyr Met
 1 5 10 15

Phe Met Arg Ala Val Asn Thr Ala Lys Lys Ser Arg Leu Thr Asp Val
 20 25 30

Thr Leu

<210> SEQ ID NO 37

<211> LENGTH: 28

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: CD28 transmembrane peptide

<400> SEQUENCE: 37

Phe Trp Val Leu Val Val Val Gly Gly Val Leu Ala Cys Tyr Ser Leu
 1 5 10 15

Leu Val Thr Val Ala Phe Ile Ile Phe Trp Val Arg
 20 25

<210> SEQ ID NO 38

<211> LENGTH: 22

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: GITR, TNF receptor member 18 transmembrane element

<400> SEQUENCE: 38

Pro Leu Gly Trp Leu Thr Val Val Leu Leu Ala Val Ala Ala Cys Val
 1 5 10 15

Leu Leu Leu Thr Ser Ala
 20

<210> SEQ ID NO 39

<211> LENGTH: 61

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: CD8 alpha hinge region

<400> SEQUENCE: 39

Leu Ser Asn Ser Ile Met Tyr Phe Ser His Phe Val Pro Val Phe Leu

-continued

```

1           5           10           15
Pro Ala Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr Pro Ala
   20           25           30
Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala Cys Arg
   35           40           45
Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp
   50           55           60

```

```

<210> SEQ ID NO 40
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CD8 beta spacer element peptide

```

```

<400> SEQUENCE: 40

```

```

Val Asp Phe Leu Pro Thr Thr Ala Gln Pro Thr Lys Lys Ser Thr Leu
1           5           10           15
Lys Lys Arg Val Cys Arg Leu Pro Arg Pro Glu Thr Gln Lys Gly Pro
   20           25           30
Leu Cys Ser Pro Ile Thr Leu Gly Leu Leu Val Ala Gly Val Leu Val
   35           40           45
Leu Leu Val Ser Leu Gly Val Ala Ile His Leu Cys Cys
   50           55           60

```

```

<210> SEQ ID NO 41
<211> LENGTH: 82
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CD4 315-396 spacer element

```

```

<400> SEQUENCE: 41

```

```

Val Val Met Arg Ala Thr Gln Leu Gln Lys Asn Leu Thr Cys Glu Val
1           5           10           15
Trp Gly Pro Thr Ser Pro Lys Leu Met Leu Ser Leu Lys Leu Glu Asn
   20           25           30
Lys Glu Ala Lys Val Ser Lys Arg Glu Lys Ala Val Trp Val Leu Asn
   35           40           45
Pro Glu Ala Gly Met Trp Gln Cys Leu Leu Ser Asp Ser Gly Gln Val
   50           55           60
Leu Leu Glu Ser Asn Ile Lys Val Leu Pro Thr Trp Ser Thr Pro Val
   65           70           75           80
Gln Pro

```

```

<210> SEQ ID NO 42
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CD28 137-152 spacer element peptide

```

```

<400> SEQUENCE: 42

```

```

Gly Lys His Leu Cys Pro Ser Pro Leu Phe Pro Gly Pro Ser Lys Pro
1           5           10           15

```

```

<210> SEQ ID NO 43
<211> LENGTH: 49

```

-continued

<212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: TNF receptor member 19 peptide

<400> SEQUENCE: 43

Arg Ser Gln Cys Met Trp Pro Arg Glu Thr Gln Leu Leu Leu Glu Val
 1 5 10 15

Pro Pro Ser Thr Glu Asp Ala Arg Ser Cys Gln Phe Pro Glu Glu Glu
 20 25 30

Arg Gly Glu Arg Ser Ala Glu Glu Lys Gly Arg Leu Gly Asp Leu Trp
 35 40 45

Val

<210> SEQ ID NO 44
 <211> LENGTH: 30
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: GPI signal sequence from CD24

<400> SEQUENCE: 44

Thr Asn Ala Thr Thr Lys Ala Ala Gly Gly Ala Leu Gln Ser Thr Ala
 1 5 10 15

Ser Leu Phe Val Val Ser Leu Ser Leu Leu His Leu Tyr Ser
 20 25 30

<210> SEQ ID NO 45
 <211> LENGTH: 32
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: GPI signal sequence from CNTN1

<400> SEQUENCE: 45

Val Ser Gln Val Lys Ile Ser Gly Ala Pro Thr Leu Ser Pro Ser Leu
 1 5 10 15

Leu Gly Leu Leu Leu Pro Ala Phe Gly Ile Leu Val Tyr Leu Glu Phe
 20 25 30

<210> SEQ ID NO 46
 <211> LENGTH: 35
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: GPI signal sequence from EFNA1

<400> SEQUENCE: 46

Pro Glu Val Arg Val Leu His Ser Ile Gly His Ser Ala Ala Pro Arg
 1 5 10 15

Leu Phe Pro Leu Ala Trp Thr Val Leu Leu Leu Pro Leu Leu Leu Leu
 20 25 30

Gln Thr Pro
 35

<210> SEQ ID NO 47
 <211> LENGTH: 35
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: GPI signal sequence from EFNA2

-continued

<400> SEQUENCE: 47

Glu Ala Pro Glu Pro Ile Phe Thr Ser Asn Asn Ser Cys Ser Ser Pro
 1 5 10 15

Gly Gly Cys Arg Leu Phe Leu Ser Thr Ile Pro Val Leu Trp Arg Leu
 20 25 30

Leu Gly Ser
 35

<210> SEQ ID NO 48

<211> LENGTH: 35

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: GPI signal sequence from EFNA3

<400> SEQUENCE: 48

Gln Val Pro Lys Leu Glu Lys Ser Ile Ser Gly Thr Ser Pro Lys Arg
 1 5 10 15

Glu His Leu Pro Leu Ala Val Gly Ile Ala Phe Phe Leu Met Thr Phe
 20 25 30

Leu Ala Ser
 35

<210> SEQ ID NO 49

<211> LENGTH: 35

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: GPI signal sequence from EFNA5

<400> SEQUENCE: 49

Glu Ser Ala Glu Pro Ser Arg Gly Glu Asn Ala Ala Gln Thr Pro Arg
 1 5 10 15

Ile Pro Ser Arg Leu Leu Ala Ile Leu Leu Phe Leu Leu Ala Met Leu
 20 25 30

Leu Thr Leu
 35

<210> SEQ ID NO 50

<211> LENGTH: 29

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: GPI signal sequence from FOLI

<400> SEQUENCE: 50

Tyr Ala Ala Ala Met Ser Gly Ala Gly Pro Trp Ala Ala Trp Pro Phe
 1 5 10 15

Leu Leu Ser Leu Ala Leu Met Leu Leu Trp Leu Leu Ser
 20 25

<210> SEQ ID NO 51

<211> LENGTH: 29

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: GPI signal sequence from LSAMP

<400> SEQUENCE: 51

-continued

```
Ser Val Arg Gly Ile Asn Gly Ser Ile Ser Leu Ala Val Pro Leu Trp
1           5           10           15
```

```
Leu Leu Ala Ala Ser Leu Leu Cys Leu Leu Ser Lys Cys
           20           25
```

```
<210> SEQ ID NO 52
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GPI signal sequence from PPB1
```

```
<400> SEQUENCE: 52
```

```
Thr Thr Asp Ala Ala His Pro Gly Arg Ser Val Val Pro Ala Leu Leu
1           5           10           15
```

```
Pro Leu Leu Ala Gly Thr Leu Leu Leu Leu Glu Thr Ala Thr Ala Pro
           20           25           30
```

```
<210> SEQ ID NO 53
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GPI signal sequence from RTN4R
```

```
<400> SEQUENCE: 53
```

```
Asp Ser Glu Gly Ser Gly Ala Leu Pro Ser Leu Thr Cys Ser Leu Thr
1           5           10           15
```

```
Pro Leu Gly Leu Ala Leu Val Leu Trp Thr Val Leu Gly Pro Cys
           20           25           30
```

What is claimed is:

1. A method of treating a subject having a disease comprising the steps of: obtaining a eukaryotic cell comprising a polynucleotide encoding a CAR, wherein the CAR is comprised of an extracellular element, a transmembrane element, and an intracellular element, wherein the transmembrane element is between the extracellular element and the intracellular element, and wherein the eukaryotic cell is modified to increase killing of a target cell by a cytotoxicity mechanism, administering the eukaryotic cell to the subject, and killing a plurality of target cells by the eukaryotic cell whereby the disease is treated.

2. The method of claim 1, wherein the polynucleotide further encode a RNA control device or a destabilizing element.

3. The method of claim 2, wherein the transgenic cell is a T-lymphocyte or a natural killer cell.

4. The method of claim 3, wherein the transgenic cell is derived from a donor.

5. The method of claim 3, wherein the donor is the subject.

6. The method of claim 3, wherein the donor is a syngeneic donor or an allogenic donor.

7. The method of claim 2, wherein the disease is a hematopoietic disorder.

8. The method of claim 7, wherein the hematopoietic disorder is a hematopoietic cancer.

9. The method of claim 8, wherein the extracellular element binds to a CD 33, a CD 34, a CD 38, a CD 44, a CD

45, a CD 45RA, a CD 47, a CD 64, a CD 66, a CD 123, a CD 133, a CD 157, a CLL-1, a CXCR4, a LeY, a PR1, a CD 168, a TIM-3, or a WT1.

10. The method of claim 9, wherein the extracellular element binds to a CD 34, a CD 41, a CD 45, a CD 90, a CD 117, a CD 123, or a CD 133.

11. The method of claim 9, wherein the extracellular element binds to a CD 123.

12. The method of claim 11, further comprising the step of administering a treatment for the hematopoietic cancer.

13. The method of claim 12, wherein the treatment for the hematopoietic cancer is selected from the group consisting of administering a chemotherapeutic agent, administering a radiation treatment, administering a T-cell with a CAR that targets the hematopoietic cancer, and administering a natural killer cell with a CAR that targets the hematopoietic cancer.

14. The method of claim 13, further comprising the step of providing the subject with a transplant of a plurality of hematopoietic stem cells.

15. The method of claim 8, wherein the disease is an AML.

16. The method of claim 7, wherein the disease is an autoimmune disease.

17. The method of claim 16, further comprising the step of providing the subject with a treatment for the autoimmune disease.

18. The method of claim 17, wherein the treatment is selected from the group consisting of administering a chemotherapeutic agent, administering a radiation treatment, administering a T-cell with a CAR that targets a memory cell

that contributes to the autoimmune disease, and administering a natural killer cell with a CAR that targets a memory cell that contributes to the autoimmune disease.

19. The method of claim **18**, wherein an extracellular domain of the CAR binds to an antigen selected from the group consisting of a CD 19, a CD 21, a CD 27, a CD 40, and a CD84.

20. The method of claim **18**, wherein an extracellular domain of the CAR binds to an antigen selected from the group consisting of a CCR5, a CCR7, a CD11a, a CD27, a CD28, a CD45RA, a CD45RO, a CD57, and a CD62L.

* * * * *