TITLE OF THE INVENTION

COMPOSITIONS AND METHODS FOR REGULATING CAR T CELLS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 61/671,518, filed July 13, 2012, the content of which is hereby incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Normal T cells can be re-directed to attack tumor by transduction with a chimeric antigen receptor (CAR) against specific cell surface targets. In one case, a CAR exhibited remarkable anti-tumor effects in patients with chronic leukemia (Porter et al., 2011, New Engl J Med, 365(8): 725-733; Kalos et al, 2011, Sci Tr Med, 3(95): 95ra73).

In that model, the T cells were genetically engineered to express an antibody fragment (called an "scFv") against CD19, an antigen that is expressed on the surface of B-cell malignancies such as chronic lymphocytic leukemia (CLL). However, the same molecule is also expressed on normal B lymphocytes. The normal function of B lymphocytes is to produce antibodies and help in T-cells to control infection. Although to date, there have been no infectious complications related specifically to B cell depletion in patients treated with genetically modified anti-CD19 T cells ("CART-19 cells"), the consequences of protracted profound B cell depletion are as yet unknown. Furthermore, multiple other CAR T cell products with new specificities are under currently under development. These new CAR T cell products may be associated with unique toxicities related to the selective depletion of bystander cells that share expression of the targeted antigen with the particular cancer type under study.

Thus, there is a need in the art to develop compositions and methods that can specifically and on demand target cells that express CAR on their surface in order to prevent the unwanted depletion of healthy bystander cells during CAR T cell therapy. The present invention satisfies this unmet need.

SUMMARY OF THE INVENTION

The invention provides a drug-molecule conjugate comprising a drug and a molecule which binds to a CAR expressed on the surface of a cell.

In one embodiment, binding of the conjugate to the CAR results in internalization of the conjugate into the cell.

In one embodiment, binding of the conjugate to the CAR results in the drug-mediated death of the cell.

In one embodiment, the cell is a T cell and wherein binding of the conjugate to the CAR results in the drug-mediated inhibition of the activation of the T cell.

In one embodiment, the molecule is selected from the group consisting of an antibody, a protein, a peptide, a nucleotide, a small molecule, and fragments thereof.

The invention provides a method for inhibiting the depletion of healthy tissue during CAR T cell therapy comprising administering a drug-molecule conjugate comprising a drug and a molecule to a subject receiving CAR T cell therapy, wherein the molecule binds to a CAR expressed on the surface of a T cell.

In one embodiment, binding of the conjugate to the CAR results in internalization of the conjugate into the cell.

In one embodiment, binding of the conjugate to the CAR results in the drug-mediated death of the cell.

In one embodiment, binding of the conjugate to the CAR results in the drug-mediated inhibition of the activation of the T cell.

In one embodiment, the molecule is selected from the group consisting of an antibody, a protein, a peptide, a nucleotide, a small molecule, and fragments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1 is a set of graphs depicting the results of experiments demonstrating the loss of surface expression of the anti-CD19 chimeric antigen receptor upon incubation with CD19 expressing targets (K562-CD19 and Nalm6).

Figure 2 is a set of graphs depicting the results of experiments demonstrating the surface and intracellular staining of CAR upon exposure to the antigen target. Top row: exposure of anti-CD19 chimeric-antigen transduced T cells to irrelevant target (left) or CD19 expressing target (right) shows that surface expression of the receptor is lost upon encounter of cognate target. Bottom row: intracellular staining demonstrates that the receptor can be found inside the cell.

DETAILED DESCRIPTION

The present invention provides compositions and methods to regulate the activity of T cells modified to express a chimeric antigen receptor (CAR). T cells that have been genetically modified to express a CAR have been used in treatments for cancers where the CAR redirects the modified T cell to recognize a tumor antigen. In some instances, it may be beneficial to effectively control and regulate CAR T cells such that they kill tumor cells while not affecting normal bystander cells. Thus, in one embodiment, the present invention also provides methods of killing cancerous cells while minimizing the depletion of normal non-cancerous cells.

In one embodiment, the present invention provides for a plurality of types of CARs expressed on a cell, where binding of a plurality of types of CARs to their target antigen is required for CAR T cell activation. In one embodiment, the methods of the invention comprise genetically modifying a T cell to express a plurality of types of CARs, where T cell activation is dependent on the binding of a plurality of types of CARs to their target antigens. For example, in one embodiment a T cell can express a first CAR targeted to a first desired antigen and a second CAR targeted to a second desired antigen. In one embodiment, activation of the modified T cell only occurs when the first CAR binds the first desired antigen and the second CAR binds to the second desired antigen. In one embodiment, dependence on the binding of a plurality of different CARs improves the specificity of CAR T cell therapies.

In one embodiment, the present invention provides an inhibitory CAR where binding of the inhibitory CAR to a normal cell results in inhibition of CAR T cell activity. In one embodiment, the inhibitory CAR is co-expressed in the same T

cell as a therapeutic tumor directed CAR. In one embodiment, the inhibitory CAR comprises an antigen binding domain that recognizes an antigen associated with a normal, non-cancerous, cell and a cytoplasmic domain. In one embodiment, the method comprises genetically modifying a T cell to express at least one inhibitory CAR and at least one therapeutic tumor directed CAR. In one embodiment, binding of the inhibitory CAR to an antigen associated with a non-cancerous cell results in the death of the CAR T cell. In one embodiment, binding of the therapeutic tumor directed CAR to a tumor antigen on a cancerous cell results in T cell activation and T cell-mediated death of the cancerous cell.

In one embodiment, the present invention provides a drug-molecule conjugate that binds to a CAR expressed on the cell surface. In one embodiment, binding of the conjugate to the CAR induces internalization of the conjugate, which allows the drug to kill the CAR T cell. The present invention also provides methods of regulating CAR T cell activity by administering the drug-molecule conjugate, where the drug-molecule conjugate leads to internalization of the CAR and death of the CAR T cell.

Definitions

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 the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

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.

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

“About” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, in some instances ±5%, in some instances ±1%, and in some instances ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

"Activation," as used herein, refers to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. Activation can also be associated with induced cytokine production, and detectable effector functions. The term "activated T cells" refers to, among other things, T cells that are undergoing cell division.

The term "antibody," as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are often of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and $F(ab)_2$, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

The term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

An "antibody heavy chain," as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

An "antibody light chain," as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations. κ and λ light chains refer to the two major antibody light chain isotypes.

By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody

protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

The term "antigen" or "Ag" as used herein is defined as 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 virtually all proteins or peptides, 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 one, or more than one, gene and that these nucleotide sequences are arranged in various combinations to 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 generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

The term "anti-tumor effect" as used herein, refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An "anti-tumor effect" can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies of the invention in prevention of the occurrence of tumor in the first place.

The term "auto-antigen" means, in accordance with the present invention, any self-antigen which is recognized by the immune system as if it were foreign. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors.

The term "autoimmune disease" as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an

inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases include but are not limited to, Addision's disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barr syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

As used herein, the term "autologous" is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

"Allogeneic" refers to a graft derived from a different animal of the same species.

"Xenogeneic" refers to a graft derived from an animal of a different species.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like.

"Co-stimulatory ligand," as the term is used herein, includes a molecule on an antigen presenting cell (e.g., an aAPC, dendritic cell, B cell, and the like) that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A co-stimulatory ligand can include, but is not limited to, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, HVEM, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also

encompasses, *inter alia*, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the T cell, such as, but not limited to, proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and a Toll ligand receptor.

A "co-stimulatory signal", as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation and/or upregulation or downregulation of key molecules.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

An "effective amount" as used herein, means an amount which provides a therapeutic or prophylactic benefit.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

As used herein, the term "exogenous" refers to any material introduced to an organism, cell, tissue or system that was produced outside an organism, cell, tissue or system.

The term "expression" as used herein is defined as the transcription and/or translation of a particular nucleotide sequence.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

"Homologous" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

The term "immunoglobulin" or "Ig," as used herein is defined as a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of proteins are IgA, IgG, IgM, IgD, and IgE. IgA is the primary antibody that is present in body secretions, such as saliva, tears, breast milk, gastrointestinal secretions and mucus secretions of the respiratory and genitourinary tracts. IgG is the most common circulating antibody. IgM is the main immunoglobulin produced in the primary immune response in most subjects. It is the most efficient immunoglobulin in agglutination, complement fixation, and other antibody responses, and is important in defense against bacteria and viruses. IgD is the immunoglobulin

that has no known antibody function, but may serve as an antigen receptor. IgE is the immunoglobulin that mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to allergen.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the nucleic acid, peptide, and/or composition of the invention or be shipped together with a container which contains the nucleic acid, peptide, and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

A "lentivirus" as used herein refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer in vivo.

By the term "modulating," as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

The term "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The term "overexpressed" tumor antigen or "overexpression" of a tumor antigen is intended to indicate an abnormal level of expression of a tumor antigen in a cell from a disease area like a solid tumor within a specific tissue or organ of the patient relative to the level of expression in a normal cell from that tissue or organ. Patients having solid tumors or a hematological malignancy characterized by overexpression of the tumor antigen can be determined by standard assays known in the art.

"Parenteral" administration of an immunogenic composition includes, e.g., subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR, and the like, and by synthetic means.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

By the term "specifically binds," as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A," the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

By the term "stimulation," is meant a primary response induced by binding of a stimulatory molecule (*e.g.*, a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex. Stimulation can mediate altered expression

of certain molecules, such as downregulation of TGF-β, and/or reorganization of cytoskeletal structures, and the like.

A "stimulatory molecule," as the term is used herein, means a molecule on a T cell that specifically binds with a cognate stimulatory ligand present on an antigen presenting cell.

A "stimulatory ligand," as used herein, means a ligand that when present on an antigen presenting cell (*e.g.*, an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a "stimulatory molecule") on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands are well-known in the art and encompass, *inter alia*, an MHC Class I molecule loaded with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

The term "subject," "patient" and "individual" are used interchangeably herein and are intended to include living organisms in which an immune response can be elicited (e.g., mammals). Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof.

As used herein, a "substantially purified" cell is a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some embodiments, the cells are cultured *in vitro*. In other embodiments, the cells are not cultured *in vitro*.

The term "therapeutic" as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

The term "therapeutically effective amount" refers to the amount of the subject compound that will elicit the biological or medical response of a tissue, system, or subject that is being sought by the researcher, veterinarian, medical doctor or other clinician. The term "therapeutically effective amount" includes that amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the signs or symptoms of the disorder or

disease being treated. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, etc., of the subject to be treated.

To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

The term "transfected" or "transformed" or "transduced" as used herein refers to a process by which exogenous nucleic acid is transferred to, or introduced into, the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

The phrase "under transcriptional control" or "operatively linked" as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from

3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The present invention provides compositions and methods for limiting the depletion of non-cancerous cells by CAR T cell therapy. As disclosed herein, therapeutic CAR T cells exhibit an antitumor property when bound to its target, whereas an inhibitory CAR results in inhibition of CAR T cell activity when the inhibitory CAR is bound to its target.

Regardless of the type of CAR, CARs are engineered to comprise an extracellular domain having an antigen binding domain fused to a cytoplasmic domain. In one embodiment, CARs, when expressed in a T cell, are able to redirect antigen recognition based upon the antigen specificity. An exemplary antigen is CD19 because this antigen is expressed on B cell lymphoma. However, CD19 is also expressed on normal B cells, and thus CARs comprising an anti-CD19 domain may result in depletion of normal B cells. Depletion of normal B cells can make a treated subject susceptible to infection, as B cells normally aid T cells in the control of infection. The present invention provides for compositions and methods to limit the depletion of normal tissue during CAR T cell therapy. In one embodiment, the present invention provides methods to treat cancer and other disorders using CAR T cell therapy while limiting the depletion of healthy bystander cells.

In one embodiment, the invention comprises controlling or regulating CAR T cell activity. In one embodiment, the invention comprises compositions and methods related to genetically modifying T cells to express a plurality of types of CARs, where CAR T cell activation is dependent on the binding of a plurality of types of CARs to their target receptor. Dependence on the binding of a plurality of types of CARs improves the specificity of the lytic activity of the CAR T cell, thereby reducing the potential for depleting normal healthy tissue.

In another embodiment, the invention comprises compositions and methods related to genetically modifying T cells with an inhibitory CAR. In one embodiment, the inhibitory CAR comprises an extracellular antigen binding domain that recognizes an antigen associated with a normal, non-cancerous, cell and an inhibitory cytoplasmic domain.

In one embodiment, the invention provides a dual CAR where a T cell is genetically modified to express an inhibitory CAR and a therapeutic tumor directed CAR. In one embodiment, binding of the inhibitory CAR to a normal, non-cancerous cell results in the inhibition of the CAR T cell. For example, in one embodiment, binding of the inhibitory CAR results in the death of the CAR T cell. In another embodiment, binding of the inhibitory CAR results in inhibiting the signal transduction of the therapeutic tumor directed CAR. In yet another embodiment, binding of the inhibitory CAR results in the induction of a signal transduction signal that prevents the modified T cell from exhibiting its anti-tumor activity. Accordingly, the dual CAR of the invention provides a mechanism to regulate the activity of the CAR T cell.

In one embodiment, the invention comprises compositions and methods related to a drug-molecule conjugate that binds to a therapeutic tumor directed CAR. In one embodiment, binding of the conjugate to the therapeutic tumor directed CAR results in the internalization of the CAR and the drug-molecule conjugate. In one embodiment, binding of the conjugate to the CAR results in the death of the CAR T cell. In another embodiment, binding of the conjugate to the CAR results in inhibiting the signal transduction of the therapeutic tumor directed CAR. In yet another embodiment, binding of the conjugate to the CAR results in the induction of a signal transduction signal that prevents the modified T cell from exhibiting its anti-tumor activity. Accordingly, the invention provides a mechanism to regulate the activity of the CAR T cell.

In one embodiment, the present invention provides methods for treating cancer and other disorders using CAR T cell therapies while minimizing the depletion of normal healthy tissue. The cancer may be a hematological malignancy, a solid tumor, a primary or a metastasizing tumor. Other diseases treatable using the compositions and methods of the invention include viral, bacterial and parasitic infections as well as autoimmune diseases.

1. Multiple CAR strategy

In one embodiment, the present invention provides compositions and methods to increase CAR T cell therapy specificity and limit depletion of normal healthy tissue by genetically modifying a T cell to express a plurality of types of CARs, wherein activation of the T cell is dependent on the binding of a plurality of

types of CARs. Dependence of the binding of a plurality of types of CARs increases specificity of the therapy and therefore limits the amount of depletion of normal healthy tissue. As described elsewhere herein, T cells modified to express a plurality of types of CARs can be generated by administering lentiviral vectors, in vitro transcribed RNA, or combination thereof, to the cells.

Chimeric Antigen Receptors

The present invention provides a chimeric antigen receptor (CAR) comprising an extracellular and intracellular domain. Compositions and methods of making CARs have been described in PCT/US11/64191, which is incorporated in its entirety by reference herein.

The extracellular domain comprises a target-specific binding element otherwise referred to as an antigen binding domain. In some embodiments, the extracellular domain also comprises a hinge domain. In one embodiment, the intracellular domain or otherwise the cytoplasmic domain comprises a costimulatory signaling region and a zeta chain portion. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. Costimulatory molecules are cell surface molecules other than antigen receptors or their ligands that are required for an efficient response of lymphocytes to antigen.

Between the extracellular domain and the transmembrane domain of the CAR, or between the cytoplasmic domain and the transmembrane domain of the CAR, there may be incorporated a spacer domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular domain or, the cytoplasmic domain in the polypeptide chain. A spacer domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids.

The present invention includes retroviral and lentiviral vector constructs expressing a CAR that can be directly transduced into a cell. The present invention also includes an RNA construct that can be directly transfected into a cell. A method for generating mRNA for use in transfection involves in vitro transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR"), a 5' cap and/or Internal Ribosome Entry Site (IRES), the gene to be expressed, and a polyA

tail, typically 50-2000 bases in length. RNA so produced can efficiently transfect different kinds of cells. In one embodiment, the template includes sequences for the CAR.

Preferably, the CAR comprises an extracellular domain, a transmembrane domain and a cytoplasmic domain. The extracellular domain and transmembrane domain can be derived from any desired source of such domains. In some instances, the hinge domain of the CAR of the invention comprises the CD8α hinge domain.

In one embodiment, the CAR of the invention comprises a target-specific binding element otherwise referred to as an antigen binding domain. The choice of moiety depends upon the type and number of ligands that define the surface of a target cell. For example, the antigen binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands for the antigen moiety domain in the CAR of the invention include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells.

In one embodiment, the CAR of the invention can be engineered to target a tumor antigen of interest by way of engineering a desired antigen binding domain that specifically binds to an antigen on a tumor cell. In the context of the present invention, "tumor antigen" or "hyperporoliferative disorder antigen" or "antigen associated with a hyperproliferative disorder," refers to antigens that are common to specific hyperproliferative disorders such as cancer. The antigens discussed herein are merely included by way of example. The list is not intended to be exclusive and further examples will be readily apparent to those of skill in the art.

Tumor antigens are proteins that are produced by tumor cells that elicit an immune response, particularly T-cell mediated immune responses. The selection of the antigen binding domain of the invention will depend on the particular type of cancer to be treated. Tumor antigens are well known in the art and include, for example, a glioma-associated antigen, carcinoembryonic antigen (CEA), β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CA IX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, M-CSF, prostase, prostate-specific antigen (PSA), PAP, NY-ESO-1, LAGE-1a, p53, prostein, PSMA, Her2/neu, survivin and telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M,

neutrophil elastase, ephrinB2, CD22, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor and mesothelin.

In one embodiment, the tumor antigen comprises one or more antigenic cancer epitopes associated with a malignant tumor. Malignant tumors express a number of proteins that can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase and GP 100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER-2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma. Some of these antigens (CEA, HER-2, CD19, CD20, idiotype) have been used as targets for passive immunotherapy with monoclonal antibodies with limited success.

The type of tumor antigen referred to in the invention may also be a tumor-specific antigen (TSA) or a tumor-associated antigen (TAA). A TSA is unique to tumor cells and does not occur on other cells in the body. A TAA associated antigen is not unique to a tumor cell and instead is also expressed on a normal cell under conditions that fail to induce a state of immunologic tolerance to the antigen. The expression of the antigen on the tumor may occur under conditions that enable the immune system to respond to the antigen. TAAs may be antigens that are expressed on normal cells during fetal development when the immune system is immature and unable to respond or they may be antigens that are normally present at extremely low levels on normal cells but which are expressed at much higher levels on tumor cells.

Non-limiting examples of TSA or TAA antigens include the following: Differentiation antigens such as MART-1/MelanA (MART-I), gp100 (Pmel 17), tyrosinase, TRP-1, TRP-2 and tumor-specific multilineage antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15; overexpressed embryonic antigens such as CEA; overexpressed oncogenes and mutated tumor-suppressor genes such as p53, Ras, HER-2/neu; unique tumor antigens resulting from chromosomal translocations; such as BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR; and viral antigens,

such as the Epstein Barr virus antigens EBVA and the human papillomavirus (HPV) antigens E6 and E7. Other large, protein-based antigens include TSP-180, MAGE-4, MAGE-5, MAGE-6, RAGE, NY-ESO, p185erbB2, p180erbB-3, c-met, nm-23H1, PSA, TAG-72, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-Catenin, CDK4, Mum-1, p 15, p 16, 43-9F, 5T4, 791Tgp72, alpha-fetoprotein, beta-HCG, BCA225, BTAA, CA 125, CA 15-3\CA 27.29\BCAA, CA 195, CA 242, CA-50, CAM43, CD68\P1, CO-029, FGF-5, G250, Ga733\EpCAM, HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90\Mac-2 binding protein\cyclophilin C-associated protein, TAAL6, TAG72, TLP, and TPS.

In a preferred embodiment, the antigen binding domain portion of the CAR targets an antigen that includes but is not limited to CD19, CD20, CD22, ROR1, Mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, MY-ESO-1 TCR, MAGE A3 TCR, and the like.

Depending on the desired antigen to be targeted, the CAR of the invention can be engineered to include the appropriate antigen bind moiety that is specific to the desired antigen target.

The antigen binding domain can be any domain that binds to the antigen including but not limited to monoclonal antibodies, polyclonal antibodies, synthetic antibodies, human antibodies, humanized antibodies, and fragments thereof. In some instances, it is beneficial for the antigen binding domain to be derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial for the antigen binding domain of the CAR to comprise a human antibody or fragment thereof. Thus, in one embodiment, the antigen biding domain portion comprises a human antibody or a fragment thereof. Alternatively, in some embodiments, a non-human antibody is humanized, where specific sequences or regions of the antibody are modified to increase similarity to an antibody naturally produced in a human.

In one embodiment of the present invention, a plurality of types of CARs is expressed on the surface of a T cell. The different types of CAR may differ in their antigen binding domain. That is, in one embodiment, the different types of CARs each bind a different antigen. In one embodiment, the different antigens are markers for a specific tumor. For example, in one embodiment, the different types of CARs each bind to a different antigen, where each antigen is expressed on a specific type of tumor. Examples of such antigens are discussed elsewhere herein.

With respect to the transmembrane domain, the CAR can be designed to comprise a transmembrane domain that is fused to the extracellular domain of the CAR. In one embodiment, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this invention may be derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, ICOS. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

The cytoplasmic domain or otherwise the intracellular signaling domain of the CAR of the invention is responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been placed in. 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 domain” refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to

include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

In one embodiment of the present invention, the effector function of the cell is dependent upon the binding of a plurality of types of CARs to their targeted antigen. For example, in one embodiment, binding of one type of CAR to its target is not sufficient to induce the effector function of the cell.

Examples of intracellular signaling domains for use in the 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 synthetic sequence that has the same functional capability.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequence: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences) and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences).

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

Examples of ITAM containing primary cytoplasmic signaling sequences that are of particular use in the invention include those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma , CD3 delta , CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. It is particularly preferred that cytoplasmic signaling molecule in the CAR of the invention comprises a cytoplasmic signaling sequence derived from CD3 zeta.

In one embodiment, the cytoplasmic domain of the CAR can be designed to comprise the CD3-zeta signaling domain by itself or combined with any other desired cytoplasmic domain(s) useful in the context of the CAR of the invention. For example, the cytoplasmic domain of the CAR can comprise a CD3-zeta chain portion and a costimulatory signaling region. The costimulatory signaling

region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. A costimulatory molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, 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.

The cytoplasmic signaling sequences within the cytoplasmic signaling portion of the CAR of the invention may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage. A glycine-serine doublet provides a particularly suitable linker.

In one embodiment, the cytoplasmic domain is designed to comprise the signaling domain of CD3-zeta. In another embodiment, the cytoplasmic domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of 4-1BB. In one embodiment of the present invention, a plurality of types of CARs is expressed on a cell, where the different types of CAR may vary in their cytoplasmic domain. In one embodiment, at least one type of CAR comprises the CD3 zeta domain, while at least one type of CAR comprises a costimulatory domain, for example the 4-1BB domain. However, the different types of CARs are not limited by any particular cytoplasmic domain. For example, each type of CAR can comprise any ITAM containing sequence, costimulatory domain, or combination thereof such that binding of each individual type of CAR is insufficient to induce effector function but binding of a plurality of types of CARs are able to induce effector function. That is, the domains of each type of CAR work together to induce effector function.

2. Inhibitory CAR strategy

The present invention provides compositions and methods for limiting the depletion of normal healthy tissue by genetically modifying a T cell to express an inhibitory CAR. In one embodiment, the inhibitory CAR of the invention comprises an extracellular domain and an intracellular domain. The extracellular domain comprises a target-specific binding element referred to as an antigen binding domain. In one embodiment, the inhibitory CAR comprises an antigen binding domain that binds to an antigen associated with normal, healthy tissue. For example, in one

embodiment, the antigen binding domain of the inhibitory CAR binds to an antigen specifically found in non-cancerous cells. As described elsewhere herein, the antigen binding domain can be any domain that binds to the antigen including but not limited to monoclonal antibodies, polyclonal antibodies, synthetic antibodies, human antibodies, humanized antibodies, and fragments thereof.

The inhibitory CAR of the invention may comprise a transmembrane domain. As described elsewhere herein, the transmembrane domain may be derived from any membrane-bound or transmembrane protein. Alternatively, the transmembrane domain may be synthetic.

The inhibitory CAR of the invention comprises an cytoplasmic domain responsible for inhibiting the activity of the CAR T cell. In one embodiment of the present invention, the inhibitory CAR is expressed in the same T cell as one or more therapeutic, anti-tumor CARs described elsewhere herein. In one embodiment, the cytoplasmic domain of the inhibitory CAR prevents the activation of the T cell, inhibits the cytolytic activity of the T cell, or inhibits the helper activity of the T cell.

The inhibitory CAR of the invention is not limited as to any specific function that inhibits CAR T cell activity. For example, the inhibitory CAR can comprise a cytoplasmic domain that, upon binding to its target antigen, induces internalization of therapeutic CARs, prevents the activation of the CAR T cell, or induces the CAR T cell to die.

In one embodiment, the cytoplasmic domain of the inhibitory CAR comprises inhibitory ITAM containing sequences.

In one embodiment, the cytoplasmic domain of the inhibitory CAR comprises a death domain (DD). As used herein, a DD refers to a region that shares sequence homology with the DD domain of DD proteins such as TNFR1, Fas, DR3, DR4/TrailR1, DR5/TrailR2, DR6, FADD, MyD88, Raidd, IRAK, IRAK-2, IRAK-M, p75NTR, Tradd, DAP kinase, RIP, NMP84, and ankyrins, and have been found herein to have binding properties similar to those of other known DD proteins.

Apoptosis-inducing members of the Tumor Necrosis Factor (TNF) receptor family recruit the proforms of caspase-family cell death proteases to liganded receptor complexes through interactions of their intracellular Death Domains (DDs) with adapter proteins (Ashkenazi and Dixit, Science 281:1305-1308 (1998); Wallach et al., Annu. Rev. Immunol. 17:331-367 (1999)). Several caspase family members are known, for example, caspase-1, caspase-2, caspase-3, caspase-4, caspase-5, caspase-6,

caspase-7, caspase-8, caspase-9, caspase-10, caspase-11, caspase-12, caspase-13, and caspase-14 (Grutter, Curr. Opin. Struct. Biol. 10:649-655 (2000)).

Death receptors such as TNF-R1 and Fas oligomerize to signal via their intracellular DDs. The signal is transported by cytosolic adapters to caspases. The Death Inducing Signaling Complex (DISC) for Fas has been shown to encompass minimally a Fas trimer, Fadd, and Caspase-8. A similar DISC complex has been found for DR4 and DR5. In the case of the TRAIL receptors, mixed complexes, for example, two DR4s plus one DR5 to form a trimer, appear to be functional. Decoy receptors, for example, DcR1, DcR2 and DcR3, which have no or incomplete death domains, can inhibit apoptosis possibly by interfering with DISC formation.

The intracellular regions of several TNFR-family members (TNFR1; p75NTR, neurotrophin receptor, also called p75NGFR, nerve growth factor receptor; Fas; DR3; DR4/TrailR1; DR5/TrailR2; DR6) contain a structure known as the "Death Domain" (DD) and induce apoptosis when bound by ligand (Ashkenazi and Dixit, Science 281:1305-1308 (1998), Wallach et al., Annu. Rev. Immunol. 17:331-367 (1999)). The mechanism of apoptosis induction by such "death receptors" involves recruitment to the receptor complex of adapter proteins, which bind the prodomains of certain caspase-family cell death proteases. Caspases are present in living cells as zymogens, typically requiring proteolytic processing for their activation. Because the proforms of caspases possess weak protease activity, however, their receptor-mediated clustering results in trans-proteolysis through the "induced proximity" mechanism (Salvesen et al., Proc. Natl. Acad. Sci. USA 96:10964-10967 (1999)).

In one embodiment, the inhibitory CAR comprises an extracellular antigen binding domain that binds to an antigen associated with normal, non-cancerous, cells and a cytoplasmic domain that comprises a death domain, or portion thereof. In one embodiment, the binding of the inhibitory CAR to its target antigen results in the apoptotic death of the CAR T cell, thereby preventing the activation of the CAR T cell and reducing the depletion of normal, healthy tissue. In one embodiment, a T cell is genetically modified to express an inhibitory CAR and one or more therapeutic, tumor-targeted CARs, as described elsewhere herein. CAR T cell binding to a tumor antigen results in the activation of the CAR T cell and elimination of the tumor, while CAR T cell binding to an antigen associated with non-cancerous tissue results in the inhibition of CAR T cell activity (e.g. inhibition of activation, apoptotic cell death, etc.). As described elsewhere herein, T cells modified to express

an inhibitory CAR and at least one tumor-directed CAR can be generated by administering lentiviral vectors, in vitro transcribed RNA, or combination thereof, to the cells.

3. Drug-molecule conjugate

The present invention provides compositions and methods to modulate CAR T cell therapy to limit depletion of normal healthy tissue by administering a drug-molecule conjugate to a subject receiving CAR T cell therapy. In one embodiment, the drug-molecule conjugate binds to the CAR, resulting in internalization of the CAR and of the drug-molecule conjugate. The present invention is based upon the observation that CARs are transiently internalized after target recognition. This behavior can thus be exploited in methods to actively, and controllably, regulate CAR T cell activity. Further, regulation of CAR T cell activity via administration of a drug-molecule conjugate as described herein, does not require further genetic modification of CAR T cells, thereby eliminating the need for undue technical complexity and increased cost required for additional genetic manipulation of the cells.

Molecule

In one embodiment, the molecule of the drug-molecule conjugate comprises a molecule that binds to a CAR expressed on a genetically modified cell. The molecule may bind any portion of the CAR. For example, the molecule can bind to the antigen binding region or linker region of the CAR. The molecule may be of any type that can bind a region of the CAR. For example, the molecule may be a peptide, nucleotide, antibody, small molecule, and the like.

In one embodiment, the molecule comprises an antibody, or fragment thereof, which is targeted to bind the extracellular domain of a CAR. In one embodiment, the antibody binds to an antigen, where the antigen is the CAR or a region of the CAR. Methods of making and using antibodies are well known in the art. For example, polyclonal antibodies useful in the present invention are generated by immunizing rabbits according to standard immunological techniques well-known in the art (see, e.g., Harlow et al., 1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, N.Y.).

However, the invention should not be construed as being limited solely to methods and compositions including these antibodies or to these portions of the antigens. Rather, the invention should be construed to include other antibodies, as that term is defined elsewhere herein, to antigens, or portions thereof. One skilled in the art would appreciate, based upon the disclosure provided herein, that the antibody can specifically bind with any portion of the CAR and the full-length or any portion of the CAR can be used to generate antibodies specific therefor. However, the present invention is not limited to using the full-length protein as an immunogen. Rather, the present invention includes using an immunogenic portion of the protein to produce an antibody that specifically binds with a specific antigen. That is, the invention includes immunizing an animal using an immunogenic portion, or antigenic determinant, of the antigen.

Further, the skilled artisan, based upon the disclosure provided herein, would appreciate that using a non-conserved immunogenic portion can produce antibodies specific for the non-conserved region thereby producing antibodies that do not cross-react with other proteins which can share one or more conserved portions. Thus, one skilled in the art would appreciate, based upon the disclosure provided herein, that the non-conserved regions of an antigen of interest can be used to produce antibodies that are specific only for that antigen and do not cross-react non-specifically with other proteins, including other types of CARs.

The skilled artisan would appreciate, based upon the disclosure provided herein, that that present invention includes use of a single antibody recognizing a single antigenic epitope but that the invention is not limited to use of a single antibody. Instead, the invention encompasses use of at least one antibody where the antibodies can be directed to the same or different antigenic protein epitopes.

The generation of polyclonal antibodies is accomplished by inoculating the desired animal with the antigen and isolating antibodies which specifically bind the antigen therefrom using standard antibody production methods such as those described in, for example, Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, N.Y.).

Monoclonal antibodies directed against full length or peptide fragments of a protein or peptide may be prepared using any well-known monoclonal antibody preparation procedures, such as those described, for example, in Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, N.Y.) and in

Tuszynski et al. (1988, Blood, 72:109-115). Quantities of the desired peptide may also be synthesized using chemical synthesis technology. Alternatively, DNA encoding the desired peptide may be cloned and expressed from an appropriate promoter sequence in cells suitable for the generation of large quantities of peptide. Monoclonal antibodies directed against the peptide are generated from mice immunized with the peptide using standard procedures as referenced herein.

Nucleic acid encoding the monoclonal antibody obtained using the procedures described herein may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. Immunol. 12:125-168), and the references cited therein. Further, the antibody of the invention may be "humanized" using the technology described in, for example, Wright et al., and in the references cited therein, and in Gu et al. (1997, Thrombosis and Hematocyst 77:755-759), and other methods of humanizing antibodies well-known in the art or to be developed.

The present invention also includes the use of humanized antibodies specifically reactive with epitopes of an antigen of interest. The humanized antibodies of the invention have a human framework and have one or more complementarity determining regions (CDRs) from an antibody, typically a mouse antibody, specifically reactive with an antigen of interest. When the antibody used in the invention is humanized, the antibody may be generated as described in Queen, et al. (U.S. Pat. No. 6,180,370), Wright et al., (supra) and in the references cited therein, or in Gu et al. (1997, Thrombosis and Hematocyst 77(4):755-759). The method disclosed in Queen et al. is directed in part toward designing humanized immunoglobulins that are produced by expressing recombinant DNA segments encoding the heavy and light chain complementarity determining regions (CDRs) from a donor immunoglobulin capable of binding to a desired antigen, such as an epitope on an antigen of interest, attached to DNA segments encoding acceptor human framework regions. Generally speaking, the invention in the Queen patent has applicability toward the design of substantially any humanized immunoglobulin. Queen explains that the DNA segments will typically include an expression control DNA sequence operably linked to the humanized immunoglobulin coding sequences, including naturally-associated or heterologous promoter regions. The expression control sequences can be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells or the expression control sequences

can be prokaryotic promoter systems in vectors capable of transforming or transfecting prokaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the introduced nucleotide sequences and as desired the collection and purification of the humanized light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow (Beychok, Cells of Immunoglobulin Synthesis, Academic Press, New York, (1979), which is incorporated herein by reference).

In one embodiment of the invention, a phage antibody library may be generated, as described in detail elsewhere herein. To generate a phage antibody library, a cDNA library is first obtained from mRNA which is isolated from cells, e.g., peripheral blood lymphocytes, which express the desired protein to be expressed on the phage surface, e.g., the desired antibody. cDNA copies of the mRNA are produced using reverse transcriptase. cDNA which specifies immunoglobulin fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying immunoglobulin genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook et al., supra.

Bacteriophage which encode the desired antibody, may be engineered such that the protein is displayed on the surface thereof in such a manner that it is available for binding to its corresponding binding protein, e.g., the antigen against which the antibody is directed, such as an antigen of interest. Thus, when bacteriophage which express a specific antibody are incubated in the presence of the corresponding antigen, the bacteriophage will bind to the antigen. Bacteriophage which do not express the antibody will not bind to the antigen. Such panning techniques are well known in the art and are described for example, in Wright et al. (supra).

Processes such as those described above, have been developed for the production of human antibodies using M13 bacteriophage display (Burton et al., 1994, Adv. Immunol. 57:191-280). Essentially, a cDNA library is generated from mRNA obtained from a population of antibody-producing cells. The mRNA encodes rearranged immunoglobulin genes and thus, the cDNA encodes the same. Amplified cDNA is cloned into M13 expression vectors creating a library of phage which

express human Fab fragments on their surface. Phage which display the antibody of interest are selected by antigen binding and are propagated in bacteria to produce soluble human Fab immunoglobulin. Thus, in contrast to conventional monoclonal antibody synthesis, this procedure immortalizes DNA encoding human immunoglobulin rather than cells which express human immunoglobulin.

The procedures just presented describe the generation of phage which encode the Fab portion of an antibody molecule. However, the invention should not be construed to be limited solely to the generation of phage encoding Fab antibodies. Rather, phage which encode single chain antibodies (scFv/phage antibody libraries) are also included in the invention. Fab molecules comprise the entire Ig light chain, that is, they comprise both the variable and constant region of the light chain, but include only the variable region and first constant region domain (CH1) of the heavy chain. Single chain antibody molecules comprise a single chain of protein comprising the Ig Fv fragment. An Ig Fv fragment includes only the variable regions of the heavy and light chains of the antibody, having no constant region contained therein. Phage libraries comprising scFv DNA may be generated following the procedures described in Marks et al. (1991, J. Mol. Biol. 222:581-597). Panning of phage so generated for the isolation of a desired antibody is conducted in a manner similar to that described for phage libraries comprising Fab DNA.

The invention should also be construed to include synthetic phage display libraries in which the heavy and light chain variable regions may be synthesized such that they include nearly all possible specificities (Barbas, 1995, Nature Medicine 1:837-839; de Kruif et al. 1995, J. Mol. Biol. 248:97-105).

In another embodiment of the invention, phage-cloned antibodies derived from immunized animals can be humanized by known techniques.

In one embodiment, the molecule of the drug-molecule conjugate of the invention comprises a peptide derived from the antigenic epitope that is targeted by the CAR. For example, if the CAR is directed against CD19, the molecule of the invention can comprise a peptide derived from the epitope of CD19 that binds to the CAR. As such, the peptide can comprise a full-length protein or portions thereof. The peptides therefore mimic the antigen targeted by the antigen binding region of the CAR.

The peptide of the present invention may be made using chemical methods. For example, peptides can be synthesized by solid phase techniques

(Roberge J Y et al (1995) Science 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography. Automated synthesis may be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

Drug

The drug-molecule conjugate of the invention comprises a drug which, in one embodiment, is internalized into the CAR T cell. As described elsewhere herein, upon binding of the conjugate to the CAR, the CAR along with the conjugate is internalized. In one embodiment, the drug regulates the activity of the CAR T cell. The type of drug used in the present invention is not limited to any specific type. Rather, any drug that regulates the activity of the CAR T cell may be used. For example, in one embodiment the drug causes the death of the CAR T cell.

Conjugate Production

The drug-molecule conjugate of the present invention may be produced in any suitable manner available in the art, although in particular embodiments, the conjugate is generated as a fusion polypeptide or is chemically conjugated, such as by use of a linker.

In embodiments wherein the drug-molecule conjugate is produced by conjugation, such as chemical conjugation or by use of a linker, the singular components are provided or obtained and are then associated by a chemical conjugation or linking method.

For example, the conjugate components may be joined via a biologically-releasable bond, such as a selectively-cleavable linker or amino acid sequence. For example, peptide linkers that include a cleavage site for an enzyme preferentially located or active within a tumor environment are contemplated. Exemplary forms of such peptide linkers are those that are cleaved by urokinase, plasmin, thrombin, Factor IXa, Factor Xa, or a metallaproteinase, such as collagenase, gelatinase, or stromelysin. Alternatively, peptides or polypeptides may be joined to an adjuvant.

Additionally, any other linking/coupling agents and/or mechanisms known to those of skill in the art can be used to combine the components of the present invention, such as, for example, antibody-antigen interaction, avidin biotin

linkages, amide linkages, ester linkages, thioester linkages, ether linkages, thioether linkages, phosphoester linkages, phosphoramide linkages, anhydride linkages, disulfide linkages, ionic and hydrophobic interactions, bispecific antibodies and antibody fragments, or combinations thereof.

It is contemplated that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and therapeutic/preventative agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability in vivo, preventing release of the targeting peptide prior to reaching the site of action. These linkers are thus one group of linking agents.

Another cross-linking reagent is 4-succinimdyloxycarbonyl-methyl-x-[2-pyridyldithio]-toluene (SMPT), which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that steric hindrance of the disulfide bond serves the function of protecting the bond from attack by thiolate anions such as glutathione which can be present in tissues and blood, and thereby aids in preventing decoupling of the conjugate prior to the delivery of the attached agent to the target site.

The SMPT cross-linking reagent, as with many other known cross-linking reagents, facilitates cross-linking of functional groups such as the SH of cysteine or primary amines (e.g., the epsilon amino group of lysine). Another type of cross-linker includes the hetero-bifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(p-azido salicylamido)ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.

In addition to hindered cross-linkers, non-hindered linkers also can be employed in accordance herewith. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include succinimidyl acetylthioacetate (SATA), N-succinimidyl3-(2-pyridyldithio) propionate SPDP and 2-iminothiolane (Wawrzynczak & Thorpe, 1987). The use of such cross-linkers is well understood in the art.

U.S. Pat. No. 4,680,338, describes bifunctional linkers useful for producing conjugates of ligands with amine-containing polymers and/or proteins,

especially for forming antibody conjugates with chelators, drugs, enzymes, detectable labels and the like. U.S. Pat. Nos. 5,141,648 and 5,563,250 disclose cleavable conjugates containing a labile bond that is cleavable under a variety of mild conditions. This linker is particularly useful in that the agent of interest may be bonded directly to the linker, with cleavage resulting in release of the active agent. Preferred uses include adding a free amino or free sulfhydryl group to a protein, such as an antibody, or a drug.

U.S. Pat. No. 5,856,456 provides peptide linkers for use in connecting polypeptide constituents to make fusion proteins, e.g., single chain antibodies. The linker is up to about 50 amino acids in length, contains at least one occurrence of a charged amino acid (preferably arginine or lysine) followed by a proline, and is characterized by greater stability and reduced aggregation. U.S. Pat. No. 5,880,270 discloses aminooxy-containing linkers useful in a variety of immunodiagnostic and separative techniques.

Another embodiment involves the use of flexible linkers.

Administration of the Chimeric Molecules

In one embodiment, the present invention comprises methods of limiting the depletion of normal, non-cancerous, cells during CAR T cell therapy. As described elsewhere herein, while CAR T cell therapy can effectively eliminate tumor cells, it is sometimes necessary to limit CAR T cell activity such that tumor cells are targeted, while normal cells are spared. In one embodiment, the method comprises administering a drug-molecule conjugate to a subject receiving CAR T cell therapy when it is determined that the CAR T cells are depleting too much normal tissue. For example, it may be determined that anti-CD19 CAR T cells are depleting an unsafe amount of normal B cells. Such determination can be made by any trained physician or health care provider.

In some embodiments, an effective amount of the conjugate of the present invention is administered to a cell. In other embodiments, a therapeutically effective amount of the conjugates of the present invention are administered to an individual for the treatment of a disease or condition.

The term “effective amount” as used herein is defined as the amount of the conjugates of the present invention that is necessary to result in a physiological change in the cell or tissue to which it is administered.

The term "therapeutically effective amount" as used herein is defined as the amount of the conjugates of the present invention that eliminates, decreases, delays, or minimizes adverse effects of the condition (i.e. excessive depletion of normal tissue caused by CAR T cell therapy). A skilled artisan readily recognizes that in many cases the conjugates may not provide a cure but may only provide partial benefit, such as alleviation or improvement of at least one symptom of the condition. In some embodiments, a physiological change having some benefit is also considered therapeutically beneficial. Thus, in some embodiments, an amount of conjugates that provides a physiological change is considered an "effective amount" or a "therapeutically effective amount."

In some embodiments of the present invention and as an advantage over known methods in the art, the conjugates are delivered as proteins and not as nucleic acid molecules to be translated to produce the desired polypeptides. As an additional advantage, in some embodiments human sequences are utilized in the conjugate of the present invention to circumvent any undesirable immune responses from a foreign polypeptide.

The conjugates of the invention may be administered to a subject per se or in the form of a pharmaceutical composition. Pharmaceutical compositions comprising the proteins of the invention may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries that facilitate processing of the proteins into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For topical administration the conjugates of the invention may be formulated as solutions, gels, ointments, creams, suspensions, etc. as are well-known in the art.

Systemic formulations include those designed for administration by injection, e.g. subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, inhalation, oral or pulmonary administration.

For injection, the conjugates of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks'

solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Alternatively, the conjugates may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the conjugates can be readily formulated by combining the conjugates with pharmaceutically acceptable carriers well known in the art. Such carriers enable the conjugates of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. For oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, e.g. lactose, sucrose, mannitol and sorbitol; cellulose preparations such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP); granulating agents; and binding agents. If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

If desired, solid dosage forms may be sugar-coated or enteric-coated using standard techniques.

Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well-known examples of delivery vehicles that may be used to deliver conjugates of the invention. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the conjugates may be delivered using a sustained-release system, such as semipermeable matrices of solid polymers containing the conjugate. Various forms of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the molecules for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the conjugates, additional strategies for molecule stabilization may be employed.

The protein embodiments of the conjugates of the invention may contain charged side chains or termini. Thus, they may be included in any of the

above-described formulations as the free acids or bases or as pharmaceutically acceptable salts. Pharmaceutically acceptable salts are those salts that substantially retain the biologic activity of the free bases and which are prepared by reaction with inorganic acids. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

The conjugates of the invention will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent a disease condition, the conjugates of the invention, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount.

For systemic administration, a therapeutically effective dose can be estimated initially from in vitro assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from in vivo data, e.g., animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the molecules which are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.001 to 100 mg/kg/day, preferably from about 0.5 to 1 mg/kg/day and any and all whole or partial integers there between. Therapeutically effective serum levels may be achieved by administering multiple doses each day.

In cases of local administration or selective uptake, the effective local concentration of the proteins may not be related to plasma concentration. One skilled in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

The amount of conjugates administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

The therapy may be repeated intermittently while symptoms detectable or even when they are not detectable. The therapy may be provided alone or in combination with other drugs.

RNA transfection

In one embodiment, the genetically modified T cells of the invention are modified through the introduction of RNA. In one embodiment, an in vitro transcribed RNA CAR can be introduced to a cell as a form of transient transfection. In another embodiment, the RNA CAR is introduced along with an in vitro transcribed RNA encoding a bispecific antibody. The RNA is produced by in vitro transcription using a polymerase chain reaction (PCR)-generated template. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. The desired template for in vitro transcription is the CAR of the present invention. In one embodiment, the template for the RNA CAR comprises an extracellular domain comprising a single chain variable domain of an anti-tumor antibody; a transmembrane domain comprising the hinge and transmembrane domain of CD8a; and a cytoplasmic domain comprises the signaling domain of CD3-zeta. By way of another example, the template comprises an inhibitory CAR having an extracellular domain comprising an antibody, or portion thereof, directed to an antigen associated with normal healthy tissue. By way of another example, the template comprises plurality type of CAR. In some instances the template comprises an inhibitory CAR and at least one type of tumor-directed CAR.

In one embodiment, the DNA to be used for PCR contains an open reading frame. The DNA can be from a naturally occurring DNA sequence from the genome of an organism. In one embodiment, the DNA is a full length gene of interest of a portion of a gene. The gene can include some or all of the 5' and/or 3' untranslated regions (UTRs). The gene can include exons and introns. In one embodiment, the DNA to be used for PCR is a human gene. In another embodiment, the DNA to be used for PCR is a human gene including the 5' and 3' UTRs. The DNA can alternatively be an artificial DNA sequence that is not normally expressed in a naturally occurring organism. An exemplary artificial DNA sequence is one that contains portions of genes that are ligated together to form an open reading frame that encodes a fusion protein. The portions of DNA that are ligated together can be from a single organism or from plurality organism.

Genes that can be used as sources of DNA for PCR include genes that encode polypeptides that provide a therapeutic or prophylactic effect to an organism or that can be used to diagnose a disease or disorder in an organism. Preferred genes are genes which are useful for a short term treatment, or where there are safety concerns regarding dosage or the expressed gene. For example, for treatment of cancer, autoimmune disorders, parasitic, viral, bacterial, fungal or other infections, the transgene(s) to be expressed may encode a polypeptide that functions as a ligand or receptor for cells of the immune system, or can function to stimulate or inhibit the immune system of an organism. In some embodiments, it is not desirable to have prolonged ongoing stimulation of the immune system, nor necessary to produce changes which last after successful treatment, since this may then elicit a new problem. For treatment of an autoimmune disorder, it may be desirable to inhibit or suppress the immune system during a flare-up, but not long term, which could result in the patient becoming overly sensitive to an infection.

PCR is used to generate a template for in vitro transcription of mRNA which is used for transfection. Methods for performing PCR are well known in the art. Primers for use in PCR are designed to have regions that are substantially complementary to regions of the DNA to be used as a template for the PCR. "Substantially complementary", as used herein, refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are complementary, or one or more bases are non-complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of a gene that is normally transcribed in cells (the open reading frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a gene that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR are generated by synthetic methods that are well known in the art. "Forward primers" are primers that contain a region of nucleotides that are substantially complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. "Upstream" is used herein to refer to a location 5, to the DNA sequence to be amplified relative to the coding strand. "Reverse primers" are primers that contain

a region of nucleotides that are substantially complementary to a double-stranded DNA template that are downstream of the DNA sequence that is to be amplified. "Downstream" is used herein to refer to a location 3' to the DNA sequence to be amplified relative to the coding strand.

Any DNA polymerase useful for PCR can be used in the methods disclosed herein. The reagents and polymerase are commercially available from a number of sources.

Chemical structures with the ability to promote stability and/or translation efficiency may also be used. The RNA preferably has 5' and 3' UTRs. In one embodiment, the 5' UTR is between zero and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to, designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the gene of interest. Alternatively, UTR sequences that are not endogenous to the gene of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the gene of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of mRNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art.

In one embodiment, the 5' UTR can contain the Kozak sequence of the endogenous gene. Alternatively, when a 5' UTR that is not endogenous to the gene of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many mRNAs is known in the art. In other embodiments the 5' UTR can be derived from an RNA virus whose RNA genome is stable in cells. In other embodiments

various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the mRNA.

To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In one preferred embodiment, the promoter is a T7 polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters. Consensus nucleotide sequences for T7, T3 and SP6 promoters are known in the art.

In a preferred embodiment, 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.

On a linear DNA template, phage T7 RNA polymerase can extend the 3' end of the transcript beyond the last base of the template (Schenborn and Mierendorf, Nuc Acids Res., 13:6223-36 (1985); Nacheva and Berzal-Herranz, Eur. J. Biochem., 270:1485-65 (2003).

The conventional method of integration of polyA/T stretches into a DNA template is molecular cloning. However polyA/T sequence integrated into plasmid DNA can cause plasmid instability, which is why plasmid DNA templates obtained from bacterial cells are often highly contaminated with deletions and other aberrations. This makes cloning procedures not only laborious and time consuming but often not reliable. That is why a method which allows construction of DNA templates with polyA/T 3' stretch without cloning highly desirable.

The polyA/T segment of the transcriptional DNA template can be produced during PCR by using a reverse primer containing a polyT tail, such as 100T tail (size can be 50-5000 T), or after PCR by any other method, including, but not limited to, DNA ligation or in vitro recombination. Poly(A) tails also provide stability to RNAs and reduce their degradation. Generally, the length of a poly(A) tail

positively correlates with the stability of the transcribed RNA. In one embodiment, the poly(A) tail is between 100 and 5000 adenosines.

Poly(A) tails of RNAs can be further extended following in vitro transcription with the use of a poly(A) polymerase, such as E. coli polyA polymerase (E-PAP). In one embodiment, increasing the length of a poly(A) tail from 100 nucleotides to between 300 and 400 nucleotides results in about a two-fold increase in the translation efficiency of the RNA. Additionally, the attachment of different chemical groups to the 3' end can increase mRNA stability. Such attachment can contain modified/artificial nucleotides, aptamers and other compounds. For example, ATP analogs can be incorporated into the poly(A) tail using poly(A) polymerase. ATP analogs can further increase the stability of the RNA.

5' caps on also provide stability to RNA molecules. In a preferred embodiment, RNAs produced by the methods disclosed herein include a 5' cap. The 5' cap is provided using techniques known in the art and described herein (Cougot, et al., Trends in Biochem. Sci., 29:436-444 (2001); Stepinski, et al., RNA, 7:1468-95 (2001); Elango, et al., Biochim. Biophys. Res. Commun., 330:958-966 (2005)).

The RNAs produced by the methods disclosed herein can also contain an internal ribosome entry site (IRES) sequence. The IRES sequence may be any viral, chromosomal or artificially designed sequence which initiates cap-independent ribosome binding to mRNA and facilitates the initiation of translation. Any solutes suitable for cell electroporation, which can contain factors facilitating cellular permeability and viability such as sugars, peptides, lipids, proteins, antioxidants, and surfactants can be included.

RNA can be introduced into target cells using any of a number of different methods, for instance, commercially available methods which include, but are not limited to, electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, Mass.) or the Gene Pulser II (BioRad, Denver, Colo.), Multiporator (Eppendort, Hamburg Germany), cationic liposorne mediated transfection using lipofection, polymer encapsulation, peptide mediated transfection, or biolistic particle delivery systems such as “gene guns” (see, for example, Nishikawa, et al. Hum Gene Ther., 12(8):861-70 (2001).

Genetically Modified T Cells

In some embodiments, the CAR sequences are delivered into cells using a retroviral or lentiviral vector. CAR-expressing retroviral and lentiviral vectors can be delivered into different types of eukaryotic cells as well as into tissues and whole organisms using transduced cells as carriers or cell-free local or systemic delivery of encapsulated, bound or naked vectors. The method used can be for any purpose where stable expression is required or sufficient.

In other embodiments, the CAR sequences and bispecific antibody sequences are delivered into cells using in vitro transcribed mRNA. In vitro transcribed mRNA CAR can be delivered into different types of eukaryotic cells as well as into tissues and whole organisms using transfected cells as carriers or cell-free local or systemic delivery of encapsulated, bound or naked mRNA. The method used can be for any purpose where transient expression is required or sufficient.

The disclosed methods can be applied to the modulation of T cell activity in basic research and therapy, in the fields of cancer, stem cells, acute and chronic infections, and autoimmune diseases, including the assessment of the ability of the genetically modified T cell to kill a target cancer cell.

The methods also provide the ability to control the level of expression over a wide range by changing, for example, the promoter or the amount of input RNA, making it possible to individually regulate the expression level. Furthermore, the PCR-based technique of mRNA production greatly facilitates the design of the chimeric receptor mRNAs with different structures and combination of their domains. For example, varying of different intracellular effector/costimulator domains on multiple chimeric receptors in the same cell allows determination of the structure of the receptor combinations which assess the highest level of cytotoxicity against multi-antigenic targets, and at the same time lowest cytotoxicity toward normal cells.

One advantage of RNA transfection methods of the invention is that RNA transfection is essentially transient and a vector-free: An RNA transgene can be delivered to a lymphocyte and expressed therein following a brief in vitro cell activation, as a minimal expressing cassette without the need for any additional viral sequences. Under these conditions, integration of the transgene into the host cell genome is unlikely. Cloning of cells is not necessary because of the efficiency of transfection of the RNA and its ability to uniformly modify the entire lymphocyte population.

Genetic modification of T cells with in vitro-transcribed RNA (IVT-RNA) makes use of two different strategies both of which have been successively tested in various animal models. Cells are transfected with in vitro-transcribed RNA by means of lipofection or electroporation. Preferably, it is desirable to stabilize IVT-RNA using various modifications in order to achieve prolonged expression of transferred IVT-RNA.

Some IVT vectors are known in the literature which are utilized in a standardized manner as template for in vitro transcription and which have been genetically modified in such a way that stabilized RNA transcripts are produced. Currently protocols used in the art are based on a plasmid vector with the following structure: a 5' RNA polymerase promoter enabling RNA transcription, followed by a gene of interest which is flanked either 3' and/or 5' by untranslated regions (UTR), and a 3' polyadenyl cassette containing 50-70 A nucleotides. Prior to in vitro transcription, the circular plasmid is linearized downstream of the polyadenyl cassette by type II restriction enzymes (recognition sequence corresponds to cleavage site). The polyadenyl cassette thus corresponds to the later poly(A) sequence in the transcript. As a result of this procedure, some nucleotides remain as part of the enzyme cleavage site after linearization and extend or mask the poly(A) sequence at the 3' end. It is not clear, whether this nonphysiological overhang affects the amount of protein produced intracellularly from such a construct.

RNA has several advantages over more traditional plasmid or viral approaches. Gene expression from an RNA source does not require transcription and the protein product is produced rapidly after the transfection. Further, since the RNA has to only gain access to the cytoplasm, rather than the nucleus, and therefore typical transfection methods result in an extremely high rate of transfection. In addition, plasmid based approaches require that the promoter driving the expression of the gene of interest be active in the cells under study.

In another aspect, the RNA construct can be delivered into the cells by electroporation. See, e.g., the formulations and methodology of electroporation of nucleic acid constructs into mammalian cells as taught in US 2004/0014645, US 2005/0052630A1, US 2005/0070841A1, US 2004/0059285A1, US 2004/0092907A1. The various parameters including electric field strength required for electroporation of any known cell type are generally known in the relevant research literature as well as numerous patents and applications in the field. See e.g., U.S. Pat. No. 6,678,556, U.S.

Pat. No. 7,171,264, and U.S. Pat. No. 7,173,116. Apparatus for therapeutic application of electroporation are available commercially, e.g., the MedPulser™ DNA Electroporation Therapy System (Inovio/Genetronics, San Diego, Calif.), and are described in patents such as U.S. Pat. No. 6,567,694; U.S. Pat. No. 6,516,223, U.S. Pat. No. 5,993,434, U.S. Pat. No. 6,181,964, U.S. Pat. No. 6,241,701, and U.S. Pat. No. 6,233,482; electroporation may also be used for transfection of cells in vitro as described e.g. in US20070128708A1. Electroporation may also be utilized to deliver nucleic acids into cells in vitro. Accordingly, electroporation-mediated administration into cells of nucleic acids including expression constructs utilizing any of the many available devices and electroporation systems known to those of skill in the art presents an exciting new means for delivering an RNA of interest to a target cell.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Internalization of CAR binding reagents as a way to modulate and/or ablate the activity of CAR transduced T cells.

A common observation for all of the CAR constructs that have been tested so far is that the CAR complex is transiently internalized after recognizing target antigen (Figure 1, Figure 2). Without wishing to be bound by any particular theory, this internalization may be required for optimal CAR-driven function. This feature of CAR may be similar to the internalization of T cell receptor complexes after binding to target cells and also the internalization observed for cell surface

antigens after binding a cognate antibody. For example, this is the mechanism of action of some clinically available drug-antibody conjugates (anti-CD33 antibody gemtuzumab ozogamicin (Mylotarg); anti-CD30 antibody brentuximab vedotin (Adcetris)) used in the treatment of hematologic malignancy. The tumor cell binds the antibody-drug conjugate, internalizes the conjugate, and the drug (calicheamycin in the case of Mylotarg, MMAE in the case of Adcetris) is released intracellularly, leading to cell death.

The extracellular domain of CAR molecules usually consists of a binding domain derived from antibodies specific for molecules expressed on the surface of target cells; typically this binding domain is synthesized using standard molecular biology-based techniques and consists of a single-chain variable fragment (scFv) which is a fusion of the variable regions of heavy and light chains of an immunoglobulin molecule that recognizes the target molecule, connected via a short linker peptide; the scFv is derived from antibodies that recognize the target molecule, generated in non-human species and in some cases "humanized" to minimize immunogenicity. These domains are responsible for the specificity of CAR.

The scFv domain of CAR is itself targeted and/or bound by other molecules, such as antibodies that are specific for the scFv, epitopes derived from the target antigen itself, or other molecules that adopt a conformation that binds to the scFv.

It is described herein that molecules are developed which specifically bind to CAR and, upon binding, are internalized by cells that express surface CAR. Further, the CAR targeting agents are linked to other molecules that disrupt cell function, such that upon CAR binding internalization CAR-expressing cells are disabled and/or eliminated. This technology allows the specific, at-will elimination or inactivation of cells which express surface CAR.

It has been demonstrated that CARs are internalized upon binding to target molecules on cells. Importantly it has been shown that this phenomenon occurs in vivo in patients treated with CAR T cells. In developing the internalization inducing reagents, an antibody which binds to the CAR is linked to an antimitotic drug using a linker using a standard biochemical procedure. This allows the demonstration that CAR-expressing T cells are specifically lysed by the addition of a drug-antibody conjugate without impacting on the residual non-engineered T cells.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A drug-molecule conjugate comprising a drug and a molecule which binds to a CAR expressed on the surface of a cell.

2. The conjugate of claim 1, wherein binding of the conjugate to the CAR results in internalization of the conjugate into the cell.

3. The conjugate of claim 1, wherein binding of the conjugate to the CAR results in the drug-mediated death of the cell.

4. The conjugate of claim 1, wherein the cell is a T cell and wherein binding of the conjugate to the CAR results in the drug-mediated inhibition of the activation of the T cell.

5. The conjugate of claim 1, wherein the molecule is selected from the group consisting of an antibody, a protein, a peptide, a nucleotide, a small molecule, and fragments thereof.

6. A method for inhibiting the depletion of healthy tissue during CAR T cell therapy comprising administering a drug-molecule conjugate comprising a drug and a molecule to a subject receiving CAR T cell therapy, wherein the molecule binds to a CAR expressed on the surface of a T cell.

7. The method of claim 6, wherein binding of the conjugate to the CAR results in internalization of the conjugate into the cell.

8. The method of claim 6, wherein the binding of the conjugate to the CAR results in the drug-mediated death of the cell.

9. The method of claim 6, wherein the binding of the conjugate to the CAR results in the drug-mediated inhibition of the activation of the T cell.

10. The method of claim 6, wherein the molecule is selected from the group consisting of an antibody, a protein, a peptide, a nucleotide, a small molecule, and fragments thereof.