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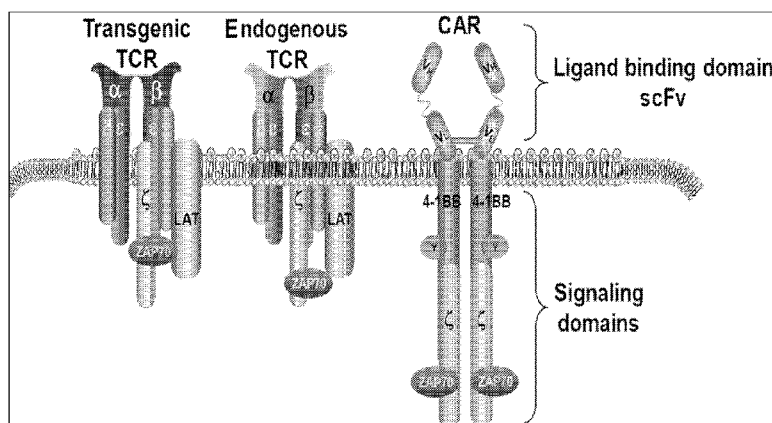


Figure 1

(57) Abstract: The invention provides compositions and methods for reducing and preventing tumor escape from immunotherapy. Specifically, the invention relates to administering a genetically modified T cell comprising both a TCR and a CAR. The invention provides an isolated nucleic acid encoding a T cell receptor (TCR) and a chimeric antigen receptor (CAR), wherein the isolated nucleic acid sequence comprises a TCR- α sequence, a TCR- β sequence, a CAR antigen binding domain and a costimulatory signaling domain sequence. In one embodiment, the CAR sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

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TITLE OF THE INVENTION
DOUBLE TRANSGENIC T CELLS COMPRISING A CAR AND A TCR
AND THEIR METHODS OF USE

5 CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial No. 61/601,905, filed February 22, 2012, the contents of which are incorporated by reference herein in their entirety.

10 BACKGROUND OF THE INVENTION

The immune system can sometimes become ineffective against chronic infections and self-like antigens, such as those associated with cancers, due to a potential loss of antigen presentation (Marincola et al., 2000, *Adv Immunol.* 74:181-273; Vitale et al., 2005, *Clin Cancer Res.* 11:67-72) or low avidity of T cell receptors (TCR) for these antigens. Among efforts to overcome such tolerance, various strategies using synthetic biology to engineer T cells for gain-of-function as a new cancer therapeutic have been proposed. Preclinical studies using engineered lymphocytes expressing antigen-reactive T cell receptors (TCR) in mice (Kessels et al., 2001, *Nat Immunol* 2:957-961) and primates (Barsov, et al., 2001, *PloS one* 6:e23703) warrant this as a promising strategy towards developing therapies for cancer and chronic infections. Concurrently, recent success using gene transfer to create antigen-specific chimeric antigen receptors (CAR) in combating chronic lymphocytic leukemia (Kalos et al., 2011, *Science translational medicine* 3:95ra73; Porter et al., 2011, *NEJM* 365:725-733) provided clinical evidence supporting the use of genetically engineered T cells to treat certain cancers. Despite this, immune evasion (Hanahan et al., 2011, *Cell* 144:646-674) caused by mutations/adaptations, is a major hallmark in cancer biology that poses a significant impediment towards successful anti-tumor therapy. Immune tolerance is another such problem in the field of immunotherapy.

30 Accordingly, there exists a continued need in the art for new compositions and methods to reduce and prevent tumor escape from immunotherapy.

SUMMARY OF THE INVENTION

The invention provides an isolated nucleic acid encoding a T cell receptor (TCR) and a chimeric antigen receptor (CAR), wherein the isolated nucleic acid sequence comprises a TCR- α sequence, a TCR- β sequence, a CAR antigen binding domain and a costimulatory signaling domain sequence.

In one embodiment, the CAR sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

In one embodiment, the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

In one embodiment, the TCR specifically binds to a tumor antigen.

In one embodiment, the TCR specifically binds a tumor antigen derived from one selected from the group consisting of: FR α , CD24, CD44, CD133, CD166, epCAM, CA-125, HE4, Ova1, estrogen receptor, progesterone receptor, HER-2/neu, uPA, PAI-1, CD19, CD20, CD22, ROR1, mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, NYEso-1, and MAGE A3.

In one embodiment, the CAR antigen binding domain specifically binds to a tumor antigen.

In one embodiment, the CAR antigen binding domain specifically binds to one selected from the group consisting of: FR α , CD24, CD44, CD133, CD166, epCAM, CA-125, HE4, Ova1, estrogen receptor, progesterone receptor, HER-2/neu, uPA, PAI-1, CD19, CD20, CD22, ROR1, mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, NYEso-1, and MAGE A3.

In one embodiment, the CAR antigen binding fragment is a Fab or a scFv.

In one embodiment, the TCR and the CAR bind to different antigens.

In one embodiment, the isolated nucleic acid further comprises the nucleic acid of a CAR transmembrane domain.

In one embodiment, wherein the TCR and the CAR are on more than one nucleic acid.

The invention also provides a vector comprising an isolated nucleic acid comprising an isolated nucleic acid encoding a T cell receptor (TCR) and a chimeric antigen receptor (CAR), wherein the isolated nucleic acid sequence comprises a TCR- α sequence, a TCR- β sequence, a CAR antigen binding domain and
5 a costimulatory signaling domain sequence.

In one embodiment, the vector is a retroviral vector or a lentiviral vector.

The invention also provides genetically modified T cell comprising an isolated nucleic acid encoding a T cell receptor (TCR) and a chimeric antigen
10 receptor (CAR), wherein the isolated nucleic acid sequence comprises a TCR- α sequence, a TCR- β sequence, a CAR antigen binding domain and a costimulatory signaling domain sequence.

In one embodiment, the nucleic acid is in vitro transcribed RNA.

The invention provides a genetically modified T cell comprising the
15 polypeptides encoded by the nucleic acid sequence comprising an isolated nucleic acid comprising an isolated nucleic acid encoding a T cell receptor (TCR) and a chimeric antigen receptor (CAR), wherein the isolated nucleic acid sequence comprises a TCR- α sequence, a TCR- β sequence, a CAR antigen binding domain and a costimulatory signaling domain sequence.

The invention provides a method of providing anti-tumor immunity in
20 a subject, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and a nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic sequence
25 and a costimulatory signaling domain nucleic acid sequence, thereby providing anti-tumor immunity in the subject.

In one embodiment, the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-
30 associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

In one embodiment, the CAR nucleic acid sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

In one embodiment, the subject is a mammal. In another embodiment, the subject is a human.

The invention provides a method of stimulating a T cell-mediated immune response to a cell population or tissue in a subject, the method comprising:
5 administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and an nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic sequence and a costimulatory signaling domain nucleic acid sequence, thereby stimulating a T cell-mediated immune
10 response in the subject.

The invention provides a method of treating cancer in a subject, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and an nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic
15 acid sequence comprises an antigen binding domain nucleic sequence and a costimulatory signaling domain nucleic acid sequence, thereby treating cancer in the subject.

The invention also provides a method of reducing cancer escape from immunotherapy in a subject, the method comprising: administering to the subject an
20 effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and an nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic sequence and a costimulatory signaling domain nucleic acid sequence, thereby treating cancer in the subject.

The invention also provides a method of generating a persisting population of genetically engineered T cells in a subject diagnosed with cancer, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and an nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic
25 acid sequence comprises an antigen binding domain nucleic sequence and a costimulatory signaling domain nucleic acid sequence, wherein the persisting population of genetically engineered T cells persists in the subject for at least one
30 month after administration.

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 schematic depicting engineered double transgenic T cell (DTTC). The endogenous TCR is a single heterodimeric (α and β chains) surface molecule. DTTCs are engineered by the introduction of genes coding for artificial TCRs and CARs with specificity to particular target tumor antigens. The transgenic TCRs are similar to the endogenous ones, but have tumor antigen specificity. CARs consist of an extra-cellular antibody-type ligand binding domain, with intra-cellular signaling domains derived from T-cell signaling proteins. CARs can target antigen in a MHC-independent fashion. LAT, linker for activation of T cells; scFv, single chain variable fragment; ZAP70, ζ -chain-associated protein kinase 70kDa. 4-1BB is a T cell co-stimulatory signal receptor (Figure adapted from June et al., 2007, J. Clin. Invest. 117:1466–1476).

Figure 2 depicts the results of a flow cytometry analysis of DTTC. T cells (equal mix of CD4 and CD8) electroporated with RNA expressing NYEso-1 TCR and with RNA expressing CD19 BBz CAR, are stained with fluorescent-tagged antibodies against the TCR and the CAR and analyzed on a flow cytometer.

Figure 3, comprising Figures 3A and 3B, depicts the creation of DTTC with specificity for NY-Eseo1 1G4 TCR and mesothelin. In Figure 3A, results of a flow cytometry analysis of DTTC. T cells (equal mix of CD4 and CD8) were electroporated with RNA expressing NYEso-1 TCR, and with RNA expressing anti-mesothelin SS1CD191BBz CAR or with a mixture of RNA for NYEso-1 TCR and SS19BBz CAR. 24 hours later, cells were stained with fluorescent-tagged antibodies against the TCR Vbeta chain and the CAR and analyzed on a flow cytometer. Cells electroporated with PBS, and non-electroporated T cells served as controls; there is native TCR Vbeta 13.1 expression on some of the polyclonal T cells; V beta 13.1 is used by the NY-Eso-1 TCR. In Figure 3B, the population of electroporated T cells

(3A) were exposed to target cells comprised of HLA negative/mesothelin+ K562-meso cells; K562-CD19 cells serve as a control. For TCR function, electroporated T cells were exposed to HLA-A2+ /NY-Eso-1+ 624.38mel cells, or a NY-ESO-1-/HLA-A2+ melanoma line 526mel cells as a control, and the function of the electroporated cells evaluated by CD107a staining.

Figure 4 is a schematic depicting an example construct having a single promoter expressing TCR- α , TCR- β and CD19BBz CAR and utilizing a P2A and T2A system (see, for example, Carey et al., 2009, PNAS 106:157-162).

Figure 5 is a schematic depicting an example construct having two promoters (one promoter driving expression of TCR- α and TCR- β utilizing a P2A system, and the other promoter driving expression of CD19BBz CAR).

Figure 6 is a schematic depicting an example bi-directional construct having two promoters (one promoter driving expression of TCR- α and TCR- β utilizing a P2A system, and the other promoter driving expression of CD19BBz CAR).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for reducing or eliminating cancer escape from immunotherapy. The cancer may be a hematological malignancy, a solid tumor, a primary or a metastasizing tumor.

In one embodiment, the invention provides a T cell genetically engineered to express both a TCR and a CAR (i.e., a double transgenic T cell (DTTC)), wherein both the TCR and the CAR exhibits an antitumor property. In some embodiments, the genetically modified T cells of the invention stably express both a TCR and a CAR. In other embodiments, the genetically modified T cells of the invention are transfected with one or more RNAs encoding both a TCR and a CAR. In some embodiments, the TCR and the CAR of a DTTC are engineered to recognize the same antigen on a tumor cell. In other embodiments, the TCR and the CAR of the DTTC are engineered to recognize different antigens on a tumor cell. Without wishing to be bound by any particular theory, it is believed that a cancer is less likely to escape immunotherapy when the DTTC of the invention has a TCR and a CAR engineered to recognize different antigens. The present invention relates generally to

the use of DTTC genetically modified to express a desired TCR and a desired CAR. T cells expressing a TCR and a CAR are referred to herein as DTTC.

5 The TCR of the invention can be engineered to comprise an extracellular MHC-dependent antigen binding domain to direct antigen recognition based upon the antigen binding specificity. The invention includes any antigen binding domain that when bound to its cognate antigen, affects a tumor cell so that the tumor cell fails to grow, is prompted to die, or otherwise is affected so that the tumor burden in a patient is diminished or eliminated.

10 In one embodiment, the CAR of the invention comprises an antigen recognition domain, a transmembrane domain, and a cytoplasmic domain. In one embodiment, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In another embodiment, 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. In some 15 embodiments, the extracellular domain also comprises a hinge domain. Preferably, the hinge domain is a CD8 α hinge domain.

20 The CAR of the invention can be engineered to comprise an extracellular domain having an antigen binding domain fused to an intracellular signaling domain of the T cell antigen receptor complex zeta chain (e.g., CD3 zeta). The CAR of the invention when expressed in a T cell is able to redirect antigen recognition based on the antigen binding specificity. The invention includes any antigen binding domain that when bound to its cognate antigen, affects a tumor cell so that the tumor cell fails to grow, is prompted to die, or otherwise is affected so that 25 the tumor burden in a patient is diminished or eliminated. The antigen binding domain is preferably fused with an intracellular domain from one or more of a costimulatory molecule and a zeta chain. Preferably, the antigen binding domain is fused with one or more intracellular domains selected from the group of a CD137 (4-1BB) signaling domain, a CD28 signaling domain, a CD3zeta signal domain, and any combination 30 thereof.

In one embodiment, the CAR of the invention comprises a CD137 (4-1BB) signaling domain. This is because the present invention is partly based on the discovery that CAR-mediated T-cell responses can be further enhanced with the

addition of costimulatory domains. For example, inclusion of the CD137 (4-1BB) signaling domain significantly increased anti-tumor activity and *in vivo* persistence of CAR T cells compared to an otherwise identical CAR T cell not engineered to express CD137 (4-1BB).

5 In one embodiment, the DTTC of the invention is generated by introducing a lentiviral vector comprising a desired TCR and a desired CAR. For example, the lentiviral vector comprising a TCR and a CAR comprising an antibody binding domain, a CD8 α hinge, a transmembrane domain, and a human 4-1BB and CD3zeta signaling domain, is introduced into a T cell. The antibody binding domain
10 of the CAR can be any domain that binds to a desired antigen, including but not limited to monoclonal antibodies, polyclonal antibodies, antibody fragments, and humanized antibodies. The DTTC of the invention are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control.

 In one embodiment the invention relates to administering a DTTC
15 expressing a TCR and a CAR for the treatment of a patient having cancer or at risk of having cancer. Preferably, autologous lymphocyte infusion is used in the treatment. Autologous PBMCs are collected from a patient in need of treatment and T cells are activated, genetically modified, and expanded using the methods described herein and known in the art and then infused back into the patient.

20

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
25 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
30 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 more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one 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 $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

As used herein, the terms “4-1BB (CD137) costimulatory domain” may refer to any sequence of 4-1BB including, for example, a stimulatory signaling domain of 4-1BB. Stimulatory signaling domains of 4-1BB and their variants are well known in the art and fully described in U.S. Patent Publication 20050113564, which is incorporated by reference herein in its entirety. Nucleic acid and amino acid sequences of 4-1BB and their variants are well known in the art and fully described in U.S. Patent Publications U.S. 20060063923; U.S. 20060029595; U.S. 20030082157; U.S. 20020168719; U.S. 20040091476; U.S. 20050113564; and U.S. 20060002904, all of which are incorporated by reference herein in their entirety. In one embodiment, the 4-1BB (CD137) costimulatory domain is a homologue, a variant, an isomer, or a functional fragment of 4-1BB (CD137). Each possibility represents a separate embodiment of the present invention.

“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 typically tetramers 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
5 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
10 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
15 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
20 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
25 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
30 not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to 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.

5 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
10 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 mistakenly recognized by the immune system as being foreign. Auto-antigens comprise, but are not limited to, cellular proteins,
15 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
20 diseases include but are not limited to, Addison'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,
25 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
30 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, bladder cancer, kidney cancer, melanoma, mesothelioma, prostate cancer, thymoma, sarcoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, uterine 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.

5 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
10 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
15 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.
20 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
25 inside an organism, cell, tissue or system.

As used herein, the term “exogenous” refers to any material introduced from or 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 driven by its promoter.

30 “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.

5 “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
10 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.
15 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
20 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
25 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
30 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
5 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
10 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”
15 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
20 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
25 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
30 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.

5 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
10 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 the
15 tumor antigen is intended to indicate an abnormal level of expression of the 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
20 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
25 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
30 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.

5 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
10 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,
15 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
20 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
25 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
30 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.

5 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
10 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
15 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
20 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
25 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.

5 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
10 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” is 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.

15 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
20 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
25 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
30 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
5 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 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
10 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
15 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
20 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
25 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
30 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

5 The present invention provides compositions and methods for reducing or eliminating cancer escape from immunotherapy. The cancer may be a hematological malignancy, a solid tumor, a primary or a metastasizing tumor.

 In one embodiment, the invention provides a T cell genetically modified to express both a TCR and a CAR (i.e., a double transgenic T cell (DTTC)),
10 wherein both the TCR and the CAR exhibits an antitumor property. In some embodiments, the genetically modified T cells of the invention stably express both a TCR and a CAR. In other embodiments, the genetically modified T cells of the invention are transfected with one or more RNAs encoding both a TCR and a CAR. In some embodiments, the TCR and the CAR of a DTTC are engineered to recognize
15 the same antigen on a tumor cell. In other embodiments, the TCR and the CAR of the DTTC are engineered to recognize different antigens on a tumor cell.

 The TCR of the invention can be engineered to comprise an extracellular antigen binding domain to direct antigen recognition based upon the antigen binding specificity. The invention includes any antigen binding domain that
20 when bound to its cognate antigen, affects a tumor cell so that the tumor cell fails to grow, is prompted to die, or otherwise is affected so that the tumor burden in a patient is diminished or eliminated.

 The CAR of the invention can be engineered to comprise an extracellular domain having an antigen binding domain fused to an intracellular
25 signaling domain of the T cell antigen receptor complex zeta chain (e.g., CD3 zeta). The CAR of the invention when expressed in a T cell is able to redirect antigen recognition based on the antigen binding specificity. The invention includes any antigen binding domain that when bound to its cognate antigen, affects a tumor cell so that the tumor cell fails to grow, is prompted to die, or otherwise is affected so that
30 the tumor burden in a patient is diminished or eliminated. The antigen binding domain is preferably fused with an intracellular domain from one or more of a costimulatory molecule and a zeta chain. Preferably, the antigen binding domain is fused with one or more intracellular domains selected from the group of a CD137 (4-1BB) signaling

domain, a CD28 signaling domain, a CD3zeta signal domain, and any combination thereof.

In one embodiment, the CAR of the invention comprises a CD137 (4-1BB) signaling domain. This is because the present invention is partly based on the discovery that CAR-mediated T-cell responses can be further enhanced with the addition of costimulatory domains. For example, inclusion of the CD137 (4-1BB) signaling domain significantly increased anti-tumor activity and *in vivo* persistence of CAR T cells compared to an otherwise identical CAR T cell not engineered to express CD137 (4-1BB).

10

T Cell Receptors

In addition to their role in combating infections, T cells have also been implicated in the destruction of cancerous cells. T cell receptors (TCRs) are closely related to antibody molecules in structure, and they are involved in antigen binding although, unlike antibodies, they do not recognize free antigen; instead, they bind antigen fragments which are bound and presented by antigen-presenting molecules. An important group of antigen-presenting molecules are the MHC class I and class II molecules that present antigenic peptides and protein fragments to T cells.

Variability in the antigen binding site of a TCR is created in a fashion similar to the antigen binding site of antibodies, and also provides specificity for a vast number of different antigens. Diversity occurs in the complementarity determining regions (CDRs) in the N-terminal domains of the disulfide-linked alpha (α) and beta (β), or gamma (γ) and delta (Δ), polypeptides of the TCR. The CDR loops are clustered together to form an MHC-antigen-binding site analogous to the antigen-binding site of antibodies, although in TCRs, the various chains each contain two additional hypervariable loops as compared to antibodies. TCR diversity for specific antigens is also directly related to the MHC molecule on the APC's surface to which the antigen is bound and presented to the TCR.

In one embodiment, the TCR of the invention binds to a specific antigenic peptide bound in an MHC molecule. In one embodiment, the TCR of the invention can be selected or engineered to target a particular tumor antigen of interest. In the context of the present invention, "tumor antigen" or "hyperproliferative 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.

5 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 targeted antigen 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
10 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
15 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.
20 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
25 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
30 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
5 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:
10 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;
15 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,
20 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.

25 In various embodiments, the TCR of the DTTC targets an antigen that includes but is not limited to FR α , CD24, CD44, CD133, CD166, epCAM, CA-125, HE4, Ova1, estrogen receptor, progesterone receptor, HER-2/neu, uPA, PAI-1, and the like.

Depending on the desired antigen to be targeted, the TCR of the
30 invention can be engineered to include the appropriate antigen binding domain that is specific to the desired antigen target. For example, if FR α is the desired antigen that is to be targeted, TCR specific for FR α can be used.

In some situations, it is preferred that there is an HLA match between the tumor cell and the DTTC of the invention. That is to say the tumor cell displays an MHC molecule of an allele for which the donor of the T-cells is HLA positive. In some embodiments, this is achieved because the tumor cell from a first individual and
5 the DTTC from a second individual have an HLA match. However, in alternative embodiments, the tumor cell and the DTTC are from the same individual.

In further embodiments of the present invention there is provided a method for preparing DTTC suitable for delivery to a patient suffering from cancer. The DTTC prepared in accordance with the invention are administered to a patient in
10 order to treat cancer in the patient. In principle, the DTTC of the invention are capable of being used for the treatment of many different types of cancer including leukemia, lymphomas such as non-Hodgkin lymphoma, multiple myeloma and the like.

Thus, in some embodiments of the present invention, pharmaceutical
15 preparations are provided comprising a DTTC of the invention and a pharmaceutically acceptable carrier, diluent or excipient, further details of which may be found in Remington's Pharmaceutical Sciences in US Pharmacopeia, 1984 Mack Publishing Company, Easton, Pa., USA.

As discussed elsewhere herein, the HLA allele of the MHC molecule
20 used to present the peptide to the T-cells is an HLA allele also expressed by the patient and therefore when the DTTC are administered to the patient, they recognize the antigenic peptide displayed on MHC molecules of that HLA allele.

In some alternative embodiments, multiple sets (e.g. 2 or 3 sets) of DTTC are provided, each DTTC being specific for a different peptide. In each case,
25 the DTTC are allogeneic, as described elsewhere herein, that is to say the HLA allele of the MHC molecule on which the peptide is displayed is an HLA allele which is not expressed in the donor individual from whom the DTTC are obtained. The peptides may all be from the same cell specific protein or may be from different proteins but specific for the same cell type. The peptide may or may not be a peptide of the
30 invention. In some embodiments, the multiple sets of DTTC are administered simultaneously but in other embodiments they are administered sequentially.

Reference has been made to the preparation and provision of DTTC. However, it is to be appreciated that the important feature of the DTTC is the T-cell

receptor (TCR) and the chimeric antigen receptor (CAR) which is displayed on the DTTCs and, more specifically, the specificity of the TCR for the complex of the peptide and the MHC molecule. In various embodiments, the nucleic acid encoding the TCR is incorporated into a vector such as a viral vector (e.g. a retroviral vector),
5 lentiviral vector, adenoviral vector or a vaccinia vector. Alternatively, a non-viral approach may be followed such as using naked DNA or lipoplexes and polyplexes or mRNA in order to transfect a T-cell with the nucleic acid encoding the TCR.

Since the DTTC of the invention also display their endogenous TCRs, it is preferred that the T-cells are pre-selected, prior to their genetic modification, to
10 eliminate T-cells that would give rise to graft-versus-host disease. In some embodiments, the T-cells are pre-selected such that the specificity of their endogenous TCRs is known. For instance, T-cells are selected which are reactive with a particular tumor antigen. In other embodiments, the T-cells are obtained from the patient and thus are naturally tolerized for the patient.

15 In some alternative embodiments, the TCR, as a whole, is not recombinantly expressed but rather the regions of the TCR which are responsible for its binding specificity are incorporated into a structure which maintains the confirmation of these regions. More specifically, complementarity determining regions (CDRs) of the TCR are maintained in the same conformation in the
20 recombinant protein.

Therefore, in some embodiments of the invention, only the TCRs, or a polynucleotide encoding the TCRs are provided. For example, in one specific embodiment, allo-restricted T-cells which are reactive to a particular peptide from a particular tumor antigen, when displayed by an MHC molecule of the HLA allele
25 HLA-A*0201 are generated. The T cells are cloned, and the TCRs from one or several T-cell clones are isolated and sequenced. A cDNA encoding the TCR is then prepared and inserted into an expression cassette or vector, which also expressed a CAR. When a patient who is HLA-A*0201 positive and suffering from cancer requires treatment, T-cells from a donor individual are transfected with the vector or
30 expression cassette or mRNA and the TCR, along with the CAR, are expressed by and displayed on the DTTC. The DTTC are then administered to the patient in order to elicit an immune response to tumor cells and eliminate them from the patient's body.

Chimeric Antigen Receptor (CAR)

The present invention provides a CAR comprising an extracellular and intracellular domain. The extracellular domain comprises a target-specific binding element otherwise referred to as an antigen binding domain. 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 antigens 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.

Antigen binding 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 “hyperproliferative 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 FR α , CD24, CD44, CD133, CD166, epCAM, CA-125, HE4, Ova1, estrogen receptor, progesterone receptor, HER-2/neu, uPA, PAI-1, CD19, CD20, CD22, ROR1, mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, NY-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 binding domain that is specific to the desired antigen target. For example, if FR α is the desired antigen that is to be targeted, an antibody for FR α can be used as the antigen bind moiety for incorporation into the CAR of the invention.

Transmembrane domain

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 TCR, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154. 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.

Preferably, the transmembrane domain in the CAR of the invention is the CD8 transmembrane domain. In some instances, the transmembrane domain of the CAR of the invention comprises the CD8 transmembrane domain.

Cytoplasmic domain

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.

Preferred 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 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 a preferred 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
5 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
10 with CD83, and the like. Thus, while the invention is exemplified primarily with 4-1BB as the co-stimulatory signaling element, other costimulatory elements are within the scope of the invention.

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
15 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 and the signaling domain of CD28. In another
20 embodiment, the cytoplasmic domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of 4-1BB. In yet another embodiment, the cytoplasmic domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD28 and 4-1BB.

25 RNA transfection

Disclosed herein are methods for producing the in vitro transcribed RNA TCRs and RNA CARs of the invention. In one embodiment, the in vitro transcribed RNA can be introduced to a cell as a form of transient transfection. The RNA is produced by in vitro transcription using a polymerase chain reaction (PCR)-
30 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 templates for in vitro transcription are the TCRs and CARs of the present invention. For example, the template for the RNA TCR comprises TCR α and TCR β . For example, the template for the RNA CAR comprises an extracellular domain comprising a single chain variable domain of an anti-tumor antibody; a
5 transmembrane domain comprising the hinge and transmembrane domain of CD8a; and a cytoplasmic domain comprises the signaling domain of CD3-zeta and the signaling domain of 4-1BB.

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
10 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
15 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 more than one organism.

20 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
25 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. It is not desirable to have prolonged ongoing stimulation of the immune system, nor necessary to produce changes which last after
30 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.

5 “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
10 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
15 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
20 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.

25 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
30 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
5 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
10 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
15 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
20 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
25 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
30 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 (Eppendorf, Hamburg Germany), cationic liposome 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)).

Vectors

The present invention encompasses a DNA construct comprising sequences of a TCR and a CAR, wherein the CAR sequence comprises the nucleic acid sequence of an antigen binding domain operably linked to the nucleic acid sequence of an intracellular domain. An exemplary intracellular domain that can be used in the CAR of the invention includes but is not limited to the intracellular domain of CD3-zeta, CD28, 4-1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4-1BB, and the like.

The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector

known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than cloned.

5 The present invention also provides vectors in which a DNA of the present invention is inserted. Vectors derived from retroviruses, such as the lentivirus, are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene or transgenes and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-
10 proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity.

In brief summary, the expression of natural or synthetic nucleic acids encoding a TCR and a CAR is typically achieved by operably linking a nucleic acid encoding the TCR and CAR polypeptide or portions thereof to one or more
15 promoters, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

The expression constructs of the present invention may also be used
20 for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. *See, e.g.*, U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties. In another embodiment, the invention provides a gene therapy vector.

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

Further, the expression vector may be provided to a cell in the form of
30 a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to,

retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 5 6,326,193).

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant 10 virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

Additional promoter elements, e.g., enhancers, regulate the frequency 15 of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the 20 thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. One example of an element known to increase expression levels is the woodchuck hepatitis post-transcriptional regulatory element (WPRE) 25 (see, for example, Klein et al., 2006, *Gene* 372:153-161).

In some embodiments, the nucleic acid construct of the invention are multicistronic constructs that permit the expression of multiple transgenes (e.g., TCR- α , TCR- β , CAR, etc.) under the control of a single promoter. In some 30 embodiments, the transgenes (e.g., TCR- α , TCR- β , CAR, etc.) are separated by a self-cleaving 2A peptide. Examples of 2A peptides useful in the nucleic acid constructs of the invention include F2A, P2A, T2A and E2A (see for example, Kim et al., *PLoS One* 6:e18556; Carey et al., 2009, *PNAS* 106:157-162; Szymczak et al., 2004, *Nature Biotechnology* 22:589-594).

In other embodiments of the invention, the nucleic acid construct of the invention is a multicistronic construct comprising two promoters; one promoter driving the expression of TCR- α and TCR- β (separated by a 2A peptide, and the other promoter driving the expression of the CAR. In some embodiments, the dual promoter constructs of the invention are uni-directional. In other embodiments, the dual promoter constructs of the invention are bi-directional.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor -1 α (EF-1 α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionein promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

In order to assess the expression of a TCR or CAR polypeptide or portions thereof, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or transduced through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory

sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

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

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus,

poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (e.g., an artificial membrane vesicle).

In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro*, *ex vivo* or *in vivo*). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a “collapsed” structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine (“DMPC”) can be obtained from Sigma, St. Louis, MO; dicetyl phosphate (“DCP”) can be obtained from K & K Laboratories (Plainview, NY); cholesterol (“Choi”) can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol (“DMPG”) and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform

or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as
5 having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the
10 lipid bilayers (Ghosh et al., 1991 *Glycobiology* 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

15 Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and
20 Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

25 Genetically Modified T Cells

In some embodiments, the TCR and CAR nucleic acids are delivered into cells using a retroviral or lentiviral vector. TCR-expressing and 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
30 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 TCR and CAR nucleic acids are delivered into cells using in vitro transcribed mRNA. In vitro transcribed mRNA CAR and

mRNA TCR 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.

5 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
10 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
15 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
20 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
25 population. Thus, cells containing an RNA construct introduced according to the disclosed method can be used in the methods of the invention described herein. For example, a lymphocyte cell population is withdrawn from a patient, transfected with different RNA constructs, and then used in the assay of the invention to assess the susceptibility of a target cancer cell to being killed by the genetically modified T cell.
30 In some embodiments, the target cancer cell and the T cell is derived from the same patient.

 In the preferred embodiment, the technology is used to evaluate personalized therapy. For example, for treatment of tumors, the patient's blood or

cells is collected by an appropriate method such as apheresis, biopsy or venapuncture. The cells are cultured for at least 24 hours during which time the cells are transduced with an appropriate CAR-containing retroviral or lentiviral vector, or transfected with an appropriate CAR-containing RNA construct. The cells can be stored frozen before
5 transduction or transfection, if necessary.

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-
10 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.
15 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
20 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
25 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
30 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.

5 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

10 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

15 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.

20

Sources of T cells

Prior to expansion and genetic modification, a source of T cells is obtained from a subject. The term “subject” is intended to include living organisms in which an immune response can be elicited (e.g., mammals). Examples of subjects

25 include humans, dogs, cats, mice, rats, and transgenic species thereof. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments of the present invention, any number of T cell lines available in the art, may be used.

30 In certain embodiments of the present invention, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. In one preferred embodiment, cells from the circulating blood of an individual are obtained by apheresis. The apheresis

product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Again, surprisingly, initial activation steps in the absence of calcium lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated “flow-through” centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics Cell Saver 5) according to the manufacturer’s instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

In another embodiment, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient or by counterflow centrifugal elutriation. A specific subpopulation of T cells, such as CD3⁺, CD28⁺, CD4⁺, CD8⁺, CD45RA⁺, and CD45RO⁺T cells, can be further isolated by positive or negative selection techniques. For example, in one embodiment, T cells are isolated by incubation with anti-CD3/anti-CD28 (*i.e.*, 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another preferred embodiment, the time period is 10 to 24 hours. In one preferred embodiment, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as

compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8⁺ T cells. Thus, by simply shortening or lengthening the time T cells are allowed to bind to the CD3/CD28 beads and/or by increasing or decreasing the ratio of beads to T cells (as described further herein), subpopulations of T cells can be preferentially selected for or against at culture initiation or at other time points during the process. Additionally, by increasing or decreasing the ratio of anti-CD3 and/or anti-CD28 antibodies on the beads or other surface, subpopulations of T cells can be preferentially selected for or against at culture initiation or at other desired time points. The skilled artisan would recognize that multiple rounds of selection can also be used in the context of this invention. In certain embodiments, it may be desirable to perform the selection procedure and use the “unselected” cells in the activation and expansion process. “Unselected” cells can also be subjected to further rounds of selection.

Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4⁺ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In certain embodiments, it may be desirable to enrich for or positively select for regulatory T cells which typically express CD4⁺, CD25⁺, CD62L^{hi}, GITR⁺, and FoxP3⁺. Alternatively, in certain embodiments, T regulatory cells are depleted by anti-CD25 conjugated beads or other similar method of selection.

For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (*e.g.*, particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (*i.e.*, increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15,

20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (*i.e.*, leukemic blood, tumor tissue, *etc.*). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8⁺ T cells that normally have weaker CD28 expression.

In a related embodiment, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (*e.g.*, particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4⁺ T cells express higher levels of CD28 and are more efficiently captured than CD8⁺ T cells in dilute concentrations. In one embodiment, the concentration of cells used is 5×10^6 /ml. In other embodiments, the concentration used can be from about 1×10^5 /ml to 1×10^6 /ml, and any integer value in between.

In other embodiments, the cells may be incubated on a rotator for varying lengths of time at varying speeds at either 2-10°C or at room temperature.

T cells for stimulation can also be frozen after a washing step. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or culture media containing 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin and 7.5% DMSO, or 31.25% Plasmalyte-A, 31.25% Dextrose 5%, 0.45% NaCl, 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin, and 7.5% DMSO or other suitable cell freezing media containing for example, Hespan and PlasmaLyte A, the cells then are frozen to -80°C at a rate of 1° per minute and stored in the vapor phase

of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at -20° C or in liquid nitrogen.

In certain embodiments, cryopreserved cells are thawed and washed as described herein and allowed to rest for one hour at room temperature prior to
5 activation using the methods of the present invention.

Also contemplated in the context of the invention is the collection of blood samples or apheresis product from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as
10 T cells, isolated and frozen for later use in T cell therapy for any number of diseases or conditions that would benefit from T cell therapy, such as those described herein. In one embodiment a blood sample or an apheresis is taken from a generally healthy subject. In certain embodiments, a blood sample or an apheresis is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet
15 developed a disease, and the cells of interest are isolated and frozen for later use. In certain embodiments, the T cells may be expanded, frozen, and used at a later time. In certain embodiments, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but prior to any treatments. In a further embodiment, the cells are isolated from a blood sample or an apheresis from a subject
20 prior to any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, cytoxan,
25 fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Curr. Opin. Immun. 5:763-
30 773, 1993). In a further embodiment, the cells are isolated for a patient and frozen for later use in conjunction with (*e.g.*, before, simultaneously or following) bone marrow or stem cell transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or

antibodies such as OKT3 or CAMPATH. In another embodiment, the cells are isolated prior to and can be frozen for later use for treatment following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan.

5 In a further embodiment of the present invention, T cells are obtained from a patient directly following treatment with a non-cellular based treatment and the T cells are engineered to comprise the CAR of the invention. In this regard, it has been observed that following certain cancer treatments, in particular treatments with drugs that damage the immune system, shortly after treatment during the period when patients would normally be recovering from the treatment, the quality of T cells
10 obtained may be optimal or improved for their ability to expand *ex vivo*. Likewise, following *ex vivo* manipulation using the methods described herein, these cells may be in a preferred state for enhanced engraftment and *in vivo* expansion. Thus, it is contemplated within the context of the present invention to collect blood cells, including T cells, dendritic cells, or other cells of the hematopoietic lineage, during
15 this recovery phase. Further, in certain embodiments, mobilization (for example, mobilization with GM-CSF) and conditioning regimens can be used to create a condition in a subject wherein repopulation, recirculation, regeneration, and/or expansion of particular cell types is favored, especially during a defined window of time following therapy. Illustrative cell types include T cells, B cells, dendritic cells,
20 and other cells of the immune system.

Activation and Expansion of T Cells

Whether prior to or after genetic modification of the T cells to express a desirable TCR and a desirable CAR, the T cells can be activated and expanded
25 generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005.

Generally, the T cells of the invention are expanded by contact with a
30 surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the T cells. In particular, T cell populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or

an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted
5 with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4⁺ T cells or CD8⁺ T cells, an anti-CD3 antibody and an anti-CD28 antibody. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, Besançon, France) can be used as can other methods commonly known in the art (Berg et al., Transplant
10 Proc. 30(8):3975-3977, 1998; Haanen et al., J. Exp. Med. 190(9):13191328, 1999; Garland et al., J. Immunol Meth. 227(1-2):53-63, 1999).

In certain embodiments, the primary stimulatory signal and the co-stimulatory signal for the T cell may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When
15 coupled to a surface, the agents may be coupled to the same surface (*i.e.*, in “cis” formation) or to separate surfaces (*i.e.*, in “trans” formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In one embodiment, the agent providing the co-stimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In
20 certain embodiments, both agents can be in solution. In another embodiment, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing Fc receptors or an antibody or other binding agent which will bind to the agents. In this regard, see for example, U.S. Patent Application Publication Nos. 20040101519 and 20060034810 for artificial antigen presenting cells (aAPCs) that
25 are contemplated for use in activating and expanding T cells in the present invention.

In one embodiment, the two agents are immobilized on beads, either on the same bead, *i.e.*, “cis,” or to separate beads, *i.e.*, “trans.” By way of example, the agent providing the primary activation signal is an anti-CD3 antibody or an antigen-binding fragment thereof and the agent providing the co-stimulatory signal is
30 an anti-CD28 antibody or antigen-binding fragment thereof; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In one embodiment, a 1:1 ratio of each antibody bound to the beads for CD4⁺ T cell expansion and T cell growth is used. In certain aspects of the present invention, a ratio of anti CD3:CD28

antibodies bound to the beads is used such that an increase in T cell expansion is observed as compared to the expansion observed using a ratio of 1:1. In one particular embodiment an increase of from about 1 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In one embodiment, the ratio of

5 CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In one aspect of the present invention, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, *i.e.*, the ratio of CD3:CD28 is less than one. In certain embodiments of the invention, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than 2:1. In one particular

10 embodiment, a 1:100 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In a further embodiment, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one preferred embodiment, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In

15 another embodiment, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet another embodiment, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

Ratios of particles to cells from 1:500 to 500:1 and any integer values in between may be used to stimulate T cells or other target cells. As those of ordinary

20 skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. For example, small sized beads could only bind a few cells, while larger beads could bind many. In certain embodiments the ratio of cells to particles ranges from 1:100 to 100:1 and any integer values in-between and in further embodiments the ratio comprises 1:9 to 9:1 and any integer values in between,

25 can also be used to stimulate T cells. The ratio of anti-CD3- and anti-CD28-coupled particles to T cells that result in T cell stimulation can vary as noted above, however certain preferred values include 1:100, 1:50, 1:40, 1:30, 1:20, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, and 15:1 with one preferred ratio being at least 1:1 particles per T cell. In one embodiment, a ratio of

30 particles to cells of 1:1 or less is used. In one particular embodiment, a preferred particle: cell ratio is 1:5. In further embodiments, the ratio of particles to cells can be varied depending on the day of stimulation. For example, in one embodiment, the ratio of particles to cells is from 1:1 to 10:1 on the first day and additional particles

are added to the cells every day or every other day thereafter for up to 10 days, at final ratios of from 1:1 to 1:10 (based on cell counts on the day of addition). In one particular embodiment, the ratio of particles to cells is 1:1 on the first day of stimulation and adjusted to 1:5 on the third and fifth days of stimulation. In another
5 embodiment, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:5 on the third and fifth days of stimulation. In another embodiment, the ratio of particles to cells is 2:1 on the first day of stimulation and adjusted to 1:10 on the third and fifth days of stimulation. In another embodiment, particles are added on a daily or every other day basis to a final ratio of 1:1 on the
10 first day, and 1:10 on the third and fifth days of stimulation. One of skill in the art will appreciate that a variety of other ratios may be suitable for use in the present invention. In particular, ratios will vary depending on particle size and on cell size and type.

In further embodiments of the present invention, the cells, such as T
15 cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In an alternative embodiment, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In a further embodiment, the beads and cells are first concentrated by application of a force, such as a magnetic force, resulting in increased ligation of cell surface markers,
20 thereby inducing cell stimulation.

By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3x28 beads) to contact the T cells. In one embodiment the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a
25 ratio of 1:1) are combined in a buffer, preferably PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (*i.e.*, 100%) may comprise the target cell of interest. Accordingly, any cell number is
30 within the context of the present invention. In certain embodiments, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (*i.e.*, increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in one embodiment, a concentration of about 2 billion

cells/ml is used. In another embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells. Such populations of cells may have therapeutic value and would be desirable to obtain in certain embodiments. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In one embodiment of the present invention, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may be cultured for 21 days. In one embodiment of the invention the beads and the T cells are cultured together for about eight days. In another embodiment, the beads and T cells are cultured together for 2-3 days. Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell culture include an appropriate media (*e.g.*, Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF β , and TNF- α or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, α -MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37° C) and atmosphere (*e.g.*, air plus 5% CO₂).

T cells that have been exposed to varied stimulation times may exhibit different characteristics. For example, typical blood or apheresed peripheral blood mononuclear cell products have a helper T cell population (T_H , $CD4^+$) that is greater than the cytotoxic or suppressor T cell population (T_C , $CD8^+$). *Ex vivo* expansion of T cells by stimulating CD3 and CD28 receptors produces a population of T cells that prior to about days 8-9 consists predominately of T_H cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of T_C cells. Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of T_H cells may be advantageous. Similarly, if an antigen-specific subset of T_C cells has been isolated it may be beneficial to expand this subset to a greater degree.

Further, in addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated T cell product for specific purposes.

Therapeutic Application

The present invention encompasses a DTTC transduced with a lentiviral vector (LV). For example, the LV encodes a TCR with binding specificity to a particular antigen and a CAR that combines an antigen recognition domain of a specific antibody with an intracellular domain of CD3-zeta, CD28, 4-1BB, or any combinations thereof. Therefore, in some instances, the DTTC can elicit a TCR-mediated T-cell response, a CAR-mediated T-cell response, or both.

The invention provides the use of a TCR and a CAR to redirect the specificity of a primary T cell to at least one tumor antigens. Thus, the present invention also provides a method for stimulating a T cell-mediated immune response to a target cell population or tissue in a mammal comprising the step of administering to the mammal a DTTC that expresses a TCR, wherein the TCR has binding specificity to a predetermined target antigen, and a CAR, wherein the CAR comprises a binding moiety that specifically interacts with a predetermined target, a zeta chain portion comprising for example the intracellular domain of human CD3zeta, and a costimulatory signaling region. In some embodiments, the TCR and the CAR have

binding specificity for the same antigen. In other embodiments, the TCR and the CAR have binding specificity to different antigens.

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

In one embodiment, the DTTC of the invention can undergo robust *in vivo* T cell expansion and can persist for an extended amount of time. In another embodiment, the DTTC of the invention evolve into specific memory T cells that can be reactivated to inhibit any additional tumor formation or growth. Without wishing to be bound by any particular theory, DTTC may differentiate *in vivo* into a central memory-like state upon encounter and subsequent elimination of target cells expressing the surrogate antigen.

Without wishing to be bound by any particular theory, the anti-tumor immunity response elicited by the DTTC may be an active or a passive immune response. In addition, the DTTC mediated immune response may be part of an adoptive immunotherapy approach in which DTTC induce an immune response specific to the antigen binding domain in the CAR, to the TCR, or to both.

Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise non-solid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the DTTC of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies *e.g.*, sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

However, the invention should not be construed to be limited to solely to the antigen targets and diseases disclosed herein. Rather, the invention should be construed to include any antigenic target that is associated with a disease where a DTTC can be used to treat the disease. By way of non-limiting example, a DTTC

comprising a TCR that binds to peptides associated with NY-ESO1 and a CAR that binds to mesothelin is useful for many solid tumor types.

The DTTC of the invention may also serve as a type of vaccine for *ex vivo* immunization and/or *in vivo* therapy in a mammal. Preferably, the mammal is a human.

With respect to *ex vivo* immunization, at least one of the following occurs *in vitro* prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a TCR and a CAR to the cells, and/or iii) cryopreservation of the cells.

Ex vivo procedures are well known in the art. Briefly, cells are isolated from a mammal (preferably a human) and genetically modified (i.e., transduced or transfected *in vitro*) with a vector expressing a TCR and a CAR. The DTTC can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the DTTC cell can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

The procedure for *ex vivo* expansion of hematopoietic stem and progenitor cells is described in U.S. Pat. No. 5,199,942, incorporated herein by reference, can be applied to the cells of the present invention. Other suitable methods are known in the art, therefore the present invention is not limited to any particular method of *ex vivo* expansion of the cells. Briefly, *ex vivo* culture and expansion of T cells comprises: (1) collecting CD34+ hematopoietic stem and progenitor cells from a mammal from peripheral blood harvest or bone marrow explants; and (2) expanding such cells *ex vivo*. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used for culturing and expansion of the cells.

In addition to using a cell-based vaccine in terms of *ex vivo* immunization, the present invention also provides compositions and methods for *in vivo* immunization to elicit an immune response directed against an antigen in a patient.

Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, the DTTC of the invention are used in the

treatment cancer. In certain embodiments, the cells of the invention are used in the treatment of patients at risk for developing cancer. Thus, the present invention provides methods for the treatment or prevention of cancer comprising administering to a subject in need thereof, a therapeutically effective amount of the DTTC of the invention.

The DTTC of the present invention may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations. Briefly, pharmaceutical compositions of the present invention may comprise a target cell population as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

When "an immunologically effective amount", "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the DTTC described herein may be administered at a dosage of 10^4 to 10^9 cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. DTTC compositions may also be administered multiple times at these dosages. The DTTC can be administered by using infusion techniques that are commonly known in immunotherapy (see, *e.g.*, Rosenberg et al., *New Eng. J.*

of Med. 319:1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

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

The administration of the DTTC compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion,
15 transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (*i.v.*) injection, or intraperitoneally. In one embodiment, the DTTC compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In
20 another embodiment, the DTTC compositions of the present invention are preferably administered by *i.v.* injection. The compositions of T cells may be injected directly into a tumor, lymph node, or site of infection.

In certain embodiments of the present invention, cells activated and expanded using the methods described herein, or other methods known in the art
25 where T cells are expanded to therapeutic levels, are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or natalizumab treatment for MS patients or efalizumab treatment for psoriasis patients
30 or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM

PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Curr. Opin. Immun. 5:763-773, 1993). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. The dose for CAMPATH, for example, will generally be in the range 1 to about 100 mg for an adult patient, usually administered daily for a period between 1 and 30 days. The preferred daily dose is 1 to 10 mg per day although in some instances larger doses of up to 40 mg per day may be used (described in U.S. Patent No. 6,120,766).

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.

The materials and methods employed in these experiments are now described.

Example 1. Construction of a double transgenic T cell expressing a TCR with NYEso-1 specificity and a mesothelin-specific CAR.

10 µg of each of in vitro transcribed RNA of anti-mesothelin CAR ss1bbz (ss1), or RNA for NY-ESO-1 1G4 TCR or the combination of the RNA (ss1+TCR) was electroporated (EP) into T cells (1×10^8 /ml). 24 hours later, transgene expression for both CAR (mIgG Fab) and TCR (vb13.1) was evaluated by flow cytometry (Figure 3A). No RNA EP and mock EP control cells are shown. To assess function, Day 1 electroporated T cells were co-cultured with target cells and the induction of CD107a on the double transgenic T cells was used to detect effector function (Figure 3B). Target cells were the mesothelin+ cell line K562-meso, using K562-CD19 as control for CAR specificity. Target cells for TCR specificity were the NY-ESO-1+/HLA-A2+ melanoma cell line 624.38mel, using NY-ESO-1negative/HLA-A2+ melanoma line 526mel as a control for CD107a staining.

Example 2: Construction of nucleic acid sequences having a TCR sequence and a CAR sequence.

A nucleic acid was constructed having the arrangement of elements as depicted in Figure 4. This nucleic acid is a single-promoter, multicistronic nucleic acid encoding TCR- α , TCR- β and CD19BBz, having a 2A peptide separating each. The sequence of this nucleic acid is:

cagctagcatggagacctgctggcctgctgatcctgtggctgcagctccaagtgggtgtccagcaagcaggaggtgac
ccagatccctgccgccctgagcgtgcccgagggcgagaacctgggtgctgaactgcagcttcaccgactccgccatctaca
acctgcagtgggtccggcaggacccccgcaaggcctgaccagcctgctgctgatccagagcagccagcgggagcag
accagcggacggctgaacgccagcctggacaagagcagcggccggagcacctgtacatcgccgccagccagcccc
5 gcgacagcggcacctacctgtgcgctgtgcccctctgtacggcggcagctacatccccacctcggcagaggcaccag
cctgatcgtgaccccctacatccagaacccccgaccccgccgtgtaccagctgcgggacagcaagagcagcgacaagtct
gtgtgcctgttcaccgacttcgacagccagaccaatgtgagccagagcaaggacagcagctgtacatcaccgacaaga
ccgtgctggacatcgggagcatggactcaagagcaacagcggcctggcctggagcaacaagagcagcttcgctgcg
ccaacgccttcaacaacagcattatccccgaggacaccttctccccagccccgagagcagctgcgacgtgaaactgggtg
10 gagaagagcttcgagaccgacaccaacctgaactccagaacctgagcgtgatcggcttcagaatcctgctgctgaaggt
ggccggattcaacctgctgatgacctgcggctgtggagcagcggctcccgggccaagagaagcggatccggcggccac
caacttcagcctgctgaagcaggccggagacgtggaagaaaacctggcctaggatgagcatggcctgctgtgctgc
ggcgcctgagcctgctgtggcaggaccctgaacgccggagtaccagacccccaaagtccaagtgctgaaaacc
ggccagagcatgacctgcagtcgcccaggacatgaaccacgagtacatgagctggtatcggcaggacccccggcatg
15 ggctgcggctgatccactactgtgggagccggaatcaccgaccaggcggaggtgccaacggctacaatgtgagcc
ggagcaccaccgaggacttccccctgcggctgctgagcgtgccccagccagaccagcgtgtacttctgcgccagcag
ctatgtgggcaacaccggcagcgtgttcttcggcagggctccaggctgacctgctggaggacctgaagaacgtgttcc
ccccgaggtggcctgttcgagcccagcaggccgagatcagccacaccagaaggccacactgggtgtgtctggcca
ccggcttctaccccgaccacgtggagctgtcctgggtgggtgaacggcaaggaggtgcacagcggcgtgtctaccgacc
20 ccagcccctgaaggagcagcccgcctgaacgacagccggtactgctgtcctccagactgagagtgagcggccacctt
tggcagaacccccggaaccacttccgggtgccaggtgcagttctacggcctgagcagagaacgacgagtggaccagcag
cgggccaagcccgtgaccagattgtgagcggcaggcctggggcagggccgactgcggcttcaccagcagagctta
ccagcagggcgtgctgagcggccacctctgtacgagatcctgctgggcaaggccacctgtacggcgtgctgggtgctg
ccctgggtgctgatggctatggtgaagcggagagcagccggggcgatcccgtgctaaacgctcagggctccggagagg
25 gcagaggaagtcttaacatgcgggtgacgtggaggagaatccgggcatatgatggctagaggatccatggccttacc
gtgaccgcttgcctcctgccgtggcctgctgctccacgccgccaggccggacatccagatgacacagactacatcctcc
ctgctgcctctctgggagacagagtcaccatcagttgcagggcaagtcaggacattagtaaataatggtatcagca
gaaaccagatggaactgttaactcctgatctaccatacatcaagattacactcaggagtccatcaagggtcagtgagcag
gggtctggaacagattattctctcaccattagcaacctggagcaagaagatattgccacttactttgccaacagggtaatac
30 gcttccgtacagttcggaggggggaccaagctggagatcacaggtggcgggtggctcggcggtggtgggtcgggtgg
cggcggatctgaggtgaaactgcaggagtcaggacctggcctggtggcgcctcacagagcctgtccgtcacatgcaact
gtctcaggggtctcattaccgactatggtgtaagctggattcggcagcctccacgaaagggtctggagtggtgggagta
atatggggtagtgaaaccacatactataattcagctctcaaatccagactgaccatcatcaaggacaactccaagagccaa

gttttctaaaaatgaacagtctgcaactgatgacacagccatttactactgtgccaacattattactacggtagctatg
 ctatggactactggggccaaggaacctcagtcaccgtctcctcaaccacgacgccagcggcgcgaccaccaacaccgg
 cggccaccatcgcgtcgcagcccctgtccctgcgcccagaggcgtgccggccagcggcggggggcgagtgcacac
 gagggggctggacttcgctgtgatattctacatctgggcgcccttggccgggacttggggctcttctcctgtactggta
 5 tcacccttactgcaaacggggcagaaagaactcctgtatatattcaacaaccatttatgagaccagtacaactactcaa
 gaggaagatggctgtagctgccgattccaagaagaagaaggagatgtgaactgagagtgaagttcagcaggagc
 gcagacgccccgcgtacaagcagggccagaaccagctctataacgagctcaatctaggacgaagagaggatcagat
 gtttggacaagagacgtggccgggaccctgagatggggggaaagccgagaaggaagaaccctcaggaaggcctgta
 caatgaactgcagaaagataagatggcggaggcctacagtgagattgggatgaaaggcgagcggcggaggggcaagg
 10 ggcacgatggcctttaccagggtctcagtacagccaccaaggacacctacgacgcccttcacatgacggcctgccccct
 cgctaagtcgacaatca (SEQ ID NO:1)

A nucleic acid was constructed having the arrangement of elements as depicted in Figure 5. This nucleic acid has two uni-directional promoters (one
 15 promoter expressing TCR- α and TCR- β with a 2A peptide between them, and the other promoter expressing CD19BBz). The sequence of this nucleic acid is:

cgtgaggctccgggtcccgtcagtgaggcagagcgcacatcggccacgtccccgagaagttggggggaggggctggc
 aattgaaccgggtcctagagaaggtggcgcggggtaaacgggaaagtgatgtcgtgtactggctccgctttttcccgag
 20 ggtgggggagaaccgtatataagtgacagtagtcgccgtgaacgttcttttcgcaacgggttccgccagaacacaggtta
 agtgccgtgtgtggttcccgcgggctggcctctttacgggttatggcccttgcgtgccttgaattacttccacctggctgca
 gtacgtgattcttgatcccagcttcgggttgaagtggtgggagagttcaggccttgcgcttaaggagccccttcgct
 cgtgcttgagttgaggcctggcctggcgctggggccgccgctgcgaatctggtggcaccttcgcgctgtctcgtcgt
 ttcgataagctctagccatttaaaatgtgatgacctgctgcgacgctttttctggcaagatagcttctaagtgcgggcaa
 25 gatctgcacactggtatttcggttttggggccgcggcgggcagggggcccgtgcgtcccagcgcacatgttcggcgag
 gcggggctgcgagcgcggccaccgagaatcgacggggtagtctcaagctggccggcctgctctggtgcctggcct
 cgcgccgctgtatcggccgctggcggaaggctggcccggctggcaccagtgcgtgagcggaaagatggc
 cgctcccggcctgctgcaggagctcaaaatggaggacgcggcgtcgggagagcggcggggtgagtcaccaca
 caaagaaaaggccttccgtcctcagccgtcgttcatgtgactccactgagtaccggcgccgtccaggcactcgt
 30 tagttctcgtctttggagtagctcgtctttaggttggggggagggtttatgcgatggagttccccactgagtggtg
 gagactgaagttaggccagcttggcacttgatgtaatttccttgaatttggcctttttgagtttggatcttggttcattctcaag
 cctcagacagtgggtcaagtttttcttccatttcaggtgtcgtgaatggagaccctgctggcctgctgatctgtggctgc
 agctccagtgggtgtccagcaagcaggaggtgaccagatccctgccgctgagcgtgcccgaggggcgagaacctgg

tgctgaactgcagcttcaccgactccgccatctacaacctgcagtggtccggcaggacccccggcaaggcctgaccagc
ctgctgctgatccagagcagccagcgggagcagaccagcggacggctgaacgccagcctggacaagagcagcggcc
ggagcacccctgtacatcgccgccagccagccccggcgacagcggccacctacctgtgcgctgtgcggcctctgtacggcg
gcagctacatccccaccttcggcagaggcaccagcctgatcgtgcaccctacatccagaacccccgaccccgctgta
5 ccagctgcgggacagcaagagcagcgacaagtctgtgtgcctgttcaccgacttcgacagccagaccaatgtgagccag
agcaaggacagcagctgtacatcaccgacaagaccgtgctggacatcgggagcatggacttcaagagcaacagcggc
gtggcctggagcaacaagagcagcttcgctgcgccaacgccttcaacaacagcattatccccgaggacaccttctccc
cagccccgagagcagctgcgacgtgaaactggtggagaagagcttcgagaccgacaccaacctgaactccagaacct
gagcgtgatcggcttcagaatcctgctgctgaagggtggccggattcaacctgctgatgacctcggctgtggagcagcg
10 gctccccggccaagagaagcggatccggcgccaccaacttcagcctgctgaagcaggccggagacgtggaagaaaac
cctggccctaggatgagcatcggcctgctgtgctgcgccgccctgagcctgctgtggcaggaccgtgaacgccggag
tgaccagacccccaaagtccaggtgctgaaaaccggccagagcatgacctgcagtgcgcccaggacatgaaccag
agtacatgagctggtatcggcaggacccccggcatggcctgcggctgatccactactctgtggagccggaatcaccga
ccaggcgaggtgccaacggctacaatgtgagccggagcaccaccgaggactccccctcgggctgctgagcgtgc
15 cccagccagaccagcgtgacttctgcgccagcagctatgtgggcaaacaccggcagctgttcttcggcaggggctcca
ggctgaccgtgctggaggacctgaagaacgtgtccccccgagggtggcctgttcgagcccagcagggccgagatca
gccacaccagaaggccacactgggtgtctggccaccggcttctacccccgaccacgtggagctgtcctggtgggtgaac
ggcaaggaggtgcacagcggcgtgtctaccgacccccagccccgaaggagcagccccctgaacgacagccggta
ctgctgtcctccagactgagagtgagcggcacccttctggcagaacccccggaaccactccggtgccaggtgcagttcta
20 cggcctgagcagagaacgacgagtgaccagcggcggcgaagcccgtgaccagattgtgagcggcaggcctgg
ggcagggccgactcgggcttcaccagcgagagctaccagcaggcgtgctgagcggcaccatcctgtacgagatcctg
ctgggcaaggccaccctgtaccgctgctggtgtctgccctggtgctgatggctatggtgaagcgggaaggacagccggg
gctaaacgcgtgagacctgcagcggatcacgagactagcctcgagaagcttgatatcgaattccacgggggttggggtg
cgcccttccaaggcagccctgggttgcgcaggagcggcgtgctctggcgtggttccgggaaacgcagcggcgccc
25 acctgggtctcgcacattctcacgtccgttcgcagcgtcaccggatcttcgccgtacccttggggccccccggcga
cgcttctgctccgccctaagtgggaagggtccttcgggttcggcgtgccggacgtgacaaacggaagccgcacgt
ctcactagtacctcgcagacggacagcggcagggaatggcagcgcgccgaccgcgatgggctgtggccaatag
cggctgctcagcggggcgcgccgagagcagcggccgggaaggggcgggtcgggaggcggggtgtggggcggtagt
gtgggccctgttctgcccgcggtgttccgattctgcaagcctccggagcgcacgtcggcagtcggctcctcgttga
30 ccgaatcaccgacctctccccagggtacctaggtcacatattgtgattaattaagagacctcgaggtacgcgtatggcta
gaggatccatggccttaccagtgaccgcttgcctcctgccgtggccttgcctcctccacgccgccaggccggacatccag
atgacacagactacatctccctgtctgcctctctgggagacagagtcacatcagttgcagggcaagtcaggacattagta
aatattfaaattggtatcagcagaaaccagatggaactgtfaaactcctgatctaccatacatcaagattacactcaggagtcc

catcaaggttcagtggcagtggtctggaacagattattctctcaccattagcaacctggagcaagaagatattgccacttac
 ttttgccaacagggaataacgcttccgtacacgttcggaggggggaccaagctggagatcacaggtggcggctggctcggg
 cggtggtgggtcgggtggcggcgatctgaggtgaaactgcaggagtcaggacctggcctggtgccctcacagag
 cctgtccgtcacatgcactgtctcaggggtctcattaccgactatggtgtaagctggattcggcagcctccacgaaagggt
 5 ctggagtggctgggagtaatatggggtagtgaaccacatactataattcagctctcaaatccagactgaccatcatcaagg
 acaactccaagagccaagttttcttaaaaatgaacagtctgcaaaactgatgacacagccatttactactgtccaaacattatt
 actacggtggtagctatgctatggactactggggccaaggaacctcagtcaccgtctcctcaaccacgacgccagcgccg
 cgaccaccaacaccggcgcccaccatcgcgtcgcagcccctgtccctgcgccagaggcgtgccggccagcggcggg
 gggcgcagtgcacacgagggggctggacttcgctgtgatatctacatctggcggccctggccgggacttgggggtcc
 10 ttctctgtcactggttatcacccttactgcaaacggggcagaaagaaactcctgtatatattcaaaacaccatttatgagacc
 agtaciaaactactcaagaggaagatggctgtagctgccgattccagaagaagaagaaggaggatgtgaactgagagtg
 aagttcagcaggagcgcagacgccccgcgtacaagcagggccagaaccagctctataacgagctcaatctaggacga
 agagaggagtacgatgttttgacaagagacgtggccgggaccctgagatggggggaaagccgagaaggaagaaccc
 tcaggaaggcctgtacaatgaactgcagaaagataagatggcggaggcctacagtgagattgggatgaaaggcgagcg
 15 ccggaggggcaaggggcacgatggcctttaccagggtctcagtacgccaccaaggacacctacgacgcccttcacatg
 caggeccctgccccctcgctaa(SEQ ID NO:2)

A nucleic acid was constructed having the arrangement of elements as depicted in Figure 6. This nucleic acid has two bi-directional promoters (one
 20 promoter expressing TCR- α and TCR- β with a 2A peptide between them, and the other promoter expressing CD19BBz). The sequence of this nucleic acid is:

ttagcgagggggcagggcctgcatgtgaagggcgtcgtaggtgtccttgggtgctgtactgagaccctggtaaaggccat
 cgtgcccttgcacctccggcgctcgcctttcatccaatctcactgtaggcctccgccatcttatctttctgcagttcattgtac
 25 aggccttctgagggttcttctctcggctttccccatctcagggtcccggccacgtctcttgcataaacatcgtactcct
 ctctcgtcctagattgagctcgttatagactgggtctggccctgctgtacggggggcgtctcgcctcctgtaactca
 ctctcagttcacatcctccttcttcttcttgaaaatcggcagctacagccatcttctcttgagtagtttactggtctcataaa
 tgggtgtttgaatatatacaggagtttcttctgccccgtttgcagtaaagggtgataaccagtgacaggagaaggacccccac
 aagtcggccaagggcgcccagatgtagatatcacaggcgaagtccagccccctcgtgtgactgcccccccgccc
 30 ctggccggcacgcctctggggcagggacaggggctgcgacgcgatggtgggcgccggtgtgtggtgcggcgct
 ggcgtcgtggtgaggagacggtgactgaggttccttggccccagtagtccatagcatagctaccaccgtagtaataatgtt
 tggcacagtagtaaatggctgtgtcatcagtttcagactgttcatttttaagaaaacttggtccttggagttgcttctgatgatg
 gtcagctctggatttgagagctgaattatagatgtggtttcactaccccatattactcccagccactccagacccttctgaggag

gctggcgaatccagcttacaccatagtcgggtaatgagaccctgagacagtgcattgtgacggacaggctctgtgagggc
gccaccaggccaggtcctgactcctgcagttcacctcagatccgccaccgaccaccgcccagaccaccgc
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CLAIMSWhat is claimed is:

1. An isolated nucleic acid encoding a T cell receptor (TCR) and a chimeric antigen receptor (CAR), wherein the isolated nucleic acid sequence comprises a TCR- α sequence, a TCR- β sequence, a CAR antigen binding domain and a costimulatory signaling domain sequence.

2. The isolated nucleic acid of claim 1, wherein the CAR sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

3. The isolated nucleic acid of claim 1, wherein the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

4. The isolated nucleic acid of claim 1, wherein the TCR specifically binds to a tumor antigen.

5. The isolated nucleic acid of claim 1, wherein the TCR specifically binds a tumor antigen derived from one selected from the group consisting of: FR α , CD24, CD44, CD133, CD166, epCAM, CA-125, HE4, Ova1, estrogen receptor, progesterone receptor, HER-2/neu, uPA, PAI-1, CD19, CD20, CD22, ROR1, mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, NYEso-1, and MAGE A3.

6. The isolated nucleic acid of claim 1, wherein the CAR antigen binding domain specifically binds to a tumor antigen.

7. The isolated nucleic acid of claim 1, wherein the CAR antigen binding domain specifically binds to one selected from the group consisting of: FR α , CD24, CD44, CD133, CD166, epCAM, CA-125, HE4, Ova1, estrogen receptor, progesterone receptor, HER-2/neu, uPA, PAI-1, CD19, CD20, CD22, ROR1,

mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, NYEso-1, and MAGE A3.

8. The isolated nucleic acid of claim 1, wherein the CAR antigen binding fragment is a Fab or a scFv.

9. The isolated nucleic acid of claim 1, wherein the TCR and the CAR bind to different antigens.

10. The isolated nucleic acid of claim 1, further comprising the nucleic acid of a CAR transmembrane domain.

11. The isolated nucleic acid of claim 1, wherein the TCR and the CAR are on more than one nucleic acid.

12. A vector comprising an isolated nucleic acid of claim 1.

13. A genetically modified T cell comprising the nucleic acid of claim 1.

14. The genetically modified T cell of claim 13, wherein the nucleic acid is in vitro transcribed RNA.

15. A genetically modified T cell comprising the polypeptides encoded by the nucleic acid sequence of claim 1.

16. A genetically modified T cell comprising the vector of claim 11.

17. The genetically modified T cell claim 16, wherein the vector is a retroviral vector or a lentiviral vector.

18. A method of providing anti-tumor immunity in a subject, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and a nucleic

acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic acid sequence and a costimulatory signaling domain nucleic acid sequence, thereby providing anti-tumor immunity in the subject.

19. The method of claim 18, wherein the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

20. The method of claim 18, wherein the CAR nucleic acid sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

21. The method of claim 18, wherein the subject is a mammal.

22. The method of claim 18, wherein the subject is a human.

23. A method of stimulating a T cell-mediated immune response to a cell population or tissue in a subject, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and an nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic acid sequence and a costimulatory signaling domain nucleic acid sequence, thereby stimulating a T cell-mediated immune response in the subject.

24. The method of claim 23, wherein the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

25. The method of claim 23, wherein the CAR nucleic acid sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

26. The method of claim 23, wherein the subject is a mammal.
27. The method of claim 23, wherein the subject is a human.
28. A method of treating cancer in a subject, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and a nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic acid sequence and a costimulatory signaling domain nucleic acid sequence, thereby treating cancer in the subject.
29. The method of claim 28, wherein the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.
30. The method of claim 28, wherein the CAR nucleic acid sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.
31. The method of claim 28, wherein the subject is a mammal.
32. The method of claim 28, wherein the subject is a human.
33. A method of reducing cancer escape from immunotherapy in a subject, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and a nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic acid sequence and a costimulatory signaling domain nucleic acid sequence, thereby treating cancer in the subject.

34. The method of claim 33, wherein the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

35. The method of claim 33, wherein the CAR nucleic acid sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

36. The method of claim 33, wherein the subject is a mammal.

37. The method of claim 33, wherein the subject is a human.

38. A method of generating a persisting population of genetically engineered T cells in a subject diagnosed with cancer, the method comprising: administering to the subject an effective amount of a genetically modified T cell comprising a nucleic acid sequence encoding a TCR, and an nucleic acid sequence encoding a chimeric antigen receptor (CAR), wherein the CAR nucleic acid sequence comprises an antigen binding domain nucleic sequence and a costimulatory signaling domain nucleic acid sequence, wherein the persisting population of genetically engineered T cells persists in the subject for at least one month after administration.

39. The method of claim 38, wherein the costimulatory signaling domain comprises the intracellular domain of a costimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, and any combination thereof.

40. The method of claim 38, wherein the persisting population of genetically engineered T cells persists in the human for at least three months after administration.

41. The method of claim 38, wherein the CAR nucleic acid sequence further comprises the nucleic acid sequence of a CD3 zeta signaling domain.

42. The method of claim 38, wherein the subject is a mammal.

43. The method of claim 38, wherein the subject is a human.

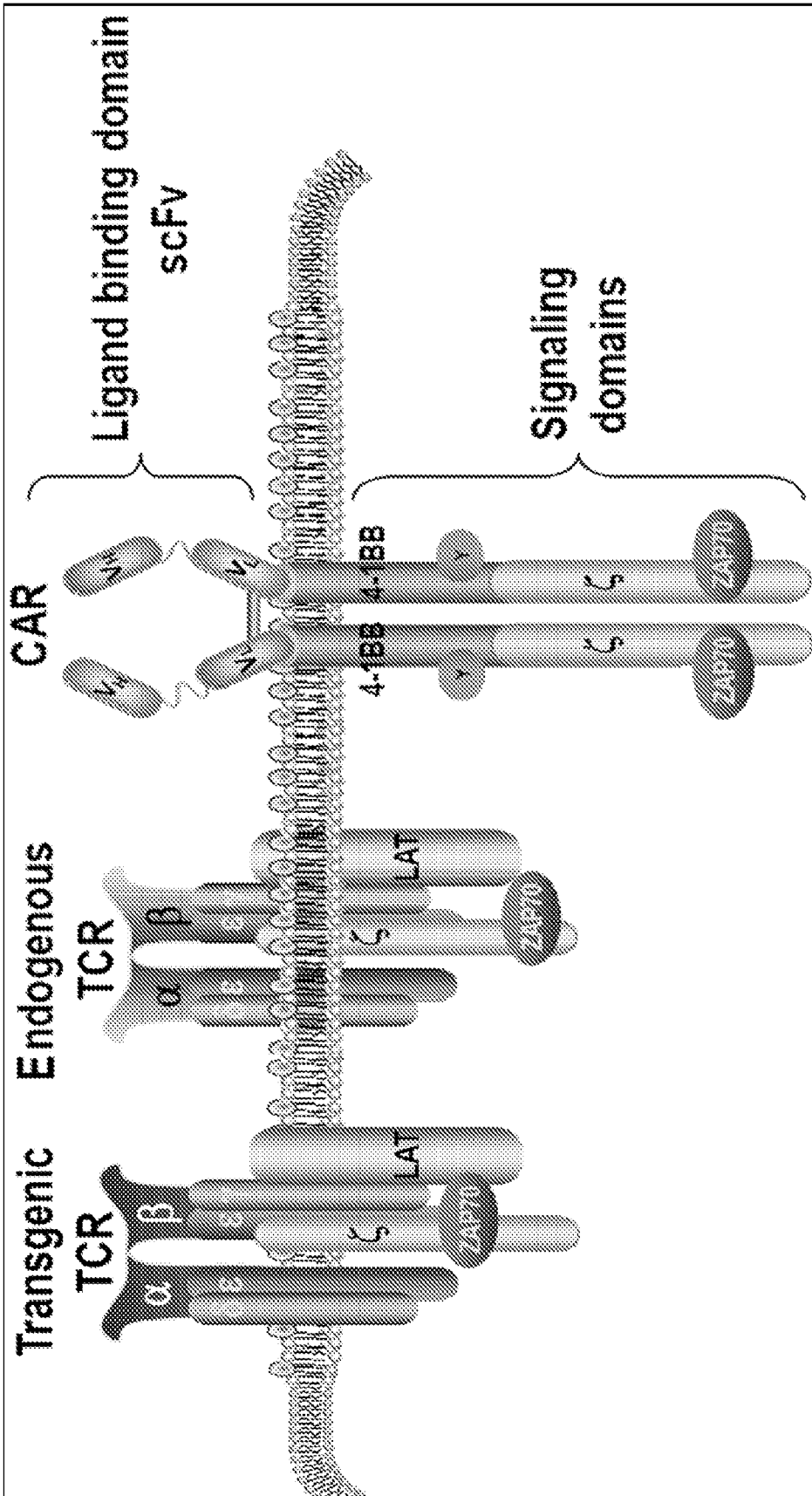


Figure 1

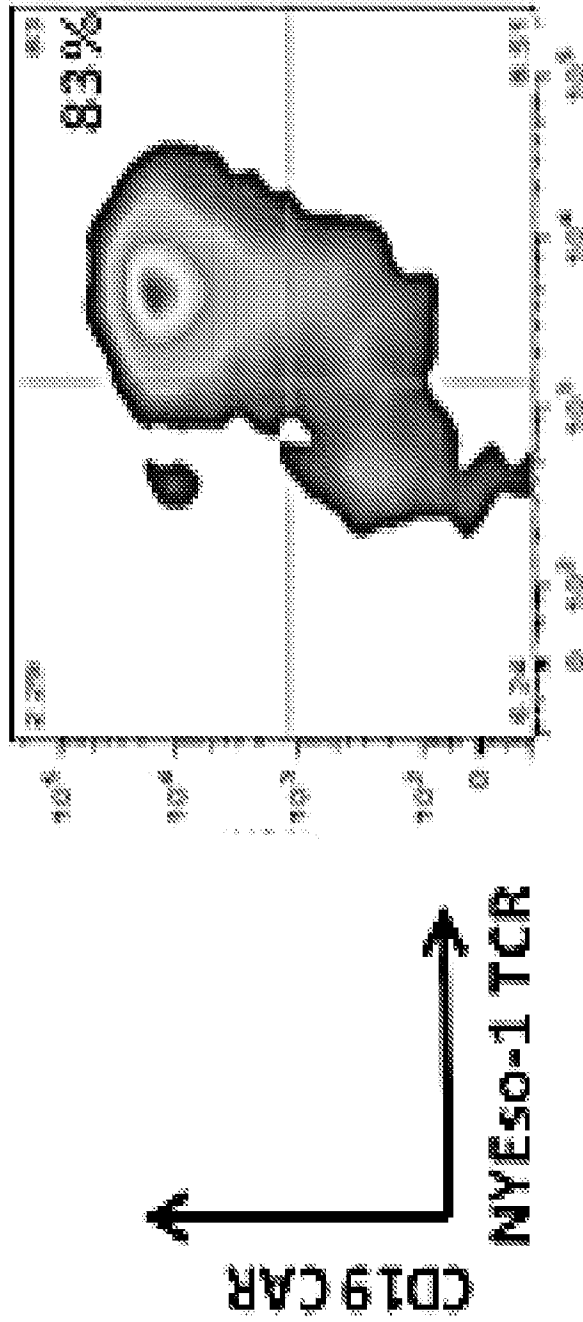


Figure 2

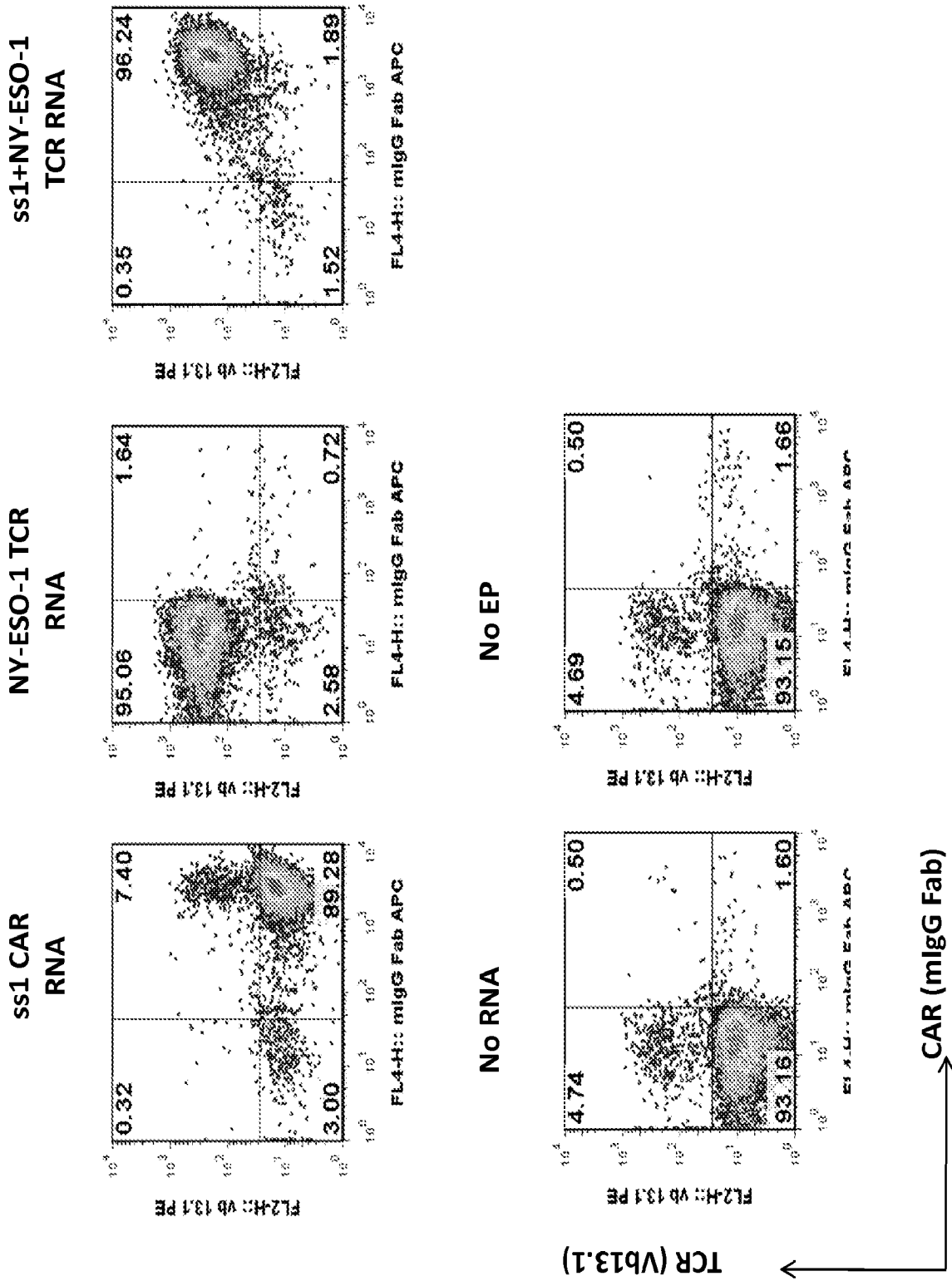
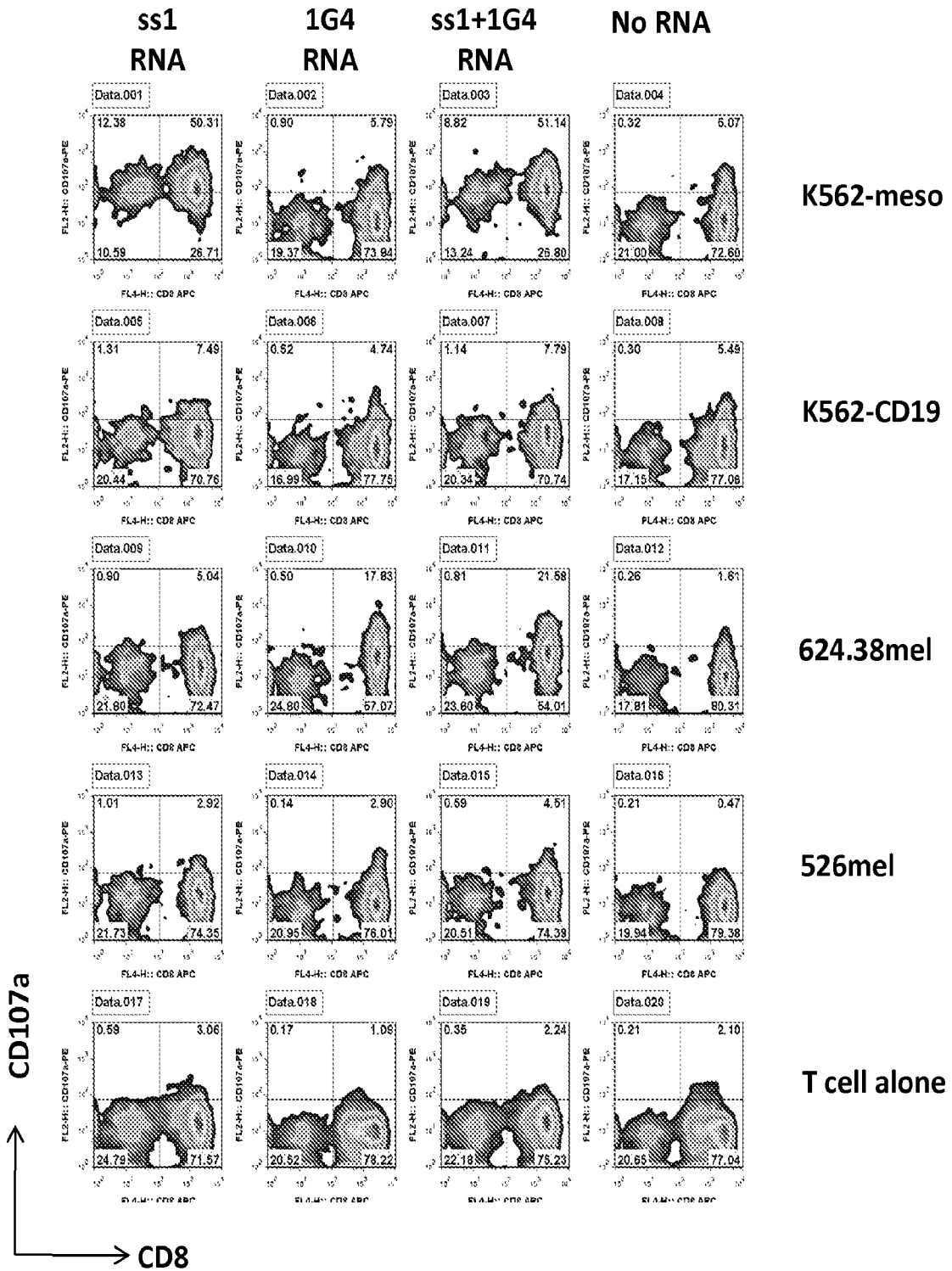


Figure 3A



Donor: DN350
EP data: 08/12/2010
FACS data: 08/13/2010

Figure 3B

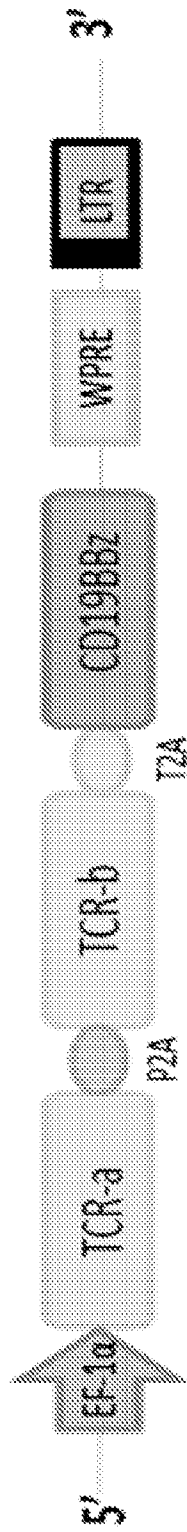


Figure 4

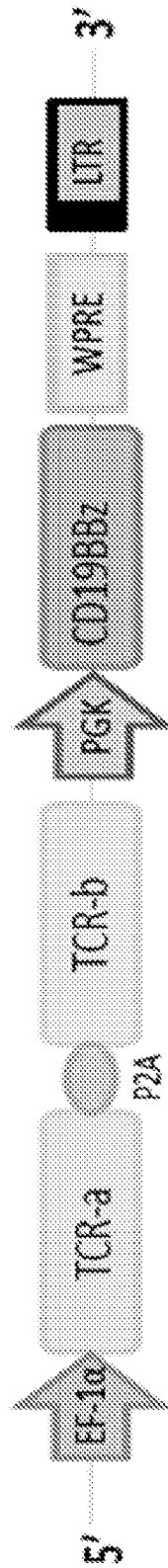


Figure 5

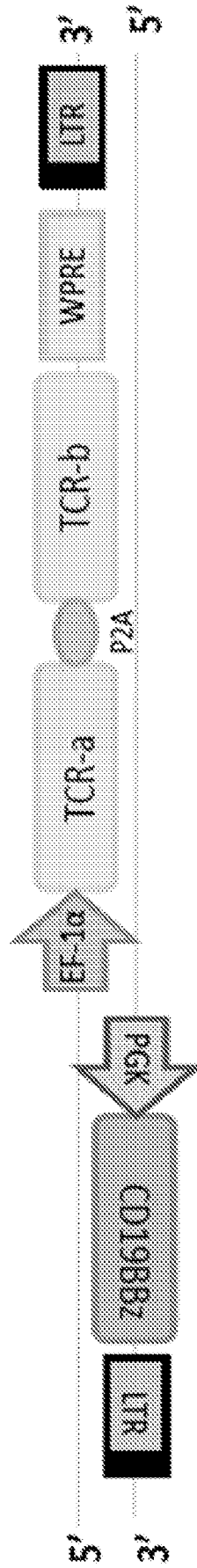


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/027357

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/705 (2013.01)

USPC - 435/7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 38/00, 48/00; A61P 35/00; C07K 14/55, 14/73, 14/705, 14/725 (2013.01)

USPC - 435/7.1, 7.2, 69.7, 172.3, 252.3, 320.1, 456; 530/350; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - A61K 38/00; C07K 14/55, 14/705, 14/7051, 14/70507, 14/70514, 14/70521, 14/70528, 2319/00 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Patbase, PubMed, Google Patents

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/059836 A2 (SENTMAN) 19 May 2011 (19.05.2011) entire document	1-3, 6-43
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Y		4, 5
Y	US 2010/0135974 A1 (ESHHAR et al) 03 June 2010 (03.06.2010) entire document	4, 5
A	US 2011/0223129 A1 (JENSEN) 15 September 2011 (15.09.2011) entire document	1-43
A	US 2005/0009180 A1 (YANG et al) 13 January 2005 (13.01.2005) entire document	1-43

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 June 2013

Date of mailing of the international search report

03 JUL 2013

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