Figure 1A. The construction of CAR4-1BBZ on transduced T cells

(Continued on nextpage)

(55) Abstract: Cytotoxic lymphocytes expressing chimeric antigen receptors (CAR) that target and bind small conjugate molecules (SCM) are disclosed, as well as methods of using the cells and the SCMs in the treatment of cancer.
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CHIMERIC ANTIGEN RECEPTOR-EXPRESSING T CELLS AS ANTI-CANCER THERAPEUTICS

BACKGROUND OF INVENTION

[0001] Immunotherapy based on adoptive transfer of lymphocytes (e.g., T cells) into a patient can play an important role in eliminating cancer. Among many different types of immunotherapeutic agents, one of the most promising therapeutic methods being developed is T cells expressing Chimeric Antigen Receptors (CAR). CARs are genetically engineered receptors that are designed to target a specific antigen of a selected tumor [1]. For example, T cells that have cytotoxic activity are transected with and grown to express CARs such that T cells expressing CARs can target and kill tumors via tumor-associated antigens.

[0002] First generation CARs are composed of two main regions. First, a recognition region, e.g., a single chain fragment variable (scFv) region derived from a tumor-targeted antibody, is used to recognize and bind tumor-associated antigens. Second, an activation signaling domain, e.g., the CD3ζ chain of T cells, serves as a T cell activation signal in CARs [2]. Although T cells transduced to express such constructs showed positive results in vitro, they have been found to have limited performance in eliminating tumor cells in clinical trials. The main limitation has been the relative inability to prolong and expand the T cells population and achieve sustained antitumor effects in vivo.

[0003] To address these problems, a co-stimulation domain (e.g. CD137, CD28 or CD134) is included in second generation CARs to achieve full, prolonged activation of T cells. Addition of a co-stimulation domain enhances the in vivo proliferation and survival of T cells containing CARs, and initial clinical data have shown that such constructs are a promising therapeutic agent in the treatment of tumors [3].

[0004] Although use of CAR-expressing T cells as an immunotherapeutic agent shows promise, there remain several challenges to overcome in order to achieve significant clinical outcomes. First, Off-target' toxicities may result due to the fact that it is difficult to target only cancer cells via tumor-associated antigens because in many cases normal cells also express the tumor-associated antigen. For example, CD19 is a tumor-associated antigen that is expressed on malignant B cells. CARs containing anti-CD19 antibody were generated and used treated to patients. Although remission of malignant B cells was found, normal B cells were depleted in the
patients as well because normal B cells also express CD19 [4]. Another example pertains to carbonic anhydrase IX (CAIX) which is overexpressed in clear cell renal carcinoma. Liver toxicity was found in subjects of the first clinical trial using CAR-targeting CAIX, likely due to the fact that CAIX is also expressed in bile duct epithelial cells and as such, the T cells targeted normal tissue as well [5].

[0005] Second, 'unregulated CAR activity' may be found where the rapid eradication of cancer cells by CARs induces a constellation of metabolic disturbances, called tumor lysis syndrome or a cytokine storm, which can be a fatal consequence to patients [1, 4, 6]. This is a result because transduced T cells expressing CARs cannot be easily regulated. Once transduced T cells are infused to patients, it is currently very difficult to regulate or control the activation of the cells.

[0006] Therefore, while CAR-expressing T cells show great promise as a tool in the treatment of cancer, the next generation of the CAR system is needed that provides reduced off-target toxicity and greater control of activation. The present invention is directed to this and other important ends.

BRIEF SUMMARY OF INVENTION

[0007] The invention relates to a Chimeric Antigen Receptor (CAR) system and methods for using the system in the treatment of subjects with cancer. The CAR system of the present invention includes cytotoxic lymphocytes expressing CARs that target a moiety that is not produced or expressed by cells of the subject being treated. This CAR system thus allows for focused targeting of the cytotoxic lymphocytes to target cells, such as cancer cells. The targeted moiety is part of a small conjugate molecule (SCM) that also comprises a ligand of a tumor cell receptor. Administration of a SCM along with the CAR-expressing cytotoxic lymphocytes results in the targeting of the cytotoxic lymphocyte response to only those cells expressing the tumor receptor to which the SCM is bound.

[0008] In a first embodiment, the invention is directed to CAR-expressing cytotoxic lymphocytes. The CAR is a fusion protein comprising a recognition region, at least one co-stimulation domain, and an activation signaling domain. The CAR has binding specificity for a selected targeted moiety or can be bound by a targeted moiety.
In certain aspects of this embodiment, the recognition region of the CAR is a single chain fragment variable (scFv) region of an antibody with binding specificity for the targeted moiety. In a particular aspect, the recognition region of the CAR is a single chain fragment variable (scFv) region of an anti-FITC antibody.

In certain aspects of this embodiment, the co-stimulation domain of the CAR is selected from the group consisting of CD28, CD137 (4-1BB), CD134 (OX40), and CD278 (ICOS).

In certain aspects of this embodiment, the activation signaling domain of the CAR is the T cell CD3ζ chain or Fc receptor γ.

In certain aspects of this embodiment, the cytotoxic lymphocytes are one or more of cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells.

In a particular aspect of this embodiment, the recognition region is a single chain fragment variable (scFv) region of an anti-FITC antibody, the co-stimulation domain is CD137 (4-1BB), and the activation signaling domain is the T cell CD3ζ chain.

In certain aspects of this embodiment, the targeted moiety is a molecule selected from the group consisting of 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin.

In certain aspects of this embodiment, the binding specificity of the CAR for the targeted moiety has an affinity of at least about 100 pM.

In a second embodiment, the invention is directed to small conjugate molecules (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand, wherein the tumor receptor ligand is folate, 2-[3-(1,3-dicarboxypropyl)ureido] pentanedioic acid (DUPA), or cholecystokinin 2 receptor (CCK2R) ligand.

Targeted moieties that may be used in the SCMs of the invention include, but are not limited to, 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin.

In certain aspects of this embodiment, the targeted moiety and the ligand are conjugated via a linker domain. Linker domains include, but are not limited to, polyethylene
glycol (PEG), polyproline, a hydrophilic amino acid, a sugar, an unnatural peptideoglycan, polyvinylpyrrolidone, and pluronic F-127. In a particular aspect, the linker domain is (PEG)$_{12}$.

[0019] In certain aspects of this embodiment, the targeted moiety is FITC.

[0020] In certain aspects of this embodiment, the targeted moiety is FITC and the linker is (PEG)$_{12}$.

[0021] In particular aspects of this embodiment, the SCM is FITC-folate, FITC-DUPA, FITC-CCK2R ligand, FITC-(PEG)$_{12}$-folate, FITC-(PEG)$_{12}$-DUPA, or FITC-(PEG)$_{12}$-CCK2R ligand.

[0022] In a third embodiment, the invention is directed to a two component cancer therapeutic comprising:

(a) a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand, wherein the tumor receptor ligand is folate, DUPA, or CCK2R ligand; and

(b) chimeric antigen receptor (CAR)-expressing cytotoxic lymphocytes, wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain, and wherein the CAR has binding specificity for the targeted moiety.

[0023] Targeted moieties that may be used in the SCMs include, but are not limited to, 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin.

[0024] In certain aspects of this embodiment, the targeted moiety and the ligand are conjugated via a linker domain. Linker domains include, but are not limited to, polyethylene glycol (PEG), polyproline, a hydrophilic amino acid, a sugar, an unnatural peptideoglycan, polyvinylpyrrolidone, and pluronic F-127. In a particular aspect, the linker domain is (PEG)$_{12}$.

[0025] In certain aspects of this embodiment, the targeted moiety is FITC.

[0026] In certain aspects of this embodiment, the targeted moiety is FITC and the linker is (PEG)$_{12}$.

[0027] In particular aspects of this embodiment, the SCM is FITC-folate, FITC-DUPA, FITC-CCK2R ligand, FITC-(PEG)$_{12}$-folate, FITC-(PEG)$_{12}$-DUPA, or FITC-(PEG)$_{12}$-CCK2R ligand.
In certain aspects of this embodiment, the recognition region of the CAR is a single chain fragment variable (scFv) region of an antibody with binding specificity for the targeted moiety. In a particular aspect, the recognition region of the CAR is a single chain fragment variable (scFv) region of an anti-FITC antibody.

In certain aspects of this embodiment, the co-stimulation domain of the CAR is CD28, CD137 (4-1BB), CD134 (OX40), or CD278 (ICOS).

In certain aspects of this embodiment, the activation signaling domain of the CAR is the T cell CD3ζ chain or Fc receptor γ.

In certain aspects of this embodiment, the cytotoxic lymphocytes are one or more of cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells.

In a particular aspect of this embodiment, the recognition region is a single chain fragment variable (scFv) region of an anti-FITC antibody, the co-stimulation domain is CD137 (4-1BB), and the activation signaling domain is the T cell CD3ζ chain.

In certain aspects of this embodiment, the binding specificity of the CAR for the targeted moiety is an affinity of at least about 100 pM.

In a fourth embodiment, the invention is directed to a method of treating cancer in a subject. In a first aspect the method comprises:

(a) culturing a population of cytotoxic lymphocytes under conditions promoting activation;

(b) transfecting the lymphocyte population of (a) with a vector encoding a chimeric antigen receptor (CAR), wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain;

(c) administering a therapeutically effective number of the transfected lymphocytes of (b) to a subject having cancer; and

(d) administering a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand to the subject, wherein the ligand is recognized and bound by a receptor on the surface of a cell of the cancer, and wherein the CAR has binding specificity for the targeted moiety or can be bound by the targeted moiety; thereby treating cancer in a subject.

In a related embodiment the method comprises:
(a) culturing a population of cytotoxic lymphocytes under conditions promoting activation;
(b) transfecting the lymphocyte population of (a) with a vector encoding a chimeric antigen receptor (CAR), wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain;
(c) administering a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand to a subject having cancer, wherein the ligand is recognized and bound by a receptor on the surface of a cell of the cancer; and
(d) administering a therapeutically effective number of the transfected T cells of (b) to the subject, and wherein the CAR has binding specificity for the targeted moiety or can be bound by the targeted moiety;
thereby treating cancer in a subject.

[0036] In a further related embodiment the method comprises:
(a) culturing a population of cytotoxic lymphocytes under conditions promoting activation;
(b) transfecting the lymphocytes population of (a) with a vector encoding a chimeric antigen receptor (CAR), wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain, and wherein the CAR has binding specificity for a targeted moiety or can be bound by the targeted moiety;
(c) incubating the lymphocytes of (b) with a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand;
(d) administering a therapeutically effective number of the transfected lymphocytes of (c) to a subject having cancer;
thereby treating cancer in a subject.

[0037] In these three related embodiments the cytotoxic lymphocytes may be autologous or heterologous cells, with respect to the subject being treated, or a combination of both.

[0038] In these three related embodiments the culturing conditions of (a) may comprise culturing the population of lymphocytes in the presence of anti-CD3 antibodies or anti-CD28 antibodies, or both.

[0039] In certain aspects of these three related embodiments, the recognition region of the CAR is a single chain fragment variable (scFv) region of an antibody with binding specificity for
the targeted moiety. In a particular aspect, the recognition region of the CAR is a single chain fragment variable (scFv) region of an anti-FITC antibody.

[0040] In certain aspects of these three related embodiments, the co-stimulation domain of the CAR is CD28, CD137 (4-1BB), CD134 (OX40), or CD278 (ICOS).

[0041] In certain aspects of these three related embodiments, the activation signaling domain of the CAR is the T cell CD3ζ chain or Fc receptor γ.

[0042] In certain aspects of these three related embodiments, the cytotoxic lymphocytes are one or more of cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells.

[0043] In certain aspects of these three related embodiments, the recognition region is a single chain fragment variable (scFv) region of an anti-FITC antibody, the co-stimulation domain is CD137 (4-1BB), and the activation signaling domain is the T cell CD3ζ chain.

[0044] In certain aspects of these three related embodiments, the targeted moiety is a molecule selected from the group consisting of 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin. In a particular aspect, the targeted moiety is FITC.

[0045] In certain aspects of these three related embodiments, the ligand is folate, DUPA, CCK2R ligand.

[0046] In certain aspects of these three related embodiments, the targeted moiety and the ligand are conjugated via a linker domain. The linker domain may be, for example, polyethylene glycol (PEG), polyproline, a hydrophilic amino acid, a sugar, an unnatural peptidoglycan, polyvinylpyrrolidone, or pluronic F-127.

[0047] In certain aspects of these three related embodiments, the targeted moiety is FITC and the linker is (PEG)_{12}.

[0048] In certain aspects of these three related embodiments, the vector is a lentivirus vector.

[0049] In certain aspects of these three related embodiments, the binding specificity of the CAR for the targeted moiety is an affinity of at least about 100 pM.

[0050] In certain aspects of these three related embodiments, the subject is a human.

[0051] In certain embodiments of these three related embodiments, the cancer is one or more of a cancer of the brain, thyroid, lung, pancreas, kidney, stomach, gastrointestinal stroma,
endometrium, breast, cervix, ovary, colon, prostate, leukemias, lymphomas, other blood-related cancers or head and neck cancer.

[0052] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described herein, which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that any conception and specific embodiment disclosed herein may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that any description, figure, example, etc. is provided for the purpose of illustration and description only and is by no means intended to define the limits the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0053] Figure 1A is a schematic showing the CAR4-1BBZ construct.

[0054] Figure 1B shows the transduction efficiency into T cells of the CAR4-1BBZ construct. 96h after transduction, the expression of CAR4-1BBZ was identified through copGFP expression by flow cytometry. As shown in the figure, approximately 30% of transduced T cells expressed CAR4-1BBZ.

[0055] Figure 1C shows the expression of CAR4-1BBZ on transduced T cells using confocal microscopy. copGFP was expressed on positive transduced T cells containing CAR4-1BBZ (arrows). However, copGFP expression was not detected on non-transduced T cells that do not express CAR4-1BBZ.

[0056] Figure 2 shows confocal microscopy of copGFP expression in transduced T cells containing CAR 4-1BBZ (top row) and FITC-folate binding on CAR-1BBZ transduced T cells (middle row). The bottom row shows the same view in the absence of fluorescence.
Figure 3 shows the binding ability of FITC-Folate conjugates to cancer cells via fluorescence microscopy. Figure 3A shows the binding of FITC-Folate conjugates to KB cancer cells. Figure 3B shows the binding of FITC-Folate conjugates to L1210A cancer cells. Figure 3C shows the binding of FITC-(PEG)2-Folate conjugates to KB cancer cells. Figure 3D shows the binding of FITC-(PEG)2-Folate conjugates to L1210A cancer cells. The competitor was 50X excess folate acid.

Figure 4 shows the results of assays to determine whether CAR-expressing T cells are cytotoxic to cancer cells in the presence or absence of FITC-Folate conjugates using KB cells (Figure 4A) or L1210A cells (Figure 4B).

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found, for example, in Benjamin Lewin, Genes VII, published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew et al. (eds.); The Encyclopedia of Molecular Biology, published by Blackwell Publishers, 1994 (ISBN 0632021829); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar technical references.

As used herein, "a" or "an" may mean one or more. As used herein when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more. Furthermore, unless otherwise required by context, singular terms include pluralities and plural terms include the singular.

As used herein, "about" refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term "about" generally refers to a range of numerical values (e.g., +/- 5-10% of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). In some instances, the term "about" may include numerical values that are rounded to the nearest significant figure.
As used herein, "treat" and all its forms and tenses (including, for example, treat, treating, treated, and treatment) refer to both therapeutic treatment and prophylactic or preventative treatment.

II. The Present Invention

The present invention is directed to a CAR system for use in the treatment of subjects with cancer. The CAR system of the present invention (e.g., cytotoxic lymphocytes expressing novel CARs and cognate small conjugate molecules (SCM)) makes use of CARs that target a moiety that is not produced or expressed by cells of the subject being treated. This CAR system thus allows for focused targeting of the cytotoxic lymphocytes to target cells, such as cancer cells. The targeted moiety is part of a small conjugate molecule (SCM) that also comprises a ligand of a tumor cell receptor. Because small organic molecules are typically used as the targeted moiety, clearance of the SCM from the bloodstream can be achieved within about 20 minutes. By administration of a SCM along with the CAR-expressing cytotoxic lymphocytes, the lymphocyte response can be targeted to only those cells expressing the tumor receptor, thereby reducing off-target toxicity, and the activation of lymphocytes can be more easily controlled due to the rapid clearance of the SCM. As an added advantage, the CAR-expressing lymphocytes can be used as a "universal" cytotoxic cell to target a wide variety of tumors without the need to prepare separate CAR constructs. The targeted moiety recognized by the CAR may also remain constant. It is only the ligand portion of the SCM that needs to be altered to allow the system to target cancer cells of different identity.

One embodiment of the invention provides an illustration of this novel CAR system. In this embodiment, and as a first component, a SCM is prepared that comprises FITC linked to a ligand of a selected tumor cell receptor. As a second component, cytotoxic T cells are transduced to express a CAR that comprises anti-FITC scFv. This CAR thus targets Fluorescein Isothiocyanate (FITC) instead of a tumor-associated antigen that might also be expressed by healthy, non-target cells. The two components are administered to a subject having cancer and the FITC-SCM (first component) is bound by the target tumor cells (through binding of the ligand portion of the molecule to cognate tumor cell receptor). The FITC portion of the SCM is then recognized and bound by the anti-FITC CAR expressed by the T cells (second component). Upon FITC binding, the anti-FITC CAR-expressing T cells are activated and the tumor cell is
killed. As will be apparent to the skilled artisan, the cytotoxic T cells cannot kill cells with first
binding to a tumor cell. As it will be further apparent, T cells will not bind to non-target cells
because the recognition region of the CAR will only recognize and bind FITC, which is not
produce or expressed by cells of the subject. The SCM thus acts as a bridge between the
cytotoxic T cells and the target tumor cells. As long as the targeted moiety of the SCM is a
moiety not found in the host, the activity of the T cells can be limited to the target cells. Further,
the activation of the CAR-expressing T cells can be regulated by limiting the amount of SCM
administered to a subject, for example, by manipulating infusion of the small conjugate molecule
if a side effect is detected. Thus, the CAR system of the present invention overcomes problems
associated with conventional CAR therapy.

Small Conjugate Molecules (SCM)

[0065] The CAR system of the present invention utilizes small conjugate molecules (SCMs)
as the bridge between cytotoxic lymphocytes and targeted cancer cells. The SCMs are conjugates
comprising a targeted moiety on one end of the molecule and a tumor receptor ligand on the
other, optionally connected by a bridge domain. The targeted moiety is a molecule that is
recognized by a CAR of a transduced lymphocyte or that can bind to a region of the CAR. The
identity of the targeted moiety is limited only in that it must be a molecule that can be recognized
and bound by CAR expressed by a lymphocyte, or recognized and bind the CAR itself, in both
cases preferably with specificity, and that it have a relatively low molecular weight. Exemplary
targeted moieties are haptons that can be recognized and bound by CARs and include small
molecular weight organic molecules such as DNP (2,4-dinitrophenol), TNP (2,4,6-
trinitrophenol), biotin, and digoxigenin, along with fluorescein and derivatives thereof, including
FITC (fluorescein isothiocyanate), NHS-fluorescein, and pentafluorophenyl ester (PFP) and
tetrafluorophenyl ester (TFP) derivatives. Suitable targeted moieties that themselves bind to one
or more regions of a CAR include knottins [16], centyrins and DARPins [7].

[0066] The tumor receptor ligands that comprise the SCMs of the present invention are
molecules recognized and bound by receptors expressed by target tumor cells, typically
expressed on the surface of the tumor cells. Suitable ligands include: 1) DUPA (DUPA-
(99m)Tc), a ligand bound by PSMA-positive human prostate cancer cells with nanomolar
affinity (K_d = 14 nM; [8]); 2) CCK2R ligand, a ligand bound by CCK2R-positive cancer cells
(e.g., cancers of the thyroid, lung, pancreas, ovary, brain, stomach, gastrointestinal stroma, and colon; [9]); 3) folate, a ligand bound by the folate receptor on cells of cancers that include cancers of the ovary, cervix, endometrium, lung, kidney, brain, breast, colon, and head and neck cancers [10].

The targeted moiety and the ligand can be directly conjugated through such means as reaction between the isothiocyanate group of FITC and free amine group of small ligands (e.g. folate, DUPA and CCK2R ligand). However, the use of a linking domain to connect the two molecules can be helpful as it can provide flexibility and stability to the SMC depending on the identity of the components comprising the SMC. Examples of suitable linking domains include: 1) polyethylene glycol (PEG); 2) polyproline; 3) hydrophilic amino acids; 4) sugars; 5) unnatural peptideo glycans; 6) polyvinylpyrrolidone; 7) pluronic F-127. Linkers lengths that are suitable include, but are not limited to, linkers having 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40, or more atoms.

While the affinity at which the ligands and cancer cell receptors bind can vary, and in some cases low affinity binding may be preferable (such as about 1 µM), the binding affinity of the ligands and cancer cell receptors will generally be at least about 100 µM, 1 nM, 10 nM, or 100 nM, preferably at least about 1 pM or 10 pM, even more preferably at least about 100 pM.

The skilled artisan will understand and recognize that various means can be used to prepare SMCs comprised of a targeted moiety, a linking domain, and a ligand. Examples are provided in the Examples included herein.

Prior to being administered to a subject, the SCMs are prepared in a pharmaceutically acceptable formulation. Such formulations may contain a pharmaceutically acceptable carrier or diluent.

Exemplary SCMs included within the scope of the invention include the following molecules.
FITC-(PEG)$_2$-DUPA
FITC-CCK2R ligand
- (PEG)$_2$-CCK2R ligand
Chimeric Antigen Receptors (CARs)

[0078] The CAR system of the present invention also utilizes cytotoxic lymphocytes engineered to express chimeric antigen receptors (CARs) that recognize and bind the targeting moiety of the SCMs. The CARs used in the CAR system comprise three domains. The first domain is the recognition region which, as the name suggests, recognizes and binds the targeting moiety. The second domain is the co-stimulation domain which enhances the proliferation and survival of the lymphocytes. The third domain is the activation signaling domain which is a cytotoxic lymphocyte activation signal. The three domains, together in the form of a fusion protein, comprise the CARs of the present invention.

[0079] As suggested above, the recognition region is the portion of a CAR that recognizes and binds a targeting moiety. The recognition regions comprising the CARs of the present invention are single chain fragment variable (scFv) regions of antibodies that bind the targeted moiety. Preferably, the scFv regions bind the targeted moiety with specificity. The identity of the antibody used in the production of the recognition region is limited only in that it binds the targeted moiety of the SCM. Thus, as non-limiting examples, scFv regions of antibodies that bind one of the following targeted moieties are included within the scope of the invention: DUPA, CCK2R ligand, folate. The scFv regions can be prepared from (i) antibodies known in the art that bind a targeted moiety, (ii) antibodies newly prepared using a selected targeted moiety as a hapten, and (iii) sequence variants derived from the scFv regions of such antibodies, e.g., scFv regions having at least about 80% sequence identity to the amino acid sequence of the scFv region from which they are derived. The use of unaltered (i.e., full size) antibodies, such as IgG, IgM, IgA, IgD or IgE, in the CAR or as the CAR is excluded from the scope of the invention.

[0080] The co-stimulation domain serves to enhance the proliferation and survival of the cytotoxic lymphocytes upon binding of the CAR to a targeted moiety. The identity of the co-stimulation domain is limited only in that it has the ability to enhance cellular proliferation and survival activation upon binding of the targeted moiety by the CAR. Suitable co-stimulation domains include: 1) CD28 [11]; 2) CD137 (4-1BB), a member of the tumor necrosis factor (TNF) receptor family [12]; 3) CD134 (OX40), a member of the TNFR-superfamily of receptors [13]; 4) CD278 (ICOS), a CD28-superfamily co-stimulatory molecule expressed on activated T cells [14]. The skilled artisan will understand that sequence variants of these noted co-
stimulation domains can be used without adversely impacting the invention, where the variants have the same or similar activity as the domain on which they are modeled. Such variants will have at least about 80% sequence identity to the amino acid sequence of the domain from which they are derived.

[0081] In some embodiments of the invention, the CAR constructs comprise two co-stimulation domains. While the particular combinations include all possible variations of the four noted domains, specific examples include: 1) CD28+CD137 (4-1BB) and 2) CD28+CD134 (OX40).

[0082] The activation signaling domain serves to activate cytotoxic lymphocytes upon binding of the CAR to a targeted moiety. The identity of the activation signaling domain is limited only in that it has the ability to induce activation of the selected cytotoxic lymphocyte upon binding of the targeted moiety by the CAR. Suitable activation signaling domains include the T cell CD3ζ chain and Fc receptor γ. The skilled artisan will understand that sequence variants of these noted activation signaling domains can be used without adversely impacting the invention, where the variants have the same or similar activity as the domain on which they are modeled. Such variants will have at least about 80% sequence identity to the amino acid sequence of the domain from which they are derived.

[0083] Constructs encoding the CARs of the invention are prepared through genetic engineering. As an example, a plasmid or viral expression vector can be prepared that encodes a fusion protein comprising a recognition region, one or more co-stimulation domains, and an activation signaling domain, in frame and linked in a 5’ to 3’ direction. However, the CARs of the present invention are not limited in this arrangement and other arrangements are acceptable and include: (i) a recognition region, an activation signaling domain, and one or more co-stimulation domains, and (ii) a recognition region, a co-stimulation domain, and an activation signaling domain, linked in a 5’ to 3’ direction. It will be understood that because the recognition region must be free to bind the targeted moiety, the placement of the recognition region in the fusion protein will generally be such that display of the region on the exterior of the cell is achieved. In the same manner, because the co-stimulation and activation signaling domains serve to induce activity and proliferation of the cytotoxic lymphocytes, the constructs will generally encode a fusion protein that displays these two domains in the interior of the cell.
The CARs may include additional elements, such as a signal peptide to ensure proper export of the fusion protein to the cells surface, a transmembrane domain to ensure the fusion protein is maintained as an integral membrane protein, and a hinge domain that imparts flexibility to the recognition region and allows strong binding to the targeted moiety.

An example of an exemplary CAR of the present invention is shown in Figure 1A where the fusion protein is encoded by a lentivirus expression vector and where "SP" is a signal peptide, the CAR is an anti-FITC CAR, a CD8a hinge is present, a transmembrane domain is present ("TM"), the co-stimulation domain is 4-IBB, and the activation signaling domain is CD3ζ. The sequence of the CAR-encoding vector is provided as SEQ ID NO:1.

In addition to the use of plasmid and viral vectors, cytotoxic lymphocytes can be engineered to express CARs of the invention through retrovirus, lentivirus (viral mediated CAR gene delivery system), sleeping beauty, and piggyback (transposon/transposase systems that include a non-viral mediated CAR gene delivery system).

While the affinity at which the CARs, expressed by the cytotoxic lymphocytes, bind to the targeted moiety can vary, and in some cases low affinity binding may be preferable (such as about 50 nM), the binding affinity of the CARs to the targeted ligand will generally be at least about 100 nM, 1 pM, or 10 pM, preferably at least about 100 pM, 1 fM or 10 fM, even more preferably at least about 100 fM.

**CAR-expressing Cytotoxic Lymphocytes**

The cells used in the CAR system of the present invention are cytotoxic lymphocytes selected from (i) cytotoxic T cells (also variously known as cytotoxic T lymphocytes, CTLs, T killer cells, cytolytic T cells, CD8+ T cells, and killer T cells), natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. Upon activation, each of these cytotoxic lymphocytes triggers the destruction of target tumor cells. For example, cytotoxic T cells trigger the destruction of target tumor cells by either or both of the following means. First, upon activation T cells release cytotoxins such as perforin, granzymes, and granulysin. Perforin and granulysin create pores in the target cell, and granzymes enter the cell and trigger a caspase cascade in the cytoplasm that induces apoptosis (programmed cell death) of the cell. Second, apoptosis can be induced via Fas-Fas ligand interaction between the T cells and target tumor cells.
The cytotoxic lymphocytes will preferably be autologous cells, although heterologous cells can also be used, such as when the subject being treated using the CAR system of the invention has received high-dose chemotherapy or radiation treatment to destroy the subject’s immune system. Under such circumstances, allogenic cells can be used.

The cytotoxic lymphocytes can be isolated from peripheral blood using techniques well known in the art, include Ficoll density gradient centrifugation followed by negative selection to remove undesired cells.

Cytotoxic lymphocytes can be engineered to express CAR constructs by transfecting a population of lymphocytes with an expression vector encoding the CAR construct. Appropriates means for preparing a transduced population of lymphocytes expressing a selected CAR construct will be well known to the skilled artisan, and includes retrovirus, lentivirus (viral mediated CAR gene delivery system), sleeping beauty, and piggyback (transposon/transposase systems that include a non-viral mediated CAR gene delivery system), to name a few examples.

Transduced cytotoxic lymphocytes are grown in conditions that are suitable for a population of cells that will be introduced into a subject such as a human. Specific considerations include the use of culture media that lacks any animal products, such as bovine serum. Other considerations include sterilized-condition to avoid contamination of bacteria, fungi and mycoplasma.

Prior to being administered to a subject, the cells are pelleted, washed, and resuspended in a pharmaceutically acceptable carrier or diluent. Exemplary formulations comprising CAR-expressing cytotoxic lymphocytes include formulations comprising the cells in sterile 290 mOsm saline, infusible cryomedia (containing Plasma-Lyte A, dextrose, sodium chloride injection, human serum albumin and DMSO), 0.9% NaCl with 2% human serum albumin or any other sterile 290 mOsm infusible materials.

Methods of Treatment

The CAR system of the present invention can be used in the treatment of a subject having cancer. The methods of treatment encompassed by the invention generally includes the steps of (i) obtaining a population of autologous or heterologous cytotoxic lymphocytes, (ii) culturing the lymphocytes under conditions that promote the activation of the cells, (iii) transfecting the lymphocytes with an expression vector encoding a CAR, (iv) administering a
formulation comprising the transfected lymphocytes to a subject having cancer, and (v) administering a formulation comprising SCM to the subject.

[0095] The invention also includes variations on this theme such, as administering the formulation comprising SMC to the subject before the formulation comprising the transfected lymphocytes, or at the same time as the formulation comprising the transfected lymphocytes. A further variation includes culturing the formulation comprising the transfected lymphocytes with the SCM prior to administration to the subject.

[0096] The population of cytotoxic lymphocytes can be obtained from a subject by means well known in the art. For example, cytotoxic T cells can be obtained by collecting peripheral blood from the subject, subjecting the blood to Ficoll density gradient centrifugation, and then using a negative T cell isolation kit (such as EasySep™ T Cell Isolation Kit) to isolate a population of cytotoxic T cells from the blood. While the population of cytotoxic lymphocytes need not be pure and may contain other blood cells such as T cells, monocytes, macrophages, natural killer cells and B cells, depending on the population being collected, preferably the population comprises at least about 90% of the selected cell type. In particular aspects, the population comprises at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 100% of the selected cell type. As indicated earlier, the population of cells may come from the subject to be treated, from one or more different subjects, or the population may be a combination of cells from the subject to be treated and one or more different subjects.

[0097] After the population of cytotoxic lymphocytes is obtained, the cells are cultured under conditions that promote the activation of the cells. The culture conditions will be such that the cells can be administered to a subject without concern for reactivity against components of the culture. For example, when the population will be administered to a human, the culture conditions will not include bovine serum products, such as bovine serum albumin. The activation of the lymphocytes in the culture can be achieved by introducing known activators into the culture, such as anti-CD3 antibodies in the case of cytotoxic T cells. Other suitable activators include anti-CD28 antibodies. The population of lymphocytes will generally be cultured under conditions promoting activation for about 1 to 4 days. The appropriate level of cellular activation can be determined by cell size, proliferation rate or activation markers by flow cytometry.

[0098] After the population of cytotoxic lymphocytes has been cultured under conditions promoting activation, the cells are transfected with an expression vector encoding a CAR. Such
vectors are described above, along with suitable means of transfection. After transfection, the resulting population of cells can be immediately administered to a subject or the cells can be culture for at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or more days, or between about 5 and 12 days, between about 6 and 13 days, between about 7 and 14 days, or between about 8 and 15 days, for example, to allow time for the cells to recover from the transfection. Suitable culture conditions with be the same as those conditions under which the cells were culture while activation was being promoted, either with or without the agent that was used to promote activation and expansion.

[0099] When the transfected cells are ready a formulation comprising the cells is prepared and administered to a subject having cancer. Prior to administration, the population of cells can be washed and resuspended in a pharmaceutically acceptable carrier or diluent to form the formulation. Such carriers and diluents include, but are not limited to, sterile 290 mOsm saline, infusible cryomedia (containing Plasma-Lyte A, dextrose, sodium chloride injection, human serum albumin and DMSO), 0.9%NaCl with 2% human serum albumin or any other sterile 290 mOsm infusible materials. Alternatively, depending on the identity of the culture media used in the previous step, the cells can be administered in the culture media as the formulation, or concentrated and resuspended in the culture media before administration. The formulation can be administered to the subject via suitable means, such as parenteral administration, e.g., intradermally, subcutaneously, intramuscularly, intraperitoneally, intravenously, or intrathecally.

[00100] The total number of cells and the concentration of cells in the formulation administered to a subject will vary depending on a number of factors including the type of cytotoxic lymphocytes being used, the binding specificity of the CAR, the identity of the targeted moiety and the ligand, the identity of the cancer or tumor to be treated, the location in the subject of the cancer or tumor, the means used to administer the formulations to the subject, and the health, age and weight of the subject being treated. However, suitable formulations comprising transduced lymphocytes include those having a volume of between about 5 ml and 200 ml, containing from about $1 \times 10^5$ to $1 \times 10^{15}$ transduced cells. Typical formulations comprise a volume of between about 10 ml and 125 ml, containing from about $1 \times 10^7$ to $1 \times 10^{10}$ transduced cells. An exemplary formulation comprises about $1 \times 10^9$ transduced cells in a volume of about 100 ml.
The final step in the method is the administration of a formulation comprising SCM to the subject. As described above, the SCM will be prepared in a formulation appropriate for the subject receiving the molecules. The concentration of SCM in a SCM formulation will vary depending on factors that include the binding specificity of the CAR, the identity of the targeted moiety and the ligand, the identity of the cancer or tumor to be treated, the location in the subject of the cancer or tumor, the means used to administer the formulations to the subject, and the health, age and weight of the subject being treated. However, suitable formulations comprising SCM include those having a volume of between about 1 ml and 50 ml and contain between about 20 µg/kg body weight and 3 mg/kg body weight SCM. Typical formulations comprise a volume of between about 5 ml and 20 ml and contain between about 0.2 mg/kg body weight and 0.4 mg/kg body weight SCM. An exemplary formulation comprises about 50 µg/kg body weight SCM in a volume of about 10 ml.

The timing between the administration of transduced lymphocyte formulation and the SCM formation may range widely depending on factors that include the type of cytotoxic lymphocytes being used, the binding specificity of the CAR, the identity of the targeted moiety and the ligand, the identity of the cancer or tumor to be treated, the location in the subject of the cancer or tumor, the means used to administer the formulations to the subject, and the health, age and weight of the subject being treated. Indeed, the SCM formation may be administered prior to, simultaneous with, or after the lymphocyte formulation. In general, the SCM formation will be administered after the lymphocyte formulation, such as within 3, 6, 9, 12, 15, 18, 21, or 24 hours, or within 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10 or more days. When the SCM formation is administered before the lymphocyte formulation, the lymphocyte formulation will generally be administered within about 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more hours. When the SCM formation and the lymphocyte formulation are added simultaneously, it is preferable that the formations are not combined and thus administered separately to the subject.

Depending on the cancer being treated the step of administering the lymphocyte formulation, or the step of administering the SCM formulation, or both, can be repeated one or more times. The particular number and order of the steps is not limited as the attending physician may find that a method can be practiced to the advantage of the subject using one or more of the following methodologies, or others not named here: (i) administering the lymphocyte formulation (A) followed by the SCM formulation (B), i.e., A then B; (ii) B then A; (iii) A then
B then A then B; (iv) A then B then A; (v) B then A then B; (vi) A then B then A; (vii) B
then A then A; (viii) B then B then A.

[00104] The formulations can be administered as single continuous doses, or they can be
divided and administered as a multiple-dose regimen depending on the reaction (i.e., side effects)
of the patient to the formulations.

[00105] The types of cancers which may be treated using the methods of the invention will be
governed based on the identity of the ligand used in the SCM. When the ligands defined above
are used (i.e., DUPA, CCK2R ligand, folate) cancers that may be treated using the CAR system
and methods of the present invention generally include solid tumors, and more specifically
include prostate cancer adenocarcinoma, hepatoma, colorectal liver metastasis, and cancers of
neuroendocrine origin.

[00106] In each of the embodiments and aspects of the invention, the subject is a human, a
non-human primate, bird, horse, cow, goat, sheep, a companion animal, such as a dog, cat or
rodent, or other mammal.

///.  Examples

1. Generation of CAR4-1BBZ and transduction of mouse T cells to express CAR

[00107] To generate modified T cells containing CAR that target cancer cells by FITC-ligand
small molecule conjugates, CAR constructs were designed and generated as shown in Figure 1.

A) Generation of chimeric antigen receptor (CAR) in lentiviral vector

[00108] An overlap PCR method was used to generate CAR constructs comprising scFv
against FITC (CAR4-1BBZ). scFv against FITC, 4M5.3 (Kd = 270 fM, 762bp) derived from
anti-fluorescence (4-4-20) antibody, was synthesized based on a previous report [15]. As shown
in Figure 1, sequence encoding the mouse CD8a signal peptide (SP, 81bp), the hinge and
transmembrane region (207bp), the cytoplasmic domain of 4-1BB (CD137, 144bp) and the CD3ζ
chain (339bp) were fused with the anti-FITC scFv by overlapping PCR. The resulting CAR
construct (CAR4-1BBZ) (1533 bp; SEQ ID NO:2) was inserted into Nhel/NotI cleaved lentiviral
expression vector pCDH-EFl-MCS-BGH-PGK-GFP-T2A-Puro (System Biosciences, Mountain
View, CA). CAR4-1BBZ expression is regulated by EF1α promoter in the lentiviral vector. The
sequence of CAR constructs in lentiviral vector (CAR4-1BBZ) was confirmed by DNA sequencing (Purdue Genomic Core Facility) and is provided in SEQ ID NO:1. copGFP expression encoded in the lentiviral vector was monitored to identify CAR4-1BBZ expression (Figure 1C).

B) Isolation and transduction of mouse T cells

T cells were isolated from mouse spleen or peripheral blood. To isolate T cells, mouse splenocytes and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation (GF. Healthcare Lifesciences). After washing away remaining Ficoll solution, T cells were isolated by EasySep™ Mouse T Cell Isolation Kit (STEM CELL technologies). Purified T cells are cultured in RPMI 1640 with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin sulfate, 10mM HEPES. To prepare lentiviral virus containing CAR4-1BBZ, 293TN packaging cell line was co-transfected with CAR4-1BBZ lentiviral vector and packaging plasmids. After 48 and 72 hours transfection, supernatants containing CAR4-1BBZ lentivirus were harvested and virus particles were concentrated for transduction. For transduction of mouse T cells, isolated T cells were activated with Dynabeads coupled with anti-CD3/CD28 antibodies (Life Technologies) for 12-24 hours in the presence of mouse IL-2 (50 units/ml), then infected with lentiviral expression vector containing CAR4-1BBZ. Mouse IL-2 (50 units/ml) was provided every other day. After 96 hours, cells were harvested and the expression of CAR on transduced T cells was identified by flow cytometry. As shown in Figure 1B, approximately 30% of transduced T cells expressed CAR4-1BBZ.

C) Flow cytometry

The expression of CAR4-1BBZ on transduced T cells was determined by flow cytometry. Since lentiviral expression backbone also encodes copGFP expression, CAR4-1BBZ transduced T cells were verified by copGFP expression. Figure 1C shows the expression of CAR4-1BBZ on transduced T cells. Transduced T cells were further confirmed by confocal microscope. copGFP was expressed on positive transduced T cells containing CAR4-1BBZ (arrows). However, copGFP expression was not detected on non-transduced T cells that do not express CAR4-1BBZ. Data was analyzed with FlowJo software.
Based on these results, it was evident that the CAR4-1BBZ constructs was successfully transduced to mouse T cells and that the transduced T cells express CAR4-1BBZ.

D) Cell culture

Two cancer cell lines were used in this study: L1210A and KB. L1210A is a murine leukemia cell line. KB is a human epidermoid carcinoma cell line. Both of them have higher folate receptor expression on cell surface.

L1210A and KB cells were cultured in folic acid-deficient RPMI medium, 10% of heat inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin sulfate were included in the culture media.

2. Generation of Small Conjugate Molecules Comprising FITC and Ligands

A) Synthesis of FITC-Folate

Folate-y-ethylenediamine was coupled to FITC isomer I (Sigma-Aldrich, St. Louis, MO) in anhydrous dimethylsulfoxide in the presence of tetramethylguanidine and diisopropylamine. The crude product was loaded onto an Xterra RP18 preparative HPLC column (Waters) and eluted with gradient conditions starting with 99% 5 mM sodium phosphate (mobile phase A, pH7.4) and 1% acetonitrile (mobile phase B) and reaching 90% A and 10% B in 10 min at a flow rate of 20 mL/min. Under these conditions, the folate-FITC main peak typically eluted at 27-50 min. The quality of folate-FITC fraction was monitored by analytical reverse-phase HPLC with a UV detector. Fractions with greater than 98.0% purity (LCMS) were lyophilized to obtain the final folate-FITC product.
**FITC-Folate**

![FITC-Folate structure](image)

B) Synthesis of FITC-(PEG)$_{12}$-Folate

[00115] Universal PEG Nova Tag™ resin (0.2 g) was loaded into a peptide synthesis vessel and washed with i-PrOH (3 x 10 mL), followed by DMF (3 x 10mL). Fmoc deprotection was carried out using 20% piperidine in DMF (3 x 10 mL). Kaiser tests were performed to assess reaction completion. To the vessel was then introduced a solution of Fmoc-Glu-(0-t-Bu)-OH (23.5 mg) in DMF, i-Pr$_2$NEt (4 equiv), and PyBOP (2 equiv). Fmoc deprotection was carried out using 20% piperidine in DMF (3 x 10 mL). To the vessel was then introduced a solution of N10-TFA-Pte-OH (22.5 mg), DMF, j-Pr$_2$NEt (4 equiv), and PyBOP (2 equiv). Argon was bubbled for 2h, and resin was washed with DMF (3 x 3 mL) and i-PrOH(3 x 3 mL). After swelling the resin in DCM, a solution of 1M HOBT in DCM/TFE (1:1) (2 x 3 mL) was added for removal of Mmt group. Argon was bubbled for lh, the solvent was removed and resin was washed with DMF (3 x 3 mL) and i-PrOH(3 x 3 mL). After swelling the resin in DMF, a solution of Fmoc-NH-(PEG)$_{12}$-COOH (46.3 mg) in DMF, j-Pr$_2$NEt (4 equiv), and PyBOP (2 equiv) was added. Argon was bubbled for 2h, and resin was washed with DMF (3 x 3 mL) and i-PrOH (3 x 3 mL). Fmoc deprotection was carried out using 20% piperidine in DMF (3 x 10 mL). Kaiser tests were performed to assess reaction completion. To the vessel was then introduced a solution of FITC (21.4 mg) in DMF, i-Pr$_2$NEt (4 equiv), then Argon was bubbled for 2h, and resin was washed with DMF (3 x 3 mL) and j-PrOH(3 x 3 mL). Then to the vessel was added 2% NH$_2$NH$_2$ in DMF (2 x 2 mL). Final compound was cleaved from resin using a TFA:H$_2$O:TIS (95:2.5:2.5) and concentrated under vacuum. The concentrated product was precipitated in Et$_2$O and dried under vacuum. The crude product was purified by using preparative RP-HPLC (mobile phase: A = 10
mM ammonium acetate pH = 7, B = ACN; method: 0% B to 30% B in 30 min at 13 mL/min).
The pure fractions were pooled and freeze-dried, furnishing the FITC-(PEG)$_{12}$-Folate.

**SCHEME 1. Synthesis of the Folate-(PEG)$_{12}$-FITC Spacer Unit:**

Reagents and conditions: (i) a) 20% piperidine, DMF; b) Fmoc-NH-Glu(OBu)-COOH, PyBOP, DIPEA, DMF; (ii) a) 20% piperidine, DMF;
b) N$^{3}$-(Trifluoroacetyl)pteroic acid, PyBOP, DIPEA, DMF; (iii) a) 1M HOBT in DCM:TEF (1:1); b) Fmoc-NH-PEG$_{12}$CH$_{2}$CH$_{2}$COOH, PyBOP, DIPEA, DMF;
(iv) a) 20% piperidine, DMF; b) FITC, DIPEA, DMF; (v) a) 2% NH$_{2}$NH$_{2}$, DMF; b) TFA, water, i-Pr$_{3}$SiH.

C) Synthesis of FITC-(PEG)$_{12}$-DUPA

[00116] Synthesis of DPA-(PEG)$_{12}$-EDA: 1,2-Diaminoethane trityl-resin (0.025 g) was loaded into a peptide synthesis vessel and washed with i-PrOH (3 x 10 mL), followed by DMF (3 x 10 mL). To the vessel was then introduced a solution of Fmoc-NH-(PEG)$_{12}$-COOH (42.8 mg) in DMF, j-Pr$_{2}$NEt (2.5 equiv), and PyBOP (2.5 equiv). The resulting solution was bubbled with Ar for 1 h, the coupling solution was drained, and the resin washed with DMF (3 x 10 mL) and i-PrOH (3 x 10 mL). Kaiser tests were performed to assess reaction completion. Fmoc deprotection was carried out using 20% piperidine in DMF (3 x 10 mL). This procedure was repeated to complete the all coupling steps (2 x 1.5 equiv of Fmoc-Phe-OH and 1.5 equiv of 8-aminooctanoic acid and 1.2 equiv of DUPA were used on each of their respective coupling steps). After the DUPA coupling, the resin was washed with DMF (3 x 10 mL) and i-PrOH (3 x 10 mL) and dried under reduced pressure. The peptide was cleaved from the resin in the peptide synthesis vessel using a cleavage mixture consisting of 95% CF$_{3}$C0$_{2}$H, 2.5% H$_{2}$O, and 2.5% triisopropylsilane. Fifteen milliliters of the cleavage mixture was added to the peptide synthesis.
vessel, and the reaction was bubbled under Ar for 15 min. The resin was treated with two
additional 10 mL quantities of the cleavage mixture for 5 min each. The cleavage mixture was
concentrated to ca. 5 mL, and ethyl ether was added to induce precipitation. The precipitate was
collected by centrifugation, washed with ethyl ether three times, and dried under high vacuum,
resulting in the recovery of DUPA-(PEG)$_{12}$-EDA as crude material.
SCHEME 1. Synthesis of the DUPA-(PEG)$_{12}$-EDA Spacer Unit

Reagents and conditions: (i) Fmoc-NH(PEG)$_2$-COOH, PyBop, DIPEA, DMF; (ii) 20% piperidine, DMF; (iii) Fmoc-Phe-OH, PyBop, DIPEA, DMF; (iv) Fmoc-8-amino octanoic acid, PyBop, DIPEA, DMF; (v) DUPA, PyBop, DIPEA, DMF; (vi) TFA, water, i-Pr$_3$SiH.
[00117]  *Synthesis of FITC-(PEG)$_{12}$-DUPA:* To a stirred solution of the crude DUPA-(PEG)$_{12}$-EDA (10 mg) and FITC (5.6 mg) in dimethylsulfoxide (DMSO, 1 mL) was added Pr$_2$NEt (5 equiv) at room temperature and stirring continued for 6 hr under argon. The reaction was monitored by LCMS and purified by preparative HPLC (mobile phase: A = 10 mM ammonium acetate pH = 7, B = ACN; method: 0% B to 50% B in 30 min at 13 mL/min). The pure fractions were pooled and freeze-dried, furnishing the FITC-(PEG)$_{12}$-DUPA.
SCHEME 2. Synthesis of the DUPA-[PEG] \_2-EDA-FITC
D) Synthesis of FITC-(PEG)$_{12}$-CCK2R ligand (Z360)

[00118] Synthesis of CCK2R ligand-(PEG)$_{12}$-EDA: 1,2-Diaminoethane trityl-resin (0.025 g) was loaded into a peptide synthesis vessel and washed with j-PrOH (3 x 10 mL), followed by DMF (3 x 10 mL). To the vessel was then introduced a solution of Fmoc-NH-(PEG)$_{12}$-COOH (42.8 mg) in DMF, i-Pr$_2$NEt (2 equiv), and PyBOP (1 equiv). The resulting solution was bubbled with Ar for 1 h, the coupling solution was drained, and the resin washed with DMF (3 x 10 mL) and j-PrOH (3 x 10 mL). Kaiser tests were performed to assess reaction completion. Fmoc deprotection was carried out using 20% piperidine in DMF (3 x 10 mL). Then added Z360 (10 mg) in DMF, i-Pr$_2$NEt (2.5 equiv), and PyBOP (2.5 equiv). After the Z360 coupling, the resin was washed with DMF (3 x 10 mL) and i-PrOH (3 x 10 mL) and dried under reduced pressure. The peptide was cleaved from the resin in the peptide synthesis vessel using a cleavage mixture consisting of 95% CF$_3$C0$_2$H, 2.5% H$_2$O, and 2.5% triisopropylsilane. Fifteen milliliters of the cleavage mixture was added to the peptide synthesis vessel, and the reaction was bubbled under Ar for 15 min. The resin was treated with two additional 10 mL quantities of the cleavage mixture for 5 min each. The cleavage mixture was concentrated to ca. 5 mL, and ethyl ether was added to induce precipitation. The precipitate was collected by centrifugation, washed with ethyl ether three times, and dried under high vacuum, resulting in the recovery of crude material.
Reagents and conditions:
(i) Fmoc-NH(PEG)$_2$-COOH, PyBop, DIPEA, DMF;
(ii) 20% piperidine, DMF;
(iii) Z360, PyBop, DIPEA, DMF;
(iv) TFA, water, $\cdot$Pr$_3$SiH.
[00119] Synthesis of FITC-(PEG)\textsubscript{12}-CCK2R ligand: To a stirred solution of the crude CCK2R ligand-(PEG)\textsubscript{12}-EDA (10 mg) and FITC (6 mg) in dimethylsulfoxide (DMSO, 1 mL) was added Pr\textsubscript{2}NEt (5 equiv) at room temperature and stirring continued for 6 hr under argon. The reaction was monitored by LCMS and purified by preparative HPLC (mobile phase: A = 10 mM ammonium acetate pH = 7, B = ACN; method: 0% B to 50% B in 30 min at 13 mL/min). The pure fractions were pooled and freeze-dried, furnishing the FITC-(PEG)\textsubscript{12}-CCK2R ligand.
SCHEME 4. Synthesis of the Z360-(PEG)₂-EDA-FITC
3. Binding of FITC-Folate and FITC-(PEG)$_{12}$-Folate conjugates to transduced T cells through anti-FITC scFv in CAR4-1BBZ

[00120] To examine the ability of the transduced T cells containing CAR4-1BBZ to bind the FITC-Folate conjugates, binding assays with the FITC-Folate conjugate and the FITC-(PEG)$_{12}$-Folate conjugate were performed. Since the excitation wavelength of FITC in the FITC-Folate conjugates overlapped with copGFP in CAR4-1BB transduced T cells, it was difficult to distinguish between FITC binding and copGFP expression. Therefore, an anti-Folate Acid (FA) monoclonal antibody and a fluorophore (excitation 640nm)-labeled anti-mouse IgG antibody were utilized.

[00121] First, the transduced T cells were incubated with FITC-Folate or FITC-(PEG)$_{12}$-Folate conjugates at room temperature for 1 hour. After washing with IX PBS, transduced T cells were incubated with anti-FA monoclonal antibody (1:15 dilution) for 1 hour. After another washing with IX PBS, transduced T cells further were incubated with fluorophore (excitation 640nm) labeled anti-mouse IgG antibody (1:50 dilution). Finally, unbound antibodies were washed away and a confocal microscope was used to confirm the FITC-Folate and FITC-(PEG)$_{12}$-Folate conjugates binding ability. All data was analyzed by Olympus Fluoview software. As shown in Figure 2, copGFP expression was observed on the transduced T cells containing CAR 4-1BBZ (top row), but not in non-transduced T cells. As shown in the middle panel, FITC-folate binding was observed on CAR-1BBZ transduced T cells, but not on non-transduced T cells. More importantly, only those transduced T cells showing copGFP expression (top row) also show FITC-Folate conjugates binding (middle row). By confocal microscopy, it was confirmed that FITC-Folate and FITC-(PEG)$_{12}$-Folate conjugates were successfully bound to anti-FITC in transduced T cells, but not to non-transduced T cells. Simultaneously, binding of FITC-Folate and FITC-(PEG)$_{12}$-Folate conjugates (arrows) were also detected on the CAR4-1BBZ transduced T cells. Based on this data, it was concluded that transduced T cells express CAR4-1BBZ, and that FITC-Folate conjugates can bind to transduced T cells through anti-FITC scFv expressed by CAR4-1BBZ transduction.

4. The binding of FITC-Folate conjugates to FR positive cancer cells

[00122] The ability of the FITC-Folate and FITC-(PEG)$_{12}$-Folate conjugates to bind folate receptor (FR) positive cancer cells was next investigated. FR positive cells lines L1210A (Mouse
lymphocytic leukemia) and KB (Mouth epidermal carcinoma), which are FR positive cell lines, were used to test the binding affinity of the FITC-Folate conjugates. Briefly, 3-4 x 10^4 cancer cells were prepared to perform binding affinity assays with FITC-Folate and FITC-(PEG)_{12}-Folate conjugates. With two different concentrations (e.g. 20nM, 70nM), the two FITC-Folate conjugates were incubated with cancer cells at room temperature for 1 hour. Since both FITC-Folate conjugates have fluorescence, the binding ability of FITC-Folate conjugates to cancer cells can be detected by fluorescence microscopy. After two washes with IX phosphate buffered saline (PBS) to remove all unbound compound, the cancer cells were observed by confocal microscope. In order to specify whether FITC-Folate conjugates bind to cancer cells via FR on the surface of cancer cells, Folate Acid was also incubated with cancer cells as a competition compound. As shown in Figures 3A, B, C, and D, the FITC-Folate conjugates can bind to both cancer cells (KB, L1210A) via FR on the cell surface. As shown in competition panel, addition of 50X excess folate acid blocked FITC-Folate binding with these cancer cells, which indicates the specific binding is between folate acid and FR on cell surface.

As shown in Figure 3A, the FITC-Folate conjugate was internalized into KB cell cytoplasm, caused by FR mediated endocytosis. However, as shown in Figure 3C, the FITC-(PEG)_{12}-Folate conjugate, which has a (PEG)_{12} linker between Folate and FITC, stayed on the extracellular surface of the KB cells.

From this data it was concluded that the FITC-Folate and FITC-(PEG)_{12}-Folate conjugates can bind to cancer cells through FR on the surface of cancer cells. It was also found that the PEG linker between Folate and FITC can prohibit the internalization of FITC-Folate conjugate. Surface localization of FITC-Folate conjugate would help increase the chance for cancer cells to be recognized by transduced T cell containing CAR4-1BBZ. If FITC-Folate conjugates are internalized via FR mediated endocytosis, transduced T cells cannot target cancer cells through FITC-Folate conjugates.

5. Cytotoxicity of anti-FITC CAR-modified T cells against Folate receptor positive (FR+) cancer cells

In order to explore whether CAR-expressing T cells can kill cancer cells, ^{51}Cr Chromium release assays were conducted. In this assay, cancer cells are labeled with ^{51}Cr. When cancer
cells are lysed, $^{51}$Cr would be released from cancer cells to supernatant. By measuring $^{51}$Cr in supernatant, the number of cancer cells killed can be determined.

First, target cancer cells L1210A were incubated in 50μL growth medium containing 50 μCi $^{51}$Chromium to get labeled. After washing with PBS X3, L1210A cells were resuspended and incubated at 37°C for 1 hour in the growth medium containing 70nM FITC-Folate conjugate or FITC-(PEG)$_{12}$-Folate conjugate. After washing away excess FITC conjugates, 5 x 10$^3$ target cancer cells were added in each well of 96-well plates. When adhesive KB cells were used as the target, they were treated similar as L1210A cells, except KB cells were grown in 96-well plates for 24 hours before $^{51}$Cr labeling and FITC-Folate conjugates binding were performed in 96-well plates. Effector T cells were then harvested, resuspended in growth media and added to the wells containing target cancer cells. The effector T cell to target cancer cell ratio was 20:1. After incubation for 4-10 hours, 20 μl aliquots of cell-free supernatant were harvested and $^{51}$Cr in the supernatants was measured with a scintillation counter or a γ-counter.

Percent specific cytolysis was calculated using following formula:

\[
\text{% cytolysis} = \frac{(\text{Experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release})}{(\text{Maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release})} \times 100
\]

Control wells contained only target cancer cells, effector T cells were never added in these wells. Maximum $^{51}$Cr release was determined by measuring $^{51}$Cr release from labeled target cells treated with 2.5% SDS to lyse all cells.

As shown in Figure 4A, in the absence of FITC-Folate conjugates, CAR-expressing T cells showed negligible activity (-5%) on target KB cell cytolysis. In the presence of FITC-Folate conjugate, CAR-expressing T cells showed -18% cancer cell cytolysis. It is implicated that FITC-Folate conjugate acts as a bridge to redirect anti-FITC CAR-expressing T cells to FR+ KB cells. Furthermore, in the presence of FITC-(PEG)$_{12}$-Folate conjugate, which has a -40 Å (PEG)$_{12}$ spacer between FITC and Folate molecules, anti-FITC CAR-expressing T cells showed much higher activity (-39%) on cancer cell cytolysis. With -40 Å distance between FITC and Folate, this conjugate can redirect CAR-expressing T cells to FR+ KB cells much better (39% vs. 18%). As the non-specific cytotoxicity control, unmodified T cells showed only 5-10% cytolysis. The existence or absence of the FITC-Folate conjugates did not show any effect on unmodified T cell cytotoxicity against FR+ KB cells.
Figure 4B shows cytotoxicity of anti-FITC CAR modified T cells against cancer cell line L1210A. In the absence of FITC-Folate conjugates, CAR-expressing T cells showed negligible activity (2-3%) on target L1210A cell cytolysis. In the presence of FITC-Folate conjugate, CAR-expressing T cells showed -29% cancer cell cytolysis. It is implicated that FITC-Folate conjugate acts as a bridge to redirect anti-FITC CAR-expressing T cells to FR+ L1210A cells. Furthermore, in the presence of the FITC-(PEG)_{12}-Folate conjugate, which has a -40 Å (PEG) spacer between FITC and Folate molecules, anti-FITC CAR-expressing T cells showed much higher activity (-51%) on FR+ L1210A cytolysis. With -40 Å distance between FITC and Folate, this conjugate can redirect CAR-expressing T cells to FR+ L1210A much better (51% vs. 29%). As the non-specific cytotoxicity control, unmodified T cells showed only 5-10% cytolysis as expected.

From this data it was concluded that anti-FITC CAR-expressing T cells do not have innate cytotoxicity against FR+ cancer cells. However, in the presence of the FITC-Folate conjugate, anti-FITC CAR-expressing T cells are redirected to FR+ cancer cells and cause specific cytolysis, and the activation of CAR-expressing T cells can be regulated by controlling of the addition of FITC-Folate conjugates. Further, the FITC-(PEG)_{12}-Folate conjugate, which has a -40 Å spacer between the FITC and the Folate molecules, can redirect anti-FITC CAR-expressing T cells to FR+ cancer cells much better than the conjugate without the spacer.

* * * *

While the invention has been described with reference to certain particular embodiments thereof, those skilled in the art will appreciate that various modifications may be made without departing from the spirit and scope of the invention. The scope of the appended claims is not to be limited to the specific embodiments described.
REFERENCES

[00133] All patents and publications mentioned in this specification are indicative of the level of skill of those skilled in the art to which the invention pertains. Each cited patent and publication is incorporated herein by reference in its entirety. All of the following references have been cited in this application:


WHAT IS CLAIMED IS:

1. A two component cancer therapeutic comprising:
   (a) a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand, wherein the tumor receptor ligand is selected from the group consisting of folate, DUPA, and CCK2R ligand; and
   (b) chimeric antigen receptor (CAR)-expressing cytotoxic lymphocytes, wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain, and wherein the CAR has binding specificity for the targeted moiety or can be bound by the targeted moiety.

2. The two component cancer therapeutic of claim 1, wherein the targeted moiety and the ligand are conjugated via a linker domain.

3. The two component cancer therapeutic of claim 1, wherein the targeted moiety is a molecule selected from the group consisting of 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin.

4. The two component cancer therapeutic of claim 2, wherein the linker domain is selected from the group consisting of polyethylene glycol (PEG), polyproline, a hydrophilic amino acid, a sugar, an unnatural peptidoeglycan, polyvinylpyrrolidone, and pluronic F-127.

5. The two component cancer therapeutic of claim 1, wherein the targeted moiety is FITC.

6. The two component cancer therapeutic of claim 4, wherein the targeted moiety is FITC and the linker is (PEG)$_{12}$.

7. The two component cancer therapeutic of claim 1, wherein the recognition region of the CAR is a single chain fragment variable (scFv) region of an antibody with binding specificity for the targeted moiety.

8. The two component cancer therapeutic of claim 5 or 6, wherein the recognition region of the CAR is a single chain fragment variable (scFv) region of an anti-FITC antibody.

9. The two component cancer therapeutic of claim 1, wherein the co-stimulation domain of the CAR is selected from the group consisting of CD28, CD137 (4-1BB), CD134 (OX40), and CD278 (ICOS).
10. The two component cancer therapeutic of claim 1, wherein the activation signaling domain of the CAR is the T cell CD3ζ chain or Fc receptor γ.

11. The two component cancer therapeutic of claim 5 or 6, wherein the recognition region is a single chain fragment variable (scFv) region of an anti-FITC antibody, wherein the co-stimulation domain is CD137 (4-1BB), and wherein the activation signaling domain is the T cell CD3C chain.

12. The two component cancer therapeutic of claim 1, wherein the cytotoxic lymphocyte are one or more cell types selected from the group consisting of cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells.

13. A small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand, wherein the tumor receptor ligand is selected from the group consisting of folate, DUPA, and CCK2R ligand.

14. The SCM of claim 13, wherein the targeted moiety and the ligand are conjugated via a linker domain.

15. The SCM of claim 13, wherein the targeted moiety is a molecule selected from the group consisting of 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin.

16. The SCM of claim 14, wherein the linker domain is selected from the group consisting of polyethylene glycol (PEG), polyproline, a hydrophilic amino acid, a sugar, an unnatural peptidoeglycan, polyvinylpyrrolidone, and pluronic F-127.

17. The SCM of claim 13, wherein the targeted moiety is FITC.

18. The SCM of claim 16, wherein the targeted moiety is FITC and the linker is (PEG)$_{12}$.

19. The SCM of claim 13, comprising FITC-folate, FITC-DUPA, FITC-CCK2R ligand, FITC-(PEG)$_{12}$-folate, FITC-(PEG)$_{12}$-DUPA, or FITC-(PEG)$_{12}$-CCK2R ligand.

20. CAR-expressing lymphocytes, wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain, and wherein the CAR has binding specificity for a selected targeted moiety or can be bound by the targeted moiety.
21. The CAR-expressing cytotoxic lymphocytes of claim 20, wherein the recognition region of the CAR is a single chain fragment variable (scFv) region of an antibody with binding specificity for the targeted moiety.

22. The CAR-expressing cytotoxic lymphocytes of claim 21, wherein the recognition region of the CAR is a single chain fragment variable (scFv) region of an anti-FITC antibody.

23. The CAR-expressing cytotoxic lymphocytes of claim 20, wherein the co-stimulation domain of the CAR is selected from the group consisting of CD28, CD137 (4-1BB), CD134 (OX40), and CD278 (ICOS).

24. The CAR-expressing cytotoxic lymphocytes of claim 20, wherein the activation signaling domain of the CAR is the T cell CD3ζ chain or Fc receptor γ.

25. The CAR-expressing cytotoxic lymphocytes of claim 20, wherein the recognition region is a single chain fragment variable (scFv) region of an anti-FITC antibody, wherein the co-stimulation domain is CD137 (4-1BB), and wherein the activation signaling domain is the T cell CD3C chain.

26. The CAR-expressing cytotoxic lymphocytes of claim 20, wherein the targeted moiety is a molecule selected from the group consisting of 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentafluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centyrin, and a DARPin.

27. The CAR-expressing cytotoxic lymphocytes of claim 20, wherein the cytotoxic lymphocyte are one or more cell types selected from the group consisting of cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells.

28. A method of treating cancer in a subject, comprising:
   (a) culturing a population of cytotoxic lymphocytes under conditions promoting activation;
   (b) transfecting the lymphocyte population of (a) with a vector encoding a chimeric antigen receptor (CAR), wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain;
   (c) administering a therapeutically effective number of the transfected lymphocytes of (b) to a subject having cancer; and
(d) administering a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand to the subject, wherein the ligand is recognized and bound by a receptor on the surface of a cell of the cancer, and wherein the CAR has binding specificity for the targeted moiety or can be bound by the targeted moiety; thereby treating cancer in a subject.

29. A method of treating cancer in a subject, comprising:
   (a) culturing a population of cytotoxic lymphocytes under conditions promoting activation;
   (b) transfecting the lymphocyte population of (a) with a vector encoding a chimeric antigen receptor (CAR), wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain;
   (c) administering a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand to a subject having cancer, wherein the ligand is recognized and bound by a receptor on the surface of a cell of the cancer; and
   (d) administering a therapeutically effective number of the transfected T cells of (b) to the subject, and wherein the CAR has binding specificity for the targeted moiety or can be bound by the targeted moiety;
   thereby treating cancer in a subject.

30. A method of treating cancer in a subject, comprising:
   (a) culturing a population of cytotoxic lymphocytes under conditions promoting activation;
   (b) transfecting the lymphocytes population of (a) with a vector encoding a chimeric antigen receptor (CAR), wherein the CAR is a fusion protein comprising a recognition region, a co-stimulation domain and an activation signaling domain, and wherein the CAR has binding specificity for a targeted moiety or can be bound by the targeted moiety;
   (c) incubating the lymphocytes of (b) with a small conjugate molecule (SCM) comprising a targeted moiety conjugated to a tumor receptor ligand;
   (d) administering a therapeutically effective number of the transfected lymphocytes of (c) to a subject having cancer;
   thereby treating cancer in a subject.
31. The method of any one of claims 28-30, wherein the lymphocytes are autologous or heterologous cells, with respect to the subject being treated.

32. The method of any one of claims 28-30, wherein the culturing conditions of (a) comprise culturing the population of lymphocytes in the presence of anti-CD3 antibodies or anti-CD28 antibodies, or both.

33. The method of any one of claims 28-30, wherein the recognition region of the CAR is a single chain fragment variable (scFv) region of an antibody with binding specificity for the targeted moiety.

34. The method of any one of claims 28-30, wherein the recognition region of the CAR is a single chain fragment variable (scFv) region of an anti-FITC antibody.

35. The method of any one of claims 28-30, wherein the co-stimulation domain of the CAR is selected from the group consisting of CD28, CD137 (4-1BB), CD134 (OX40), and CD278 (ICOS).

36. The method of any one of claims 28-30, wherein the activation signaling domain of the CAR is the T cell CD3ζ chain or Fc receptor γ.

37. The method of any one of claims 28-30, wherein the recognition region is a single chain fragment variable (scFv) region of an anti-FITC antibody, wherein the co-stimulation domain is CD137 (4-1BB), and wherein the activation signaling domain is the T cell CD3ζ chain.

38. The method of any one of claims 28-30, wherein the targeted moiety is a molecule selected from the group consisting of 2,4-dinitrophenol (DNP), 2,4,6-trinitrophenol (TNP), biotin, digoxigenin, fluorescein, fluorescein isothiocyanate (FITC), NHS-fluorescein, pentfluorophenyl ester (PFP), tetrafluorophenyl ester (TFP), a knottin, a centytrin, and a DARPin.

39. The method of any one of claims 28-30, wherein the ligand is selected from the group consisting of folate, DUPA, and CCK2R ligand.

40. The method of any one of claims 28-30, wherein the targeted moiety and the ligand are conjugated via a linker domain.

41. The method of claim 40, wherein the linker domain is selected from the group consisting of polyethylene glycol (PEG), polyproline, a hydrophilic amino acid, a sugar, an unnatural peptideoglycan, polyvinylpyrrolidone, and pluronic F-127.
42. The method of claim 38, wherein the targeted moiety is FITC.
43. The method of claim 41, wherein the targeted moiety is FITC and the linker is (PEG)$_{12}$.
44. The method of claim 42 or 43, wherein the ligand is selected from the group consisting of folate, DUPA, and CCK2R ligand.
45. The method of any one of claims 28-30, wherein the vector is a lentivirus vector.
46. The method of any one of claims 28-30, wherein the cytotoxic lymphocytes are one or more cell types selected from the group consisting of cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells.
47. The method of any one of claims 28-30, wherein the subject is a human.
48. The method of any one of claims 28-30, wherein the cancer is one or more of a cancer of the brain, thyroid, lung, pancreas, kidney, stomach, gastrointestinal stroma, endometrium, breast, cervix, ovary, colon, prostate, leukemias, lymphomas, other blood-related cancers, or head and neck cancer.
Figure 1A. The construction of CAR4-1BBZ on transduced T cells

A

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1kb

2kb

RSV 5'LTR

AmpR

pCDH-EF1-MCS- BGH
PGK-GFP-T2A-Puro
Cat.# CD550A-1

EF1α

PGK

BGH poly-A

SV40 poly-A

SV40 ORI

3'LTR

WPRE

pureT

MCS

5' NheI

EcoRI

BamHI

SalI

BamHI

NotI 3'
Figure 1B and C. The expression of CAR4-1BBZ on transduced T cells.
Figure 2. Binding of FITC-Folate and FITC-(PEG)$_{12}$-Folate conjugates to CAR-transduced T cells
Figure 3A. The binding of FITC-Folate conjugate (EC17) to cancer cells (KB)

Figure 3B. The binding of FITC-Folate conjugate (EC17) to cancer cells (L1210A)
Figure 3C. The binding of FITC-(PEG)$_{12}$-Folate conjugate to cancer cells (KB)

Figure 3D. The binding of FITC-(PEG)$_{12}$-Folate conjugate to cancer cells (L1210A)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
A61K 31/4985(2006.01)i, A61K 31/352(2006.01)i, A61K 31/551(2006.01)i, A61P 35/00(2006.01)i

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)
A61K 31/4985; A61K 39/395; A61K 31/4188; A61K 31/552; A61K 31/551; A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: chimeric antigen receptor, FITC, scFv, T cell, conjugate, folate, DUPA, CCK2R

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>CN 102775500 A (ZHENG, J.) 14 November 2012 See abstract and claims 1-6.</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* 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: 28 April 2014 (28.04.2014)

Date of mailing of the international search report: 28 April 2014 (28.04.2014)

Name and mailing address of the ISA/KR
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Form PCT/ISA/2.10 (second sheet) (July 2009)
**INTERNATIONAL SEARCH REPORT**

**Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 28-48
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 28-48 pertain to a method for treatment of the human by therapy, and thus relate to a subject matter which this international Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.: 
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 44
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6-4(a).

**Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☒ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☒ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.
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