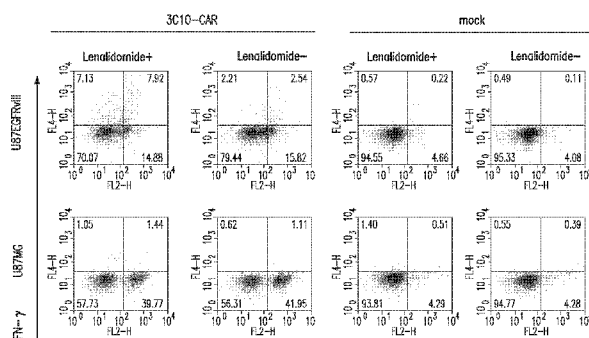
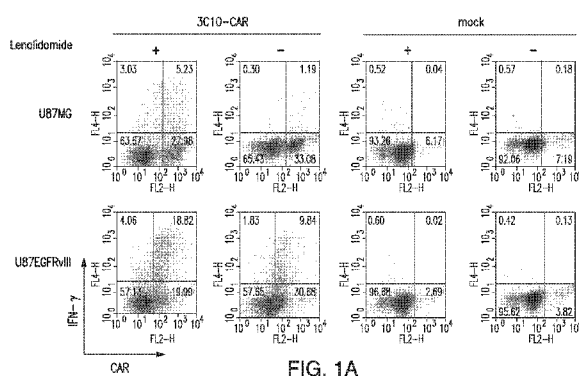




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[Continued on next page]

(54) Title: USES OF LENALIDOMIDE AND CAR T-CELLS



(57) Abstract: Provided herein are methods of using lenalidomide and modified T lymphocytes for the treatment of diseases, such as a cancer or a tumor expressing EGFRvIII, and related uses of lenalidomide for modulating T lymphocyte activation and/or proliferation.



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USES OF LENALIDOMIDE AND CAR T-CELLS

1. FIELD

[0001] The disclosure herein relates to the field of immunology therapy, and more specifically, to the use of lenalidomide and modified T lymphocytes for the treatment of diseases and related uses of lenalidomide for modulating T lymphocyte activation and/or proliferation.

2. SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 7, 2016, is named 14247-006-228_Sequence_Listing.txt and is 1,765 bytes in size.

3. BACKGROUND

[0003] Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults and the median overall survival time is less than 15 months, despite the currently available multimodal therapy (Stupp *et al.*, *N Engl J Med* 2005; 352: 987–996; and Stupp *et al.*, *Lancet Oncol* 2009; 10: 459–466). Thus far, a variety of immunotherapeutic approaches including cancer vaccines and antibody-mediated and cell-based therapies have been tested to treat cancers (Rosenberg, *Nature* 2001; 411: 380–384; Johnson *et al.*, *Curr Neurol Neurosci Rep* 2010; 10: 259–266; and Chung *et al.*, *J Immunol Res* 2014; 2014: 326545). However, these therapies have major drawbacks such as major histocompatibility complex restriction, nonspecific killing and technical difficulties for expansion *ex vivo* (Chung *et al.*, *J Immunol Res* 2014; 2014: 326545).

[0004] Thus, there exists a need in the art for compositions and methods of treatment that target neoplastic cells, such as cancer cells and tumor cells, including GBM. The disclosure provided herein includes such compositions and methods with related advantages.

4. SUMMARY

[0005] Provided herein are methods of treating a subject (*e.g.*, a human subject) having a disease associated with expression of EGFRvIII. The methods can comprise administering to the subject an effective amount of lenalidomide and modified T lymphocytes expressing a chimeric antigen receptor (CAR) that targets EGFRvIII expressing cells, such as cancer cells

or tumor cells. The administration of lenalidomide improves or enhances the activity of the modified T lymphocytes against the EGFRvIII expressing cells. Such modified T lymphocytes, in some aspects, express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0006] Accordingly, in a first aspect, provided herein is a method of treating a subject having a disease associated with expression of EGFRvIII, comprising administering to the subject an effective amount of lenalidomide and modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0007] In some embodiments, the disease associated with expression of EGFRvIII is a cancer or a tumor. The cancer or tumor can be a metastatic cancer or a metastatic tumor. Alternatively, the cancer or tumor is a primary cancer or a primary tumor. Such a primary cancer or primary tumor can arise from or be located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof of the subject. In a specific embodiment, the tumor is a glioma, which can be, but is not limited to, an astrocytoma. In another specific embodiment, the astrocytoma is a glioblastoma multiforme.

[0008] In some embodiments, the disease associated with expression of EGFRvIII is a cancer or tumor selected from anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, medulloepithelioma, ependymblastoma, medulloblastoma, supratentorial primitive neuroectodermal tumor, and atypical teratoid/rhabdoid tumor. In a specific embodiment, the disease associated with expression of EGFRvIII is glioblastoma multiform.

[0009] In some embodiments, the effective amount of lenalidomide administered to the subject is from about 0.01 mg/kg to about 0.50 mg/kg, or from about 0.05 mg/kg to about 0.10 mg/kg, or from about 0.06 mg/kg to about 0.09 mg/kg. Alternatively, or in addition, the effective amount of lenalidomide is from about 0.10 mg to about 150 mg, or from about 2.5 mg to about 25 mg. In some specific embodiments, the effective amount of lenalidomide is selected from about 2.5 mg, about 5 mg, about 10 mg, about 15 mg, about 20 mg, and about 25 mg.

[0010] In certain embodiments, the effective amount of lenalidomide and CAR is an amount sufficient to cause a reduction in the rate of growth of an EGFRvIII-expressing tumor, to cause a cessation in growth of an EGFRvIII-expressing tumor, or to cause a reduction in size of an EGFRvIII-expressing tumor, *e.g.*, over 1, 2, or 3 weeks, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 months after said administering.

[0011] In some embodiments, the lenalidomide administered to the subject is administered daily for a predetermined period of time following an initial administration of lenalidomide. Additionally, the lenalidomide and modified T lymphocytes can be administered simultaneously or sequentially. In some embodiments, the modified T lymphocytes are administered before the lenalidomide is administered, following which the lenalidomide is administered 1 day, 2 days, 3 days or more after administration of the modified T lymphocytes.

[0012] In some embodiments, the likelihood of the subject's relative survival increases by at least 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%.

[0013] Also provided herein are methods of increasing the release of interferon gamma by modified T lymphocytes. Such methods can comprise contacting the modified T lymphocytes with an effective amount of lenalidomide. In some embodiments, the modified T lymphocytes used in the disclosed methods express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0014] Accordingly, in a second aspect, provided herein is a method of increasing the release of interferon gamma by modified T lymphocytes, comprising contacting the modified T lymphocytes with an effective amount of lenalidomide, wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain. In some embodiments, the release of interferon gamma is increased by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 30%, 40% or 50%.

[0015] Further provided herein are methods of enhancing the cytotoxicity of modified T lymphocytes. Such methods can comprise contacting the modified T lymphocytes with an effective amount of lenalidomide. In some embodiments, the modified T lymphocytes

express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0016] Accordingly, in a third aspect, provided herein is a method of enhancing the cytotoxicity of modified T lymphocytes, comprising contacting the modified T lymphocytes with an effective amount of lenalidomide, wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% (*e.g.*, as determined in a cell killing assay with U87-EGFRvIII cells as targets). In certain embodiments, the effective amount of lenalidomide is 0.1 μ M, 0.2 μ M, 0.3 μ M, 0.4 μ M, 0.5 μ M, 0.6 μ M, 0.7 μ M, 0.8 μ M, 0.9 μ M, 1.0 μ M, 1.1 μ M, 1.2 μ M, 1.3 μ M, 1.4 μ M, 1.5 μ M, 1.6 μ M, 1.7 μ M, 1.8 μ M, 1.9 μ M, 2.0 μ M, 2.5 μ M, 3.0 μ M, 3.5 μ M, 4.0 μ M, 4.5 μ M, or 5.0 μ M, lenalidomide if the contacting is *in vitro*, or 0.01 mg/kg to about 0.50 mg/kg, or about 0.05 mg/kg to about 0.10 mg/kg, or about 0.06 mg/kg to about 0.09 mg/kg if said contacting is *in vivo*.

[0017] Also provided herein are methods of increasing the proliferation of a population of T lymphocytes. Such methods can comprise contacting the population of T lymphocytes with an effective amount of lenalidomide. In some embodiments, the population of T lymphocytes comprises modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain. In certain embodiments, proliferation of the T lymphocytes is increased *in vitro*. In certain other embodiments, proliferation of the T lymphocytes is increased *in vivo*.

[0018] Accordingly, in a fourth aspect, provided herein is a method of increasing the proliferation of a population of T lymphocytes, comprising contacting the population of T lymphocytes with an effective amount of lenalidomide, wherein the population of T lymphocytes comprise modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0019] Provided herein are methods of enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells. Such methods can comprise contacting the modified T lymphocytes and the cancer cells or tumor cells with an effective amount of lenalidomide, wherein the cancer cells or tumor cells express EGFRvIII. In some

embodiments, the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0020] Accordingly, in a fifth aspect, provided herein is a method of enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells, comprising contacting the modified T lymphocytes and the cancer cells or tumor cells with an effective amount of lenalidomide, wherein the cancer cells or tumor cells express EGFRvIII, and wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[0021] In some embodiments, the cancer cells or tumor cells expressing EGFRvIII are metastatic cancer cells or metastatic tumor cells. Alternatively, the cancer cells or tumor cells are primary cancer cells or primary tumor cells. Such primary cancer cells or primary tumor cells can arise from or can be located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof. In a specific embodiment, the tumor cells are glioma brain tumor cells, which can be, but is not limited to, astrocytoma brain tumor cells. In another specific embodiment, the astrocytoma brain tumor cells are glioblastoma multiforme brain tumor cells.

[0022] In some embodiments, the cancer cells or tumor cells expressing EGFRvIII are selected from anaplastic astrocytoma cells, giant cell glioblastoma cells, gliosarcoma cells, anaplastic oligodendroglioma cells, anaplastic ependymoma cells, choroid plexus carcinoma cells, anaplastic ganglioglioma cells, pineoblastoma cells, medulloepithelioma cells, ependymoblastoma cells, medulloblastoma cells, supratentorial primitive neuroectodermal tumor cells, and atypical teratoid/rhabdoid tumor cells.

[0023] In some embodiments, the CAR-expressing modified T lymphocytes used in the disclosed methods comprise a CAR having an anti-EGFRvIII binding domain, wherein the anti-EGFRvIII binding domain comprises an antibody or antibody fragment that includes an anti-EGFRvIII binding domain. In a specific embodiment, the antibody fragment is a single-chain variable fragment (scFV).

[0024] In some embodiments, the CAR-expressing modified T lymphocytes used in the disclosed methods comprise a CAR having a transmembrane domain, wherein the transmembrane domain comprises a transmembrane domain selected from alpha, beta or zeta

chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD8. In some embodiments, the anti-EGFRvIII binding domain is joined to the transmembrane domain by a linker, spacer or hinge polypeptide. Such a linker, spacer or hinge polypeptide, in some embodiments, is the hinge region of CD8 α chain.

[0025] In some embodiments, the CAR-expressing modified T lymphocytes used in the disclosed methods comprise a CAR having an intracellular domain, wherein the intracellular domain is an intracellular domain of a protein that is expressed on the surface of T lymphocytes and triggers activation or proliferation of T lymphocytes. In a specific embodiment, the intracellular signaling domain comprises the signaling domain of one or more T lymphocyte costimulatory protein and/or the signaling domain of T-cell receptor zeta chain (CD3 ζ). Such T lymphocyte costimulatory protein can be selected from OX40, CD2, CD27, CD28, CD5, CD30, CD40, ICAM-1, LFA-1, ICOS and 4-1BB. In a further specific embodiment, the intracellular signaling domain comprises the signaling domain of CD28, 4-1BB and CD3 ζ .

[0026] In a specific embodiment, the CAR-expressing modified T lymphocytes used in the disclosed methods comprise a CAR having an anti-EGFRvIII binding domain, a transmembrane domain and an intracellular domain, wherein the anti-EGFRvIII binding domain comprises an scFV, wherein the transmembrane domain comprises the transmembrane domain of CD8, wherein the anti-EGFRvIII binding domain is joined to the transmembrane domain by the hinge region of CD8 α chain, and wherein the intracellular domain comprises the signaling domain of CD28, 4-1BB and CD3 ζ .

5. BRIEF DESCRIPTION OF THE DRAWINGS

[0027] **FIG. 1A** and **1B** show the results from transduction of lentivirus-mediated 3C10-CAR and an intracellular IFN- γ assay. CAR PBMCs or mock PBMCs (1×10^5 cells) were incubated with 0.5×10^6 U87-EGFRvIII cells or U87MGs. PBMCs pretreated with lenalidomide and non-treated PBMCs were used. The approximate transduction efficiency of 3C10-CAR was 30–40%. After gating the CD8-FITC population, the proportion of IFN- γ and CAR double-positive cells was 9.84% in the 3C10-CAR PBMCs that were coincubated with U87-EGFRvIII, and 18.82% in the lenalidomide-pretreated 3C10-CAR PBMCs. **FIG. 1A**

shows the results for the first experiment, and **FIG. 1B** shows the results for the second experiment.

[0028] **FIG. 2** shows the results of a calcein assay. Even without lenalidomide treatment, PBMCs transduced with 3C10-CAR lysed ~50% of the U87-EGFRvIII cells at an E:T ratio of 25:1, and lysis occurred in an E:T ratio-dependent manner. Mock PBMCs did not demonstrate this killing effect. PBMCs transduced with 3C10-CAR and mock PBMCs were pretreated with 1 μ m lenalidomide for 48 h. 3C10-CAR PBMCs pretreated with lenalidomide lysed ~80% of the U87-EGFRvIII cells at an E:T ratio of 25:1. Mock PBMCs treated with lenalidomide were not effective, similar to those without lenalidomide treatment (left). CD3⁺ cells were selectively separated from PBMCs using CD3 microbeads to exclude the influence of natural killer (NK) cell activation. The selected CD3⁺ cells were transduced with viral supernatant to express 3C10-CAR (CD3-selected 3C10-CAR), and then a calcein assay was performed. 3C10-CAR CD3 T cells exerted a similar cytotoxicity to 3C10-CAR PBMCs. Additionally, lenalidomide enhanced the cytotoxicity of the 3C10-CAR CD3 T cells (center). Against the parental U87MGs without EGFRvIII expression, lenalidomide-pretreated 3C10-CAR PBMCs exhibited no killing effect, similar to the results for the other effector cells (i.e., original 3C10-CAR cells and mock PBMCs with or without lenalidomide treatment) (right). *P<0.05. Error bars, s.e.m.

[0029] **FIG. 3** shows the schema for the intracranial glioma xenograph mice experiments.

[0030] **FIG. 4** shows exemplary bioluminescence imaging of intracranial glioma xenograph mice. Tumors began to be eradicated from days 7 to 14 in the 3C10-CAR+lenalidomide group, and from days 14 to 21 in the 3C10-CAR group. *Significant difference (P<0.05) between the 3C10-CAR+lenalidomide group and the other three groups. Error bars, s.e.m.

[0031] **FIG. 5** shows the survival curve of treated intracranial glioma xenograph mice. Although 50% of the mice treated with 3C10-CAR PBMCs died near day 20, all mice treated with 3C10-CAR+lenalidomide were eventually tumor-free. (**) Represents a significant difference (P<0.05) between the 3C10-CAR⁺ and 3C10-CAR⁻ lenalidomide groups, whereas (*) represents a significant difference (P<0.05) between the 3C10-CAR⁻ lenalidomide group and the two mock-treated groups.

[0032] **FIG. 6** shows 3C10-CAR T cells migrated to intracranial EGFRvIII-expressing glioblastoma. Seven days after the inoculation of tumor cells, 3C10-CAR PBMCs or mock

PBMCs were infused. Simultaneously, lenalidomide or PBS was given for 20 consecutive days. The brain sections were stained with anti-human CD3 antibody. A number of human CD3⁺ cells were observed in the tumors of 3C10-CAR-treated mice, but none were seen the tumors of mock-CAR-treated mice. The number of CD3⁺ cells and DAPI⁺ cells in three fields was counted. Lenalidomide treatment significantly increased the numbers of migrated CD3⁺ T cells from those in the tumors without lenalidomide treatment. Scale bars, 200 μ m. Error bars, s.e.m.

[0033] FIG. 7 shows the results of a WST-1 assay. Lenalidomide increased the growth of both types of PBMCs, regardless of CAR transduction, in a dose-dependent manner.

[0034] FIG. 8 shows immunofluorescence of CD11a and F-actin in the junction of CAR T cells and tumor cells. Tumor cells labeled with CellTracker Blue CMAC (blue); 3C10-CAR, F-actin and CD11a were colocalized in the connection between the tumor cells and 3C10-CAR PBMCs.

[0035] FIG. 9 shows F-actin polymerization quantified using a relative recruitment index (RRI). The thickness of the F-actin polymers was significantly higher in the lenalidomide (+) group compared with that in the lenalidomide (−) group. Arrows indicate immune synapses. *P<0.05, Error bars, s.e.m.

[0036] FIG. 10 shows PD1 and CTLA-4 expression analysis by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Error bars, S.E.M. ns, Not significant.

6. DETAILED DESCRIPTION

[0037] The methods and compositions provided herein are based, in part, on the discovery that lenalidomide can be used in conjunction with modified T lymphocytes that target EGFRvIII expressing cancers or tumors (*e.g.*, GBM) to improve or enhance the activity of the modified T lymphocytes against the EGFRvIII expressing cancers or tumors. Accordingly, in certain embodiments, the methods provided herein include treating a subject (*e.g.*, a human subject) having a disease associated with expression of EGFRvIII that includes administering lenalidomide and modified T lymphocytes that target EGFRvIII expressing cells. In some embodiments, the methods provided herein are further based, in part, on the discovery that lenalidomide can be used to: (i) increase the release of interferon gamma by modified T lymphocytes; (ii) enhance the cytotoxicity of modified T lymphocytes; (iii) increase the

proliferation of a population of T lymphocytes; and/or (iv) enhance immune synapse formation of modified T lymphocytes to cancer cells or tumor cells.

6.1. Definitions

[0038] All patents, applications, published applications and other publications are incorporated by reference in their entirety. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art. For purposes of interpreting this specification, the following description of terms will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any description of terms set forth conflicts with any document incorporated herein by reference, the description of term set forth below shall control.

[0039] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification can mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0040] The term “about” or “approximately” means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term “about” or “approximately” means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term “about” or “approximately” means within 50%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range.

[0041] As used herein, “administering,” “administer” or “administration” refers to the act of injecting or otherwise physically delivering a substance as it exists outside the body into a patient, such as by mucosal, intradermal, intravenous, intramuscular delivery and/or any other method of physical delivery described herein or known in the art. When a disease or a symptom thereof is being treated, administration of the substance typically occurs after the onset of disease or symptoms thereof.

[0042] As used herein, the term “antibody,” or grammatical variations thereof, refers to immunoglobulin polypeptide(s) capable of binding to an antigen. An antibody can be a full-length antibody or less than full-length. If an antibody is less than full length (*e.g.*, an antibody fragment), it includes at least one binding site. In some such embodiments, the binding site comprises at least one, and preferably at least two sequences with structure of

antibody variable regions. In some embodiments, the term “antibody” encompasses any protein having a binding domain that is homologous or largely homologous to an immunoglobulin-binding domain. In particular embodiments, the term “antibody” encompasses polypeptides having a binding domain that shows at least 99% identity with an immunoglobulin-binding domain. In some embodiments, the antibody is any protein having a binding domain that shows at least 70%, at least 80%, at least 85%, at least 90% or at least 95% identity with an immunoglobulin-binding domain. Antibody polypeptides in accordance with the present invention may be prepared by any available means, including, for example, isolation from a natural source or antibody library, recombinant production in or with a host system, chemical synthesis, etc., or combinations thereof. In some embodiments, an antibody is monoclonal or polyclonal. In some embodiments, an antibody may be a member of any immunoglobulin class, including any of the human classes IgG, IgM, IgA, IgD and IgE. In certain embodiments, an antibody is a member of the IgG immunoglobulin class. In some embodiments, the term “antibody” refers to any derivative of an antibody that possesses the ability to bind to an antigen of interest. In some embodiments, an antibody is multispecific (*e.g.*, an antibody that binds to at least two different epitopes, antigens or cells). In some embodiments, an antibody is monospecific (*e.g.*, an antibody that binds to one epitope, antigen or cell). In some embodiments, an antibody fragment comprises multiple chains that are linked together, for example, by disulfide linkages. In some embodiments, an antibody is a human antibody. In some embodiments, an antibody is a humanized antibody. In some embodiments, humanized antibodies include chimeric immunoglobulins, immunoglobulin chains or antibody fragments that contain minimal sequence from non-human immunoglobulin. In some embodiments, humanized antibodies are human immunoglobulin (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In particular embodiments, antibodies for use in the present disclosure bind to EGFRvIII.

[0043] An “antibody fragment” refers to a portion of a full-length antibody, for example, the antigen binding or variable region of the intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules (*e.g.*, single-chain Fv); and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-

binding site, and a residual “Fc” fragment, a designation reflecting the ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

[0044] An “antigen” is a predetermined target to which an antibody or antibody fragment can selectively bind. An antigen can be a polypeptide, carbohydrate, nucleic acid, lipid, hapten or other naturally occurring or synthetic compound. In some embodiments, the target antigen is a polypeptide, including, for example, an EGFRvIII.

[0045] The terms “binds” or “binding” as used herein refer to an interaction between molecules to form a complex. Interactions can be, for example, non-covalent interactions including hydrogen bonds, ionic bonds, hydrophobic interactions, and/or van der Waals interactions. A complex can also include the binding of two or more molecules held together by covalent or non-covalent bonds, interactions or forces. The strength of the total non-covalent interactions between a single antigen-binding site on an antibody and a single epitope of a target molecule, such as EGFRvIII, is the affinity of the antibody or functional fragment for that epitope. The ratio of association (k_1) to dissociation (k_{-1}) of an antibody to a monovalent antigen (k_1/k_{-1}) is the association constant K , which is a measure of affinity. The value of K varies for different complexes of antibody and antigen and depends on both k_1 and k_{-1} . The association constant K for an antibody provided herein can be determined using any method provided herein or any other method well known to those skilled in the art. The affinity at one binding site does not always reflect the true strength of the interaction between an antibody and an antigen. When complex antigens containing multiple, repeating antigenic determinants, such as EGFRvIII, come in contact with antibodies containing multiple binding sites, the interaction of antibody with antigen at one site will increase the probability of a reaction at a second site. The strength of such multiple interactions between a multivalent antibody and antigen is called the avidity. The avidity of an antibody can be a better measure of its binding capacity than is the affinity of its individual binding sites. For example, high avidity can compensate for low affinity as is sometimes found for pentameric IgM antibodies, which can have a lower affinity than IgG, but the high avidity of IgM, resulting from its multivalence, enables it to bind antigen effectively.

[0046] The term “cancer” refers to any malignant neoplasm characterized by the proliferation of cells that can invade surrounding tissue and/or metastasize to new body sites. Cancer includes, but is not limited to, blood born tumors and solid tumors. Blood born tumors

include lymphomas, leukemias and myelomas. Lymphomas and leukemias are malignancies arising among white blood cells. The term “tumor” refers to both benign and malignant tumors, which can be classified according to the type of tissue in which they are found. For example, fibromas are neoplasms of fibrous connective tissue, and melanomas are abnormal growths of pigment (melanin) cells. Malignant tumors originating from epithelial tissue, *e.g.*, in skin, bronchi, and stomach, are termed carcinomas. Malignancies of epithelial glandular tissue such as are found in the breast, prostate, and colon, are known as adenocarcinomas. Malignant growths of connective tissue, *e.g.*, muscle, cartilage, lymph tissue, and bone, are called sarcomas. Through the process of metastasis, tumor cell migration to other areas of the body establishes neoplasms in areas away from the “primary cancer” or “primary tumor,” which is the site of initial appearance.

[0047] A “chimeric antigen receptor” or “CAR,” also known in the art as an artificial T cell receptor, chimeric T cell receptor, or chimeric immunoreceptor, refers an artificial membrane-bound protein that can direct a T lymphocyte to an antigen, and stimulate the T lymphocyte to kill a cell displaying the antigen. See, *e.g.*, Eshhar, U.S. Patent No. 7,741,465. At a minimum, the CAR comprises: (i) an extracellular domain that binds to an antigen, *i.e.* an antigen binding domain, such as an antigen on a cell; (ii) a transmembrane domain, and (iii) an intracellular (cytoplasmic) signaling domain that transmits a primary activation signal to an immune cell. All other conditions being satisfied, when the CAR is expressed on the surface of, *e.g.*, a T lymphocyte, and the extracellular domain of the CAR binds to an antigen, the intracellular signaling domain transmits a signal to the T lymphocyte to activate and/or proliferate, and, if the antigen is present on a cell surface, to kill the cell expressing the antigen. Because T lymphocytes require two signals, a primary activation signal and a costimulatory signal, in order to activate, CARs can also comprise a costimulatory domain such that binding of the antigen to the extracellular domain results in transmission of both a primary activation signal and a costimulatory signal. In some embodiments, the intracellular signaling domain further includes one or more signaling domains from at least one costimulatory protein.

[0048] Thus, in some embodiments, the CAR can include an antigen binding domain, a transmembrane domain and an intracellular signaling domain that is from at least one (one, two, three or more) costimulatory proteins and a signaling domain from a protein that is expressed on the surface of T lymphocytes and triggers activation and/or proliferation. In

some embodiments, the CAR can further include a leader sequence at the N-terminus of the extracellular antigen binding domain, wherein the leader sequence is optionally cleaved from the antigen binding domain (*e.g.*, scFv) during cellular processing and localization of the CAR to the cellular membrane.

[0049] A “cytotoxic cell” includes CD8⁺ T cells, natural-killer (NK) cells, and neutrophils, which cells are capable of mediating cytotoxicity responses.

[0050] The term “cytotoxicity” refers to the quality of being toxic to cells. Accordingly, “enhancing cytotoxicity” refers to increasing the quality of being toxic to cells in comparison to control. In the context of the present disclosure, enhancing the cytotoxicity of T lymphocytes can include increasing the ability of the T lymphocytes to recognize and/or kill cells that express EGFRvIII, such as cancer cells or tumor cells.

[0051] The terms “disease,” “disorder,” and “condition” are used inclusively and refer to an abnormal condition of an organism that impairs bodily functions, associated with specific symptoms and signs. In the context of this invention the disease may be “associated with expression of EGFRvIII.” Examples include, but are not limited to, a cancer or a tumor. Such a cancer or tumor can be metastatic or primary. In certain embodiments, a subject amenable to treatment includes a subject presently exhibiting symptoms or diagnosed with the disease, disorder or condition.

[0052] As used herein, a “disease associated with EGFRvIII” or an “EGFRvIII-mediated disease” means any disease, disorder or condition in which EGFRvIII, or a mutant thereof, is known or suspected to play a role. This includes, but is not limited to, a disease, disorder or condition associated with cells that express EGFRvIII including, tumor cells (*e.g.*, glioblastoma multiforme or another EGFRvIII-expressing cancer). Accordingly, another embodiment of the present disclosure relates to preventing, treating, stabilizing or lessening the severity or progression of one or more diseases in which EGFRvIII, or a mutant thereof, is known or suspected to play a role. Specifically, the present disclosure relates to a method of treating a subject having disease associated with EGFRvIII, such as a cancer or a tumor, wherein the method includes administering to a subject lenalidomide in combination with modified T lymphocytes that recognize and kill EGFRvIII expressing cells.

[0053] An “effective amount” is an amount sufficient to affect a beneficial or desired result. In certain embodiments, for example, the effective amount (*e.g.*, lenalidomide and EGFRvIII-

targeted CAR) is an amount sufficient to cause a reduction in the rate of growth of an EGFRvIII-expressing tumor, to cause a cessation in growth of an EGFRvIII-expressing tumor, or to cause a reduction in size of an EGFRvIII-expressing tumor over a period of time (*e.g.*, 1, 2, or 3 weeks, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 months) after administration. An effective amount can be administered in one or more administrations, applications or dosages. Such delivery is dependent on a number of variables including the time period for which the individual dosage unit is to be used, the bioavailability of the therapeutic agent, the route of administration, etc. It is understood, however, that specific dose levels of the therapeutic agents disclosed herein (*e.g.*, lenalidomide and modified T lymphocytes) for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, and diet of the subject, the time of administration, the rate of excretion, the drug combination, and the severity of the particular disease, disorder or condition being treated and form of administration. Treatment dosages generally may be titrated to optimize safety and efficacy. Dosage-effect relationships from *in vitro* and/or *in vivo* tests initially can provide useful guidance on the proper doses for administration. In certain embodiments, the effective amount of lenalidomide, modified T lymphocytes, or compositions thereof is an amount sufficient to: (i) increase the likelihood of a subject's relative survival by at least 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%; (ii) increase the release of interferon gamma by modified T lymphocytes by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 30%, 40% or 50%; and/or (iii) increase the cytotoxicity of modified T lymphocytes by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%, as compared to control. In certain embodiments, an effective amount of lenalidomide is an amount that, when administered in one or more doses to a subject in need thereof, increases the level of cytotoxic activity of a T lymphocyte expressing a CAR by least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%, as compared to the cytotoxic activity of the T lymphocyte in the absence of lenalidomide. Determination of these parameters is well within the skill of the art. These considerations, as well as effective formulations and administration procedures are well known in the art and are described in standard textbooks.

[0054] The term "EGFRvIII binding domain" refers to a polypeptide(s) (*e.g.*, an antibody or antibody fragment) that recognizes and specifically binds to EGFRvIII. In some embodiments, the EGFRvIII binding domain is a single-chain Fv (scFv).

[0055] The phrase “epidermal growth factor variant III” or “EGFRvIII” refers to a deletion mutant of the epidermal growth factor receptor (EGFR), also known as Δ EGFR, Δ 801EGFR and de2-7 EGFR, which is described in U.S. Pat. Nos. 6,455,498, 6,127,126, 5,981,725, 5,814,317, 5,710,010, 5,401,828, and 5,212,290. The EGFRvIII deletion mutant has a deletion of exons 2 to 7 of the EGFR gene, which can be the result of an apparent splice joining of exon 1 to exon 8 or a chromosomal deletion. EGFRvIII is a common truncated receptor found in human tumors, including, but not limited to, non-small-cell lung cancer (NSCLC), high and low grade gliomas, pediatric gliomas, medullablastomas, breast cancer, ovarian cancer and prostate cancer, but is rarely observed in normal tissue.

[0056] As used herein, “expression” refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0057] “Glioma” is a type of tumor that occurs in the brain and spinal cord. Gliomas begin in the gluey supportive cells (glial cells) that surround nerve cells and help them function. Three types of glial cells can produce tumors, from which gliomas are classified. The three types of gliomas include: (i) astrocytomas, including astrocytoma, anaplastic astrocytoma and glioblastoma multiform (GBM); (ii) ependymomas, including anaplastic ependymoma, myxopapillary ependymoma and subependymoma; (iii) oligodendrogliomas, including oligodendroglioma, anaplastic oligodendroglioma and anaplastic oligoastrocytoma. Gliomas are one of the most common types of primary brain tumors.

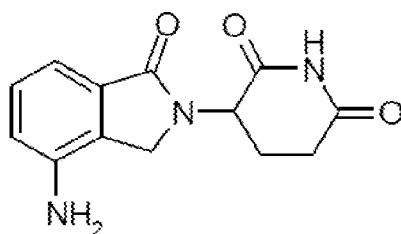
[0058] An “immune synapse” or “immunological synapse” refers to any stable, flattened interface between a T lymphocyte or natural killer cell and a target cell that the T lymphocyte or natural killer cell is in the process of recognizing. In the context of the present disclosure, at the synapse are molecular clusters of T-cell receptors and peptide-loaded major histocompatibility complex molecules on the surface of allophycocyanins that are enriched in F-actin (see, *e.g.*, Dustin, M.L. and Depoil, D., *Nat Rev Immunol* 2011; 11: 672). A mature immune synapse forms a specific pattern of receptor segregation, with a central cluster of T-cell receptors surrounded by a ring of integrin family adhesion molecules such as lymphocyte function-associated antigen-1 (see, *e.g.*, Grakoui, A. *et al.*, *Science* 1999; 285: 221–227). Accordingly, “enhancing immune synapse formation” refers to increasing the total number

synapses formed by a population of T lymphocytes, increasing the number of molecular clusters at a given synapse, or increasing the binding of the molecules present at the synapse.

[0059] The term “intracellular signaling domain” refers to a polypeptide that transmits a signal to the host cell (*e.g.*, T lymphocyte). Intracellular signaling domains suitable for use in a CAR of the present disclosure includes any desired signaling domain that provides a distinct and detectable signal (*e.g.*, increased production of one or more cytokines by the cell; change in transcription of a target gene; change in activity of a protein; change in cell behavior; cellular proliferation; cellular differentiation; cell survival; or modulation of cellular signaling responses) in response to activation of the CAR (*e.g.*, activated by binding to EGFRvIII). In some embodiments, the intracellular signaling domain includes at least one (*e.g.*, one, two, three, or more) intracellular domain of a protein that is expressed on the surface of T lymphocytes and triggers activation and/or proliferation of T lymphocytes. In some embodiments, the intracellular signaling domain further includes at least one (one, two, three or more) signaling domains from at least one costimulatory protein. For example, chimeric CD28 or 4-1BB can be used with CD3 ζ to transmit a signal, or all three can be used together.

[0060] As used herein, the term “lenalidomide,” also known in the art as REVLIMID, refers to 3-(4-amino-1-oxo 1,3-dihydro-2*H*-isoindol-2-yl) piperidine-2,6-dione.

Lenalidomide has the following chemical structure:



[0061] The empirical formula for lenalidomide is C₁₃H₁₃N₃O₃, and the gram molecular weight is 259.3. Lenalidomide is an off-white to pale-yellow solid powder. It is soluble in organic solvent/water mixtures, and buffered aqueous solvents. Lenalidomide is more soluble in organic solvents and low pH solutions. Solubility was significantly lower in less acidic buffers, ranging from about 0.4 to 0.5 mg/ml. Lenalidomide has an asymmetric carbon atom and can exist as the optically active forms S(-) and R(+), and is produced as a racemic mixture with a net optical rotation of zero.

[0062] Lenalidomide is commercially available as REVLIMID in 2.5 mg, 5 mg, 10 mg, 15 mg, 20 mg and 25 mg capsules for oral administration. Each capsule contains lenalidomide as the active ingredient and the following inactive ingredients: lactose anhydrous, microcrystalline cellulose, croscarmellose sodium, and magnesium stearate.

[0063] A “linker,” “spacer,” or “hinge” refers to polypeptide that provides structural flexibility and spacing to flanking polypeptide regions. In the context of the present disclosure, such a polypeptide links an antigen binding domain to a transmembrane domain of a CAR. In certain embodiments, the linker, spacer or hinge is flexible enough to allow the EGFRvIII binding domain to orient in different directions and to facilitate recognition of EGFRvIII. The linker, spacer or hinge may be of natural occurrence or non-natural occurrence, including, but not limited to, an altered hinge region as described in U.S. Pat. No. 5,677,425, a complete hinge region from an antibody of a different class or subclass from that of the CH1 domain, or regions from CD8 (*e.g.*, CD8 α chain), CD28 or another receptor that provides a similar function in providing flexibility and spacing to flanking regions.

[0064] A “modified T lymphocyte” refers to a T lymphocyte that has been genetically altered to have a nucleic acid sequence introduced, deleted or mutated in comparison to a naturally occurring T lymphocyte. In the context of the present disclosure, a nucleic acid sequence encoding a CAR, for example, can be introduced to a T lymphocyte. The source of a modified T lymphocyte can be the subject himself/herself or another subject, preferably from a subject from who is immune compatible with the subject who will be receiving the modified T lymphocytes.

[0065] As used herein, the term “pharmaceutically acceptable carrier” encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin (1975) Remington's Pharm. ScL, 15th Ed. (Mack Publ. Co., Easton).

[0066] The terms “proliferation,” “proliferate,” or any grammatical equivalent thereof, when used in reference to cells, refers to an increase in number. “Increasing the proliferation” of cells includes growing by rapid production of new cells.

[0067] “Relative survival” refers to the ratio of the proportion of observed survivors in a cohort of cancer patients to the proportion of expected survivors in a comparable set of cancer free individuals. The formulation is based on the assumption of independent competing causes of death. The relative survival of a disease is calculated by dividing the overall survival after diagnosis by the survival as observed in a similar population that was not diagnosed with that disease. A similar population is composed of individuals with at least age and gender similar to those diagnosed with the disease.

[0068] The term “responsiveness” or “responsive” when used in reference to a treatment refers to the degree of effectiveness of the treatment in lessening or decreasing the symptoms of a disease, disorder, or condition being treated. For example, the term “increased responsiveness” when used in reference to a treatment of a patient refers to an increase in the effectiveness in lessening or decreasing the symptoms of the disease when measured using any methods known in the art. In certain embodiments, the increase in the effectiveness is at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, or at least about 50%.

[0069] “Single-chain Fv” or “scFv” antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains, which enables the scFv to form the desired structure for antigen binding.

[0070] The terms “subject,” “individual” or “patient” are used interchangeably herein, and refer to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, rats, rabbits, simians, bovines, ovines, porcines, canines, felines, farm animals, sport animals, pets, equines, and primates, particularly humans. In some embodiments, the subject is a female. In some embodiments, the subject is a male. In further embodiments, the patient is a child. In some embodiments, the patient is a patient in need of treating a disease associated with EGFRvIII expression. In some embodiments, a subject is a human having a cancer or a tumor.

[0071] A “T lymphocyte” or “T cell” includes all types of immune cells expressing CD3 including T-helper cells ($CD4^+$ cells), cytotoxic T-cells ($CD8^+$ cells), T-regulatory cells (Treg) and gamma-delta T cells.

[0072] A “transmembrane domain” refers to a hydrophobic alpha helix polypeptide that spans the cell membrane of the T lymphocyte. The transmembrane domain of the CAR can be interposed between the antigen binding domain and the intracellular signaling domain. Where the CAR includes a linker, spacer or hinge polypeptide, the transmembrane domain is interposed between the linker, spacer or hinge polypeptide and the intracellular domain, such that the CAR comprises, in order from the amino terminus (N-terminus) to the carboxyl terminus (C-terminus): an antigen binding domain; a linker, spacer or hinge polypeptide; a transmembrane domain; and an intracellular domain. Any transmembrane domain that provides for insertion of a polypeptide into the cell membrane of a eukaryotic (*e.g.*, mammalian) cell is suitable for use. In some embodiments, the transmembrane domain from the most membrane proximal component of the intracellular signaling domain is used.

[0073] As used herein the terms “treat,” “treating” and “treatment” contemplate an action that occurs while a subject is suffering from the specified disease, disorder or condition, which reduces the severity or symptoms of the disease, disorder or condition, or retards or slows the progression or symptoms of the disease, disorder or condition.

[0074] The practice of the embodiments provided herein will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and immunology, which are within the skill of those working in the art. Such techniques are explained fully in the literature. Examples of particularly suitable texts for consultation include the following: Sambrook *et al.*, *Molecular Cloning; A Laboratory Manual* (2d ed.), 1989; Glover, ed. *DNA Cloning*, Volumes I and II, 1985; Gait, ed., *Oligonucleotide Synthesis*, 1984; Hames & Higgins, eds. *Nucleic Acid Hybridization*, 1984; Hames & Higgins, eds., *Transcription and Translation*, 1984; Freshney, ed., *Animal Cell Culture*, 1986; *Immobilized Cells and Enzymes*, IRL Press, 1986; *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes, *Protein Purification: Principles and Practice* (2d ed.; Springer Verlag, N.Y.), 1987; Weir and Blackwell, eds. *Handbook of Experimental Immunology*, Volumes I-IV, 1986; Harlow *et al.*, in *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY, 1999; and Harlow *et al.*, in *Antibodies: A Laboratory Manual*, Cold Spring Harbor, New York, 1989.

6.2. Chimeric Antigen Receptor (CAR)

6.2.1. CAR Structure

[0075] CARs are engineered receptors that graft an arbitrary specificity onto an immune effector cell. These receptors can be used to graft the specificity of a monoclonal antibody onto a T lymphocyte using retroviral vector expression techniques. The most common form of these molecules is fusions of scFv from monoclonal antibodies, fused to CD3-zeta transmembrane and an intracellular signaling domain. Such molecules result in the transmission of a zeta signal in response to recognition by the scFv of its target. “First-generation” CARs can have the intracellular domain from the CD3 ζ chain, which is the primary transmitter of signals from endogenous TCRs. “Second-generation” CARs add intracellular signaling domains from various costimulatory protein receptors (*e.g.*, CD28, 4-1BB, ICOS) to the cytoplasmic tail of the CAR to provide additional signals to the T lymphocyte. More recent, “third-generation” CARs combine multiple signaling domains, such as CD3 ζ -CD28-4-1BB or CD3 ζ -CD28-OX40, to further augment potency (*see, e.g.*, Zhao, *et al.*, *J. Immunol.*, 183:5563-5574 (2009); Pule, *et al.*, *Mol. Ther.*, 12:933-941 (2005); and Zhong, *et al.*, *Mol. Ther.*, 18:413-420 (2010)).

[0076] In some embodiments, provided herein is a CAR expressed by a modified T lymphocyte, which results in the modified T lymphocyte targeting EGFRvIII expressing cells, such as cancer cells or tumor cells. Such a CAR includes, at a minimum, an antigen binding domain (*e.g.*, anti-EGFRvIII scFv), a transmembrane domain (*e.g.*, transmembrane domain of CD8), and an intracellular signaling domain that provides a primary activation signal to the T lymphocyte (*e.g.*, signaling domain of CD3 ζ). Because T lymphocytes require two signals, a primary activation signal and a costimulatory signal, in order to activate, a CAR useful in the disclosed methods can further comprise a costimulatory domain (*e.g.*, signaling domain of CD28 or 4-1BB) such that binding of the antigen to the extracellular domain results in transmission of both a primary activation signal and a costimulatory signal.

[0077] In some embodiments, the intracellular signaling domain of the CAR includes a signaling domain from a costimulatory protein in addition to a primary signaling domain. Thus, the CAR can include an antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising at least one costimulatory signaling domain and a signaling domain from a protein that is expressed on the surface of T lymphocytes and triggers activation and/or proliferation. In some embodiments, the CAR includes an antigen

binding domain, a transmembrane domain and an intracellular signaling domain comprising at least two costimulatory signaling domains and a signaling domain from a protein that is expressed on the surface of T lymphocytes and triggers activation and/or proliferation. In some embodiments, the CAR includes an antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising at least three costimulatory signaling domains and a signaling domain from a protein that is expressed on the surface of T lymphocytes and triggers activation and/or proliferation. When the intracellular signaling domain comprises more than one signaling domain (*e.g.*, a primary signaling domain and two costimulatory signaling domains), the order of the domains from the amino terminus (N-terminus) to the carboxyl terminus (C-terminus) can be in any order. Exemplary orders of the costimulatory and primary signaling domains include from N-terminus to C-terminus: (i) two costimulatory domains and the primary signaling domain; (ii) the primary signaling domain and two costimulatory domains; or (iii) one costimulatory domain, one primary signaling domain and one costimulatory domain. It is recognized that additional variations of the signaling domains of a CAR will depend upon the number and/or types of domains present in the intracellular signaling domain, and can be readily determined by a skilled artisan.

[0078] In some embodiments, the CAR includes a linker, spacer or hinge polypeptide (*e.g.*, CD8 α chain) that is inserted between the transmembrane domain and the antigen binding domain. Such CAR comprises, in order from N-terminus to C-terminus: an antigen binding domain; a linker, spacer or hinge polypeptide; a transmembrane domain; and an intracellular domain.

[0079] In some embodiments, the CAR can further include a leader sequence at the N-terminus of the extracellular antigen binding domain, wherein the leader sequence is optionally cleaved from the antigen binding domain (*e.g.*, scFv) during cellular processing and localization of the CAR to the cellular membrane. Inclusion of such a leader sequence can increase cellular processing of the CAR and localization of the CAR to the cellular membrane of the T lymphocyte.

[0080] In a specific embodiment, the CAR-expressing modified T lymphocytes used in the disclosed methods comprise a CAR having an anti-EGFRvIII binding domain, a transmembrane domain and an intracellular domain, wherein the anti-EGFRvIII binding domain comprises an scFV, wherein the transmembrane domain comprises the transmembrane domain of CD8, wherein the anti-EGFRvIII binding domain is joined to the

transmembrane domain by the hinge region of CD8 α chain, and wherein the intracellular domain comprises the signaling domain of CD28, 4-1BB and CD3 ζ .

6.2.2. CAR Extracellular Domain

[0081] The extracellular domain of the CAR described herein binds to an antigen of interest. In certain embodiments of any of the CARs described herein, the extracellular domain comprises a receptor, or a portion of a receptor, that binds to the antigen (*e.g.*, EGFRvIII). The extracellular domain may be, *e.g.*, a receptor, or a portion of a receptor, that binds to the antigen (*e.g.*, EGFRvIII). In certain embodiments, the extracellular domain comprises, or is, an antibody or an antibody fragment. In specific embodiments, the extracellular domain comprises, or is, an scFv. The scFv can comprise, for example, a V_L linked to V_H by a flexible linker, wherein said V_L and V_H are from an antibody that binds the antigen (*e.g.*, EGFRvIII).

[0082] In some embodiments, provided herein is a CAR comprising an extracellular domain that recognizes and binds EGFRvIII when expressed by a target cell, such as a cancer or tumor cell. Such an extracellular domain, in some embodiments, is an anti-EGFRvIII binding domain that comprises an antibody or antibody fragment, as described herein. In a specific embodiment, the antibody fragment is an scFv. In a more specific embodiment, the antibody fragment is an scFv derived from the antibody 3C10 (*see, e.g.*, Takasu *et al.*, *J. Neurooncol.* 63(3):247-56 (2003)).

[0083] The antigen to which the extracellular domain of the CAR binds can be EGFRvIII expressed by a cancer cell or tumor cell. The cell may be, *e.g.*, a cell in a solid tumor, or a cell of a blood cancer. In some embodiments, the EGFRvIII is expressed on a cancer cell or tumor cell that arises from or is located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof. In more specific embodiments, the tumor cells are glioma brain tumor cells. In still more specific embodiments, the tumor cell is an astrocytoma brain tumor cell. In still further more specific embodiments, the tumor cell is a glioblastoma multiforme brain tumor cell. In additional embodiments, the cancer cells or tumor cells expressing the EGFRvIII are anaplastic astrocytoma cells, giant cell glioblastoma cells, gliosarcoma cells, anaplastic oligodendroglioma cells, anaplastic ependymoma cells, choroid plexus carcinoma cells, anaplastic ganglioglioma cells, pineoblastoma cells, medulloepithelioma cells, ependymoblastoma cells, medulloblastoma cells, supratentorial primitive neuroectodermal

tumor cells, or atypical teratoid/rhabdoid tumor cells. Accordingly, in some embodiments, the cancer cells or tumor cells are anaplastic astrocytoma cells. In some embodiments, the cancer cells or tumor cells are giant cell glioblastoma cells. In some embodiments, the cancer cells or tumor cells are gliosarcoma cells. In some embodiments, the cancer cells or tumor cells are anaplastic oligodendroglioma cells. In some embodiments, the cancer cells or tumor cells are anaplastic ependymoma cells. In some embodiments, the cancer cells or tumor cells are choroid plexus carcinoma cells. In some embodiments, the cancer cells or tumor cells are anaplastic ganglioglioma cells. In some embodiments, the cancer cells or tumor cells are pineoblastoma cells. In some embodiments, the cancer cells or tumor cells are medulloepithelioma cells. In some embodiments, the cancer cells or tumor cells are ependymblastoma cells. In some embodiments, the cancer cells or tumor cells are medulloblastoma cells. In some embodiments, the cancer cells or tumor cells are supratentorial primitive neuroectodermal tumor cells. In some embodiments, the cancer cells or tumor cells are atypical teratoid/rhabdoid tumor cells. In some embodiments, the cancer cells or tumor cells expressing EGFRvIII are metastatic cancer cells or metastatic tumor cells. Alternatively, the cancer cells or tumor cells are primary cancer cells or primary tumor cells.

[0084] Methods of generating a CAR having an extracellular domain that binds EGFRvIII are well known in the art. Exemplary methods for generating such a CAR are found in Ohno *et al.*, *Cancer Sci* 2010; 101: 2518–2524, Ohno *et al.*, *J Immunother Cancer* 2013; 1: 21, and U.S. Patent Application Publication No. 2014-0322275, published October 30, 2014, which is incorporated herein by reference. Additionally, methods for generating anti-EGFRvIII antibodies and antibody fragments useful for generating a CAR having an extracellular domain that binds EGFRvIII are described in U.S. Patent Application Publication No. 20150315288, published November 5, 2015, International Patent Application No. WO 2001/062931, published August 30, 2001, Kuan *et al.*, *Endocr Relat Cancer*, 8(2):83-96 (2001), Kuan *et al.*, *Brain Tumor Pathol*, 17(2):71-78 (2000), Kuan *et al.*, *International Journal of Cancer*, 88(6):962-969 (2000), Landry *et al.*, *Journal of Molecular Biology*, 308(5):883-893 (2001), Reist *et al.*, *Nuclear Medicine and Biology*, 26(4):405-411 (1999), Reist *et al.*, *Nuclear Medicine and Biology*, 24(7):639-647 (1997), Wikstrand *et al.*, *Cancer Immunology, Immunotherapy*, 50(12):639-652 (2002), Wikstrand *et al.*, *Cancer Research*, 55(14):3140-3148 (1995), Wikstrand *et al.*, *J. Neurovirol*, 4(2):148-158 (1998), Wikstrand *et al.*, *J. Neurovirol*, 4(2):148-158 (1998), Jungbluth *et al.*, *Proc Natl Acad Sci USA*, 100(2):639-44 (2003), Mamot *et al.*, *Cancer Research*, 63:3154-3161 (2003)), and

Nakayashiki *et al.*, *Jpn. J. Cancer Res.*, 91(10):1035-43 (2000), which describe various antibodies directed against EGFRvIII and variants thereof. The antibodies and antibody fragments described in the art can be adopted for incorporation into a CAR using well known methods, such as those described in Example 1.

6.2.3. CAR Transmembrane Domain

[0085] In some embodiments, provided herein is a CAR comprising a transmembrane domain that is thermodynamically stable in a cell membrane. The transmembrane region can be any transmembrane region that can be incorporated into a functional CAR. In certain embodiments, the transmembrane domain of the CAR is from an immune system protein that normally transmits a signal (inhibitory signal or activation signal) to the immune cell (*e.g.*, T lymphocyte). In a specific embodiment, the transmembrane domain is selected from alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. Accordingly, in a specific embodiment, the transmembrane domain comprises the transmembrane domain of alpha chain of the T-cell receptor (TCR- α). In a specific embodiment, the transmembrane domain comprises the transmembrane domain of beta chain of the T-cell receptor (TCR- β). In a specific embodiment, the transmembrane domain comprises the transmembrane domain of zeta chain of the T-cell receptor (CD3 ζ). In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD28. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD3 epsilon. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD45. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD4. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD5. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD8. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD9. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD16. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD22. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD33. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD37. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD64. In a specific embodiment, the transmembrane domain comprises the

transmembrane domain of CD80. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD86. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD134. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD137. In a specific embodiment, the transmembrane domain comprises the transmembrane domain of CD154.

[0086] In specific embodiments of the CAR described herein, in which the transmembrane domain of the polypeptide is from alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 or CD154, the transmembrane domain is from a mammalian source, *e.g.*, human, primate, or rodent, *e.g.*, murine. In some embodiments, the transmembrane domain does not comprise amino acids from the intracellular domain, extracellular domain, or either intracellular or extracellular domain described herein.

[0087] In some embodiments of the CAR described herein, the antigen binding domain (*e.g.*, anti-EGFRvIII binding domain) is joined to the transmembrane domain by a linker, spacer or hinge polypeptide. The linker, spacer or hinge may be of natural occurrence or non-natural occurrence, including, but not limited to, an altered hinge region as described in U.S. Pat. No. 5,677,425, a complete hinge region from an antibody of a different class or subclass from that of the CH1 domain, or regions from CD8 (*e.g.*, CD8 α chain), CD28 or another receptor that provides a similar function in providing flexibility and spacing to flanking regions. Such a linker, spacer or hinge polypeptide, in some embodiments, is the hinge region of CD8 α chain.

[0088] In constructing the CAR described herein, in certain embodiments, human sequences may be combined with non-human sequences. For example, a CAR comprising human extracellular and intracellular domain amino acid sequences may comprise a transmembrane domain from a non-human species; *e.g.*, may comprise a murine transmembrane domain.

[0089] The amino acid sequences that constitute the transmembrane domain, linker, spacer or hinge of any of the above proteins, including the nucleic acid sequences that encode the amino acid sequences, are well known in the art. Exemplary amino acid and/or nucleic acid sequences for such a domain and/or nucleic acid sequences that can be used to generate such a domain, which is useful for generating a CAR described herein, can be found in U.S. Patent

Application Publication Nos. 2016-0030479, published February 4, 2016, 2015-0152181, published June 4, 2015, 2015-0368342, published December 24, 2015, 2015-0031624, published January 29, 2015, 2015-0030597, January 29, 2015, 2014-0328812, published November 6, 2014, 2014-0322275, published October 30, and 2014, 2014-0106449, published April 17, 2014, and Carpenito, *et al.*, *Proc. Natl. Acad. Sci. USA*, 106:3360-65 (2009), Zhao, *et al.*, *J. Immunol.*, 183:5563-5574 (2009), Pule, *et al.*, *Mol. Ther.*, 12:933-941 (2005), Zhong, *et al.*, *Mol. Ther.*, 18:413-420 (2010), and Huang, *et al.*, *Mol. Ther.*, 16(3):580-9 (2008), which are incorporated herein by reference. Using such disclosures and what is known in the art, it is understood that a skilled artisan can generate a CAR described herein, which is used in a method described herein.

6.2.4. CAR Intracellular Domain

[0090] In some embodiments, provided herein is a CAR comprising an intracellular domain that can direct a T lymphocyte to an antigen, and stimulate the T lymphocyte to kill a cell displaying the antigen. In some embodiments, the intracellular signaling domain that transmits a primary activation signal to the T lymphocyte. Such an intracellular signaling domain transmits a signal to the T lymphocyte to activate and/or proliferate, and, if the antigen is present on a cell surface, to kill the cell expressing the antigen.

[0091] In some embodiments, provided herein is a CAR comprising an intracellular domain of a protein that is expressed on the surface of T lymphocytes and triggers activation or proliferation of T lymphocytes. In a specific embodiment, the intracellular signaling domain comprises the signaling domain of CD3 ζ . In other embodiments, the intracellular signaling domain comprises a signaling domain from CD2, CD5, CD28, TCR η chain, Fc ϵ R1 γ or β chains, MB1 (Ig α) chain, B29 (Ig β) chain, a CD3 polypeptide (ϵ , δ , Δ), a syk family tyrosine kinase (*e.g.*, Syk, ZAP70), or a src family tyrosine kinase (Lck, Fyn, Lyn). In a further specific embodiment, the intracellular signaling domain comprises the signaling domain of one or more T lymphocyte costimulatory proteins and/or the signaling domain of CD3 ζ . Such T lymphocyte costimulatory protein can be selected from the costimulatory domains of CD134 (OX40), CD2, CD27, CD28, CD5, CD30, CD40, ICAM-1, LFA-1, ICOS and CD137 (4-1BB), and combinations thereof. Accordingly, in some embodiments, the intracellular signaling domain comprises the signaling domain of CD2. In some embodiments, the intracellular signaling domain comprises the signaling domain of CD27. In some embodiments, the intracellular signaling domain comprises the signaling domain of OX40.

In some embodiments, the intracellular signaling domain comprises the signaling domain of CD28. In some embodiments, the intracellular signaling domain comprises the signaling domain of CD5. In some embodiments, the intracellular signaling domain comprises the signaling domain of CD30. In some embodiments, the intracellular signaling domain comprises the signaling domain of CD40. In some embodiments, the intracellular signaling domain comprises the signaling domain of ICAM-1. In some embodiments, the intracellular signaling domain comprises the signaling domain of LFA-1. In some embodiments, the intracellular signaling domain comprises the signaling domain of ICOS. In some embodiments, the intracellular signaling domain comprises the signaling domain of 4-1BB.

[0092] In some embodiments, the intracellular signaling domain comprises two signaling domains selected from OX40, CD2, CD27, CD28, CD5, CD30, CD40, ICAM-1, LFA-1, ICOS and 4-1BB. Exemplary combinations of signaling domains include, but are not limited to: CD28 and 4-1BB; OX40 and CD27; ICOS and 4-1BB; CD5 and CD2; and ICAM-1 and LFA-1.

[0093] In some embodiments, the intracellular signaling domain comprises three signaling domains, which can include any combination of signaling domain that transmits a primary activation signal to the T lymphocyte and signaling domain of one or more T lymphocyte costimulatory protein. Exemplary combinations of signaling domains include, but are not limited to: CD28, 4-1BB and CD3 ζ ; OX40, CD27 and CD3 ζ ; ICOS, 4-1BB and CD3 ζ ; CD5, CD2 and CD3 ζ ; and ICAM-1, LFA-1 and CD3 ζ .

[0094] The amino acid sequences that constitute an intracellular signaling domain that transmits a primary activation signal to the T lymphocyte and/or an intracellular signaling domain of a T lymphocyte costimulatory protein as described above, including the nucleic acid sequences that encode the amino acid sequences, are well known in the art. Exemplary amino acid and/or nucleic acid sequences for such a domain and/or nucleic acid sequences that can be used to generate such a domain, which is useful for generating a CAR described herein, can be found in U.S. Patent Application Publication Nos. 2016-0030479, published February 4, 2016, 2015-0152181, published June 4, 2015, 2015-0368342, published December 24, 2015, 2015-0031624, published January 29, 2015, 2015-0030597, January 29, 2015, 2014-0328812, published November 6, 2014, 2014-0322275, published October 30, 2014, 2014-0106449, published April 17, 2014, and Carpenito, *et al.*, *Proc. Natl. Acad. Sci. USA*, 106:3360-65 (2009), Zhao, *et al.*, *J. Immunol.*, 183:5563-5574 (2009), Pule, *et al.*,

Mol. Ther., 12:933-941 (2005), Zhong, *et al.*, *Mol. Ther.*, 18:413-420 (2010), and Huang, *et al.*, *Mol. Ther.*, 16(3):580-9 (2008), which are incorporated herein by reference. Using such disclosures and what is known in the art, it is understood that a skilled artisan can generate a CAR described herein, which is used in a method described herein.

6.3. CAR T-Cells

[0095] Provided herein are genetically modified cells, for example immune cells, such as T lymphocytes, *e.g.*, human T lymphocytes, that comprise a CAR described herein. In a specific embodiment, provided herein is a cell, *e.g.*, a T lymphocyte, expressing a CAR, wherein the CAR comprises an antigen binding domain (*e.g.*, anti-EGFRvIII binding domain) as described herein, a transmembrane domain as described herein, and an intracellular signaling domain as described herein.

[0096] In any of the embodiments herein, wherein the modified cells are T lymphocytes, the T lymphocytes may be CD4⁺ T lymphocytes or CD8⁺ T lymphocytes. In some embodiments, the T lymphocytes may be, without genetic modification, and specific for a particular antigen (*e.g.*, EGFRvIII). The T lymphocytes may be genetically modified to express one or more CAR that targets the T lymphocyte to a specific antigen (*e.g.*, EGFRvIII).

[0097] In a specific embodiment, the cells comprising the polypeptides provided herein are T lymphocytes. The T lymphocytes comprising the polypeptides provided herein may be naive T lymphocytes or MHC-restricted T lymphocytes. In certain embodiments, the T lymphocytes are tumor infiltrating lymphocytes (TILs). In certain embodiments, the T lymphocytes have been isolated from a tumor biopsy, or have been expanded from T lymphocytes isolated from a tumor biopsy. In certain other embodiments, the T lymphocytes have been isolated from, or are expanded from T lymphocytes expanded from, peripheral blood, cord blood, or lymph.

[0098] The immune cells, *e.g.*, T lymphocytes, used in the present methods are preferably autologous to an individual to whom the T lymphocytes are to be administered. In certain other embodiments, the T lymphocytes are allogeneic to an individual to whom the T lymphocytes are to be administered. Where allogeneic T lymphocytes are used to prepare T lymphocytes, it is preferable to select T lymphocytes that will reduce the possibility of graft-versus-host disease (GVHD) in the individual. For example, in certain embodiments, virus-specific T lymphocytes are selected for preparation of T lymphocytes; such lymphocytes will

be expected to have a greatly reduced native capacity to bind to, and thus become activated by, any recipient antigens. In certain embodiments, recipient-mediated rejection of allogeneic T lymphocytes can be reduced by co-administration to the host of one or more immunosuppressive agents, *e.g.*, cyclosporine, tacrolimus, sirolimus, cyclophosphamide, or the like.

[0099] In one embodiment, T lymphocytes are obtained from an individual, optionally then expanded, and then transformed with a polynucleotide sequence encoding CAR described herein, and optionally then expanded. In another embodiment, T lymphocytes are obtained from an individual, optionally then expanded, and then transformed with a polynucleotide encoding a CAR described herein, and optionally then expanded. Cells containing a polynucleotide encoding a CAR described herein may be selected using one or more selectable markers.

[00100] T lymphocytes, and T lymphocytes comprising a CAR that includes a CD3 ζ signaling domain and a costimulatory domain (*e.g.*, CD28) can be expanded using antibodies to CD3 and the corresponding costimulatory protein, *e.g.*, antibodies attached to beads, or to the surface of a cell culture plate; see, *e.g.*, U.S. Patent Nos. 5,948,893; 6,534,055; 6,352,694; 6,692,964; 6,887,466; and 6,905,681.

[00101] Methods for generating modified T lymphocytes generally involves genetically modifying the cells with an expression vector, or an RNA (*e.g.*, *in vitro* transcribed RNA), comprising nucleotide sequences encoding a CAR of the present disclosure. The genetic modification can be carried out *in vivo*, *in vitro*, or *ex vivo*. In some embodiments, the genetic modification is carried out *ex vivo*.

[00102] The present disclosure also provides a polynucleotide that comprises a nucleotide sequence encoding the CAR of the present disclosure. The polynucleotide will, in some embodiments, be DNA, including, *e.g.*, a recombinant expression vector. The polynucleotide will, in some embodiments, be RNA, *e.g.*, *in vitro* synthesized RNA.

[00103] In some embodiments, a polynucleotide provides for production of a CAR of the present disclosure in a T lymphocyte. In some embodiments, a polynucleotide provides for amplification of the CAR-encoding nucleotide sequence. The nucleotide sequence encoding the CAR of the present disclosure can be operably linked to a transcriptional control element, *e.g.*, a promoter, and enhancer, etc. Suitable promoter and enhancer elements are known in

the art. For expression in a eukaryotic cell, suitable promoters include, but are not limited to, light and/or heavy chain immunoglobulin gene promoter and enhancer elements; cytomegalovirus immediate early promoter; herpes simplex virus thymidine kinase promoter; early and late SV40 promoters; promoter present in long terminal repeats from a retrovirus; mouse metallothionein-I promoter; and various art-known tissue specific promoters.

[00104] Suitable reversible promoters, including reversible inducible promoters are known in the art. Such reversible promoters may be isolated and derived from many organisms, *e.g.*, eukaryotes and prokaryotes. Modification of reversible promoters derived from a first organism for use in a second organism, *e.g.*, a first prokaryote and a second a eukaryote, is well known in the art. Such reversible promoters, and systems based on such reversible promoters but also comprising additional control proteins, include, but are not limited to, alcohol regulated promoters (*e.g.*, alcohol dehydrogenase I (aldA) gene promoter, promoters responsive to alcohol transactivator proteins (AldR), etc.), tetracycline regulated promoters, (*e.g.*, promoter systems including TetActivators, TetON, TetOFF, etc.), steroid regulated promoters (*e.g.*, rat glucocorticoid receptor promoter systems, human estrogen receptor promoter systems, retinoid promoter systems, thyroid promoter systems, ecdysone promoter systems, mifepristone promoter systems, etc.), metal regulated promoters (*e.g.*, metallothionein promoter systems, etc.), pathogenesis-related regulated promoters (*e.g.*, salicylic acid regulated promoters, ethylene regulated promoters, benzothiadiazole regulated promoters, etc.), temperature regulated promoters (*e.g.*, heat shock inducible promoters (*e.g.*, HSP-70, HSP-90, soybean heat shock promoter, etc.), light regulated promoters, synthetic inducible promoters, and the like.

[00105] In some cases, the promoter is a CD8 cell-specific promoter or a CD4 cell-specific promoter. For example, a CD4 gene promoter can be used; see, *e.g.*, Salmon *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:7739; and Marodon *et al.* (2003) Blood 101:3416. As another example, a CD8 gene promoter can be used.

[00106] A nucleotide sequence encoding a CAR of the present disclosure can be present in an expression vector and/or a cloning vector. Where a subject CAR comprises two separate polypeptides, nucleotide sequences encoding the two polypeptides can be cloned in the same or separate vectors. An expression vector can include a selectable marker, an origin of replication, and other features that provide for replication and/or maintenance of the vector. Suitable expression vectors include, *e.g.*, plasmids, viral vectors, and the like.

[00107] Large numbers of suitable vectors and promoters are known to those of skill in the art; many are commercially available for generating a subject recombinant constructs. The following vectors are provided by way of example: pWLneo, pSV2cat, pOG44, PXR1, pSG (Stratagene) pSVK3, pBPV, pMSG and pSVL (Pharmacia).

[00108] Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleotide sequences encoding the desired proteins (*e.g.*, CAR). Suitable expression vectors include, but are not limited to, viral vectors (*e.g.* viral vectors based on vaccinia virus; poliovirus; adenovirus (see, *e.g.*, Li *et al.*, Invest Ophthalmol Vis Sci 35:2543 2549, 1994; Borrás *et al.*, Gene Ther 6:515 524, 1999; Li and Davidson, PNAS 92:7700 7704, 1995; Sakamoto *et al.*, H Gene Ther 5:1088 1097, 1999; WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655); adeno-associated virus (see, *e.g.*, Ali *et al.*, Hum Gene Ther 9:81 86, 1998, Flannery *et al.*, PNAS 94:6916 6921, 1997; Bennett *et al.*, Invest Ophthalmol Vis Sci 38:2857 2863, 1997; Jomary *et al.*, Gene Ther 4:683 690, 1997, Rolling *et al.*, Hum Gene Ther 10:641 648, 1999; Ali *et al.*, Hum Mol Genet 5:591 594, 1996; Srivastava in WO 93/09239, Samulski *et al.*, J. Vir. (1989) 63:3822-3828; Mendelson *et al.*, Virol. (1988) 166:154-165; and Flotte *et al.*, PNAS (1993) 90:10613-10617); SV40; herpes simplex virus; human immunodeficiency virus (see, *e.g.*, Miyoshi *et al.*, PNAS 94:10319 23, 1997; Takahashi *et al.*, J Virol 73:7812 7816, 1999); a retroviral vector (*e.g.*, Lentivirus, Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus); and the like.

[00109] As noted above, in some embodiments, a polynucleotide comprising a nucleotide sequence encoding the CAR of the present disclosure will in some embodiments be RNA, *e.g.*, *in vitro* synthesized RNA. Methods for *in vitro* synthesis of RNA are known in the art, and any known method can be used to synthesize RNA comprising a nucleotide sequence encoding the CAR of the present disclosure. Methods for introducing RNA into a host cell are known in the art (see, *e.g.*, Zhao *et al.* *Cancer Res.* 15:9053 (2010)). Introducing RNA into a host cell can be carried out *in vitro*, *ex vivo* or *in vivo*. For example, a host cell (*e.g.*, a T lymphocyte) can be electroporated *in vitro* or *ex vivo* with RNA comprising a nucleotide sequence encoding the CAR of the present disclosure.

[00110] It is also contemplated that the polynucleotide encoding the CAR of the present disclosure can be introduced into T lymphocytes as naked DNA. Methods of stably transfecting T lymphocytes by electroporation using naked DNA are known in the art (see, *e.g.*, U.S. Pat. No. 6,410,319). Naked DNA generally refers to the DNA encoding a CAR of the present disclosure contained in a plasmid expression vector in proper orientation for expression. Advantageously, the use of naked DNA may reduce the time required to produce T lymphocytes expressing the CAR of the present disclosure.

[00111] Once it is established that the transfected or transduced T lymphocyte (*i.e.*, becomes a modified T lymphocyte) is capable of expressing the CAR as a surface membrane protein with the desired regulation and at a desired level, it can be determined whether the CAR is functional in the host cell to provide for the desired signal induction. Subsequently, the modified T lymphocyte are reintroduced or administered to the subject in combination with lenalidomide to activate the anti-EGFR responses in the subject. To facilitate administration, the modified T lymphocytes of the present disclosure can be made into a pharmaceutical composition or made implant appropriate for administration *in vivo*, with appropriate carriers or diluents, which further can be pharmaceutically acceptable. The means of making such a composition or an implant have been described in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 16th Ed., Mack, ed. (1980)). Where appropriate, the transduced T lymphocyte can be formulated into a preparation in semisolid or liquid form, such as a capsule, solution, injection, inhalant, or aerosol, in the usual ways for their respective route of administration. Means known in the art can be utilized to prevent or minimize release and absorption of the composition until it reaches the target tissue or organ, or to ensure timed-release of the composition. Desirably, however, a pharmaceutically acceptable form is employed that does not ineffectuate the lymphocytes expressing the CAR. Thus, desirably the transduced T lymphocytes can be made into a pharmaceutical composition containing a balanced salt solution, preferably Hanks' balanced salt solution, or normal saline.

[00112] Additionally, the modified T lymphocytes disclosed herein may be formulated in any pharmaceutically-acceptable solution, preferably a solution suitable for the delivery of living cells, *e.g.*, saline solution (such as Ringer's solution), gelatins, carbohydrates (*e.g.*, lactose, amylose, starch, or the like), fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidine, etc. Such preparations are preferably sterilized prior to addition of the cells, and

may be mixed with auxiliary agents such as lubricants, preservatives, stabilizers, emulsifiers, salts for influencing osmotic pressure, buffers, and coloring. Pharmaceutical carriers suitable for use in formulating the cells are known in the art and are described, for example, in WO 96/05309.

6.4. Methods of Treatment

[00113] Provided herein are methods of treating a subject having a disease associated with expression of EGFRvIII. The methods can comprise administering to the subject an effective amount of lenalidomide and modified T lymphocytes expressing a CAR that targets EGFRvIII expressing cells, such as cancer cells or tumor cells. The administration of lenalidomide improves or enhances the activity of the modified T lymphocytes against the EGFRvIII expressing cells. Such modified T lymphocytes, in some aspects, express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00114] In some embodiments, provided herein is a method of treating a subject having a disease associated with expression of EGFRvIII, comprising administering to the subject an effective amount of lenalidomide and modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00115] In some embodiments, the disease associated with expression of EGFRvIII is a cancer. In some embodiments, the disease associated with expression of EGFRvIII is a tumor. The cancer or tumor can be metastatic. Alternatively, the cancer or tumor can be a primary cancer or a primary tumor. Such a primary cancer or primary tumor can arise from or be located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof of the subject. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the brain. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the spinal cord. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the central nervous system. In a specific embodiment, the primary cancer or primary tumor arises from or is located in a lung. In a specific embodiment, the primary cancer or primary tumor arises from or is located in a breast. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the prostate. In a specific embodiment, the primary cancer or primary tumor arises from or is located in an

ovary. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the colon. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the rectum. In a specific embodiment, the primary cancer or primary tumor arises from or is located in the bladder. In a specific embodiment, the tumor is a glioma. In a more specific embodiment, the tumor is an astrocytoma. In a still more specific embodiment, the tumor is a glioblastoma multiforme.

[00116] Additionally, in some embodiments, the disease associated with expression of EGFRvIII is a cancer or tumor selected from glioblastoma multiforme, anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, medulloepithelioma, ependymoblastoma, medulloblastoma, supratentorial primitive neuroectodermal tumor, and atypical teratoid/rhabdoid tumor. Accordingly, in some embodiments, the disease associated with expression of EGFRvIII is glioblastoma multiforme. In some embodiments, the disease associated with expression of EGFRvIII is anaplastic astrocytoma. In some embodiments, the disease associated with expression of EGFRvIII is giant cell glioblastoma. In some embodiments, the disease associated with expression of EGFRvIII is gliosarcoma. In some embodiments, the disease associated with expression of EGFRvIII is anaplastic oligodendroglioma. In some embodiments, the disease associated with expression of EGFRvIII is anaplastic ependymoma. In some embodiments, the disease associated with expression of EGFRvIII is choroid plexus carcinoma. In some embodiments, the disease associated with expression of EGFRvIII is anaplastic ganglioglioma. In some embodiments, the disease associated with expression of EGFRvIII is pineoblastoma. In some embodiments, the disease associated with expression of EGFRvIII is medulloepithelioma. In some embodiments, the disease associated with expression of EGFRvIII is ependymoblastoma. In some embodiments, the disease associated with expression of EGFRvIII is medulloblastoma. In some embodiments, the disease associated with expression of EGFRvIII is supratentorial primitive neuroectodermal tumor. In some embodiments, the disease associated with expression of EGFRvIII is atypical teratoid/rhabdoid tumor.

[00117] In some embodiments, the effective amount of lenalidomide is from about 0.01 mg/kg to about 0.50 mg/kg, or from about 0.05 mg/kg to about 0.10 mg/kg, or from about 0.06 mg/kg to about 0.09 mg/kg. In some embodiments, the effective amount of lenalidomide is from about 0.01 mg/kg to about 0.50 mg/kg. In some embodiments, the

effective amount of lenalidomide is from about 0.05 mg/kg to about 0.10 mg/kg. In some embodiments, the effective amount of lenalidomide is from about 0.06 mg/kg to about 0.09 mg/kg.

[00118] In some embodiments, the effective amount of lenalidomide is from about 0.10 mg to about 150 mg. In some embodiments, the effective amount of lenalidomide is from about 2.5 mg to about 25 mg. In some specific embodiments, the effective amount of lenalidomide is selected from about 2.5 mg, about 5 mg, about 10 mg, about 15 mg, about 20 mg, and about 25 mg. Accordingly, in some embodiments, the effective amount of lenalidomide is about 2.5 mg. In some embodiments, the effective amount of lenalidomide is about 5 mg. In some embodiments, the effective amount of lenalidomide is about 10 mg. In some embodiments, the effective amount of lenalidomide is about 15 mg. In some embodiments, the effective amount of lenalidomide is about 20 mg. In some embodiments, the effective amount of lenalidomide is about 25 mg.

[00119] In some embodiments, the lenalidomide administered to the subject is administered daily for a predetermined period of time following an initial administration of lenalidomide. Such a predetermined period of time can be readily determined by a skilled physician and will depend upon a number of factors including, but not limited to, the amount of lenalidomide employed, the age, body weight, general health, sex, and diet of the subject, the time of administration, the rate of excretion, the combination of modified T lymphocytes and lenalidomide employed, form of administration and the severity of the particular disease associated with EGFRvIII being treated.

[00120] In some embodiments, the lenalidomide and modified T lymphocytes are administered simultaneously. In some embodiments, the lenalidomide and modified T lymphocytes are administered sequentially. In some embodiments, the modified T lymphocytes are administered before the lenalidomide is administered. In some embodiments, the lenalidomide can be administered 1 day, 2 days, 3 days or more after administration of the modified T lymphocytes. For example, the lenalidomide can be administered for a predetermined number of days, weeks, or months.

[00121] In some embodiments, the method of treating provided herein results in the likelihood of the subject's relative survival increasing, including by at least 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. In some embodiments, the likelihood of the subject's relative survival increases by at least 10%. In some embodiments, the

likelihood of the subject's relative survival increases by at least 20%. In some embodiments, the likelihood of the subject's relative survival increases by at least 25%. In some embodiments, the likelihood of the subject's relative survival increases by at least 30%. In some embodiments, the likelihood of the subject's relative survival increases by at least 40%. In some embodiments, the likelihood of the subject's relative survival increases by at least 50%. In some embodiments, the likelihood of the subject's relative survival increases by at least 60%. In some embodiments, the likelihood of the subject's relative survival increases by at least 70%. In some embodiments, the likelihood of the subject's relative survival increases by at least 80%. In some embodiments, the likelihood of the subject's relative survival increases by at least 90%. In some embodiments, the likelihood of the subject's relative survival increases by at least 100%.

[00122] The lenalidomide and modified T lymphocytes of the present disclose can be used alone or in combination with other well-established agents useful for treating cancer. Whether delivered alone or in combination with other agents, the lenalidomide and modified T lymphocytes of the present disclose can be delivered via various routes and to various sites in a subject, particularly human, body to achieve a particular effect. One skilled in the art will recognize that, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. For example, intravenous delivery or directed injection into the brain of the modified T lymphocytes and oral administration of Lenalidomide may be advantageously used over inhalation for the treatment of a glioma brain tumor (*e.g.*, GBM). Local or systemic delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intratumoral, peritumoral, intramuscular, intratracheal, intracranial, subcutaneous, intradermal, topical application, intravenous, intraarterial, rectal, nasal, oral, and other enteral and parenteral routes of administration.

[00123] An effective amount lenalidomide and sufficient number of the modified T lymphocytes of the present disclosure in their respective pharmaceutical compositions, which is introduced into the subject, can be an amount such that long-term, specific, anti-tumor responses are established to: (i) reduce the size of a tumor; or (ii) eliminate tumor growth or regrowth, that would otherwise result in the absence of such treatment. The amount of modified T lymphocytes and lenalidomide introduced into the subject can cause at least a

10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 100% decrease in tumor size when compared to otherwise same conditions wherein the modified T lymphocytes and/or lenalidomide is not present.

[00124] Accordingly, in general, the concentration of modified T lymphocytes should be sufficient to provide in the subject being treated at least from about 1×10^6 to about 1×10^9 modified T lymphocytes, even more desirably, from about 1×10^7 to about 5×10^8 modified T lymphocytes, although any suitable amount can be utilized either above (*e.g.*, greater than 5×10^8 cells), or below (*e.g.*, less than 1×10^7 cells). In certain embodiments, the modified T lymphocytes are formulated into individual doses, wherein said individual doses comprise at least, at most, or about 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 , 1×10^8 , 5×10^8 , 1×10^9 , 5×10^9 , 1×10^{10} , 5×10^{10} , or 1×10^{11} T lymphocytes. The dosing schedule can be based on well-established cell-based therapies (see, *e.g.*, Topalian and Rosenberg (1987) *Acta Haematol.* 78 Suppl 1:75-6; U.S. Pat. No. 4,690,915) or an alternate continuous infusion strategy can be employed.

[00125] Administration of the anti-EGFRvIII CAR T cells can be on the same dosing schedule as lenalidomide, or a different dosing schedule. The anti-EGFRvIII CAR T cells can be administered on the same day as the lenalidomide, or on a different day.

[00126] These values provide general guidance of the range of modified T lymphocytes and lenalidomide to be utilized by a skilled physician upon optimizing the method of the present disclosure. The recitation herein of such ranges by no means precludes the use of a higher or lower amount of a component, as might be warranted in a particular application. A skilled physician readily can make any necessary adjustments in accordance with the exigencies of the particular situation.

6.5. Methods of Using Lenalidomide

[00127] Provided herein are methods of increasing the release of interferon gamma by modified T lymphocytes. Such methods can comprise, contacting the modified T lymphocytes with an effective amount of lenalidomide. In some embodiments, the modified T lymphocytes used in the disclosed methods express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00128] In some embodiments, provided herein is a method of increasing the release of interferon gamma by modified T lymphocytes comprising, contacting the modified T lymphocytes with an effective amount of lenalidomide, wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00129] In some embodiments, the release of interferon gamma is increased by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% , 20%, 21%, 22%, 23%, 24%, 25%, 30%, 40% or 50%. Accordingly, in some embodiments, the release of interferon gamma is increased by at least 5%. In some embodiments, the release of interferon gamma is increased by at least 6%. In some embodiments, the release of interferon gamma is increased by at least 7%. In some embodiments, the release of interferon gamma is increased by at least 8%. In some embodiments, the release of interferon gamma is increased by at least 9%. In some embodiments, the release of interferon gamma is increased by at least 10%. In some embodiments, the release of interferon gamma is increased by at least 11%. In some embodiments, the release of interferon gamma is increased by at least 12%. In some embodiments, the release of interferon gamma is increased by at least 13%. In some embodiments, the release of interferon gamma is increased by at least 14%. In some embodiments, the release of interferon gamma is increased by at least 15%. In some embodiments, the release of interferon gamma is increased by at least 16%. In some embodiments, the release of interferon gamma is increased by at least 17%. In some embodiments, the release of interferon gamma is increased by at least 18%. In some embodiments, the release of interferon gamma is increased by at least 19%. In some embodiments, the release of interferon gamma is increased by at least 20%. In some embodiments, the release of interferon gamma is increased by at least 21%. In some embodiments, the release of interferon gamma is increased by at least 22%. In some embodiments, the release of interferon gamma is increased by at least 23%. In some embodiments, the release of interferon gamma is increased by at least 24%. In some embodiments, the release of interferon gamma is increased by at least 25%. In some embodiments, the release of interferon gamma is increased by at least 30%. In some embodiments, the release of interferon gamma is increased by at least 40%. In some embodiments, the release of interferon gamma is increased by at least 50%.

[00130] Also provided herein are methods of enhancing the cytotoxicity of modified T lymphocytes. Such methods can comprise, contacting the modified T lymphocytes with an effective amount of lenalidomide. In some embodiments, the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00131] In some embodiments, provided herein is a method of enhancing the cytotoxicity of modified T lymphocytes comprising, contacting the modified T lymphocytes with an effective amount of lenalidomide, wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00132] In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. Accordingly, in some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 5%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 10%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 15%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 20%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 25%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 30%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 40%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 50%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 60%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 70%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 80%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 90%. In some embodiments, the cytotoxicity of the modified T lymphocytes is increased by at least 100%.

[00133] Also provided herein are methods of increasing the proliferation of a population of T lymphocytes. Such methods can comprise, contacting the population of T lymphocytes with an effective amount of lenalidomide. In some embodiments, the population of T lymphocytes comprises modified T lymphocytes expressing a CAR, wherein the CAR

comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00134] In some embodiments, provided herein is a method of increasing the proliferation of a population of T lymphocytes comprising, contacting the population of T lymphocytes with an effective amount of lenalidomide, wherein the population of T lymphocytes comprise modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[00135] Accordingly, provided herein is a method of increasing the proliferation of a population of T lymphocytes comprising, contacting the population of T lymphocytes with an effective amount of lenalidomide. In some embodiments, the population of T lymphocytes comprises modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[00136] Exemplary methods of assessing the cytokine production (*e.g.*, interferon gamma), cytotoxicity, and proliferation of T lymphocytes are described herein in Examples 4, 5 and 8, respectively. Additional methods of assessing these parameters are well known in the art. For example, methods of assessing cell proliferation and cytokine production have been previously described by Milone *et al.*, *Molecular Therapy* 17(8): 1453-1464 (2009). Briefly, assessment of proliferation is performed in microtiter plates by mixing washed T cells with target cells, such as U87MG, BHK or CHO cells expressing EGFRvIII or EGFR wildtype (wt) or CD32 and CD137 (KT32-BBL) for a final T-cell:target cell ratio of 1:1. Anti-CD3 and anti-CD28 monoclonal antibodies are added to cultures with KT32-BBL cells to serve as a positive control for stimulating T-cell proliferation since these signals support long-term CD8⁺ T cell expansion *ex vivo*. T cells can be enumerated in cultures using COUNTBRIGHT fluorescent beads (Invitrogen, Carlsbad, Calif.) and flow cytometry as described by the manufacturer. CAR⁺ T cells can be detected with biotinylated recombinant EGFRvIII protein and a secondary avidin-PE conjugate. CD4⁺ and CD8⁺ expression on T cells can also be simultaneously detected with specific monoclonal antibodies (BD Biosciences).

[00137] Cytokine measurements can be performed on supernatants collected 24 hours following re-stimulation using the human TH1/TH2 cytokine cytometric bead array kit (BD Biosciences, San Diego, Calif.) according the manufacturer's instructions. Fluorescence can

be assessed using a FACScalibur flow cytometer, and data is analyzed according to the manufacturer's instructions.

[00138] As another example, cytotoxicity can be assessed by a standard ⁵¹Cr-release assay. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009). Briefly, target cells (U87MG, BHK or CHO cells expressing EGFRvIII or EGFR wildtype (wt) can be loaded with ⁵¹Cr (as NaCrO₄, New England Nuclear, Boston, Mass.) at 37 °C for 2 hours with frequent agitation, washed twice in complete RPMI and plated into microtiter plates. T cells are mixed with target cells in the wells in complete RPMI at varying ratios of effector cell:target cell (E:T). Additional wells containing media only (spontaneous release, SR) or a 1% solution of triton-X 100 detergent (total release, TR) are also prepared. After 4 hours of incubation at 37 °C, supernatant from each well is harvested. Released ⁵¹Cr is then measured using a gamma particle counter (Packard Instrument Co., Waltham, Mass.). Each condition is performed in at least triplicate, and the percentage of lysis is calculated using the formula: % Lysis=(ER-SR)/(TR-SR), where ER represents the average ⁵¹Cr released for each experimental condition. Alternative cytotoxicity assays may also be used, such as flow based cytotoxicity assays, as described in Example 8.

[00139] Also provided herein are methods of enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells. Such methods can comprise, contacting the modified T lymphocytes and the cancer cells or tumor cells with an effective amount of lenalidomide, wherein the cancer cells or tumor cells express EGFRvIII. In some embodiments, the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[00140] In some embodiments, provided herein is a method of enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells comprising, contacting the modified T lymphocytes and the cancer cells or tumor cells with an effective amount of lenalidomide, wherein the cancer cells or tumor cells express EGFRvIII, and wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.

[00141] In some embodiments, the cancer cells or tumor cells expressing EGFRvIII are metastatic cancer cells or metastatic tumor cells. Alternatively, the cancer cells or tumor cells are primary cancer cells or primary tumor cells. Such primary cancer cells or primary tumor

cells can arise from or can be located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof. Accordingly, in some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the brain. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the spinal cord. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the central nervous system. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the a lung. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the a breast. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the prostate. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in an ovary. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the colon. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the rectum. In some embodiments, the primary cancer cells or primary tumor cells can arise from or can be located in the bladder. In a specific embodiment, the tumor cells are glioma brain tumor cells. In as more specific embodiment, the tumor cells are astrocytoma brain tumor cells. In another specific embodiment, the tumor cells are glioblastoma multiforme brain tumor cells.

[00142] In some embodiments, the cancer cells or tumor cells expressing EGFRvIII are selected from anaplastic astrocytoma cells, giant cell glioblastoma cells, gliosarcoma cells, anaplastic oligodendroglioma cells, anaplastic ependymoma cells, choroid plexus carcinoma cells, anaplastic ganglioglioma cells, pineoblastoma cells, medulloepithelioma cells, ependymoblastoma cells, medulloblastoma cells, supratentorial primitive neuroectodermal tumor cells, and atypical teratoid/rhabdoid tumor cells

[00143] An exemplary method of assessing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells is described herein in Example 9. Additional methods of assessing immune synapse formation are well known in the art and can be readily applied by a skilled artisan.

[00144] In some embodiments, the methods described herein can be performed *in vivo* or *in vitro*. For example, in some aspects, the method for enhancing increasing the release of interferon gamma by modified T lymphocytes, the method for enhancing the cytotoxicity of

modified T lymphocytes, the method for increasing the proliferation of a population of T lymphocytes, the method for enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells, includes a method wherein the contacting takes place *in vitro*. In some aspects, the method for enhancing increasing the release of interferon gamma by modified T lymphocytes, the method for enhancing the cytotoxicity of modified T lymphocytes, the method for increasing the proliferation of a population of T lymphocytes, the method for enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells, includes a method wherein the contacting takes place *in vivo*.

7. EXAMPLES

[00145] The examples provided herein show the immunomodulatory abilities of lenalidomide, especially the augmentation of immunological synapses. The examples also show the efficacy of EGFRvIII-targeting CAR T-cell therapy for cancers and tumors, such as GBM.

7.1. Example 1: Construction of a Third-generation 3C10-CAR with a Lentiviral Vector

[00146] The monoclonal antibody 3C10 was originally established using mice immunized against a synthetic 14-amino-acid peptide named Pep3, which was characteristically created at the EGFRvIII-specific fusion junction between amino-acid residues, including a novel glycine residue. Its corresponding scFv antibody was then produced. Using gene sequence data for a third-generation CAR (Ohno M. *et al.*, *J Immunother Cancer* 2013; 1: 21), 3C10-CAR cDNA was generated by gene synthesis (Genscript, Piscataway, NJ, USA). A mock vector was designed to harbor the scrambled sequence of the scFv portion that has shown no functional activity against glioma, breast cancer, colon cancer and pancreatic cancer cell lines.

7.2. Example 2: Preparation of Lentiviral Vector and T-cell Transduction

[00147] Human embryonic kidney 293T cells (8×10^6) were cultured in 175-cm² flasks. At 24 h, self-inactivating vectors, pMDLg/pRRE, pRSV-Rev and pMD2.G, were co-transfected using X-tremeGENE 9 (Roche Applied Science, Roche, Penzberg, Germany). The supernatant was collected at 48 h, mixed with PEG-it Virus Precipitation Solution ($5 \times$) (System Biosciences, Mountain View, CA, USA) and incubated for 24 h at 4 °C. The supernatant/PEG-it mixture was then centrifuged at 1500 g for 30 min at 4 °C, and the pellet

was resuspended in 1/10th of the original volume using cold, sterile medium at 4 °C and stored at -80 °C.

[00148] Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (centrifuged at 1000 g for 20 min at 20 °C). PBMCs were cultured in AIM-V medium (Life Technologies) supplemented with 10% human serum in the presence of interleukin-2 (IL-2; 50 U ml⁻¹; PeproTech, Rocky Hill, NJ, USA) and anti-CD3 monoclonal antibody (catalog no.16-0037-85, 100 ng ml⁻¹; eBioscience, San Diego, CA, USA) for 24 h. PBMCs were harvested, washed once and resuspended and cultured at a density of 1.0×10^6 cells per ml for 24 h. For transduction, thawed viral supernatant having the lentiviral vector of Example 1 was replaced to the medium and the cells were cultured for 24 h, and the medium was then refreshed and the cells were cultured for a further 48 h.

7.3. Example 3: GBM Cell Lines

[00149] The human GBM cell line U87-EGFRvIII stably expressing EGFRvIII and its parental U87MG cell lines described throughout the Examples herein were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) and penicillin/streptomycin (Life Technologies) at 37 °C with 5% CO₂. U87-EGFRvIII cells expressing luciferase (U87-EGFRvIII-LUC) stably expressing luciferase described throughout the Examples herein were generated by transduction with a recombinant lentivirus coding for the luciferase of *Photinuspyralis*. Two days after transduction, the cells were selected in blasticidin for 2 weeks and were cloned by limiting dilution thereafter.

7.4. Example 4: Lenalidomide Increases IFN- γ Release by 3C10-CAR T cells

[00150] In this experiment, the effector cells (3C10-CAR T cells, mock T cells) were incubated with or without lenalidomide (1 μ m) for 48 h and intracellular interferon- γ (IFN- γ) staining was performed. Previously, the third generation of 3C10-CAR T cells was shown to specifically target U87-EGFRvIII cells (Ohno M. *et al.*, *J Immunother Cancer* 2013; 1: 21).

[00151] IFN- γ staining was performed using the Cytotfix/Cytoperm plus GolgiStop Kit (BD Biosciences, Bedford, MA, USA). The effector cells (1×10^5 cells) were incubated with 0.5×10^6 U87-EGFRvIII cells or U87MG cells (Example 3) in 200 μ l AIM-V medium along with GolgiStop in a round-bottomed 96-well plate. Following a 4 h incubation period at 37 °C, the cells were incubated with biotin-SP-AffiniPure F(ab)' 2 fragment-specific goat anti-mouse

immunoglobulin G (catalog no. 115-066-006; Jackson Immuno Research Laboratories, West Grove, PA, USA) at 4 °C for 30 min. After washing, cells were stained with streptavidin-phycoerythrin (PE) (catalog no. 554061; BD Biosciences) and fluorescein isothiocyanate (FITC) mouse anti-human CD8 (catalog no. 555634; BD Biosciences) and incubated for 30 min at 4 °C. The cells were further washed and fixed by adding fixation/permeabilization solution and then incubated at 4 °C for 20 min. Finally, the cells were stained with allophycocyanin mouse anti-human IFN- γ (catalog no. 554702; BD Biosciences) and incubated at 4 °C for 30 min. After washing, cells suspended in 1% paraformaldehyde (Wako, Osaka, Japan) were analyzed by FACSCalibur (BD Biosciences). T cells treated with IL-2 were used as a positive control. After the CD8-FITC-positive population was gated, allophycocyanin and PE positivity was analyzed.

[00152] All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's t-test. Differences were considered significant when the P-value was <0.05 . A post hoc test for the detection of linear trends was used, and $P<0.05$ was considered to be significant.

[00153] First, the transduction efficiency of the lentiviral vector, as described in Example 2, which transduces the 3C10-CAR into CD8 T cells was evaluated by FACS (fluorescence-activated cell sorting) analysis. The transduction efficiency of lentivirus-mediated 3C10-CAR to CD8 T cells was 30–40%, whereas that of the mock CAR was 3–8% (**FIG. 1A**).

[00154] Next, intracellular staining of IFN- γ in 3C10-CAR PBMCs was performed. CAR PBMCs or mock PBMCs (1×10^5 cells) were incubated with 0.5×10^6 U87-EGFRvIII cells or U87MG cells, as described above. PBMCs pretreated with lenalidomide and non-treated PBMCs were used. The proportion of IFN- γ and CAR double-positive cells was 9.84% in 3C10-CAR PBMCs, which were coincubated with U87-EGFRvIII, and 18.82% in lenalidomide-pretreated 3C10-CAR PBMCs (**FIG. 1A**). This experiment was repeated two times and similar results were obtained each time (compare **FIG. 1A** and **FIG 1B**). These results show that lenalidomide treatment increases IFN- γ release by 3C10-CAR T cells.

7.5. Example 5: Lenalidomide Enhances 3C10-CAR T cell Cytolytic Effects on EGFRvIII-expressing Glioma Cells

[00155] In this experiment, the effects of lenalidomide on the killing of 3C10-CAR T cells were evaluated.

[00156] Target (T) cells (U87-EGFRvIII, U87MG) were suspended at a final concentration of 1.0×10^6 cells per ml and incubated with 10 μ m calcein-AM (Dojindo, Kumamoto, Japan) for 30 min at 37 °C, with occasional shaking. After two washes, cells were adjusted to a concentration of 1.0×10^5 cells per ml, and 1.0×10^4 cells (100 μ l) were placed into a round-bottomed 96-well plate. The effector and target cells were then added to each well at various E:T ratios and incubated at 37 °C for 4 h. The plate was centrifuged at low speed (1000 g, 5 min), and 75 μ l of supernatant was carefully aspirated and loaded into a lumaplate. The fluorescence was recorded using a 490-nm excitation filter and a 520-nm emission filter. Only target cells in medium with 1.5 μ l of 10% sodium dodecyl sulfate were used for maximum release. The percentage of specific lysis was calculated as follows: (experimental release–spontaneous release)/(maximum release–spontaneous release) $\times$ 100 (%). To exclude the influence of activation of natural killer (NK) cells, CD3+ cells were selected from the PBMCs using CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Selected CD3+ cells were transduced with viral supernatant to express 3C10-CAR (3C10-CAR-CD3 cells).

[00157] All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's t-test. Differences were considered significant when the P-value was <0.05. A post hoc test for the detection of linear trends was used, and P<0.05 was considered to be significant.

[00158] The above calcein assay confirmed that even without lenalidomide treatment, PBMCs transduced with 3C10-CAR lysed ~50% of the U87-EGFRvIII cells at an E:T ratio of 25:1, and lysis occurred in an E:T ratio-dependent manner. Mock PBMCs did not demonstrate this killing effect.

[00159] The effector cells (3C10-CAR-CD3 cells, mock-CD3 cells) were also incubated with or without lenalidomide (1 μ m) for 48 h, and the calcein assay was performed as mentioned above. The assay was repeated three times. 3C10-CAR PBMCs pretreated with

lenalidomide lysed ~80% of the U87-EGFRvIII cells at an E:T ratio of 25:1. Mock PBMCs treated with lenalidomide were not effective, similar to the results for those without lenalidomide (**FIG. 2**, left).

[00160] CD3⁺ cells were selectively isolated from PBMCs using CD3 microbeads to exclude the influence of activation of natural killer cells. The selected CD3⁺ cells were transduced with viral supernatant, as described in Example 2, to express 3C10-CAR (3C10-CAR CD3 T cells). 3C10-CAR CD3 T cells exerted a similar cytotoxicity to that of 3C10-CAR PBMCs. Additionally, lenalidomide enhanced the cytotoxicity of 3C10-CAR CD3 T cells (**FIG. 2**, center). Lenalidomide-pretreated 3C10-CAR PBMCs exhibited no killing effect on the parental U87MGs with no EGFRvIII expression, similar to the results seen for the other effector cells (i.e., original 3C10-CAR cells and mock PBMCs with or without lenalidomide) (**FIG. 2**, right). These results show that lenalidomide treatment enhances the 3C10-CAR T cells cytolytic effects on EGFRvIII-expressing glioma cells.

7.6. Example 6: Lenalidomide Increases the Antitumor Effect of 3C10-CAR T cells in Intracranial Glioma Xenograft Mice

[00161] NOD/Shi-scid, IL-2R γ -null (NOG) 5- to 6-week-old female mice (Central Institute for Experimental Animals, Kawasaki, Japan) were used for individual experiments. Animals were handled in the Animal Facility at Nagoya University in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Mice were anesthetized with an intraperitoneal injection of pentobarbital (somnopentyl; 150–160 mg kg⁻¹ body weight; Kyoritsu, Tokyo, Japan). The mice were shaved and an incision was made in the scalp, and then a burr hole was made in the skull, 3-mm lateral to the midline and 4-mm posterior to the bregma, using an 18-gauge needle. Using a stereotactic apparatus, the U87-EGFRvIII-LUC cells (5.0×10^4 cells) suspended in 5 μ l of phosphate-buffered saline (PBS) were injected at a depth of 2 mm below the dura matter. A sterile Hamilton syringe fitted with a 26-gauge needle was used with a microsyringe pump. Mice bearing established tumors were randomly assigned to two different experimental groups. Seven days after the inoculation, 2.0×10^6 3C10-CAR PBMCs (13 mice) or mock PBMC cells (12 mice) were infused into the tail vein. Lenalidomide (5 mg kg⁻¹) (13 mice) or PBS (12 mice) was given intraperitoneally for 35 consecutive days, beginning after the PBMC infusion (Sakamaki, I. *et al.*, *Leukemia* 2014; 28: 329–337).

[00162] U87-EGFR ν III-LUC cells within the brain were monitored noninvasively by bioluminescence imaging using the *in vivo* imaging system, IVIS (Xenogen, Advanced Molecular Vision, Lincolnshire, UK). Mice were injected intraperitoneally with 200 μ l of a freshly thawed aqueous solution of d-luciferin potassium salt (15 mg ml⁻¹) (Sigma-Aldrich), anesthetized with isoflurane and imaged for bioluminescence using a 1-min exposure time. Optical images were analyzed using the IVIS living image software package (Summit pharmaceuticals International, Tokyo, Japan). Overall survival following tumor inoculation was monitored.

[00163] The schema for the animal experiments described herein are summarized in **FIG. 3**. U87-EGFR ν III-LUC cells (5.0×10^4 cells) were inoculated into the brains of NOG mice, as described above. On day 7, 3C10-CAR or mock PBMCs were injected via the tail vein. Starting on the next day, lenalidomide was administered intraperitoneally for 35 consecutive days. Bioluminescence imaging, as described above, was performed every week, and overall survival time was evaluated.

[00164] All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's t-test. Differences were considered significant when the P-value was <0.05. A post hoc test for the detection of linear trends was used, and P<0.05 was considered to be significant. In the mouse survival studies, a log-rank test was used to determine significant differences in survival curves among groups.

[00165] In two mock CAR groups, regardless of the treatment with or without lenalidomide, the tumors grew exponentially, and all mice died by day 20 (**FIG. 4** and **FIG. 5**). In the 3C10-CAR group without lenalidomide, the tumors began to be eradicated from days 14 to 21, but 50% of the animals failed to benefit from the effect of the 3C10-CAR treatment and died by day 20. However, in the 3C10-CAR group with lenalidomide treatment, the tumors began to be eradicated from days 7 to 14, and all mice were eventually tumor-free (**FIG. 5**). These results show a marked improvement in the survival time of the mice when they were cotreated with 3C10-CAR T cells and lenalidomide *in vivo*, resulting in a tumor-free rate of 100%.

[00166] Without being bound by theory, one possible mechanism of action for this result is that lenalidomide augmented the absolute number of 3C10-CAR T cells (see, Example 7), resulting in enhancement of their cytolytic effects (see, Example 5) and IFN- γ release (see,

Example 4). As a second mechanism, immune synapse formation was induced between a 3C10-CAR T-cell and U87-EGFRvIII cells. Immune synapses are molecular clusters at the contact site between T-cell receptors and peptide-loaded major histocompatibility complex molecules on the surface of allophycocyanins that are enriched in F-actin (Dustin, M.L. and Depoil, D., *Nat Rev Immunol* 2011; 11: 672). A mature immune synapse forms a specific pattern of receptor segregation, with a central cluster of T-cell receptors surrounded by a ring of integrin family adhesion molecules such as lymphocyte function-associated antigen-1 (Grakoui, A. *et al.*, *Science* 1999; 285: 221–227). Ramsay *et al.* (Ramsay, A.G. *et al.*, *J Clin Invest* 2008; 118: 2427–2437; and Ramsay, A.G. *et al.*, *Blood* 2009; 114: 4713–4720) reported that lenalidomide repaired and enforced immune synapse formation in patients with hematological malignancies. However, shown here for the first time is that immune synapses formed between 3C10-CAR T cells and U87-EGFRvIII cells via the display of F-actin, lymphocyte function-associated antigen-1 and CAR polarization at the contact site, and illustrated that lenalidomide enhanced immune synapse formation, by pictorial means.

[00167] Additionally, thalidomide previously failed to show sufficient efficacy in the treatment of patients with high-grade gliomas (Giglio P. *et al.*, *Cancer* 2012; 118: 3599–3606; Alexander BM, *et al.*, *J Neurooncol* 2013; 111: 33–39). However, based on the above experiments, lenalidomide, a thalidomide derivative with theoretically greater efficacy and fewer side effects, is shown to be efficacious when used in combination with CAR T-cell therapy.

7.7. Example 7: 3C10-CAR T cells Migrate to EGFRvIII-expressing Glioblastoma

[00168] NOG 5- to 6-week-old female mice were stereotactically inoculated with U87-EGFRvIII cells (1.0×10^5 cells per 5 μ l PBS) as described in Example 6. Seven days after the inoculation, 2.0×10^6 3C10-CAR PBMCs or mock PBMCs were infused into the tail vein. Simultaneously, lenalidomide (5 mg kg⁻¹) or PBS was given intraperitoneally for 20 consecutive days, and then the brain tissue was harvested and embedded in optimum cutting temperature compound (Sakura Finetek, Tokyo, Japan). Six-micrometer-thick frozen sections were prepared with a cryostat (CM3050S; Leica, Wetzlar, Germany). After drying, the sections were heated in a microwave oven for 3 min and fixed with 4% paraformaldehyde (Wako, Osaka, Japan) for 15 min at room temperature. The sections were then dipped in PBS containing 0.1% Triton X-100 (PBST; Sigma-Aldrich) at room temperature for 20 min. Then, the sections were blocked with PBST containing 1.5% normal goat serum (Vector

Laboratories, Burlingame, CA, USA) at room temperature for 1 h and were subsequently incubated with rabbit anti-human CD3 antibody (catalog no. RM9107; Thermo Lab Vision, Fremont, CA, USA) at a 1:100 dilution in blocking reagent overnight at 4 °C. After two washes with PBST, the slides were incubated with Alexa Flour 546 goat anti-rabbit immunoglobulin G antibody (catalog no. A11035; Invitrogen, Eugene, OR, USA) at a 1:200 dilution in blocking reagent for 30 min at room temperature, and cell nuclei were counterstained with 2 µg ml⁻¹ of DAPI (4',6-diamidino-2-phenylindole) (Dojindo). After three washes, the slides were mounted using Fluorescence Mounting Medium (Dako, Carpinteria, CA, USA). Fluorescence images were acquired using an IX71 inverted microscope with a cooled CCD (Olympus, Tokyo, Japan). Three fields were randomly selected, and the number of CD3⁺ cells and DAPI⁺ cells was counted.

[00169] All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's t-test. Differences were considered significant when the P-value was <0.05. A post hoc test for the detection of linear trends was used, and P<0.05 was considered to be significant.

[00170] A number of human CD3⁺ cells were observed in the tumors of the 3C10-CAR-treated mice, but none were seen in the tumors of mock-CAR-treated mice. Lenalidomide treatment significantly increased the numbers of migrated CD3⁺ T cells from those in the tumors of the mice treated without lenalidomide (**FIG. 6**).

7.8. Example 8: Lenalidomide Increases the Number of T cells

[00171] In this experiment, lenalidomide was evaluated for its ability to increase the number of effector cells.

[00172] T-cell proliferation was quantified spectrophotometrically using the metabolic proliferation reagent WST-1 (Roche Applied Science) according to the manufacturer's instructions. For the WST-1 proliferation assays, 2×10^4 3C10-CAR T cells and the same number of non-transduced T cells were cultured in 96-well plates for 24 h in 100 µl of AIM-V medium with different concentrations of lenalidomide (10, 1, 0.1 and 0 µM). Cells were incubated at 37 °C and 5% CO₂ for 3 days. The WST-1 absorbance of the samples, using a background control as a blank (media), was measured at 450 nm using a Multiskan Ascent microplate enzyme-linked immunosorbent assay reader (Thermo Scientific, Rockford, IL,

USA). The WST-1 data were plotted using the results from three independent wells per assay. The assay was repeated three times.

[00173] All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's t-test. Differences were considered significant when the P-value was <0.05 . A post hoc test for the detection of linear trends was used, and $P < 0.05$ was considered to be significant. In the mouse studies, a log-rank test was used to determine significant differences in survival curves among groups.

[00174] As expected, the number of third-generation CAR PBMCs with costimulatory signals for self-proliferation was higher compared with that of the control PBMCs. Of note, lenalidomide treatment increased the growth of both types of PBMCs, regardless of CAR treatment, in a dose-dependent manner (**FIG. 7**). Thus, lenalidomide increased the proliferation of T cells.

7.9. Example 9: Lenalidomide Enhances Immune Synapse Formation

[00175] When effector T cells conjugate with target cells, F-actin polymerizes and CD11a (lymphocyte function-associated antigen-1) accumulates in the junction, which is known as an immune synapse. In this experiment, lenalidomide was evaluated for its ability to enhance tight connections between effector T cells and target tumor cells (Huppa, J.B., *Nat Rev Immunol* 2003; 3: 973).

[00176] Previous to dilution to an appropriate concentration (10 μm) with Dulbecco's modified Eagle's medium, U87-EGFRvIII and U87MG cells, as described in Example 3, were stained with CellTracker Blue CMAC (Life Technologies) to visually distinguish them from T cells, and incubated for 30 min at 37 °C. The solution was exchanged for fresh Dulbecco's modified Eagle's medium and the cells were incubated for a further 30 min at 37 °C.

[00177] The glioma cells (1.5×10^5 cells) were collected and cocultured in a round-bottomed 96-well plate for 2 h at 37 °C with an equal number of 3C10-CAR T cells, which had been incubated with lenalidomide (1 μm) in advance. The cells were gently collected and plated onto poly-l-lysine-coated coverslips (BD Biosciences). To visualize the immune synapse, the F-actin Visualization Biochem Kit (Cytoskeleton, Denver, CO, USA) was used, following the manufacturer's instructions. Subsequently, F(ab)'₂ fragment-specific goat anti-

mouse immunoglobulin G and streptavidin (SA)-FITC, CD11a-FITC were used to stain 3C10-CAR and lymphocyte function-associated antigen-1 (CD11a), respectively.

[00178] Cell conjugation and polarization were analyzed using a BZ-X700 (Keyence, Osaka, Japan). This method was repeated three times.

[00179] Quantitative images were analyzed, as described previously (Ramsay, A.G., *J Clin Invest* 2008; 118: 2427–2437). The relative recruitment index was calculated using the Hybrid Cell Count software (BZ-H3C; Keyence). Regions of interest were placed around the immune synapse sites and the regions of the T cell not in contact with GBM cells, and in the background.

[00180] The relative recruitment index was calculated using the following formula: (mean fluorescence intensity (MFI) at synapse–background)/(mean fluorescence intensity at the T-cell regions not in contact with GBM cells–background). Thirty conjugates that had been incubated with or without lenalidomide were analyzed.

[00181] **FIG. 8** shows tumor cells labeled with CellTracker Blue CMAC; 3C10-CAR, F-actin and CD11a were colocalized in the connections between the tumor cells and 3C10-CAR PBMCs, suggesting the formation of immune synapses between CAR T cells and tumor cells. To address the question of whether lenalidomide strengthens immune synapses, F-actin polymerization was quantified using an relative recruitment index. The thickness of the F-actin polymers was significantly higher in the lenalidomide (+) group compared with that in the lenalidomide (–) group (**FIG. 9**).

7.10. Example 10: Lenalidomide Does Not Alter Expression of Cytotoxic T-lymphocyte-associated Antigen 4 (CTLA-4) and Programmed Cell Death Protein 1 (PD-1) on CAR T Cells In Vitro

[00182] PD-1 and CTLA-4 expression was examined using quantitative RT-PCR. Total RNA was prepared from 3C10-CAR-T cells and control T cells using an RNeasy Mini kit (Qiagen, Ilden, Germany). First-strand complementary DNA (cDNA) was synthesized using a ReverTra Ace qPCT RT Master Mix with gDNA remover (ToyoBo, Osaka, Japan). RT-PCT was performed using the StepOne Plush Real-Time PCT System (Applied Biosystems, Foster city, CA) and THUNDERBIRD SYBER qPCR Mix (Toyobo). Respective expression levels of CTLA-4 and PD-1 were normalized to that of GAPDH in each sample using the $\Delta\Delta CT$ method, followed by valuing samples without lenalidomide (LEN-) as 1.0.

[00183] The primers used for the above experiment include:

- PD1#1-F: CGGCCAGGATGGTTCTTAG (SEQ ID NO: 1)
- PD1#1-R: ACGAAGCTCTCCGATGTGTT (SEQ ID NO: 2)
- PD1#2-F: GTGCTGCTAGTCTGGGTCCT (SEQ ID NO: 3)
- PD1#2-R: AATCCAGCTCCCCATAGTCC (SEQ ID NO: 4)
- CTLA4-F: GGGCATAGGCAACGGAACCCA (SEQ ID NO: 5)
- CTLA4-R: GGGGGCATTTCACATAGACCCCTG (SEQ ID NO: 6)
- GAPDH RT-F: CATGTTTCGTCATGGGTGTGAACCA (SEQ ID NO: 7)
- GAPDH RT-R: ATGGCATGGACTGTGGTCATGAGT (SEQ ID NO: 8)

[00184] All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's t-test. Differences were considered significant when the P-value was <0.05 . A post hoc test for the detection of linear trends was used, and $P<0.05$ was considered to be significant.

[00185] Lenalidomide did not alter the expression of CTLA-4 and PD-1 on CAR-T cells (**FIG. 10**).

EQUIVALENTS

[00186] The present disclosure is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the subject matter provided herein, in addition to those described, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

[00187] Various publications, patents and patent applications are cited herein, the disclosures of which are incorporated by reference in their entireties.

What is claimed is:

1. A method of treating a human subject having a disease associated with expression of epidermal growth factor variant III (EGFRvIII), comprising administering to the subject an effective amount of lenalidomide and modified T lymphocytes expressing a chimeric antigen receptor (CAR), wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.
2. The method of claim 1, wherein the disease is glioblastoma multiforme.
3. The method of claim 1, wherein the disease is a cancer or a tumor.
4. The method of claim 3, wherein the cancer or tumor is a metastatic cancer or a metastatic tumor.
5. The method of claim 3, wherein the cancer or tumor is a primary cancer or a primary tumor.
6. The method of claim 5, wherein the primary cancer or primary tumor arises from or is located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof of the subject.
7. The method of claim 3, wherein the tumor is a glioma.
8. The method of claim 7, wherein the glioma is an astrocytoma.
9. The method of claim 3, wherein the cancer or tumor is selected from the group consisting of anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, medulloepithelioma, ependymoblastoma, medulloblastoma, supratentorial primitive neuroectodermal tumor, and atypical teratoid/rhabdoid tumor.
10. The method of any one of claims 1 to 9, wherein the effective amount of lenalidomide is from about 0.01 mg/kg to about 0.50 mg/kg, or from about 0.05 mg/kg to about 0.10 mg/kg, or from about 0.06 mg/kg to about 0.09 mg/kg.

11. The method of any one of claims 1 to 9, wherein the effective amount of lenalidomide is from about 0.10 mg to about 150 mg, or from about 2.5 mg to about 25 mg.
12. The method of any one of claims 1 to 9, wherein the effective amount of lenalidomide is selected from the group consisting of about 2.5 mg, about 5 mg, about 10 mg, about 15 mg, about 20 mg, and about 25 mg.
13. The method of any one of claims 10 to 12, wherein the lenalidomide is administered daily for a predetermined period of time following an initial administration of lenalidomide.
14. The method of any one of claims 1 to 13, wherein the lenalidomide and modified T lymphocytes are administered simultaneously.
15. The method of any one of claims 1 to 13, wherein the lenalidomide and modified T lymphocytes are administered sequentially.
16. The method of any one of claims 1 to 13, wherein the modified T lymphocytes are administered before the lenalidomide is administered.
17. The method of claim 16, wherein the lenalidomide is administered 1 day, 2 days, 3 days or more after administration of the modified T lymphocytes.
18. The method of any one of claims 1 to 17, wherein the likelihood of the subject's relative survival increases by at least 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%.
19. A method of increasing the release of interferon gamma by modified T lymphocytes comprising, contacting the modified T lymphocytes with an effective amount of lenalidomide, wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.
20. The method of claim 19, wherein the contacting takes place *in vitro*.
21. The method of claim 19, wherein the contacting takes place *in vivo*.

22. The method of claim 19, wherein the release of interferon gamma is increased by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% , 20%, 21%, 22%, 23%, 24%, 25%, 30%, 40% or 50%.
23. A method of enhancing the cytotoxicity of modified T lymphocytes comprising, contacting the modified T lymphocytes with an effective amount of lenalidomide, wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.
24. The method of claim 23, wherein the cytotoxicity of the modified T lymphocytes is increased by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%.
25. A method of increasing the proliferation of a population of T lymphocytes comprising, contacting the population of T lymphocytes with an effective amount of lenalidomide.
26. The method of claim 25, wherein the population of T lymphocytes comprise modified T lymphocytes expressing a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.
27. A method of enhancing immune synapse formation of modified T lymphocytes to cancer cells or tumor cells comprising, contacting the modified T lymphocytes and the cancer cells or tumor cells with an effective amount of lenalidomide, wherein the cancer cells or tumor cells express EGFRvIII, and wherein the modified T lymphocytes express a CAR, wherein the CAR comprises an anti-EGFRvIII binding domain, a transmembrane domain, and an intracellular signaling domain.
28. The method of claim 27, wherein the contacting takes place *in vitro*.
29. The method of claim 27, wherein the contacting takes place *in vivo*.
30. The method of claim 27, wherein the cancer cells or tumor cells are metastatic cancer cells or metastatic tumor cells.
31. The method of claim 27, wherein the cancer cells or tumor cells are primary cancer cells or primary tumor cells.

32. The method of claim 31, wherein the primary cancer cells or primary tumor cells arise from or are located in the brain, the spinal cord, the central nervous system, a lung, a breast, the prostate, an ovary, the colon, the rectum, the bladder, or any combination thereof.
33. The method of claim 27, wherein the tumor cells are glioma brain tumor cells.
34. The method of claim 33, wherein the glioma brain tumor cells are astrocytoma brain tumor cells.
35. The method of claim 34, wherein the astrocytoma brain tumor cells are glioblastoma multiforme brain tumor cells.
36. The method of claim 27, wherein the cancer cells or tumor cells are selected from the group consisting of anaplastic astrocytoma cells, giant cell glioblastoma cells, gliosarcoma cells, anaplastic oligodendroglioma cells, anaplastic ependymoma cells, choroid plexus carcinoma cells, anaplastic ganglioglioma cells, pineoblastoma cells, medulloepithelioma cells, ependymoblastoma cells, medulloblastoma cells, supratentorial primitive neuroectodermal tumor cells, and atypical teratoid/rhabdoid tumor cells.
37. The method of any one of claims 1 to 24 and 26 to 36, wherein the anti-EGFRvIII binding domain comprises an antibody or antibody fragment that includes an anti-EGFRvIII binding domain.
38. The method of claim 37, wherein the antibody fragment is an single-chain variable fragment (scFv).
39. The method of any one of claims 1 to 24 and 26 to 38, wherein the transmembrane domain comprises a transmembrane domain selected from the group consisting of alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154.
40. The method of claim 39, wherein the transmembrane domain comprises the transmembrane domain of CD8.
41. The method of any one of claims 1 to 24 and 26 to 40, wherein the anti-EGFRvIII binding domain is joined to the transmembrane domain by a linker, spacer or hinge polypeptide sequence.

42. The method of claim 41, wherein the hinge polypeptide sequence is the hinge region of CD8 α chain.
43. The method of any one of claims 1 to 24 and 26 to 42, wherein the intracellular domain is an intracellular domain of a protein that is expressed on the surface of T lymphocytes and triggers activation or proliferation of T lymphocytes.
44. The method of claim 43, wherein the intracellular signaling domain comprises the signaling domain of one or more T lymphocyte costimulatory protein and/or the signaling domain of T-cell receptor zeta chain (CD3 ζ).
45. The method of claim 44, wherein the T lymphocyte costimulatory protein is OX40, CD2, CD27, CD28, CD5, CD30, CD40, ICAM-1, LFA-1, ICOS or 4-1BB.
46. The method of claim 45, wherein the intracellular signaling domain comprises the signaling domains of CD28, 4-1BB and CD3 ζ .
47. The method of any one of claims 1 to 24 and 26 to 36, wherein the anti-EGFRvIII binding domain comprises an scFV, wherein the transmembrane domain comprises the transmembrane domain of CD8, wherein the anti-EGFRvIII binding domain is joined to the transmembrane domain by the hinge region of CD8 α chain, and wherein the intracellular domain comprises the signaling domain of CD28, 4-1BB and CD3 ζ .

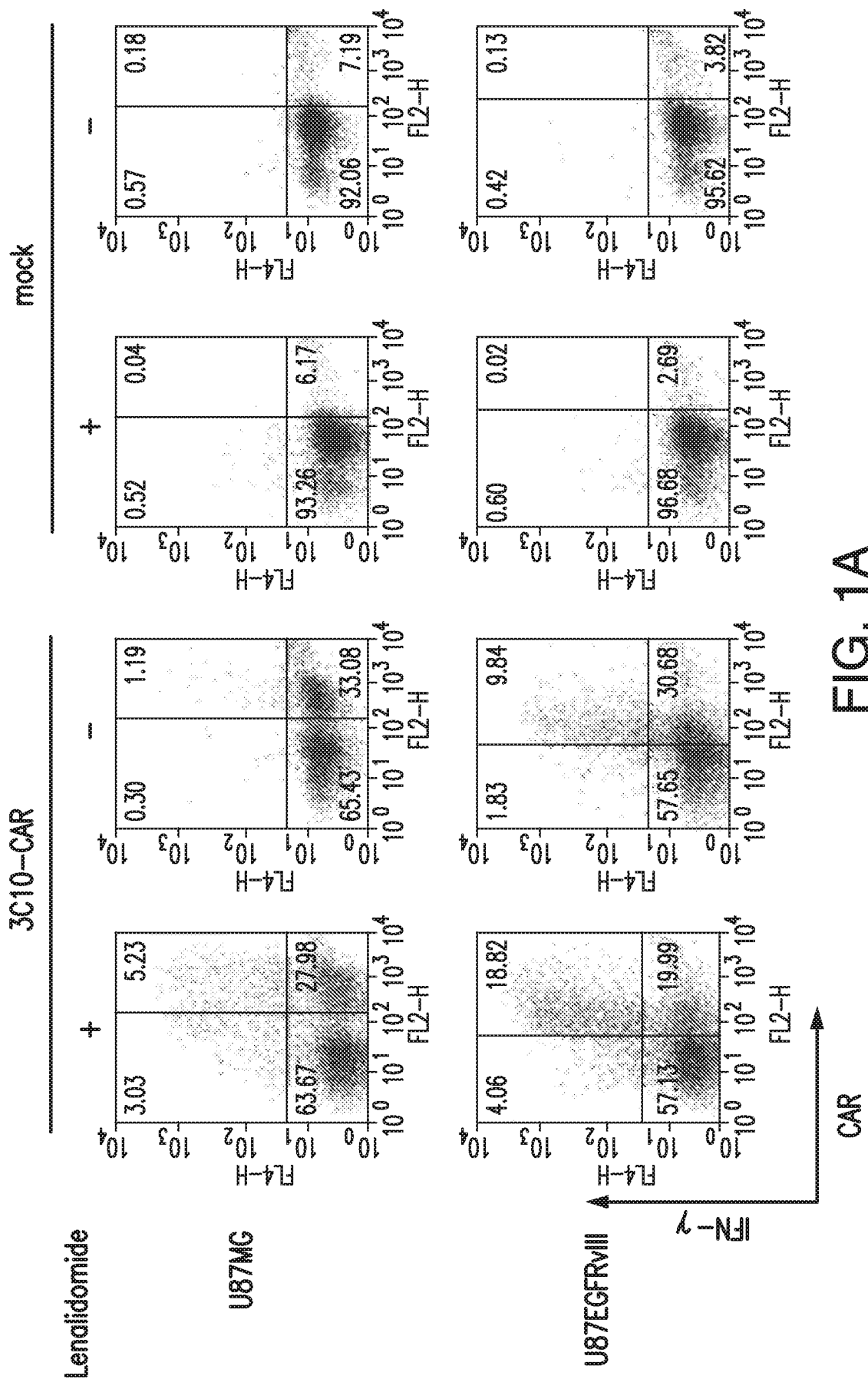


FIG. 1A

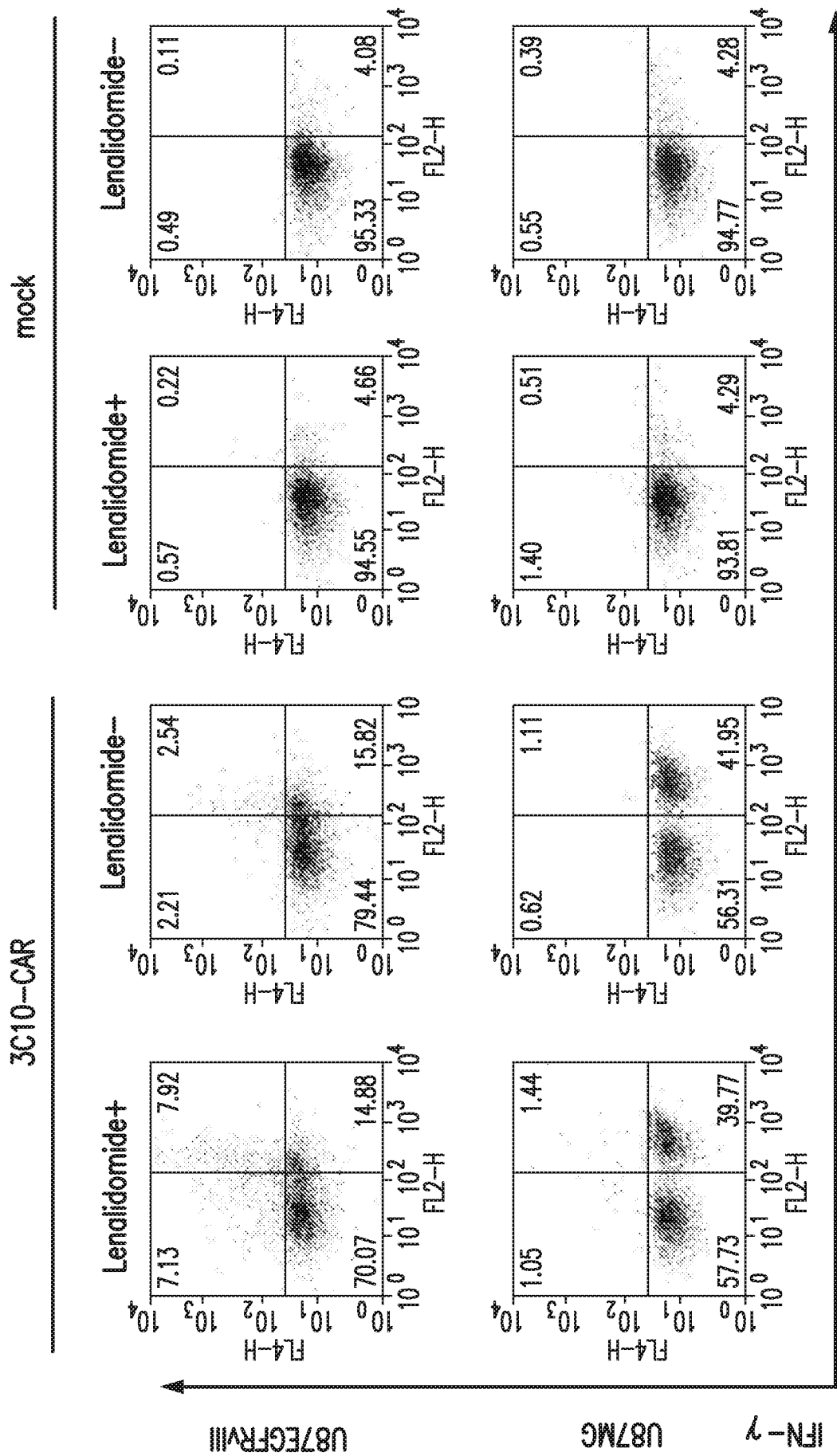


FIG. 1B

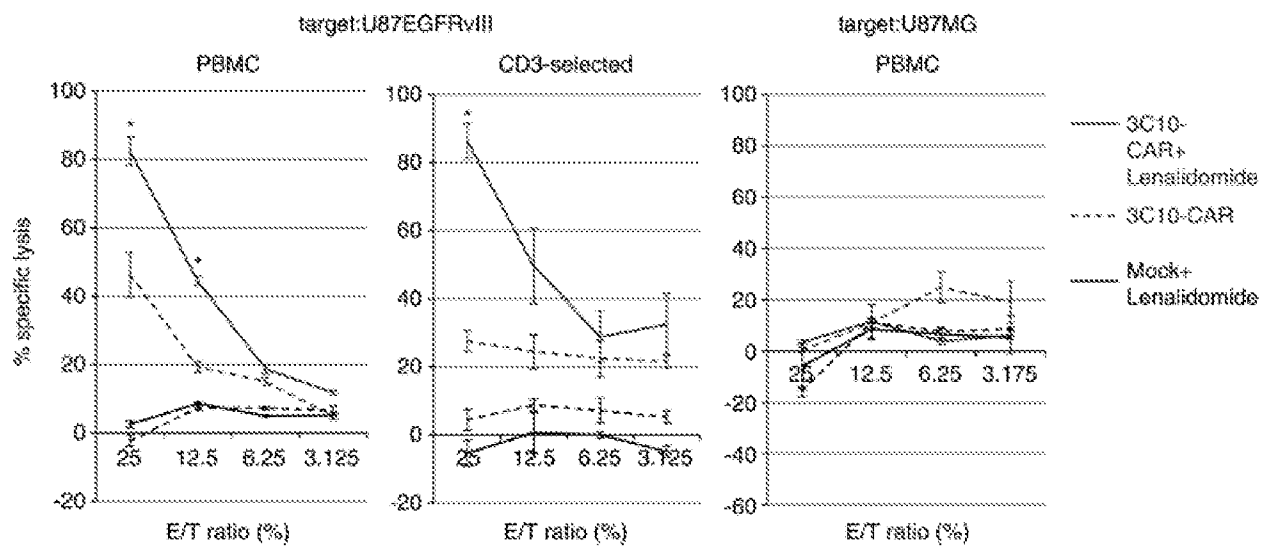


FIG. 2

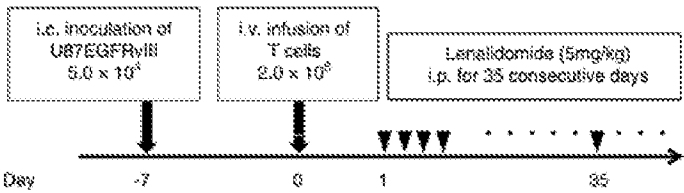


FIG. 3

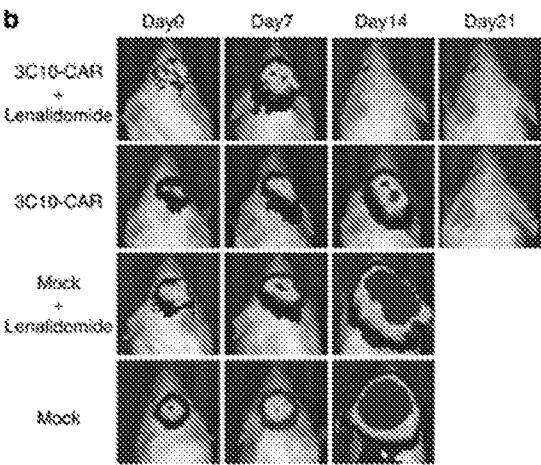


FIG. 4

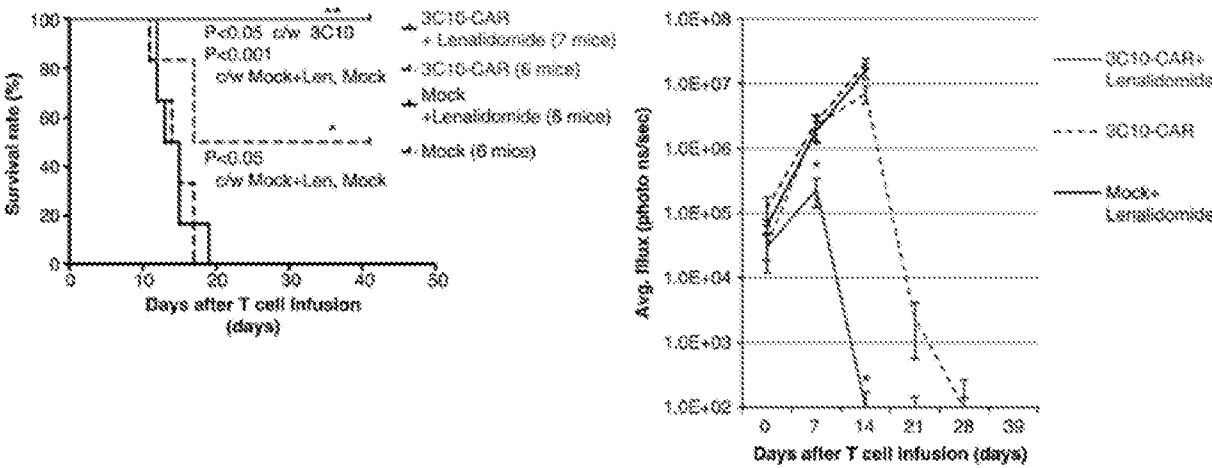
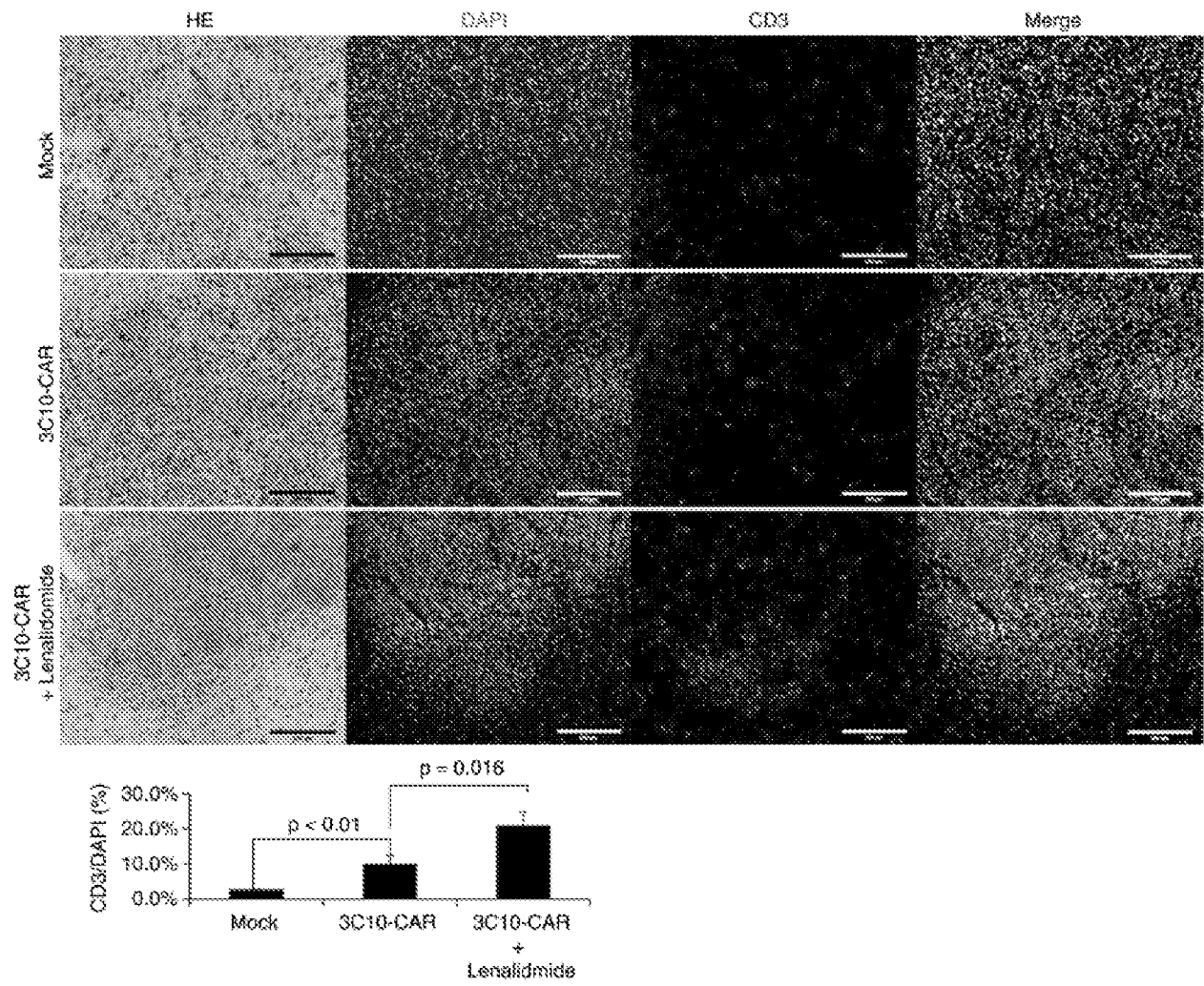


FIG. 5

**FIG. 6**

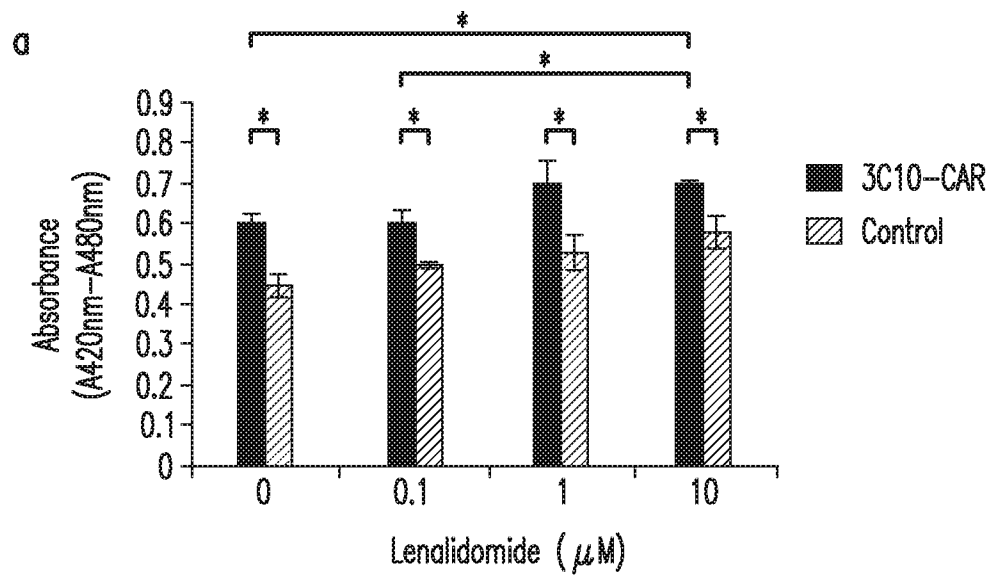


FIG. 7

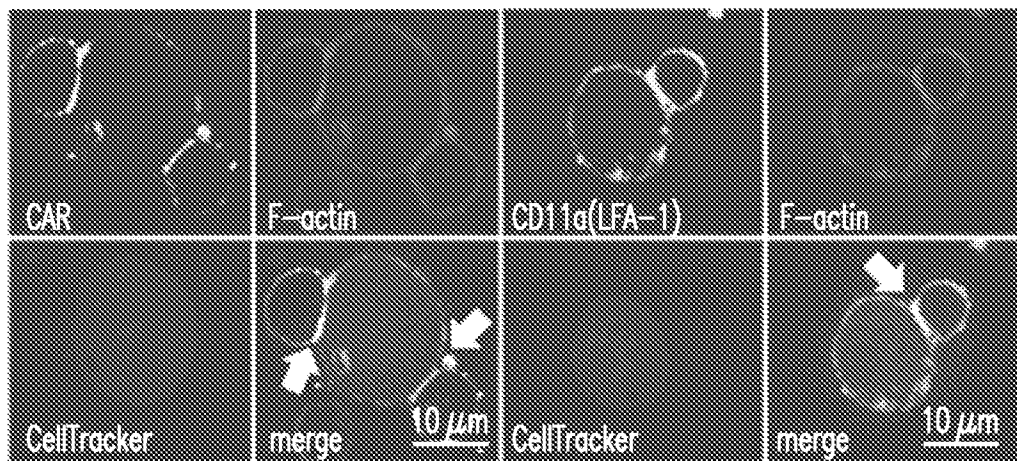


FIG. 8

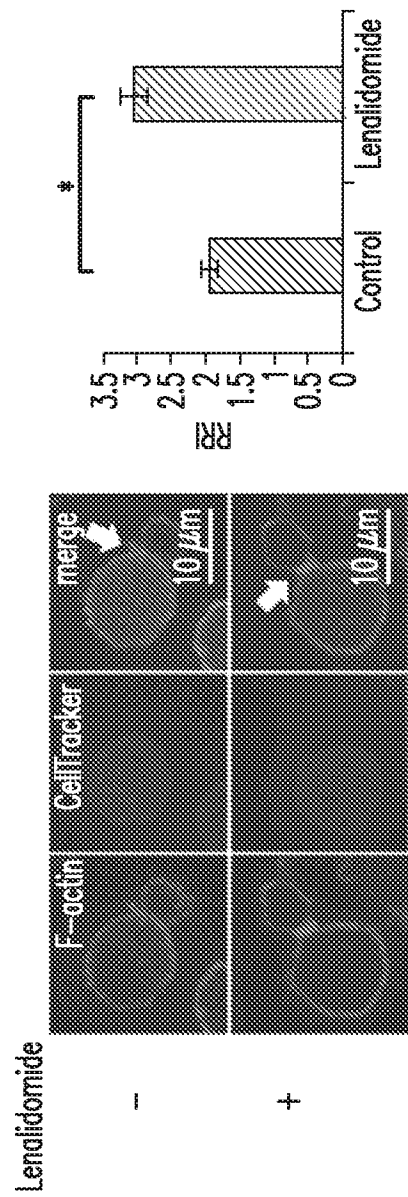


FIG. 9

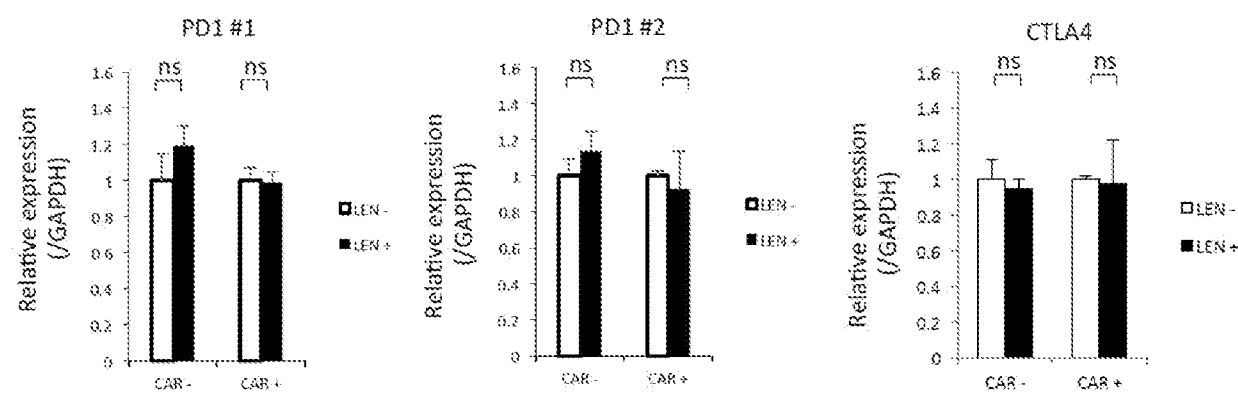


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/26731

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

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.: 13-18 and 37-47
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 additional fees, this Authority did not invite payment of 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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/26731

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 16/22, 16/28 (2016.01)

CPC - C07K 16/3053, 16/2863, 16/28; A61K 35/17

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): C07K 16/00, 16/22, 16/28 (2016.01)

CPC: A61K 35/17, 38/00; C07K 16/28, 16/2863, 16/3053

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

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

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); EBSCO Discovery; PubMed; Google; Google Scholar; Google Patents; treat, glioblastoma multiforme, lenalidomide, CAR, anti-EGFRviii

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2014/0377221 A1 (DNATRIX INC., et al.) December 25, 2014; abstract; paragraphs [0008], [0036], [0048], [0066], [017b], [0177], [0193]	25 --- 1-9, 10/1-9, 11/1-9, 12/1-9, 19-24, 26
Y	US 2014/0322275 A1 (BROGDON, J et al.) October 30, 2014; abstract; paragraphs [0008], [0036], [0059], [0212], [0260], [0287], [0296], [0298], [0304], [0305], [0330], [0365], [0368]	1-9, 10/1-9, 11/1-9, 12/1-9, 19-24, 26-36
Y	US 2011/0319277 A1 (PARK, ES et al.) December 29, 2011; abstract; paragraphs [0010], [0094], [0096]	27-36



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

17 June 2016 (17.06.2016)

Date of mailing of the international search report

15 JUL 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

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