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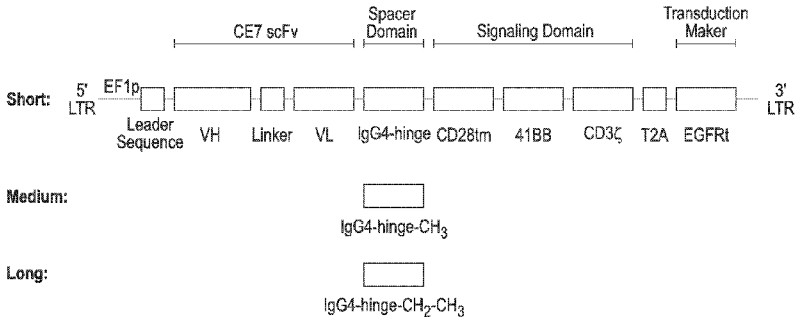

FIG. 13A

(57) Abstract: The present invention provides nucleic acids, vectors, host cells, methods and compositions to confer and/or augment immune responses mediated by cellular immunotherapy, such as by adoptively transferring CD8+ central memory T cells or combinations of central memory T cells with CD4+ T cells that are genetically modified to express a chimeric receptor. In some alternatives the genetically modified host cell comprises a nucleic acid comprising a polynucleotide coding for a ligand binding domain, a polynucleotide comprising a customized spacer region, a polynucleotide comprising a transmembrane domain, and a polynucleotide comprising an intracellular signaling domain. In some alternatives, the ligand binding domains binds to CD171.

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METHOD AND COMPOSITIONS FOR CELLULAR IMMUNOTHERAPY

RELATED APPLICATIONS

[0001] The present application claims the benefit of priority to U.S. Provisional Patent Application No. 61/977,751, filed April 10, 2014, U.S. Provisional Patent Application No. 61/986,479, filed April 30, 2014, U.S. Provisional Patent Application No. 62/058,973, filed October 2, 2014, U.S. Provisional Patent Application No. 62/088,363, filed December 5, 2014, U.S. Provisional Patent Application No. 62/089,730 filed December 9, 2014, and U.S. Provisional Patent Application No. 62/090,845, filed December 11, 2014. The entire disclosures of the aforementioned applications are expressly incorporated by reference in their entireties.

REFERENCE TO SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled SCRI-065WO_SEQUENCE_LISTING.TXT, created April 1, 2014, which is 114kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present application relates to the field of biomedicine and specifically methods and compositions for use in cellular immunotherapy, including those useful for cancer therapy. In particular, alternatives of the invention relate to methods and compositions for carrying out cellular immunotherapy comprising T cells modified with tumor targeting receptors.

BACKGROUND OF THE INVENTION

[0004] Approaches to cancer immunotherapy, whereby T cells are genetically modified to express synthetic chimeric antigen receptors (CARs), are the subject of considerable early phase human clinical trials. Whereas dramatic anti-tumor potency is observed in patients treated with CD19-specific or CD19-targeting CAR T cells for B cell

lineage malignancies, such as acute lymphoblastic leukemia and non-Hodgkin lymphomas, the challenges to achieve similar responses in patients harboring solid tumors are considerable. At present, the development and clinical testing of CAR redirected T cell adoptive therapy in cancer patients is largely empiric and constrained by a variety of technical parameters that impact feasibility of executing clinical phase I trials involving heavily pretreated patients with bulky refractory tumors. Two parameters related to cell products that can be defined with greater precision are T lymphocyte subset composition and the tuning of CAR signaling for functional outputs that maximize their anti-tumor activity. In some alternatives, the therapeutic activity of CAR-expressing central memory T cells has been examined and shown that a stable antigen-experienced component of the T cell repertoire having stem cell like features has the capacity to repopulate long lived functional memory niches following adoptive transfer. Anti-CD19 CAR-expressing cells have been produced, following enrichment for $CD45RO^{+}CD62L^{+}$ T_{CM} cells using immunomagnetic selection, and assessed in the clinic.

[0005] Moving beyond the targeting of CD19 expressing B cell malignancies, a challenge for the field can be identifying and vetting of cell surface target molecules on tumor cells that are amenable to CAR T cell recognition with tolerable "on" target "off" tumor reactivity. Once identified, however, approaches to tune new CARs for signaling outputs that are compatible with CD4 and CD8 T cell activation have not been entirely satisfactory. Parameters that are generally perceived as central to CAR development are the affinity of the target molecule CAR antigen binding domain, typically but not exclusively an antibody scFv, and the signaling modules of the cytoplasmic domain.

[0006] There is a need to identify methods for determining elements of chimeric receptor design that are important for therapeutic activity, and to enhance or improve chimeric receptors for targeting specific antigens, and cell populations to genetically modify and adoptively transfer that provide enhanced survival and efficacy *in vivo*. Among the provided alternatives are those addressing such needs.

SUMMARY OF THE INVENTION

[0007] Despite the therapeutic efficacy of chimeric antigen receptor (CAR) redirected T cell immunotherapy in leukemia and lymphoma patients, methods and compositions are needed to achieve similar clinical responses in solid tumors. CAR

development can in some cases be biased towards selecting constructs that elicit the highest magnitude of T cell functional outputs, for example, based on *in vitro* readouts. Different CAR extracellular spacers and cytoplasmic signaling domain variants can be combined to tune the magnitude of CD8+ CTL activation for tumor cell cytolysis and cytokine secretion. In studies described herein, CAR constructs that displayed the highest activity in such *in vitro* assays also displayed the lowest anti-tumor activity *in vivo*, whereas CARs tuned for moderate signaling potency mediated tumor inactivation and/or eradication. It was observed that recursive CAR triggering can have rendered CTLs expressing hyperactive CARs highly susceptible to activation induced cell death (AICD) possibly as a result of augmented FasL expression. CAR tuning using combinations of extracellular spacers and cytoplasmic signaling modules, e.g., by varying properties of such portions independently, in order to limit AICD, can promote enhanced or improved clinical activity against solid tumors. Described herein are impacts of features of the extracellular spacer joining the antigen binding and transmembrane domains, such as length of the spacer, in contributing to CAR T cell performance. In some alternatives, CAR spacers are selected in order to adjust and/or enhance or improve the biophysical synapse distance between the CAR-expressing cell, e.g., T cell, and targeted cell, e.g., tumor cell, for example, to achieve a synapse distance compatible with and/or optimal for immune cell, e.g., T cell, activation.

[0008] The alternatives described herein relate to the respective contributions of both extracellular spacer length and cytoplasmic signaling moiety selection on the performance of a CAR that can target and/or is specific for a tumor selective epitope on CD171 (L1-CAM) that is recognized by monoclonal antibody CE7 and has been tested as a first generation CAR in a clinical pilot study. Using *in vitro* functional assays for CAR redirected effector potency, a quantitative hierarchy of effector outputs based on spacer dimension in the context of second and third generation cytoplasmic signaling domains was observed. In one alternative, a striking discordance in CAR T cell performance *in vitro* versus *in vivo* due to activation induced cell death (AICD) of the most functionally potent CAR formats was shown. These alternatives reveal clinically relevant parameters for inspection in the development of CAR T cell immunotherapy for solid tumors. Given that each new scFv and target molecule defines a unique distance from the tumor cell plasma

membrane, the adjustment of CAR spacers are unique to each construct and derive via empiric testing of libraries of spacer length variants.

[0009] In one aspect, the present disclosure relates to methods and compositions to confer and/or augment immune responses mediated by cellular immunotherapy, such as by adoptively transferring tumor-specific, genetically modified populations of immune cells, such as genetically modified subsets of CD8+ or CD4+ T cells alone, or in combination. The disclosure provides for chimeric receptor nucleic acids, and vectors and host cells including such nucleic acids, as well as chimeric receptors encoded thereby. The nucleic acid sequence that encodes such a chimeric receptor generally links together a number of modular components that can be excised and replaced with other components in order to customize a chimeric receptor that can target and/or is specific for efficient cell activation and recognition of a specific target molecule or an epitope on the target molecule.

[0010] Adoptive immunotherapy using chimeric antigen receptor (CAR) expressing cells can be useful for treating, ameliorating, and/or inhibiting proliferation of a cancer. In some alternatives, a CAR directed to an epitope of the antigen CD171 (L1CAM) is used. Such CAR constructs are useful to treat, ameliorate, or inhibit any disease or disorder or malignancy in which cells express CD171. In some alternatives, the disease or disorder is a cancer or tumor that expresses CD171 (L1CAM). In some alternatives, the cancer that expresses CD171 is neuroblastoma (NB). CD171 is expressed in 100% of high risk NB. Other cancers that express CD171 include melanoma, cervical carcinoma, ovarian cancer, uterine carcinoma, pancreatic cancer, colon carcinoma, renal carcinoma, and glioblastoma.

[0011] CD171, also known as L1CAM, is a 200-kDa transmembrane glycoprotein. It is a neuronal cell adhesion molecule involved in axon guidance and cell migration, with a strong implication in treatment-resistant cancer. CD171 belongs to the immunoglobulin superfamily of recognition molecules, and participates in heterophilic interactions with other adhesion molecules such as laminin, integrins, proteoglycans and CD24. In some alternatives, CD171 comprises, consists essentially of or consists of an epitope that is predominantly found on tumor cells. In some alternatives, the epitope is found on the extracellular domain of a glycosylated CD171 and not found on

unglycosylated CD171. In some alternatives, the epitope is referred to as CE7 or the epitope recognized by the antibody deemed CE7.

[0012] Neuroblastoma is the most common extracranial solid tumor that arises during infancy. It is an embryonal malignancy of the sympathetic nervous system arising from neuroblasts (pluripotent sympathetic cells). In the developing embryo, these cells invaginate, migrate along the neuraxis, and populate the sympathetic ganglia, adrenal medulla, and other sites. The patterns of distribution of these cells correlate with the sites of primary neuroblastoma presentation. Age, stage, and biological features encountered in tumor cells are important prognostic factors and are used for risk stratification and treatment assignment. The differences in outcome for patients with neuroblastoma are striking. Patients with low-risk and intermediate-risk neuroblastoma have excellent prognosis and outcome. However, those with high-risk disease continue to have very poor outcomes despite intensive therapy. Unfortunately, approximately 70-80% of patients older than 18 months present with metastatic disease, usually in the lymph nodes, liver, bone, and bone marrow. Less than half of these patients are cured, even with the use of high-dose therapy followed by autologous bone marrow or stem cell rescue. Thus, the CAR transduced lymphocytes described herein are useful in the treatment, amelioration, or inhibition of neuroblastoma in subjects.

[0013] In some alternatives, a CAR directed to CD171 comprises components that enhance the *in vivo* activity and/or survival and/or persistence of the CAR or cells expressing the same. In some alternatives, a ligand binding domain comprises an antibody or antigen binding fragment, which specifically binds and/or targets an epitope of CD171, such as one found more often on tumor cells than healthy cells. In other alternatives, a spacer region is included, which is a short extracellular spacer. In some alternatives, an intracellular signaling domain contains a single co-stimulatory domain and a single intracellular signaling domain, and excludes other signaling domains.

[0014] Some alternatives relate to a chimeric receptor nucleic acid that comprises a polynucleotide coding for a ligand binding domain, and/or chimeric receptors encoded by the same, wherein the ligand is a molecule expressed on cancer or tumor cells, a polynucleotide encoding a polypeptide spacer, a polynucleotide encoding a transmembrane domain; and a polynucleotide encoding an intracellular signaling domain. In some alternatives, the spacer joins or is found between the ligand binding domain and

the transmembrane domain. In some alternatives, the polypeptide spacer comprises a hinge region, such as a hinge region from an antibody molecule, such as a hinge region containing an amino acid sequence X_1PPX_2P. Such a hinge region may be linked to other amino acid sequences, including one or more constant regions of an antibody, including but not limited to CH2 and CH3 regions, e.g., CH3 only sequences, of the Ig Fc. It has been surprisingly found that the length of the spacer region can be customized for individual target molecules, for better tumor or target cell recognition and/or effector function and/or persistence of cells expressing the receptor, especially *in vivo*.

[0015] In some alternatives, the length of the spacer is less than or is at 229, 200, 150, 120, 119, 100, 50, 40, 30, 20, 19, 18, 17, 16, 15, 14, 13, or 12 amino acids in length (but not less than 2 or 1 amino acids in length) or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer length is at 15 or at 12 amino acids in length. In some alternatives, the spacer length is short, intermediate (also referred to herein as "medium"), or long. In some alternatives, the short, intermediate, or long spacer is the short, intermediate, or long spacer as shown in **Tables 7** and **8**.

[0016] In some alternatives, the length of the linker is determined or influenced by the distance of the epitope of the antigen to which the chimeric receptor binds, relative to the surface plasma membrane of the cell being targeted, such as the tumor cell.

[0017] In some alternatives, a CAR directed to CD171 comprises a polynucleotide encoding a short spacer region having 15 amino acids or less (but not less than 1 or 2 amino acids), such as 15, 14, 13, or 12 amino acids, or 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 amino acids or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the short spacer comprises, consists essentially of, or consists of an amino acid sequence of X_1PPX_2P. In some alternatives, a CAR directed to an epitope of CD171 recognized by CE7, or an epitope having a similar distance on CD171 with respect to the surface plasma membrane, comprises a spacer region less than 100, 50, 40, 20, 15, or 12 amino acids in length, such as a short spacer region having 15 amino acids or less (but not less than 1 or 2 amino acids), such as 15, 14, 13, or 12 amino acids, or 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 amino acids or a length within a range defined by any two of the aforementioned lengths.

[0018] Another aspect of the disclosure provides an isolated chimeric receptor nucleic acid comprising: a polynucleotide coding for a ligand binding domain, wherein the

ligand is a tumor specific antigen, or any other molecule expressed on a target cell population that is suitable to mediate recognition, inactivation and/or elimination by a lymphocyte; a polynucleotide coding for a polypeptide spacer, wherein the polypeptide spacer is of a customized length, wherein the spacer is optimized; a polynucleotide coding for a transmembrane domain; and a polynucleotide coding for one or more intracellular signaling domains. The disclosure includes expression vectors and host cells comprising the isolated chimeric receptor as described herein, as well. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0019] Another aspect of the disclosure provides a chimeric receptor polypeptide comprising a ligand binding domain, wherein the ligand is a tumor specific antigen or any other molecule that is expressed on a target cell population and can be targeted to mediate recognition and elimination by lymphocytes; a polypeptide spacer wherein the polypeptide spacer is 15 amino acids or less (but not less than 1 or 2 amino acids), such as 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 amino acids or a length within a range defined by any two of the aforementioned lengths; a transmembrane domain; and one or more intracellular signaling domains. In some alternatives, the polypeptide spacer comprises a hinge region containing the amino acid sequence X_1PPX_2P.

[0020] In another aspect, the present disclosure provides compositions to confer and/or augment immune responses mediated by cellular immunotherapy, such as by adoptively transferring tumor-specific, subset specific genetically modified CD4+ expressing T cells, wherein the CD4+ expressing T cells confer and/or augment the ability of CD8+ expressing T cells to sustain anti-tumor reactivity and increase and/or maximize tumor-specific proliferation. In some alternatives, the CD4+ expressing T cells are genetically modified to express a chimeric receptor nucleic acid and/or chimeric receptor polypeptide, as described herein.

[0021] In another aspect, the present disclosure provides compositions to confer and/or augment immune responses mediated by cellular immunotherapy, such as by adoptively transferring tumor-specific, subset specific genetically modified CD8+ expressing T cells. In some alternatives, the CD8+ expressing T cells express a chimeric receptor nucleic acid and/or chimeric receptor polypeptide, as described herein.

[0022] In another alternative, aspects of the present invention provide an adoptive cellular immunotherapy composition having a genetically modified CD8+ expressing cytotoxic T lymphocyte cell preparation to confer and/or augment immune responses, wherein the cytotoxic T lymphocyte cell preparation comprises CD8+ expressing T cells that express a chimeric receptor comprising a ligand binding domain for a ligand associated with the disease or disorder, a customized spacer region, a transmembrane domain; and an intracellular signaling domain of a T cell or other receptors, such as a co-stimulatory domain, and/or a genetically modified helper T lymphocyte cell preparation, wherein the helper T lymphocyte cell preparation has CD4+ expressing T cells that express a chimeric receptor comprising an antibody variable domain that can target and/or is specific for the ligand associated with the disease or disorder, a customized spacer region, a transmembrane domain; and one or more intracellular signaling domains.

[0023] In some alternatives, the present disclosure provides a method of treating, ameliorating, or inhibiting a cancer, such as NB, in a patient, a method of inhibiting or delaying progression and/or metastasis of a cancer, such as NB, in a patient, a method of inhibiting or reducing the presence of a tumor or cancer cell, such as NB, in a patient, and/or a method of inhibiting or reducing a target population of CD171 expressing cells in a patient in need thereof. Such methods involve administering to said subject or said patient a genetically modified cytotoxic T lymphocyte cell preparation that provides a cellular immune response, wherein the cytotoxic T lymphocyte cell preparation comprises CD8+ expressing T cells that have a chimeric receptor encoded by a polynucleotide coding for a ligand binding domain, wherein the ligand is a tumor specific antigen, or any other molecule expressed on a target cell population (e.g., CD171) that is suitable to mediate recognition and elimination by a lymphocyte; a polynucleotide coding for a polypeptide spacer, wherein the polypeptide spacer is of a customized length, wherein the spacer provides for enhanced T cell proliferation, enhanced *in vivo* cellular activities and/or cytokine production (e.g., *in vivo*) as compared to a reference chimeric receptor; a polynucleotide coding for a transmembrane domain; and a polynucleotide coding for one or more intracellular signaling domains.

[0024] In some alternatives, the ligand binding domain is an extracellular antibody variable domain that can target and/or is specific for a ligand associated with the

disease or disorder. An alternative includes a genetically modified helper T lymphocyte cell preparation, wherein the helper T lymphocyte cell preparation comprises CD4+ expressing T cells that have a chimeric receptor comprising an a polynucleotide coding for a ligand binding domain, wherein the ligand is a tumor specific antigen or a tumor targeting antigen, or any other molecule expressed on a target cell population that is suitable to mediate recognition and elimination by a lymphocyte; a polynucleotide coding for a polypeptide spacer, wherein the polypeptide spacer is of a customized length, wherein the spacer provides for enhanced T cell proliferation, enhanced *in vivo* cellular activities and/or cytokine production as compared to a reference chimeric receptor; a polynucleotide coding for a transmembrane domain; and a polynucleotide coding for one or more intracellular signaling domains. In some alternatives, a ligand binding domain comprises an antibody or antigen binding fragment thereof, which can target and/or specifically bind an epitope of CD171 more often found on tumor cells rather than healthy cells. In other alternatives, a spacer region comprises a short extracellular spacer. In some alternatives, a signaling domain contains a single signaling domain and excludes other signaling domains.

[0025] In some alternatives, the genetically modified CD8+ and genetically modified CD4+ expressing T cell populations are co-administered. In some alternatives, the T cells are autologous or allogeneic T cells. Various modifications of the methods described herein are possible. For example, the chimeric receptor that is expressed by the CD4+ expressing T cell and the CD8+ expressing T cell can be the same or different.

[0026] Another alternative relates to a method of manufacturing an adoptive immunotherapy composition by obtaining a chimeric receptor modified tumor-specific or tumor-targeting CD8+ expressing cytotoxic T lymphocyte cell preparation that elicits a cellular immune response and expresses an antigen-reactive chimeric receptor, wherein the modified cytotoxic T lymphocyte cell preparation comprises CD8+ expressing T cells that have a chimeric receptor comprising a ligand binding domain, wherein the ligand is a tumor specific antigen or tumor targeting antigen, or any other molecule expressed on a target cell population that is suitable to mediate recognition and elimination by a lymphocyte; a polypeptide spacer, wherein the polypeptide spacer is of a customized length, wherein the spacer provides for enhanced T cell proliferation, enhanced *in vivo* cellular activities and/or cytokine production, as compared to a reference chimeric receptor; a transmembrane domain; and one or more intracellular signaling domains;

and/or obtaining a modified naïve or memory CD4+ expressing T helper cell, wherein the modified helper T lymphocyte cell preparation comprises CD4+ expressing cells that have a chimeric receptor comprising a ligand binding domain, wherein the ligand is a tumor specific antigen or a tumor targeting antigen, or any other molecule expressed on a target cell population that is suitable to mediate recognition and elimination by a lymphocyte; a polypeptide spacer, wherein the polypeptide spacer is of a customized length, wherein the spacer is optimized; a transmembrane domain; and one or more intracellular signaling domains. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, a ligand binding domain comprises an antibody or antigen binding fragment, which specifically binds and/or targets an epitope of CD171 more often found on tumor cells rather than healthy cells. In other alternatives, a spacer region comprises a short extracellular spacer, which can be 15 amino acids or less (but not less than 1 or 2 amino acids), such as 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 amino acids or a length within a range defined by any two of the aforementioned lengths. In some alternatives, a signaling domain contains a single signaling domain and excludes other signaling domains.

[0027] Some alternatives also relate to a nucleic acid encoding a chimeric receptor. In some alternatives, said nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer interposed between the ligand binding domain and a transmembrane domain, c) a polynucleotide coding for the transmembrane domain, and d) a polynucleotide coding for an intracellular signaling domain, wherein expression of the chimeric receptor in a population of immune cells results in increased survival and/or persistence of the immune cells over time, following encounter with CD171 and/or an increase in therapeutic efficacy, upon administration to a subject having a CD171-expressing tumor, said increase being relative to expression of said reference chimeric receptor having a longer polypeptide spacer. In some alternatives, the increased survival and/or persistence over time comprises a relative reduction in antigen-induced cell death as measured in an *in vitro* stress test assay, comprising exposing the cells expressing said chimeric receptor to cells expressing CD171 over multiple, successive rounds and/or the increased survival and/or persistence over time comprises an increase in persistence of

the cells *in vivo* following administration to a subject having a CD171-expressing tumor and/or said longer polypeptide spacer is at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 times greater in length. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer is less than or equal to 200, 150, 100, 50, or 20 amino acids in length (but not less than 1 or 2 amino acids) or a length within a range defined by any two of the aforementioned lengths.

[0028] Some alternatives relate to a chimeric receptor nucleic acid, wherein the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO: 17), ERKCCVECPPCP (SEQ ID NO: 18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO: 19), ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO: 21), YGPPCPPCP (SEQ ID NO: 51), KYGPPCPPCP (SEQ ID NO: 52), or EVVKYGPPCPPCP (SEQ ID NO: 53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO: 21), YGPPCPPCP (SEQ ID NO: 51), KYGPPCPPCP (SEQ ID NO: 52), or EVVKYGPPCPPCP (SEQ ID NO: 53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-

40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, and NKG2C, B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence.

[0029] In some alternatives, a chimeric receptor nucleic acid is provided, wherein the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence.

[0030] In some alternatives, a chimeric receptor polypeptide is provided, wherein the chimeric receptor polypeptide is coded for by a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a

length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence.

[0031] In some alternatives, an expression vector comprising an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence.

[0032] In some alternatives, a host cell comprising an expression vector is provided. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0033] In some alternatives, a composition comprising a host cell in a pharmaceutically acceptable excipient is provided. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In

some alternatives, the composition comprises a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells or a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+ and another host cell wherein the host cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+ or a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO.

[0034] In some alternatives, a method for preparing a host cell is provided wherein the method comprises a) providing a library of nucleic acids coding for a chimeric receptor, wherein each of the plurality of nucleic acids code for a chimeric receptor that differs in length, b) introducing each of the plurality of the nucleic acids into a separate isolated T lymphocyte population and expanding each T lymphocyte population in vitro. c) administering each genetically modified T lymphocyte population into an animal model bearing a tumor and determining whether a genetically modified T lymphocyte population has anti-tumor efficacy and d) selecting a nucleic acid coding for the chimeric receptor that provides for anti-tumor efficacy. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of

CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid

further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the method further comprises introducing the selected nucleic acid coding for the chimeric receptor into a host cell.

[0035] In some alternatives, a method for preparing a host cell of any one of claims is provided, wherein the method comprises a) introducing a nucleic acid or an expression vector into a lymphocyte population that has a CD45RA-, CD45RO+, and CD62L+ phenotype and b) culturing the cells in the presence of anti-CD3 and/or anti CD28, and at least one homeostatic cytokine until the cells expand sufficiently for use as a cell infusion. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central

memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the lymphocyte is CD8+ or CD4+.

[0036] In some alternatives, a use of the host cell or a composition of claims is provided, wherein the use is for treatment of cancer or a solid tumor expressing CD171. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected

from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the composition comprises a host cell in a pharmaceutically acceptable excipient is provided. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected

from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the composition comprises a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells or a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+ and another host cell wherein the host cell is a central memory T cell, wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+ or a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the cancer is a neuroblastoma. In some alternatives, the solid tumor is selected from the group consisting of a breast cancer, brain cancer, colon cancer, renal cancer, pancreatic cancer, and ovarian cancer.

[0037] In some alternatives, a method of performing cellular immunotherapy in a subject having cancer or a tumor is provided, wherein the method comprises administering a composition or a host cell to the subject. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In

some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the composition comprises a host cell in a pharmaceutically acceptable excipient is provided. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic. In some alternatives, the chimeric receptor nucleic acid comprises a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer

comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the composition comprises a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells or a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+ and another host cell wherein the host cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+ or a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO. In some alternatives, the cancer is neuroblastoma. In some alternatives, the tumor is selected from the group consisting of a breast cancer, brain cancer, colon cancer, renal cancer, pancreatic

cancer, and ovarian cancer. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0038] Another alternative relates to a chimeric receptor polypeptide coded for by a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling

domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the spacer provides for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0039] Another alternative relates to an expression vector comprising a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7,

NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the spacer provides for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0040] In some alternatives, a host cell comprising an expression vector is provided. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of

ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the spacer provides for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0041] In some alternatives, a composition comprising host cells in provided. In some alternatives, the composition comprises host cells comprising an expression vector. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide

coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives of the composition, the said host cells comprise a population of CD8+ cells

consisting essentially of, or having been enriched for naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells or bulk CD8+ T cells. In some alternatives, the said population of CD8+ cells consists essentially of or has been enriched for CD8+ central memory T cells positive for CD45RO, CD62L, and CD8. In some alternatives, said host cells comprise a population of CD4+ cells consisting essentially of or having been enriched for CD4+ T helper lymphocytes selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, said population of CD4+ cells consists essentially of or has been enriched for naïve CD4+ T cells positive for CD45RA, CD62L, and CD4 and negative for CD45RO. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0042] In another alternative, a composition comprising host cell or composition of and a pharmaceutically acceptable excipient is provided. In some alternatives, the host cell comprises an expression vector. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some

alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP (EPKSCDTPPPCPRCP)$_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L, and/or CD4 and/or negative for CD45RO. In some alternatives, the composition comprises host cells comprising an expression vector. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor

nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP (EPKSCDTPPPCPRCP)$_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In

some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives of the composition, the said host cells comprise a population of CD8+ cells consisting essentially of, or having been enriched for naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells or bulk CD8+ T cells. In some alternatives, the said population of CD8+ cells consists essentially of or has been enriched for CD8+ central memory T cells positive for CD45RO, CD62L, and/or CD8. In some alternatives, said host cells comprise a population of CD4+ cells consisting essentially of or having been enriched for CD4+ T helper lymphocytes selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, said population of CD4+ cells consists essentially of or has been enriched for naïve CD4+ T cells positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the composition comprises a host cell wherein the host cell is a CD8+ T cytotoxic lymphocyte cell or the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8, and another host cell wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0043] In another alternative, an *in vitro* method for preparing a host cell is provided. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets

CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell

wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the method comprises a) providing a library comprising a plurality of nucleic acids, each coding for a chimeric receptor, wherein each of a plurality of chimeric receptors coded by the plurality of nucleic acids differs in length, b) introducing each of the plurality of the nucleic acids into a separate lymphocyte population and expanding each T lymphocyte population *in vitro*, thereby generating a plurality of genetically modified T lymphocyte populations, c) administering each of the plurality of genetically modified T lymphocyte populations into an animal model bearing a tumor and has assessing a readout of anti-tumor efficacy and d) selecting a nucleic acid coding for a chimeric receptor exhibiting anti-tumor efficacy in vitro and/or in an animal model. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less 15 amino acids in length (but not less than 1 or 2 amino acids). In some

alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP (EPKSCDTPPPCPRCP)$_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the plurality of nucleic acids code for an expression vector. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are

any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP (EPKSCDTPPPCPRCP)$_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the method further comprises introducing the selected nucleic acid coding for the chimeric receptor into a host cell. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0044] In some alternatives, an *in vitro* method for selecting a chimeric receptor for targeting an antigen is provided, wherein the method comprises incubating each of a plurality of cell populations with donor cells expressing the antigen in a series of sequential recursive rounds, wherein the plurality of cell populations comprises nucleic acids encoding a plurality of chimeric receptors of varying lengths, each chimeric receptor

specifically binding to and/or targets the antigen and assessing expansion, activation, and/or survival of each of the plurality of cell populations, and selecting the chimeric receptor based on said assessment. In some alternatives, the series of sequential rounds comprises at least three rounds of incubation, wherein the cell populations are harvested between rounds. In some alternatives, the assessment is carried out by detecting survival or number or percentage of surviving cells of each population following the sequential rounds of incubation, wherein the selected chimeric receptor is expressed by a population for which survival or cell number or percentage is increased relative to another of the plurality of cell populations. In some alternatives, the cells of the populations and cells expressing the antigen are incubated at a ratio of 1:1 at the start of each round. In some alternatives, the chimeric receptors of varying lengths comprise spacers of different lengths, each spacer joining an antigen binding domain and a transmembrane domain of the chimeric receptor; and/or the chimeric receptors of varying lengths comprise chimeric receptors having varying numbers of intracellular costimulatory domains, each intracellular domain individually from a different natural costimulatory molecule. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0045] In another alternatives, an *in vitro* method for preparing a host cell is provided, wherein the method comprises: introducing a nucleic acid, as set forth above, or an expression vector, as set forth above, into a lymphocyte population that has a CD45RA, CD45RO+, and/or CD62L+ phenotype and culturing the cells in the presence of anti-CD3 and/or anti CD28, and at least one homeostatic cytokine until the cells expand sufficiently for use as a cell infusion. In some alternatives, the host cell comprises an expression vector, as set forth above. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid, as set forth above. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1

and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the chimeric receptor nucleic acid comprises:

a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In

some alternatives, the lymphocyte is CD8+ or CD4+. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0046] In some alternatives, a use of a host cell or composition in the treatment or inhibition of cancer or a solid tumor expressing CD171 is provided. In some alternatives, the cancer is a neuroblastoma. In some alternatives, the solid tumor is selected from the group consisting of a breast cancer, brain cancer, colon cancer, renal cancer, pancreatic cancer, and ovarian cancer. In some alternatives, the host cell comprises an expression vector, as set forth herein. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7,

NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell, wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, a composition comprises host cells. In some alternatives, the composition comprises host cells comprising an expression vector, as set forth herein. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid, as set forth herein. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a

human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives of the composition, the said host cells comprise a population of CD8+ cells consisting essentially of, or having been enriched for naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells or bulk CD8+ T cells. In some alternatives, the said population of CD8+ cells consists essentially of or has been enriched for CD8+ central memory T cells positive for CD45RO, CD62L, and CD8. In some alternatives, said host cells comprise a population of CD4+ cells consisting essentially of or having been enriched for CD4+ T helper lymphocytes selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, said population of CD4+ cells consists essentially of or has been enriched for naïve CD4+ T cells positive for CD45RA, CD62L and CD4 and negative for CD45RO. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a

central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the composition further comprises a pharmaceutically acceptable excipient. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

[0047] In some alternatives, a method of performing cellular immunotherapy in a subject having cancer or a tumor is provided, wherein the method comprises: administering a composition or a host cell of claims to the subject. In some alternatives, the cancer is neuroblastoma. In some alternatives, the tumor is selected from the group consisting of a breast cancer, brain cancer, colon cancer, renal cancer, pancreatic cancer, and ovarian cancer. In some alternatives, the host cell comprises an expression vector, as set forth herein. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid, as set forth herein. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19),

ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell, wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, a composition comprises host cells. In some alternatives, the composition comprises host cells comprising an expression vector. In some alternatives, the expression vector comprises a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, which ligand binding domain specifically binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of less than 100 amino acids in length (but not less than 1 or 2 amino acids), which spacer joins the ligand binding domain and a transmembrane domain of the chimeric receptor, c) a polynucleotide coding

for the transmembrane domain of the chimeric receptor and a polynucleotide coding for an intracellular signaling domain of the chimeric receptor. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P, in which X_1 and X_2, independently, are any amino acid. In some alternatives, X_1 and/or X_2 is cysteine. In some alternatives, the spacer is less than 15 amino acids in length (but not less than 1 or 2 amino acids). In some alternatives, the spacer is 12, 13, 14, or 15 amino acids in length or a length within a range defined by any two of the aforementioned lengths. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the spacer comprises, consists of, or consists essentially of EPKSCDKTHTCPPCP (SEQ ID NO:17), ERKCCVECPPCP (SEQ ID NO:18), ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19), ESKYGPPCPSCP (SEQ ID NO:20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the spacer consists of or consists essentially of ESKYGPPCPSCP (SEQ ID NO: 20), ESKYGPPCPPCP (SEQ ID NO:21), YGPPCPPCP (SEQ ID NO:51), KYGPPCPPCP (SEQ ID NO:52), or EVVKYGPPCPPCP (SEQ ID NO:53). In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the intracellular signaling domain does not further comprise an intracellular portion of another costimulatory molecule and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of a molecule selected from the group consisting of CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3, and/or wherein the intracellular signaling domain does not comprise an intracellular signaling portion of CD28. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell. In some alternatives of the composition, the said host cells comprise a population of CD8+ cells consisting essentially of, or having been enriched for naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells or bulk CD8+ T cells. In some alternatives, the said population of CD8+ cells consists essentially of or has been enriched

for CD8+ central memory T cells positive for CD45RO, CD62L, and/or CD8. In some alternatives, said host cells comprise a population of CD4+ cells consisting essentially of or having been enriched for CD4+ T helper lymphocytes selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, said population of CD4+ cells consists essentially of or has been enriched for naïve CD4+ T cells positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO, CD62L, and/or CD8. In some alternatives, the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA, CD62L and/or CD4 and/or negative for CD45RO. In some alternatives, the composition further comprises a pharmaceutically acceptable excipient. In some alternatives, the host cell is a precursor T cell. In some alternatives, the host cell is a hematopoietic stem cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0048] **Figure 1** shows isolation of central memory T cells from peripheral blood mononuclear cells. **A)** shows a flow cytometry profile of cells presorted for CD4 and CD8 markers. **B)** shows a flow cytometry profile of cells after sorting for the presence of CD8. **C)** shows a flow cytometry profile of cells after depletion of the CD8 + cell population for cells positive for CD45RA. **D)** shows a flow cytometry profile of a cell population enriched for CD8, depleted for CD45RA, enriched for CD45RO, and enriched for CD62L.

[0049] **Figure 2A** shows flow cytometry profiles of CD8 central memory cells transduced with a CAR construct having a short, medium or long spacer. The expression of anti CD171 CAR is detected by an antibody that binds to a F(ab). The inset graphs for each panel shows the % of cells expressing CD8 and the truncated EGFR present in each construct in each cell population. **Panel 1** is mock infected cells and exhibits no expression of F(ab) or EGFRt. **Panel 2** shows expression of the short construct as determined by expression of CD8, EGFRt, and F(ab). **Panel 3** shows expression of the intermediate

construct as determined by expression of CD8, EGFRt, and F(ab). **Panel 4** shows expression of the long construct as determined by expression of CD8, EGFRt, and F(ab). Expression of each of the constructs in CD8 cells is similar regardless of whether the short (**Panel 2**), medium (**Panel 3**), or long spacer (**Panel 4**) is present in the construct.

[0050] **Figure 2B** shows a western blot using an antibody directed to CD3 zeta chain showing equivalent expression of each construct having a short, medium, or long spacer in transduced CD8 cells. Lane 2 shows expression of the short construct. Lane 3 shows expression of the intermediate construct. Lane 4 shows expression of the long construct.

[0051] **Figure 2C** shows cytolytic activity of mock infected CD8 cells (●) or CD8 cells transduced with a construct with a short spacer (■), an intermediate (▲) or a long spacer (▼) against SK-N-BE 2 neuroblastoma cell line (Be2) or control cell line TML(EBV-transformed TMLCLs were made from PBMCs as previously described (Pelloquin F, Lamelin JP, Lenoir GM. In vitro cell dev biol 1986; 22(12):689-694). **Panel 1** shows lack of cytolytic activity of any of the CD8 cells against the control cell line TML. The inset graph shows that control TML cell line does not express CD171. **Panel 2** shows that the cells transduced with the long construct (▼) are more effective at killing CD171 expressing neuroblastoma cells than the cells transduced with the intermediate (▲) or short (■) constructs. The inset graph shows that the neuroblastoma cell line Be2 expresses CD171. **Panel 3** shows that TML control cells incubated with CD8 cells that can target and/or are specific for antigen CD3 (Okt3) as well as transduced with each of the constructs were killed by the effector cells that can target and/or are specific for CD3 despite the presence of the construct.

[0052] **Figure 2D** shows cytokine production by CD8 cells transduced with a construct with a short spacer, an intermediate spacer or a long spacer when contacted with Be2 neuroblastoma cells or control TML. **Panel 1** shows that CD8 transduced cells with the long spacer (L) produced more IFNγ than CD8 cells transduced with the intermediate (I) and short spacer (S) when contacted with Be2 neuroblastoma cells. **Panel 2** shows that CD8 transduced cells with the long spacer (L) produced more IL-2 than CD8 cells transduced with the intermediate (I) and short spacer (S) when contacted with Be2 neuroblastoma cells. **Panel 3** shows that CD8 transduced cells with the long spacer (L)

produced more TNFα than CD8 cells transduced with the intermediate (I) and short spacer (S) when contacted with Be2 neuroblastoma cells.

[0053] **Figure 2E** shows tumor cell survival and proliferation of the intracranial neuroblastoma xenograft tumor model in NSG mice. The graph on the left shows that labelled neuroblastoma cells in mice treated with CD8 cells transduced with a short construct showed very little expression of the label (bottom line) as compared to CD8 cells transduced with the long construct (second line from the top) or control mice treated with sham transduced T cells (top line). The graph on the right shows survival of mice having a xenograft neuroblastoma tumor treated with CD8 cells transduced with a short, intermediate, or long spacer region. The mice treated with CD8 cells transduced with a construct with a short spacer (top line) survived much longer than intermediate (second line) or long spacer (third line from the top).

[0054] **Figure 2F** shows that tumors from mice treated with CD8 cells transduced with a construct with a long spacer exhibited greater numbers of CD3+ cells in the tumor.

[0055] **Figure 2G** shows that tumors from mice treated with CD8 cells transduced with a construct with a long spacer expressed more caspase 3 in the tumor.

[0056] **Figure 3A, 3B and 3C** shows activation status and viability of transduced CAR T cells after exposure to tumor cells for 24 hours (round 1); T cells are then harvested from round 1 cell culture and transferred to a new culture for another 24 hours (round 2); and T cells are then harvested from round 2 cell culture and transferred to a new culture for another 24 hours round 3). Between the transfers the amount of viable T cells was kept the same between the different T cell lines.

[0057] **Figure 3A** shows that CD8 cells transduced with a construct with a long spacer region (L) had a greater % of cells with activated cell surface markers CD25/CD69 as compared to those cells transduced with a construct with a short spacer(S) at each round of transfer.

[0058] **Figure 3B** shows that CD8 cells transduced with a construct with a long (▼) or intermediate spacer region (▲) had a greater % of dead cells (top 2 lines) as compared to those cells transduced with a construct with a short spacer (■).

[0059] **Figure 3C** shows that CD8 cells transduced with a construct with a long or intermediate spacer region caused greater expression of FasR expression in tumor cells compared to those cells transduced with a construct with a short spacer.

[0060] **Figure 4A, 4B, 4C, 4D, 4E, and 4F** shows CD8 central memory T cells transduced with a CAR construct with a short spacer region but having an additional intracellular signaling region from CD28 so that the construct includes costimulatory signaling regions from CD28 and 4-1BB together with the CD3 zeta signaling domain.

[0061] **Figure 4A** shows flow cytometry profiles of CD8 central memory cells transduced with a CAR construct having a CD28cyto/4-1BB costimulatory domain and a construct having a 4-1BB costimulatory domain. The expression of anti- CD171 CAR is detected by an antibody that binds to a F(ab). The inset graphs for each panel shows the % of cells expressing CD8 and the truncated EGFR present in each construct in each cell population. **Panel 1** is mock infected cells and exhibits no expression of F(ab) or EGFRt. **Panel 2** shows expression of the short construct with the 4-1BB costimulatory domain as determined by expression of CD8, EGFRt, and F(ab). **Panel 3** shows expression of the short construct with the a CD28cyto/4-1BB costimulatory domain as determined by expression of CD8, EGFRt, and F(ab). Expression of each of the constructs in CD8 cells is similar.

[0062] **Figure 4B** shows cytolytic activity of CD8 cells transduced with a construct comprising a short spacer, costimulatory domain 4-1BB, and signaling domain CD3ζ and a construct comprising a short spacer, costimulatory domain CD28 cyto, costimulatory domain 4-1BB, and signaling domain CD3ζ against SK-N-BE 2 neuroblastoma cell line (Be2) The graph shows that the cells transduced with the construct with the costimulatory domain CD28cyto (top line ♦) are more effective at killing CD171 expressing neuroblastoma cells than the cells transduced with a construct lacking the CD28 cyto (2nd line from the top ▼))

[0063] **Figure 4C** shows cytokine production by transduced T cells when contacted with Be2 neuroblastoma cells or control TML. Be2 or TML cells are contacted with CD8 cells transduced with a construct comprising a short spacer, costimulatory domain 4-1BB, and signaling domain CD3ζ and a construct comprising a short spacer, costimulatory domain CD28 cyto, costimulatory domain 4-1BB, and signaling domain CD3ζ. The graph shows that CD8 transduced cells with the construct with the CD28cyto

costimulatory domain (CD28cyto) in contact with Be2 cells produced more IFNγ than CD8 cells transduced with the construct without the CD28cyto costimulatory domain (4-1BB).

[0064] **Figure 4D** shows tumor cell survival and proliferation of the intracranial neuroblastoma xenograft tumor model in NSG mice. The graph shows survival of mice having a xenograft neuroblastoma tumor treated with CD8 cells transduced with a construct comprising a short spacer, costimulatory domain 4-1BB, and signaling domain CD3ζ(4-1BB) and a construct comprising a short spacer, costimulatory domain CD28 cyto, costimulatory domain 4-1BB, and signaling domain CD3ζ (CD28 cyto). A greater percentage of mice treated with CD8 cells transduced with a construct without CD28cyto costimulatory domain(4-1BB) survived than mice treated with cells transduced with the construct with the CD28cyto costimulatory domain (CD28 cyto).

[0065] **Figure 4E** shows activation status and viability of transduced CAR T cells after exposure to tumor cells for 24 hours (round 1), T cells are then harvested from round 1 cell culture and transferred to a new culture for another 24 hours (round 2) and T cells are then harvested from round 2 cell culture and transferred to a new culture for another 24 hours (round 3). The graph shows that CD8 cells transduced with a construct with CD28 cyto costimulatory domain (CD28 cyto) had a greater % of cells with activated cell surface markers CD25/CD69, as compared to those cells transduced with a construct without CD28 cyto costimulatory domain (4-1BB).

[0066] **Figure 4F** shows that CD8 cells transduced with a construct with CD28cyto costimulatory domain (▲) had a greater % of dead cells as compared to those cells transduced with a construct without CD28cyto costimulatory domain (■).

[0067] **Figure 5** shows the sequences for the Ce7scFv-IgG4hinge-CH2-CH3 CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (long construct) (SEQ ID NO: 54). Tildes show the beginning and end of the coding sequence for each component of the construct.

[0068] **Figure 6** shows the plasmid map including the sequences for the Ce7scFv-IgG4hinge-CH2-CH3-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (long construct).

[0069] **Figure 7** shows the sequences for the CE7scFv-IgG4hinge-CH3-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (intermediate) (SEQ ID NO: 55). Tildes show the beginning and end of the coding sequence for each component of the construct.

[0070] **Figure 8** shows the plasmid map including the sequences for the CE7scFv-IgG4hinge-CH3-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (intermediate).

[0071] **Figure 9** shows the sequences for CE7scFv-IgG4hinge-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (short) (SEQ ID NO: 56). Tildes show the beginning and end of the coding sequence for each component of the construct.

[0072] **Figure 10** shows the plasmid map for the sequences for CE7scFv-IgG4hinge-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (short).

[0073] **Figure 11** shows the sequence for construct CE7scFv-IgG4hinge-CD28tm/cyto-4-1BB-zeta-T2A-EGFRt-epHIV7 (short) with two costimulatory domains (SEQ ID NO: 57). Tildes show the beginning and end of the coding sequence for each component of the construct.

[0074] **Figure 12** shows the plasmid map including the sequence for construct CE7scFv-IgG4hinge-CD28tm/cyto-4-1BB-zeta-T2A-EGFRt-epHIV7 (short) with two costimulatory domains.

[0075] **Figures 13A-13H** shows the CAR extracellular spacer tunes anti-tumor effector outputs of $CD8^+$ expressing CTLs. **Figure 13A** shows the schematic of CD171-specific and/or targeting 2G-CAR extracellular domain spacer variants. **Figure 13B** shows the Human $CD8^+$ $T_{E(CM)}$ cell surface expression of 2G SS, MS or LS spacer variants and EGFRt detected with anti-murine FAB and cetuximab, respectively. **Figure 13C** shows the 2G-CAR expression levels detected by a CD3-ζ specific Western Blot. **Figure 13D** shows the 2G-CAR induced levels of phospho-ERK upon co-culture with $CD171^+$ Be2 neuroblastoma tumor cells at an E:T ratio of 1:1 ($n \geq 3$ per condition). **Figure 13E** shows the 2G-CAR activation induced CD137 surface expression upon tumor co-culture as in Figure 13D. **Figure 13F** shows the Anti-tumor lytic activity of spacer variant 2G-CAR CTLs determined by 4-hour chromium release assay. Fold specific lysis of LS and MS spacers relative to SS 2G-CAR CTLs at an E:T ratio of 10:1. **Figure 13G** shows the Stimulation of cytokine secretion in mixed 2G-CAR CTL mixed tumor (Be2) cultures ($n \geq 5$ per condition). Fold cytokine production comparison is relative to SS 2G-CAR, as in **Figure 13H**.

[0076] **Figures 14A-14H** shows the inverse correlation of CAR spacer-dependent CTL functional potency *in vitro* and anti-tumor activity *in vivo*. **Figure 14A** shows the schema of intracranial NSG mouse neuroblastoma xenograft therapy model and

biophotonic signal of ffLuc$^+$ Be2 tumors at day +6 following stereotactic implantation. **Figure 14B** shows the Biophotonic Be2 tumor signal response to intratumorally infused 2G-CAR CD8$^+$T$_{E(CM)}$ spacer variants (n=6 mice per group). LS 2G-CAR cohort was euthanized on day 20 due to tumor related animal distress. **Figure 14C** shows the Kaplan Meier survival of treated cohorts from **Figure 14B**. **Figure 14D** shows the quantitation of intratumoral 2G-CAR T cells at time of symptomatic tumor progression. T cell density determined by counting human CD3$^+$ cells and reported as total number per 40 hpf's (data representative of individual tumor analysis). **Figure 14E** shows the timeline of tumor retrieval from NSG mice bearing Be2 i.c. xenografts and treated with 2G-CAR CTLs for subsequent IHC/IF inspection. **Figures 14F1 and 14F2** shows the representative tumor and contralateral hemisphere IF images for co-staining of co-localized SS 2G-CAR CTLs for CD3, Ki67 and activated caspase 3. **Figure 14G** shows the IF quantitation of persisting 2G-CAR spacer variant CTLs three days after intratumoral implantation. N=total human CD3+ cells per 40 hpf as in **Figure 14D**. **Figure 14H** shows the Percentage of CD3+ T cells that co-express granzyme B (left panel), Ki67 (middle panel), and activated caspase 3 (right panel) (n=ave. number cells/40hpf from analysis of two individual engrafted mice).

[0077] **Figures 15A-15H** shows the recursive antigen exposure *in vitro* results in differential FasL mediated AICD based on CAR spacer dimension. **Figure 15A** shows the schema of *in vitro* stress test assay for analysis of CAR T cell functional status and viability upon repetitive stimulation with tumor cells. **Figure 15B** shows the quantitation of residual viable fLuc$^+$Be2 tumor cells after successive rounds of 2G-CAR transfer (%tumor viability=average of 3 independent experiments). **Figure 15C** shows the glow cytometric quantitation of CD25 and CD69 surface expression following successive rounds of co-culture with Be2 cells at an effector:stimulator (E:S) ratio of 1:1 (%CD25$^+$CD69$^+$values=average of 2 independent experiments). **Figure 15D** shows the 2G-CAR T cell viability determination by Guava Viacount assay after each round. %Dead T cells values derived as in Figure 15C. **Figure 15E** shows the frequency of FasL$^+$2G-CAR CTLs before and after 8-hour co-culture with Be2 (E:S 1:1; each data point is derived from an independent experiment). **Figure 15F** shows the Fold-induction of FasL mRNA transcription measured by rt-qPCR upon co-culture of MS and LS 2G-CAR spacer variants relative to SS 2G-CAR CTLs normalized to beta-actin (average of 5 independent experiments). **Figure 15G** shows the Frequency of activated caspase 3$^+$2G- CAR CTLs

following 16-hour co-culture with Be2 (E:S 1:1; values=average of 4 independent experiments). **Figure 15H** shows the effect of siRNA knockdown of Fas or FasL on apoptosis induction in LS 2G-CAR CTLs after 3 rounds. Average viability determination by Guava Viacount assay performed in 3 independent experiments ("|+" condition is mock electroporated T cells, "scr" condition scrambled siRNA).

[0078] **Figures 16A-16F** shows the augmented co-stimulation via a third generation CD28:4-1BB:zeta cytoplasmic domain results in enhanced effector function outputs *in vitro*. **Figure 16A** shows the schematic of 2G- versus 3G-CAR composition. **Figure 16B** shows the human $CD8^{+}T_{E(CM)}$ cell surface expression of 2G- versus 3G-CAR(SS) and EGFRt detected with anti-murine FAB and cetuximab, respectively. **Figure 16C** shows the 2G- and 3G-CAR(SS) expression levels detected by of CD3-ζ specific Western Blot. Figure 16D shows the 2G- versus 3G-CAR(SS) activation induced CD137 surface expression upon tumor co-culture. **Figure 16E** shows the anti-tumor lytic activity of 2G- versus 3G-CAR(SS) CTLs determined by 4-hour chromium release assay. Fold specific lysis of SS-3G relative to SS-2G CTL at an E:T ratio of 10:1 (average of 3 independent experiments). **Figure 16F** shows the stimulation of cytokine secretion in 2G- versus 3G-CAR(SS) CTL tumor (Be2) co-cultures (n ≥6 per condition). Fold cytokine production comparison is relative to 2G-CAR(SS) as in (E).

[0079] **Figures 17A-17D** shows that 3G-CAR(SS) CTLs do not exhibit enhanced anti-tumor activity *in vivo*. **Figure 17A** shows the Kaplan Meier survival curves of Be2 engrafted NSG mice treated with 2G- versus 3G-CAR(SS) CTLs (n=5-6 per group, sham transduced CTL control in black). **Figure 17B** shows the Kaplan Meier survival curves for SK-N-DZ engrafted mice treated as in **Figure 17A**. **Figure 17C** shows the 2G- versus 3G-CAR(SS) T cell intratumoral persistence 3 days following adoptive transfer. N= number of $CD3^{+}$ cells per 40hpf in two independently derived tumor specimens. **Figure 17D** shows the IF detection of granzyme B (left panel) and activated caspase 3 + (right panel) CD3+ CTL as described in **Figure 17C**.

[0080] **Figure 18A-18E** shows that the recursive antigen exposure *in vitro* results in differential FasL mediated AICD based on CAR cytoplasmic signaling in the context of a short spacer extracellular domain. **Figure 18A** shows the flow cytometric quantitation of CD25 and CD69 surface expression by 2G-versus 3G-CAR(SS) CTLs following successive rounds of co-culture with Be2 cells at an E:S of 1:1 (%$CD25^{+}CD69^{+}$

values derived from average of two independent experiments). **Figure 18B** shows 2G- versus 3G-CAR(SS) T cell viability determination by Guava Viacount assay after each round of tumor co-culture. % Dead T cells values derived as previously described. **Figure 18C** shows the frequency of $FasL^+$ 2G- versus 3G-CAR(SS) CTLs before and after 8-hour co-culture with Be2 cells at an E:S ratio of 1:1 (each data point of 5 per 2G-CAR spacer variant is derived from an independently conducted experiment). **Figure 18D** shows the fold induction of FasL mRNA transcription measured by rt-qPCR upon co-culture of 2G- versus 3G-CAR(SS) CTLs normalized to beta-actin (average results from 5 independently conducted experiments). **Figure 18E** shows the frequency of cytosolic activated caspase 3^+ 2G- versus 3G-CAR(SS) CTLs following 16-hour co-culture with Be2 at an E:S ratio of 1:1 (values ave. of 4 independent experiments). + (**Figure 18D**) and activated caspase 3+ (**Figure 18E**) CD3+ CTL as described in **Figure 18C**.

[0081] **Figures 19A-19C** shows phenotypic similarity between isolated $CD45RO^+CD62L^+$ human Tcm expanded following lentiviral transduction with spacer variant 2G-CARs. **Figure 19A** shows the timeline of CD8 T_{CM} isolation, transduction and expansion prior to experimental use. **Figure 19B** shows the Immunomagnetic isolation method and purity of $CD8^+$ T_{CM} cells (CD45RO+ CD62L+) from PBMC. **Figure 19C** shows the phenotype of CD8+ $T_{E(CM)}$ transduced with different spacer variants of 2G-CAR at time of experimental use.

[0082] **Figures 20A-20D** shows the *in vitro* and *in vivo* anti-tumor activity of CD171-specific and/or targeting 2G-CAR spacer variant $CD8^+$ $T_{E(CM)}$ CLTs against $CD171^{low}$ SK-N-DZ human neuroblastoma xenografts. **Figure 20A** shows the lytic potency of 2G-CAR spacer variants in 4-hour CRA against SK-N-DZ. **Figure 20B** shows SK-N-DZ stimulation of IFNγ secretion by spacer variant 2G-CAR CTLs. **Figure 20C** shows Biophotonic SK-N-DZ tumor signal response to intratumorally infused 2G-CAR(SS, MS or LS) CD8 $^+T_{E(CM)}$ (n=5 per group). **Figure 20D** shows the Kaplan Meier survival of treated cohorts.

[0083] **Figure 21** shows the expression of Fas and FasL after siRNA knockdown. **Panel A** shows the frequency of Fas^+ LS 2G-CAR CTLs after siRNA knockdown of Fas relative to LS 2G-CAR CTLs treated with scr siRNA (%Fas^+ values derived from average of 4 independent experiments). **Panel B** shows the frequency of $FasL^+$ LS 2G-CAR CTLs after siRNA knockdown of FasL as described in **Panel A**.

DETAILED DESCRIPTION

[0084] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

[0085] "Activation", as used herein, refers to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation, cytokine production or expression of cell surface markers such as CD69 and CD25, or detectable effector functions.

[0086] "Activation Induced cell death" as used herein refers to a state of a T cell that is activated but is not able to proliferate for more than 2 generations and exhibits markers of apoptosis.

[0087] "Antigen" or "Ag" as used herein refers to a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. It is readily apparent that an antigen can be generated synthesized, produced recombinantly or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

[0088] "Anti-tumor effect" as used herein, refers to a biological effect, which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or a decrease of various physiological symptoms associated with the cancerous condition. An "anti-tumor effect" can also be manifested by a decrease in recurrence or an increase in the time before recurrence.

[0089] "Chimeric receptor" as used herein refers to a synthetically designed receptor comprising a ligand binding domain of an antibody or other protein sequence that binds to a molecule associated with the disease or disorder and is linked via a spacer domain to one or more intracellular signaling domains of a T cell or other receptors, such as a costimulatory domain. Chimeric receptor can also be referred to as artificial T cell receptors, chimeric T cell receptors, chimeric immunoreceptors, or chimeric antigen receptors (CARs). These CARs are engineered receptors that can graft an arbitrary specificity onto an immune receptor cell. Chimeric antigen receptors or "CARs" are

considered by some investigators to include the antibody or antibody fragment, the spacer, signaling domain, and transmembrane region. However, due to the surprising effects of modifying the different components or domains of the CAR, such as the epitope binding region (for example, antibody fragment, scFv, or portion thereof), spacer, transmembrane domain, and/ or signaling domain), in some contexts, in the present disclosure, the components of the CAR are described independently. The variation of the different elements of the CAR can, for example, lead to stronger binding affinity for a specific epitope.

[0090] "Co-stimulatory domain," as the term is used herein refers to a signaling moiety that provides to T cells a signal which, in addition to the primary signal provided by for instance the CD3 zeta chain of the TCR/CD3 complex, mediates a T cell response, including, but not limited to, activation, proliferation, differentiation, cytokine secretion, and the like. A co-stimulatory domain can include all or a portion of, but is not limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen-l (LFA-l), CD2, CD7, LIGHT, NKG2C, B7-H3, and/or a ligand that specifically binds with CD83 and/or targets CD83. In some alternatives, the co-stimulatory domain is an intracellular signaling domain that interacts with other intracellular mediators to mediate a cell response including activation, proliferation, differentiation and cytokine secretion, and the like.

[0091] "Coding for," as used herein, refers to the property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other macromolecules such as a defined sequence of amino acids. Thus, a gene codes for a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. A "nucleic acid sequence coding for a polypeptide" includes all nucleotide sequences that are degenerate versions of each other and that code for the same amino acid sequence.

[0092] "Cytotoxic T lymphocyte "(CTL) as used herein refers to a T lymphocyte that expresses CD8 on the surface thereof (e.g., a $CD8^+$ expressing T cell, also referred to as a CD8+ T cell or a CD8 T cell, all of which can be used interchangeably). In some alternatives such cells are preferably "memory" T cells (T_M cells) that are antigen-experienced. In similar fashion, CD4+ expressing T cells may be referred to as CD4+ T cells or CD4 T cells interchangeably.

[0093] "Central memory" T cell (or "T_{CM}") as used herein refers to an antigen experienced CTL that expresses CD62L or CCR-7 and/or CD45RO on the surface thereof, and does not express or has decreased expression of CD45RA as compared to naive cells. In some alternatives, central memory cells are positive for expression of CD62L, CCR7, CD28, CD127, CD45RO, and/or CD95, and/or have decreased expression of CD54RA as compared to naïve cells.

[0094] "Effector memory" T cell (or "T_{EM}") as used herein refers to an antigen experienced T cell that does not express or has decreased expression of CD62L on the surface thereof as compared to central memory cells, and does not express or has decreased expression of CD45RA as compared to naïve cell. In some alternatives, effector memory cells are negative for expression of CD62L and/or CCR7, as compared to naïve cells or central memory cells, and have variable expression of CD28 and/or CD45RA.

[0095] "Naïve" T cells as used herein refers to a non-antigen experienced T lymphocyte that expresses CD62L and/or CD45RA, and/or does not express CD45RO- as compared to central or effector memory cells. In some alternatives, naïve CD8+ T lymphocytes are characterized by the expression of phenotypic markers of naïve T cells including CD62L, CCR7, CD28, CD127, and/or CD45RA.

[0096] "Effector T_E" T cells as used herein refers to a antigen experienced cytotoxic T lymphocyte cells that do not express or have decreased expression of CD62L, CCR7, CD28, and/or are positive for granzyme B and/or perforin, as compared to central memory or naïve T cells.

[0097] "T cell precursors" as described herein refers to lymphoid precursor cells that can migrate to the thymus and become T cell precursors, which do not express a T cell receptor. All T cells originate from hematopoietic stem cells in the bone marrow. Hematopoietic progenitors (lymphoid progenitor cells) from hematopoietic stem cells populate the thymus and expand by cell division to generate a large population of immature thymocytes. The earliest thymocytes express neither CD4 nor CD8, and are therefore classed as double-negative (CD4−CD8−) cells. As they progress through their development, they become double-positive thymocytes (CD4+CD8+), and finally mature to single-positive (CD4+CD8− or CD4−CD8+) thymocytes that are then released from the thymus to peripheral tissues.

[0098] About 98% of thymocytes die during the development processes in the thymus by failing either positive selection or negative selection, whereas the other 2% survive and leave the thymus to become mature immunocompetent T cells.

[0099] The double negative (DN) stage of the precursor T cell is focused on producing a functional β-chain whereas the double positive (DP) stage is focused on producing a functional α-chain, ultimately producing a functional αβ T cell receptor. As the developing thymocyte progresses through the four DN stages (DN1, DN2, DN3, and DN4), the T cell expresses an invariant α-chain but rearranges the β-chain locus. If the rearranged β-chain successfully pairs with the invariant α-chain, signals are produced which cease rearrangement of the β-chain (and silence the alternate allele) and result in proliferation of the cell. Although these signals require this pre-TCR at the cell surface, they are dependent on ligand binding to the pre-TCR. These thymocytes will then express both CD4 and CD8 and progresses to the double positive (DP) stage where selection of the α-chain takes place. If a rearranged β-chain does not lead to any signaling (e.g. as a result of an inability to pair with the invariant α-chain), the cell may die by neglect (lack of signaling).

[0100] "Hematopoietic stem cells" or "HSC" as described herein, are precursor cells that can give rise to myeloid cells such as, for example, macrophages, monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells and lymphoid lineages (such as, for example, T-cells, B-cells, NK-cells). HSCs have a heterogeneous population in which three classes of stem cells exist, which are distinguished by their ratio of lymphoid to myeloid progeny in the blood (L/M).

[0101] "Targeting," "target" as described herein refers to an ability to bind to a ligand in which a molecule is specific for. "Specific" or "Specificity" can refer to the characteristic of a ligand for the binding partner or alternatively, the binding partner for the ligand, and can include complementary shape, charge and hydrophobic specificity for binding. Specificity for binding can include stereospecificity, regioselectivity and chemoselectivity.

[0102] "Enriched" and "depleted" as used herein to describe amounts of cell types in a mixture refers to the subjecting of the mixture of the cells to a process or step which results in an increase in the number of the "enriched" type and a decrease in the number of the "depleted' cells. Thus, depending upon the source of the original population

of cells subjected to the enriching process, a mixture or composition may contain 60, 70, 80, 90, 95, or 99 percent or more (in number or count) of the "enriched" cells and 40, 30, 20, 10, 5 or 1 percent or less (in number or count) of the "depleted" cells.

[0103] "Epitope" as used herein refers to a part of an antigen or molecule that is recognized by the immune system including antibodies, T cells, and/ or B cells. Epitopes usually have at least 7 amino acids and can be linear or conformational.

[0104] "Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide or nucleic acid that has been identified and separated and/or recovered from a component of its natural environment. Preferably, the isolated polypeptide or nucleic acid is free of association with all components with which it is naturally associated. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide or nucleic acid, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes.

[0105] "Intracellular signaling domain" as used herein refers to all or a portion of one or more domains of a molecule (here the chimeric receptor molecule) that provides for activation of a lymphocyte. Intracellular domains of such molecules mediate a signal by interacting with cellular mediators to result in proliferation, differentiation, activation and other effector functions. In some alternatives, such molecules include all or portions of CD28, CD3, or 4-1BB, or combinations thereof. Intracellular signaling domains include costimulatory domains.

[0106] "Ligand" as used herein refers to a substance that binds specifically to another substance to form a complex and/or targets another substance. Examples of ligands include epitopes on antigens, molecules that bind to receptors, substrates, inhibitors, hormones, and activators. "Ligand binding domain" as used herein refers to substance or portion of a substance that binds to a ligand. Examples of ligand binding domains include antigen binding portions of antibodies, extracellular domains of receptors, and active sites of enzymes.

[0107] "Operably linked" as used herein refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional

relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

[0108] "Percent (%) amino acid sequence identity" with respect to the chimeric receptor polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence for each of the ligand binding domain, spacer, transmembrane domain, and/or the lymphocyte activating domain, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software.

[0109] Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For example, % amino acid sequence identity values generated using the WU-BLAST-2 computer program [Altschul et al., Methods in Enzymology, 266:460-480 (1996)] uses several search parameters, most of which are set to the default values. Those that are not set to default values (i.e., the adjustable parameters) are set with the following values: overlap span=1, overlap fraction=0.125, word threshold (T)=11 and scoring matrix=BLOSUM62. A % amino acid sequence identity value is determined by dividing (a) the number of matching identical amino acid residues between the each or all of the polypeptide amino acid sequence of the reference chimeric receptor sequence provided in **Table 2** and the comparison amino acid sequence of interest as determined by WU-BLAST-2 by (b) the total number of amino acid residues of the polypeptide of interest.

[0110] "Chimeric receptor variant polynucleotide" or "chimeric receptor variant nucleic acid sequence" as used herein refers to a polypeptide-encoding nucleic acid molecule as defined below having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity with the polynucleotide acid

sequence shown in **Figures 5, 7, 9**, or **11** or a specifically derived fragment thereof, such as polynucleotide coding for an antigen binding domain, a polynucleotide encoding a spacer domain, a polynucleotide coding for a transmembrane domain and/ or a polynucleotide coding for a lymphocyte stimulatory domain. Ordinarily, a chimeric receptor variant of polynucleotide or fragment thereof will have at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity, more preferably at least 81% nucleic acid sequence identity, more preferably at least 82% nucleic acid sequence identity, more preferably at least 83% nucleic acid sequence identity, more preferably at least 84% nucleic acid sequence identity, more preferably at least 85% nucleic acid sequence identity, more preferably at least 86% nucleic acid sequence identity, more preferably at least 87% nucleic acid sequence identity, more preferably at least 8% nucleic acid sequence identity, more preferably at least 89% nucleic acid sequence identity, more preferably at least 90% nucleic acid sequence identity, more preferably at least 91% nucleic acid sequence identity, more preferably at least 92% nucleic acid sequence identity, more preferably at least 93% nucleic acid sequence identity, more preferably at least 94% nucleic acid sequence identity, more preferably at least 95% nucleic acid sequence identity, more preferably at least 96% nucleic acid sequence identity, more preferably at least 97% nucleic acid sequence identity, more preferably at least 98% nucleic acid sequence identity and yet more preferably at least 99% nucleic acid sequence identity with the nucleic acid sequence as shown in **Figures 5, 7, 9**, or **11** or a derived fragment thereof. Variants do not encompass the native nucleotide sequence. In this regard, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of chimeric receptor variant polynucleotides having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity to the nucleotide sequence of **Figures 5, 7, 9,** or **11**.

[0111] "Substantially purified" refers to a molecule that is essentially free of other molecule types or a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell, which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells.

[0112] "Not substantially found" when used in reference the presence of a tumor antigen or other molecules on normal cells refers to the percentage of a normal cell

type that has the antigen or molecule, and / or the density of the antigen on the cells. In some alternatives, not substantially found means that the antigen or molecule is found on less than 50% of normal cell type and/or at a 50% less density as compared to the amount of cells or antigen found on a tumor cell or other diseased cell.

[0113] "T cells" or "T lymphocytes" as used herein may be from any mammalian, preferably primate, species, including monkeys, dogs, and humans. In some alternatives the T cells are allogeneic (from the same species but different donor) as the recipient subject; in some alternatives the T cells are autologous (the donor and the recipient are the same); in some alternatives the T cells arc syngeneic (the donor and the recipients are different but are identical twins).

[0114] "Specific" or "Specificity" can refer to the characteristic of a ligand for the binding partner or alternatively, the binding partner for the ligand, and can include complementary shape, charge and hydrophobic specificity for binding. Specificity for binding can include stereospecificity, regioselectivity and chemoselectivity.

[0115] In some alternatives, a spacer is described such that the spacer is specific for a ligand. A spacer specific for a ligand can refer to a specific polypeptide length that can allow enhanced binding or targeting of the ligand binding domain for its specific or targeted ligand such that the spacer provides an increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0116] "Targeting" as described herein, refers to the recognition of a unique part of the foreign target, such as for example, a protein epitope. Targeting can also refer to binding or recognition to a specific region of a protein, which can be referred to as an antigen. Similar antigenic sites can also be recognized by a targeting antibody, which can lead to the ability of the antibody to react with similar antigenic sites on different proteins, thus leading to a cross-reactive antibody.

[0117] "Cellular activities" can refer to humoral responses, cell-based immune responses, cellular responses, maturation pathways, growth pathways, and/or responsiveness of particular cell populations. In some alternatives, a chimeric antigen receptor is provided, wherein the chimeric antigen receptor comprises a spacer. In some alternatives, the spacer provides for enhanced or improved T cell proliferation, enhanced and/or decrease in *in vivo* cellular activities, and/ or cytokine production.

[0118] Provided herein are chimeric receptor nucleic acids, and vectors and host cells including such nucleic acids. The chimeric receptor nucleic acid comprises a number of modular components that can be excised and replaced with other components in order to customize the chimeric receptor for targeting a specific target molecule. The disclosure provides that one of the modular components is the spacer component. It has been surprisingly found that the length of the spacer region affects the *in vivo* efficacy of the T cells modified to express the chimeric receptor and can be customized for individual target molecules for enhanced therapeutic activity.

[0119] In one aspect, methods and nucleic acid constructs are provided to design a chimeric receptor that has enhanced or improved tumor recognition, increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor, especially *in vivo*, and/or that results in increased survival of cells bearing the chimeric receptor following specific binding or targeting of the receptor to the antigen, as compared with a reference chimeric receptor.

[0120] In some alternatives, the reference chimeric receptor is a chimeric receptor that is otherwise identical to the subject chimeric receptor, aside from having one or more modifications in a spacer, such as in the polypeptide spacer joining the ligand binding domain, e.g., antibody fragment, of the receptor and the transmembrane and/or intracellular portions of the receptor. For example, in some alternatives, the reference chimeric receptor is identical to the subject receptor aside from having a spacer that is different in length or sequence, for example, a spacer that is longer, such as at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 times the number of amino acids in length, as compared to that of the subject chimeric receptor.

[0121] In some alternatives, a library of nucleic acids is provided, wherein each nucleic acid codes for a spacer region that differs from the others in sequence and/or length. Each of the nucleic acids can then be used to form a chimeric receptor nucleic acid construct that can be tested *in vivo* (in an animal model) and/or *in vitro* so that a spacer can be selected that provides for enhanced or improved tumor recognition, increased T cell proliferation and/or cytokine production in response to the ligand.

[0122] In some alternatives, a chimeric receptor nucleic acid comprises a polynucleotide coding for a ligand binding domain, wherein the ligand is a tumor antigen, a polynucleotide coding for a customized polypeptide spacer, wherein the spacer is

optimized. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0123] The design of a chimeric receptor can be customized depending on the type of tumor, the target antigen or molecule present on the tumor, the affinity of the antibody for the target molecule, the flexibility needed for the antigen binding domain, and/or the intracellular signaling domain. In some alternatives, a number of chimeric receptor constructs are tested *in vitro* and in *in vivo* models to determine the ability of T cells modified with the receptor to kill tumor cells in immunodeficient mice and to proliferate and persist after adoptive transfer.

[0124] Depending on whether the target molecule is present on a subject's tumor cells, the chimeric receptor includes a ligand binding domain that specifically binds to and/or targets that target molecule. In some alternatives, a subject's tumor cells are characterized for cell surface tumor molecules. The target molecule may be selected based on a determination of its presence on a particular subject's tumor cells. In some alternatives, a target molecule or an epitope thereof is selected that is a cell surface molecule found predominantly on tumor cells and not found on normal tissues to any substantial degree. In some alternatives, an antibody is selected to bind to an epitope on the targeted cell surface molecule. In some alternatives, the target molecule is CD171. In some alternatives, the chimeric receptor specifically binds to and/or targets an epitope on CD171 recognized by the antibody known as CE7, and/or an epitope of CD171 or of another antigen, which epitope is of the same or similar distance from the surface plasma membrane of a CD171+ cell. In some alternatives, the antigen has an extracellular portion of a similar size or length as that of CD171, and thus a similar chimeric receptor configuration is appropriate.

[0125] In addition, the spacer region of the chimeric receptor may be varied to enhance or improve T cell recognition of the ligand on the target cell. In some alternatives, a spacer domain is selected from a short spacer domain of 15 amino acids or less (but not less than 1 or 2 amino acids), such as 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 amino acids or a number of amino acids within a range defined by any two of the aforementioned lengths. In some alternatives, a spacer domain is selected from an intermediate spacer domain of 119 amino acids or less (but not less than 1 or 2 amino acids), such as 119, 115,

110, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, or 15 amino acids or a number of amino acids within a range defined by any two of the aforementioned lengths. In some alternatives, a spacer domain is selected from a long spacer of 229 amino acids or less (but not less than 1 or 2 amino acids), such as 229, 225, 220, 215, 210, 205, 200, 195, 190, 185, 180, 175, 170, 165, 160, 155, 150, 145, 140, 135, 130, 125, or 120 amino acids or a number of amino acids within a range defined by any two of the aforementioned lengths. In some alternatives, a spacer is a hinge region of a Fc receptor or a modified hinge region. In some alternatives, a spacer region is a hinge region in combinations with a CH2 or CH3 or both. In some alternatives, a spacer comprises an amino acid sequence X_1PPX_2P. In alternative a spacer region does not include a full length Fc receptor, and/or does not include a CH2 and/or a CH3 domain. In some such alternatives, the spacer does include an antibody hinge region.

[0126] A variety of combinations of primary and costimulatory intracellular signaling domain may be employed to enhance the *in vivo* efficacy of the chimeric receptor. In some alternatives, different constructs of the chimeric receptor can be tested in an *in vivo* animal model to determine efficacy for tumor killing. In some alternatives, a costimulatory intracellular signaling domain is selected from the group consisting of CD28 and modified versions thereof, 4-1BB and modified versions thereof and/or combinations thereof. Other costimulatory domains, such as OX40 may be incorporated. In some alternatives, the costimulatory intracellular signaling portion of the receptor includes only intracellular domains derived from a single costimulatory molecule, such as only 4-1BB or only CD28, and does not further contain such a domain from another such molecule.

[0127] In some alternatives, chimeric receptor-modified, e.g., CD171 specific and/or targeting chimeric receptor-modified cells, e.g., cytotoxic T cells prepared from sort purified CD8+ central memory T cells, are administered in the presence or absence of CD4+ chimeric receptor-modified cells, e.g., CD171 specific and/or targeting chimeric receptor-modified, T cells. In some alternatives, tumor-specific or tumor targeting CD4+ expressing T cells exert anti-tumor reactivity and provide help to tumor-specific CD8+ expressing T cells *in vitro* and *in vivo*. In a specific alternative, tumor-specific or tumor-targeting CD4+ expressing T cells or $CD4^+$ expressing T cells selected from the naïve or the central memory subsets are utilized alone or in combination with $CD8^+$ T_{CM}.

[0128] Adoptive immunotherapy using chimeric antigen receptor (CAR) expressing T cells in some alternatives is useful for treating or inhibiting cancer. In some alternatives, a CAR directed to an epitope of the antigen CD171 (L1CAM) is prepared. Such CAR constructs are useful to treat or inhibit any cancer that expresses CD171 (L1CAM). In some alternatives, one cancer that expresses CD171 is neuroblastoma (NB). CD171 is expressed in 100% of high risk NB. Other cancers thought to over express CD171 include melanoma, cervical carcinoma, ovarian cancer, uterine carcinoma, pancreatic cancer, colon carcinoma, renal carcinoma, and glioblastoma.

[0129] The disclosure provides a chimeric receptor nucleic acid useful for transforming or transducing lymphocytes for use in adoptive immunotherapy. In some alternatives, the nucleic acid contains a number of modular components that provide for easy substitution of elements of the nucleic acid. While not meant to limit the scope of the disclosure, it is believed that the chimeric receptor for each tumor antigen is desirably customized in terms of components in order to provide for *in vivo* efficacy and efficient expression in mammalian cells. For example, in a specific alternative, for efficacy of a chimeric receptor comprising a scFV that binds to a CD171 L1CAM epitope, such as one that is the same or similar to that recognized the antibody deemed CE7, or an epitope on CD171 or other antigen of the same relative distance from the surface plasma membrane, a spacer that is 15 amino acids or less (but not less than 1 or 2 amino acids) is employed. In some alternatives, an expression vector comprises a chimeric nucleic acid as described herein. Polypeptides encoded by all of or a portion of the chimeric receptor nucleic acids are also included herein.

[0130] In some alternatives, the chimeric receptor nucleic acid comprises a polynucleotide coding for a ligand binding domain. In some alternatives, the ligand binding domain specifically binds to and/or targets CD171, or a tumor restricted epitope of CD171. In some alternatives, the ligand binding domain is an antibody or fragment thereof. A nucleic acid sequence coding for an antibody or binding fragment thereof can readily be determined. In a specific alternative, the polynucleotide codes for a single chain Fv that specifically binds and/or targets CD171 (L1CAM). An exemplary antibody is the CE7 antibody. An exemplary nucleic acid sequence for the antibody CE7 scFv is provided in Figure 5. The sequences of other antibodies are known to or can readily be determined by those of skill in the art.

[0131] Tumor antigens are proteins that are produced by tumor cells that elicit an immune response. The selection of the ligand binding domain of the invention will depend on the type of cancer to be treated or inhibited, and may target tumor antigens or other tumor cell surface molecules. A tumor sample from a subject may be characterized for the presence of certain biomarkers or cell surface markers. For example, neuroblastoma cells from a subject are characterized for the presence of CD171 (L1CAM). Other cancer or tumor cells may also be characterized for the presence of CD171 (L1CAM) and can be treated or inhibited with the compositions described herein. In some alternatives a target molecule is a cell surface molecule that is found on tumor cells and is not substantially found on normal tissues, or restricted in its expression to non-vital normal tissues.

[0132] Once a tumor cell surface molecule that might be targeted with a chimeric receptor is identified, an epitope of the target molecule is selected and characterized. L1CAM is a cell membrane molecule involved in cell adhesion of neurons. L1CAM has several domains including amino acids 1-19 signal peptide, amino acids 35-125 Ig-like C2 type 1, amino acids 139-226 Ig-like C2 type 2, amino acids 240-328 Ig-like C2 type 3, amino acids 333-420 Ig-like C2 type 4, amino acids 425-507 Ig-like C2 type 5, amino acids 518-607 Ig-like C2 type 6, amino acids 615-712 fibronectin type III 1, amino acids 717-810 fibronectin type III 2, amino acids 814-916 fibronectin type III 3, amino acids 920-1015 fibronectin type III 4, and amino acids 1016-1115 fibronectin type III 5. Epitopes can be found in any of those domains. One epitope that has been characterized is known as the CE7 epitope. The CE7 epitope is an epitope found more often on tumor cells as compared to normal tissues. In some alternatives, an epitope of CD171 is selected that is found more often on tumor cells than healthy cells.

[0133] Antibodies that specifically bind and/or targets a tumor cell surface molecule can be prepared using methods of obtaining monoclonal antibodies, methods of phage display, methods to generate human or humanized antibodies, or methods using a transgenic animal or plant engineered to produce human antibodies. Phage display libraries of partially or fully synthetic antibodies are available and can be screened for an antibody or fragment thereof that can bind to the target molecule. Phage display libraries of human antibodies are also available. In some alternatives, antibodies specifically bind, and/or target a tumor cell surface molecule and do not cross react with nonspecific components such as bovine serum albumin or other unrelated antigens. Once identified, the amino acid

sequence or polynucleotide sequence coding for the antibody can be isolated and/or determined.

[0134] Antibodies or antigen binding fragments include all or a portion of polyclonal antibodies, a monoclonal antibody, a human antibody, a humanized antibody, a synthetic antibody, a chimeric antibody, a bispecific antibody, a minibody, and a linear antibody. Antibody fragments comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody and can readily be prepared. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. A number of anti CD171 antibodies are known and are commercially available.

[0135] In some alternatives, a number of different antibodies that bind to a particular tumor cell surface molecule can be isolated and characterized. In some alternatives, the antibodies are characterized based on epitope specificity and/or targeting of the targeted molecule. In addition, in some cases, antibodies that bind to the same epitope can be selected based on the affinity of the antibody for that epitope. In some alternatives, an antibody has an affinity of at least 1 mM, and preferably <50 nM. In some alternatives, an antibody is selected that has a higher affinity for the epitope as compared to other antibodies. For example, an antibody is selected that has at least a 2 fold, at least a 5 fold, at least a 10 fold, at least a 20 fold, at least a 30 fold, at least a 40 fold, or at least a 50 fold greater affinity than a reference antibody that binds to the same epitope.

[0136] In some alternatives, a polynucleotide coding for a ligand binding domain is operably linked to a polynucleotide coding for a spacer region. In some alternatives, the polynucleotide coding for a ligand binding domain may also have one or more restriction enzyme sites at the 5' and/or 3' ends of the coding sequence in order to provide for easy excision and replacement of the polynucleotide with another polynucleotide coding for a ligand binding domain coding for a different antigen or that has different binding characteristics. For example, a restriction site, NheI, is encoded upstream of the leader sequence; and a 3' RsrII located within the hinge region allows subcloning of any desirable scFv into a chimeric receptor vector. In some alternatives, the polynucleotide is codon optimized for expression in mammalian cells.

[0137] In some alternatives, the polynucleotide coding for a ligand binding domain is operably linked to a signal peptide. In some alternatives the signal peptide is a signal peptide for granulocyte colony stimulating factor. Polynucleotides coding for other signal peptides such as CD8 alpha can be utilized.

[0138] In some alternatives, the polynucleotide coding for a ligand binding domain is operably linked to a promoter. A promoter is selected that provides for expression of the chimeric antigen receptor in a mammalian cell. In a specific alternative the promoter is the elongation growth factor promoter (EF-1). Another example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV 40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MuMoLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Inducible promoters are also contemplated. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter. A specific alternative of a polynucleotide coding for a ligand binding domain is shown in **Figure 5** as the scFv from an antibody that specifically binds and/or targets epitope Ce7 on CD171.

[0139] In some alternatives, the chimeric receptor nucleic acid comprises a polynucleotide coding for a spacer region. It has been surprisingly found that the length of the spacer region affects the *in vivo* efficacy of the T cells modified to express the chimeric receptor and can be customized for individual target molecules for optimal tumor or target cell recognition. In some alternatives, the chimeric receptor nucleic acid comprises a polynucleotide coding for a customizable spacer region selected from a library of polynucleotides coding for spacer regions. In some alternatives, a spacer length is selected based upon the location of the epitope, affinity of the antibody for the epitope, and/or the ability of the T cells expressing the chimeric receptor to proliferate in vitro and/or in vivo in response to antigen recognition.

[0140] Typically a spacer region is found between the ligand binding domain and the transmembrane domain of the chimeric receptor. In some alternatives, a spacer

region provides for flexibility of the ligand binding domain, allows for high expression levels in lymphocytes. A CD171specific and/or targeting chimeric receptor having a spacer domain of 229 amino acids had less *in vivo* antitumor activity than a CD171-specific and/or targeting chimeric receptor with a short spacer region comprised of 15 amino acids or less (but not less than 1 or 2 amino acids).

[0141] In some alternatives, a spacer region has at least 10 to 229 amino acids, 10 to 200 amino acids, 10 to 175 amino acids, 10 to 150 amino acids, 10 to 125 amino acids, 10 to 115 amino acids, 10 to 100 amino acids, 10 to 75 amino acids, 10 to 50 amino acids, 10 to 40 amino acids, 10 to 30 amino acids, 10 to 20 amino acids, or 10 to 15 amino acids, or a length within a range defined by any two of the aforementioned amino acid lengths. In some alternatives, a spacer region has 15 amino acids or less (but not less than 1 or 2 amino acids), 119 amino acids or less (but not less than 1 or 2 amino acids), or 229 amino acids or less (but not less than 1 or 2 amino acids).

[0142] In some alternatives, the spacer region is derived from a hinge region of an immunoglobulin like molecule. In some alternatives, a spacer region comprises all or a portion of the hinge region from a human IgG1, human IgG2, a human IgG3, or a human IgG4, or modified variant thereof, and may contain one or more amino acid substitutions. Exemplary sequences of the hinge regions are provided in **Table 6**. In some alternatives, a portion of the hinge region includes the upper hinge amino acids found between the variable heavy chain and the core, and the core hinge amino acids including a polyproline region. Typically, the upper hinge region has 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In some cases, the spacer region comprises an amino acid sequence of X_1PPX_2P (SEQ ID NO: 1). In some alternatives, X_1 is a cysteine, glycine, or arginine and X_2 is a cysteine or a threonine.

[0143] In some alternatives, hinge region sequences can be modified in one or more amino acids in order to avoid undesirable structural interactions such as dimerization. In a specific alternative, the spacer region comprises a portion of a modified human hinge region from IgG4, for example, as shown in **Table 1** or **Table 6**. A representative of a polynucleotide coding for a portion of a modified IgG4 hinge region is provided in **Table 1.** In some alternatives, a hinge region can have at least 90%, 92%, 95%, or 100% sequence identity with a hinge region amino acid sequence identified in **Table 1** or **Table**

6. In a specific alternative, a portion of a human hinge region from IgG4 has an amino acid substitution in the core amino acids from CPSP to CPPC.

[0144] In some alternatives, all or a portion of the hinge region is combined with one or more domains of a constant region of an immunoglobulin. For example, a portion of a hinge region can be combined with all or a portion of a CH2 or CH3 domain or variant thereof. In some alternatives, the spacer region does not include the 47-48 amino acid hinge region sequence from CD8 alpha, a full length Fc receptor, and/or the spacer region consisting of an extracellular portion of the CD28 molecule.

[0145] In some alternatives, a short spacer region has 15 amino acids or less (but not less than 1 or 2 amino acids) and comprises all or a portion of a IgG4 hinge region sequence or variant thereof, an intermediate spacer region has 119 amino acids or less (but not less than 1 or 2 amino acids) and comprises all or a portion of a IgG4 hinge region sequence and a CH3 region or variant thereof, and a long spacer has 229 amino acids or less (but not less than 1 or 2 amino acids) and comprises all or a portion of a IgG4 hinge region sequence, a CH2 region, and a CH3 region or variant thereof.

[0146] A polynucleotide coding for a spacer region can be readily prepared by synthetic or recombinant methods from the amino acid sequence. In some alternatives, a polynucleotide coding for a spacer region is operably linked to a polynucleotide coding for a transmembrane region. In some alternatives, the polynucleotide coding for the spacer region may also have one or more restriction enzyme sites at the 5' and/or 3' ends of the coding sequence in order to provide for easy excision and replacement of the polynucleotide with another polynucleotide coding for a different spacer region. In some alternatives, the polynucleotide coding for the spacer region is codon optimized for expression in mammalian cells, preferably humans.

[0147] In some alternatives, a library of polynucleotides, each coding for different spacer region is provided. In some alternatives, the spacer region is selected from the group consisting of a hinge region sequence from IgG1, IgG2, IgG3, or IgG4 or portion thereof, a hinge region sequence from IgG1, IgG2, IgG3, or IgG4 in combination with all or a portion of a CH2 region or variant thereof, a hinge region sequence from IgG1, IgG2, IgG3, or IgG4 in combination with all or a portion of a CH3 region or variant thereof, and a hinge region sequence from IgG1, IgG2, IgG3, or IgG4 in combination with all or a portion of a CH2 region or variant thereof, and a CH3 region or variant thereof. In some

alternatives, a short spacer region is a modified IgG4 hinge sequence having 15 amino acids or less (but not less than 1 or 2 amino acids), an intermediate sequence is a IgG4 hinge sequence with a CH3 sequence having 119 amino acids or less (but not less than 1 or 2 amino acids) (SEQ ID NO:49); or a IgG4 hinge sequence with a CH2 and CH3 region having 229 amino acids or less (but not less than 1 or 2 amino acids).

[0148] In some alternatives, a method of selecting a spacer region for a chimeric receptor is provided herein. Surprisingly some chimeric receptor constructs, although effective to activate T cells and direct their killing of tumor cells *in vitro*, were not effective in vivo. In addition, the side effect profile of the chimeric receptor modified T cells can be such as to result in more cells undergoing activation induced cell death or causing an increase in *in vivo* cytokines. In some alternatives, a method comprises providing a plurality of chimeric receptor nucleic acids, wherein the chimeric receptor nucleic acids differ only in the spacer region; introducing each of the chimeric receptor nucleic acids into a separate T lymphocyte population; expanding each separate lymphocyte population *in vitro*, and introducing each lymphocyte population into an animal bearing a tumor to determine the anti-tumor efficacy of each of the chimeric receptors when expressed in T cells, and selecting a chimeric receptor that provides anti-tumor efficacy as compared to each of the other separate lymphocyte populations modified with each of the other chimeric receptors.

[0149] Animal models of different tumors are known. Anti-tumor efficacy can be measured by identifying a decrease in tumor volume, by determining animal death, persistence of the genetically modified T cells *in vivo*, activation of genetically modified T cells (for example, by detecting an increase in expression of CD25 and/CD69), and/or proliferation of genetically modified T cells *in vivo*. In some alternatives, a chimeric receptor is selected that provides for the best anti-tumor efficacy *in vivo* as determined by one or more of these parameters. Lack of anti-tumor efficacy can be determined by lack of persistence of the genetically modified lymphocytes in vivo, animal death, an increase in apoptosis as measured by an increase in induction of caspase -3, and/or a decrease in proliferation of genetically modified lymphocytes.

[0150] In some alternatives, a chimeric receptor is selected that provides for at least 30% of the cells proliferating through two generations *in vitro* and/or *in vivo*. In other

alternatives a chimeric receptor is not selected if it results in at least 50% of the cells undergoing activation induced cell death in 72 hours.

[0151] In some alternatives, providing a plurality of chimeric receptor nucleic acids, wherein the chimeric receptor nucleic acids differ only in the spacer region comprises providing a chimeric receptor construct comprising a polynucleotide coding for a ligand binding domain, wherein the ligand is a tumor specific and/or tumor targeting antigen, or any other molecule expressed on a target cell population that is suitable to mediate recognition and elimination by a lymphocyte; a polynucleotide coding for a first polypeptide spacer having a defined restriction site at the 5' and 3' end of the coding sequence for the first polypeptide spacer; a polynucleotide coding for a transmembrane domain; and a polynucleotide coding for one or more intracellular signaling domains.

[0152] In some alternatives, a method further comprises providing one or more polynucleotides, each encoding a different spacer region. Exemplary constructs are provided in **Figure 5, 7,** and **9**. In some alternatives, a method further comprises replacing the polynucleotide coding for the spacer region with a polynucleotide encoding a different spacer region to form a chimeric receptor nucleic acid with a different spacer region. The method can be repeated to form any number of chimeric receptor nucleic acids, each differing in the spacer region. In some alternatives, the chimeric receptor nucleic acids differ from one another only in the spacer region.

[0153] In some alternatives, the chimeric receptor nucleic acid comprises a polynucleotide coding for a transmembrane domain. The transmembrane domain provides for anchoring of the chimeric receptor in the membrane. In some alternatives, the transmembrane domain that naturally is associated with one of the domains in the chimeric receptor is used. In some cases, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0154] The transmembrane domain may be derived either from a natural or a synthetic source. When the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3, CD45, CD4, CD8, CD9, CD16, CD22; CD33, CD37, CD64, CD80, CD86, CD134,

CD137 and/or CD154. In a specific alternative, the transmembrane domain comprises the amino acid sequence of the CD28 transmembrane domain as shown in **Table 1**. A representative polynucleotide sequence coding for the CD28 transmembrane domain is shown in **Table 1** (SEQ ID NO:5).

[0155] A transmembrane domain may be synthetic or a variant of a naturally occurring transmembrane domain. In some alternatives, synthetic or variant transmembrane domains comprise predominantly hydrophobic residues such as leucine and valine. In some alternatives, a transmembrane domain can have at least 80%, 85%, 90%, 95%, or 100% amino acid sequence identity with a transmembrane domain as shown in **Table 1** or **Table 3**. Variant transmembrane domains preferably have a hydrophobic score of at least 50 as calculated by Kyte-Doolittle.

[0156] A polynucleotide coding for a transmembrane domain can be readily prepared by synthetic or recombinant methods. In some alternatives, a polynucleotide coding for a transmembrane domain is operably linked to a polynucleotide coding for a intracellular signaling region. In some alternatives, the polynucleotide coding for a transmembrane domain may also have one or more restriction enzyme sites at the 5' and/or 3' ends of the coding sequence in order to provide for easy excision and replacement of the polynucleotide coding for a transmembrane domain with another polynucleotide coding for a different transmembrane domain. In some alternatives, the polynucleotide coding for a transmembrane domain is codon optimized for expression in mammalian cells.

[0157] In some alternatives, the chimeric receptor nucleic acid comprises a polynucleotide coding for an intracellular signaling domain. The intracellular signaling domain provides for activation of one function of the transduced cell expressing the chimeric receptor upon binding to the ligand expressed on tumor cells. In some alternatives, the intracellular signaling domain contains one or more costimulatory domains. In some alternatives, the intracellular signaling domain is a portion of and/or a variant of an intracellular signaling domain that provides for activation of at least one function of the transduced cell.

[0158] Examples of intracellular signaling domains for use in a chimeric receptor of the disclosure include the cytoplasmic sequences of the CD3 zeta chain, and/or co-receptors that act in concert to initiate signal transduction following chimeric receptor engagement, as well as any derivative or variant of these sequences and any synthetic

sequence that has the same functional capability. T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequence: those that initiate antigen-dependent primary activation and provide a T cell receptor like signal (primary cytoplasmic signaling sequences) and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences). Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as receptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing primary cytoplasmic signaling sequences include those derived from CD3 zeta, FcR gamma, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and/or CD66d. In some alternatives, the primary signaling intracellular domain can have at least 80%, 85%, 90%, or 95% sequence identity to CD3zeta having a sequence provided in **Table 1**. In some alternatives, variants of CD3 zeta retain at least one, two, three or all ITAM regions as shown in **Table 5**.

[0159] In a preferred alternative, the intracellular signaling domain of the chimeric receptor can be designed to comprise the CD3-zeta signaling domain by itself or combined with any other desired cytoplasmic domain(s). For example, the intracellular signaling domain of the chimeric receptor can comprise a CD3-zeta chain and a costimulatory signaling region.

[0160] The costimulatory signaling region refers to a portion of the chimeric receptor comprising the intracellular domain of a costimulatory molecule. A costimulatory molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for a response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD 137), OX40, CD30, CD40, lymphocyte function-associated antigen-1 (LFA-l), CD2, CD7, LIGHT, NKG2C, B7-H3, and/or a ligand that specifically binds with CD83 and/or targets CD83. In some alternatives, the costimulatory signaling domain can have at least 80%, 85%, 90%, or 95% amino acid sequence identity to the intracellular domain of CD28 as shown in **Table 3** or to 4-1BB having a sequence provided in **Table 4**. In some alternatives, a variant of the CD28 intracellular domain comprises an amino acid substitution at positions 186-187, wherein LL is substituted with GG.

[0161] The intracellular signaling sequences of the chimeric receptor may be linked to each other in a random or specified order. Optionally, a short oligo- or

polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage. In one alternative, the intracellular signaling domains comprises all or a portion of the signaling domain of CD3-zeta or variant thereof and all or a portion of the signaling domain of CD28 or a variant thereof. In another alternative, the intracellular signaling domain comprises all or a portion of the signaling domain of CD3-zeta or variant thereof and all or a portion of the signaling domain of 4-lBB or variant thereof. In yet another alternative, the intracellular signaling domain comprises all or a portion of the signaling domain of CD3-zeta or variant thereof, all or a portion of the signaling domain of CD28 or variant thereof, and all or a portion of the signaling domain of 4-lBB or variant thereof. In a specific alternative, the amino acid sequence of the intracellular signaling domain comprising a variant of CD3zeta and a portion of the 4-1BB intracellular signaling domain is provided in **Table 1**. A representative nucleic acid sequence is provided in **Table 1**.

[0162] In some alternatives, a polynucleotide coding for an intracellular signaling domain comprises a 4-1BB intracellular domain linked to a portion of a CD3-zeta domain. In other alternatives, the intracellular signaling domain includes only a single costimulatory domain and does not include dual costimulatory domains, such as CD28 cyto combined with 4-1BB costimulatory domains.

[0163] In some alternatives, a polynucleotide coding for an intracellular signaling domain that comprises a CD28 domain linked to a 4-1BB intracellular domain linked to a portion of a CD3-zeta domain is provided in **Figure 11**. A polynucleotide coding for an intracellular signaling domain can be readily prepared by synthetic or recombinant methods from the amino acid sequence. In some alternatives, the polynucleotide coding for an intracellular signaling domain may also have one or more restriction enzyme sites at the 5' and/or 3' ends of the coding sequence in order to provide for easy excision and replacement of the polynucleotide coding for an intracellular signaling domain with another polynucleotide coding for a different intracellular signaling domain. In some alternatives, the polynucleotide coding for an intracellular signaling domain is codon optimized for expression in mammalian cells, preferably humans.

[0164] In some alternatives, the chimeric receptor nucleic acid optionally further comprises a polynucleotide sequence coding for a marker. A marker sequence preferably encodes a cell surface expressed marker that can allow for selection of transduced cells, and/or identification of transduced cells. In some alternatives, the marker

sequence is operably linked to a polynucleotide sequence coding for a linker sequence. In some alternatives, the linker sequence is a cleavable linker sequence.

[0165] A number of different marker sequences can be employed. Typically a marker sequence has a functional characteristic that allows for selection of transduced cells and/or detection of transduced cells. In some alternatives, the marker sequence is compatible with transduction of human lymphocytes.

[0166] The positive selectable marker may be a gene, which upon being introduced into the host cell, expresses a dominant phenotype permitting positive selection of cells carrying the gene. Genes of this type are known in the art, and include, inter alia, hygromycin-B phosphotransferase gene (hph) which confers resistance to hygromycin B, the amino glycoside phosphotransferase gene (neo or aph) from Tn5 which codes for resistance to the antibiotic G418, the dihydrofolate reductase (DHFR) gene, the adenosine deaminase gene (ADA), and/or the multi-drug resistance (MDR) gene.

[0167] In some alternatives, a chimeric receptor nucleic acid further comprises a polynucleotide coding for a marker. In some alternatives, the marker sequence encodes a truncated epidermal growth factor receptor, which is expressed at the cell surface. An exemplary polynucleotide for the truncated epidermal growth factor receptor is shown in Table 1. In some alternatives, the polynucleotide coding for the marker is operably linked to a polynucleotide coding for a linker sequence. In a specific alternative, the linker sequence is a cleavable linker sequence T2A, as shown in **Table 1**. An exemplary polynucleotide sequence coding for the T2A linker is provided in **Table 1**.

[0168] A polynucleotide coding for marker can be readily prepared by synthetic or recombinant methods from the amino acid sequence. In some alternatives a polynucleotide coding for a marker is operably linked to a polynucleotide coding for an intracellular signaling domain. In some alternatives, the polynucleotide coding for a marker may also have one or more restriction enzyme sites at the 5' and/or 3' ends of the coding sequence in order to provide for easy excision and replacement of the polynucleotide coding for a marker with another polynucleotide coding for a different marker. In some alternatives, the polynucleotide coding for a marker is codon optimized for expression in mammalian cells.

[0169] The compositions described herein provide for CD4+ and/or CD8+ T lymphocytes. T lymphocytes can be collected in accordance with known techniques and

enriched or depleted by known techniques such as affinity binding to antibodies such as flow cytometry and/or immunomagnetic selection. After enrichment and/or depletion steps, in vitro expansion of the desired T lymphocytes can be carried out in accordance with known techniques (including but not limited to those described in US Patent No. 6,040,177 to Riddell et al.), or variations thereof that will be apparent to those skilled in the art. In some alternatives, the T cells are autologous T cells obtained from the patient.

[0170] For example, the desired T cell population or subpopulation may be expanded by adding an initial T lymphocyte population to a culture medium in vitro, and then adding to the culture medium feeder cells, such as non-dividing peripheral blood mononuclear cells (PBMC), (e.g., such that the resulting population of cells contains at least 5, 10, 20, or 40 or more PBMC feeder cells for each T lymphocyte in the initial population to be expanded); and incubating the culture (e.g. for a time sufficient to expand the numbers of T cells). The non-dividing feeder cells can comprise gamma-irradiated PBMC feeder cells. In some alternatives, the PBMC are irradiated with gamma rays in the range of 3000 to 3600 rads to prevent cell division. The order of addition of the T cells and feeder cells to the culture media can be reversed if desired. The culture can typically be incubated under conditions of temperature and the like that are suitable for the growth of T lymphocytes. For the growth of human T lymphocytes, for example, the temperature will generally be at least 25 degrees Celsius, preferably at least 30 degrees, more preferably 37 degrees.

[0171] The T lymphocytes expanded include $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ helper T lymphocytes that may be specific for an antigen present on a human tumor or a pathogen. Optionally, the expansion method may further comprise the step of adding non-dividing EBV-transformed lymphoblastoid cells (LCL) as feeder cells. LCL can be irradiated with gamma rays in the range of 6000 to 10,000 rads. The LCL feeder cells may be provided in any suitable amount, such as a ratio of LCL feeder cells to initial T lymphocytes of at least 10:1.Optionally, the expansion method may further comprise the step of adding anti-CD3 and/or anti CD28 antibody to the culture medium (*e.g.*, at a concentration of at least 0.5 ng/ml). Optionally, the expansion method may further comprise the step of adding IL-2 and/or IL-15 to the culture medium (*e.g.*, wherein the concentration of IL-2 is at least 10 units/m1). After isolation of T lymphocytes both

cytotoxic and helper T lymphocytes can be sorted into naïve, memory, and effector T cell subpopulations either before or after expansion.

[0172] CD8+ T cells can be obtained by using standard methods. In some alternatives, CD8+ T cells are further sorted into naïve, central memory, and effector memory cells by identifying cell surface antigens that are associated with each of those types of CD8+ T cells. In some alternatives, memory T cells are present in both CD62L+ and CD62L- subsets of CD8+ peripheral blood lymphocytes. PBMC are sorted into CD62L-CD8+ and/or CD62L+CD8+ fractions after staining with anti-CD8 and anti-CD62L antibodies. In some alternatives, the expression of phenotypic markers of central memory T_{CM} include CD45RO, CD62L, CCR7, CD28, CD3, and/or CD127 and are negative or low for granzyme B and/or CD45RA. In some alternatives, central memory T cells are CD45RO+, CD62L+, or CD8+ T cells. In some alternatives, effector T_E are negative for CD62L, CCR7, CD28, and/or CD127, and positive for granzyme B and/or perforin. In some alternatives, naïve CD8+ T lymphocytes are characterized by the expression of phenotypic markers of naïve T cells including CD62L, CCR7, CD28, CD3, CD127, and/or CD45RA.

[0173] Whether a cell or cell population is positive for a particular cell surface marker can be determined by flow cytometry using staining with a specific antibody for the surface marker and an isotype matched control antibody. A cell population negative for a marker refers to the absence of significant staining of the cell population with the specific antibody above the isotype control, positive refers to uniform staining of the cell population above the isotype control. In some alternatives, a decrease in expression of one or markers refers to loss of 1 log10 in the mean fluorescence intensity and/or decrease of percentage of cells that exhibit the marker of at least 20% of the cells, 25% of-the cells, 30% of the cells, 35% of the cells, 40% of the cells, 45% of the cells, 50% of the cells, 55% of the cells, 60% of the cells, 65% of the cells, 70% of the cells, 75% of the cells, 80% of the cells, 85% of the cells, 90% of the cell, 95% of the cells, and 100% of the cells and any % between 20 and 100% when compared to a reference cell population. In some alternatives, a cell population positive for one or markers refers to a percentage of cells that exhibit the marker of at least 50% of the cells, 55% of the cells, 60% of the cells, 65% of the cells, 70% of the cells, 75% of the cells, 80% of the cells, 85% of the cells, 90% of

the cell, 95% of the cells, and 100% of the cells and any % between 50 and 100% when compared to a reference cell population.

[0174] CD4+ T helper cells are sorted into naïve, central memory, and effector cells by identifying cell populations that have cell surface antigens. CD4+ lymphocytes can be obtained by standard methods. In some alternatives, naïve CD4+ T lymphocytes are CD45RO-, CD45RA+, CD62L+, and/or CD4+ T cells. In some alternatives, central memory CD4+ cells are CD62L+ and/or CD45RO+. In some alternatives, effector CD4+ cells are CD62L- and/or CD45RO-.

[0175] In some alternatives, populations of CD4+ and CD8+ that are antigen specific can be obtained by stimulating naïve or antigen specific T lymphocytes with antigen. For example, antigen-specific T cell lines or clones can be generated to Cytomegalovirus antigens by isolating T cells from infected subjects and stimulating the cells *in vitro* with the same antigen. Naïve T cells may also be used. Any number of antigens from tumor cells may be utilized as targets to elicit T cell responses. In some alternatives, the adoptive cellular immunotherapy compositions are useful in the treatment of a disease or disorder including a solid tumor, hematologic malignancy, breast cancer or melanoma.

[0176] In some alternatives it may be desired to introduce functional genes into the T cells to be used in immunotherapy in accordance with the present disclosure. For example, the introduced gene or genes may enhance or improve the efficacy of therapy by promoting the viability and/or function of transferred T cells; or they may provide a genetic marker to permit selection and/or evaluation of *in vivo* survival or migration; or they may incorporate functions that enhance or improve the safety of immunotherapy, for example, by making the cell susceptible to negative selection in vivo as described by Lupton S. D. et al., *Mol. and Cell Biol.,* 11:6 (1991); and Riddell et al., *Human Gene Therapy* 3:319-338 (1992); see also the publications of PCT/US91/08442 and PCT/US94/05601 by Lupton et al. describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable marker with a negative selectable marker. This can be carried out in accordance with known techniques *(see, e.g.,* US Patent No. 6,040,177 to Riddell et al. at columns 14-17) or variations thereof that will be apparent to those skilled in the art based upon the present disclosure.

[0177] In some alternatives, T cells are modified with chimeric receptors, as described herein. In some alternatives, the T cells are obtained from the subject to be treated, in other alternatives, the lymphocytes are obtained from allogeneic human donors, preferably healthy human donors. Preferably, the T cells containing the chimeric antigen receptors, as described herein, are derived from thymocytes (naturally arising in humans), as well as, those that are derived from engineered precursors, such as iPS cells.

[0178] In some alternatives, chimeric receptors comprise a ligand binding domain that specifically binds and/or targets a tumor cell surface molecule, a polypeptide spacer region, a transmembrane domain and an intracellular signaling domain, as described herein. In some alternatives, the ligand binding domain is a single-chain antibody fragment (scFv) that is derived from the variable heavy (VH) and variable light (VL) chains of a monoclonal antibody (mAb). Costimulatory signals can also be provided through the chimeric receptor by fusing the costimulatory domain of CD28 and/or 4-1BB to the CD3ζ chain. Chimeric receptors are specific and/or target cell surface molecules independent from HLA, thus overcoming the limitations of TCR-recognition including HLA-restriction and low levels of HLA-expression on tumor cells.

[0179] In some alternatives, the same or a different chimeric receptor can be introduced into each of population of CD4+ and CD8+ T lymphocytes. In some alternatives, the chimeric receptor in each of these populations has a ligand binding domain that specifically binds to and/or targets the same ligand on the tumor or infected cell. The cellular signaling modules can differ. In some alternatives, the intracellular signaling domain of the CD8+ cytotoxic T cells is the same as the intracellular signaling domain of the CD4+ helper T cells. In other alternatives, the intracellular signaling domain of the CD8+ cytotoxic T cells is different than the intracellular signaling domain of the CD4+ helper T cells.

[0180] In some alternatives each of the CD4 or CD8 T lymphocytes can be sorted in to naïve, central memory, effector memory or effector cells prior to transduction as described herein. In alternative alternatives, each of the CD4 or CD8 T lymphocytes can be sorted in to naïve, central memory, effector memory, or effector cells after transduction.

[0181] Various transduction techniques have been developed, which utilize recombinant infectious virus particles for gene delivery. This represents a currently preferred approach to the transduction of T lymphocytes of the present invention. The viral

vectors which have been used in this way include virus vectors derived from simian virus 40, adenoviruses, adeno-associated virus (AAV), lentiviral vectors, and/or retroviruses. Thus, gene transfer and expression methods are numerous but essentially function to introduce and express genetic material in mammalian cells. Several of the above techniques have been used to transduce hematopoietic or lymphoid cells, including calcium phosphate transfection, protoplast fusion, electroporation, and/or infection with recombinant adenovirus, adeno-associated virus and/or retrovirus vectors. Primary T lymphocytes have been successfully transduced by electroporation and by retroviral or lentiviral infection.

[0182] Retroviral and lentiviral vectors provide a highly efficient method for gene transfer into eukaryotic cells. Moreover, retroviral or lentiviral integration takes place in a controlled fashion and results in the stable integration of one or a few copies of the new genetic information per cell.

[0183] In some alternatives it may be useful to include in the T cells a positive marker that enables the selection of cells of the negative selectable phenotype *in vitro*. The positive selectable marker may be a gene that upon being introduced into the host cell expresses a dominant phenotype permitting positive selection of cells carrying the gene. Genes of this type are known in the art, and include, inter alia, hygromycin-B phosphotransferase gene (hph), which confers resistance to hygromycin B, the amino glycoside phosphotransferase gene (neo or aph) from Tn5, which codes for resistance to the antibiotic G418, the dihydrofolate reductase (DHFR) gene, the adenosine deaminase gene (ADA), and/or the multi-drug resistance (MDR) gene.

[0184] A variety of methods can be employed for transducing T lymphocytes, as is well known in the art. In some alternatives, transduction is carried out using lentiviral vectors. In some alternatives, CD4+ and CD8+ T cells each can separately be modified with an expression vector encoding a chimeric receptor to form defined populations. In some alternatives, these cells are then further sorted into subpopulations of naïve, central memory and effector cells as described above by sorting for cell surface antigens unique to each of those cell populations. In addition, CD4+ or CD8+ T cell populations may be selected by their cytokine profile or proliferative activities. For example, CD4+ T lymphocytes that have enhanced production of cytokines such as IL-2, IL-4, IL-10, TNFα, and/or IFNγ as compared to sham transduced cells or transduced CD8+ cells when

stimulated with antigen can be selected. In other alternatives, naïve or central memory CD4+ T cells that have enhanced production of IL-2 and/or TNFα are selected. Likewise, CD8+ cells that have enhanced IFNγ production are selected as compared to sham transduced CD8+ cells.

[0185] In some alternatives, CD4+ and CD8+ T cells that proliferate in response to antigen or tumor targets are selected. For example, CD4+ T cells that proliferate vigorously when stimulated with antigen or tumor targets as compared to sham transduced cells, or CD8+ transduced cells are selected. In some alternatives, CD4+ and CD8+ T cells are selected that are cytotoxic for antigen bearing cells. In some alternatives, CD4+ T cells are expected to be weakly cytotoxic, as compared to CD8+ T cells.

[0186] In a preferred alternative, transduced lymphocytes, such as CD8+ central memory cells, are selected that provide for tumor cell killing in vivo using an animal model established for the particular type of cancer. Such animal models are known to those of skill in the art and exclude human beings. As described herein, not all chimeric receptor constructs transduced into lymphocytes confer the ability to kill tumor cells in vivo despite the ability to become activated and kill tumor cells in vitro. In particular, for some target molecules T cells having chimeric receptor constructs with a long spacer region were less effective at killing tumor cells in vivo as compared to T cells having a chimeric receptor with short spacer region.

[0187] The disclosure contemplates that combinations of CD4+ and CD8+ T cells will be utilized in the compositions. In one alternative, combinations of chimeric receptor transduced CD4+ cells can be combined with chimeric receptor transduced CD8+ cells of the same ligand specificity or combined with $CD8^+$ T cells that are specific and/or targets for a distinct tumor ligand. In other alternatives, chimeric receptor transduced CD8+ cells are combined with chimeric receptor transduced CD4+ cells specific for and/or can target a different ligand expressed on the tumor. In yet another alternative, chimeric receptor modified CD4+ and CD8+ cells are combined. In some alternatives CD8+ and CD4+ cells can be combined in different ratios for example, a 1:1 ratio of CD8+ and CD4+, a ratio of 10:1 of CD8+ to CD4+, or a ratio of 100:1 of CD8+ to CD4+. In some alternatives, the combined population is tested for cell proliferation *in vitro* and/or *in vivo*, and the ratio of cells that provides for proliferation of cells is selected.

[0188] As described herein, the disclosure contemplates that CD4+ and CD8+ T cells can be further separated into subpopulations, such as naïve, central memory, and effector memory cell populations. As described herein, in some alternatives, naïve CD4+ cells are CD45RO-, CD45RA+, CD62L+, CD4+ positive T cells. In some alternatives, central memory CD4+ cells are CD62L positive and CD45RO positive. In some alternatives, effector CD4+ cells are CD62L negative and CD45RO positive. Each of these populations may be independently modified with a chimeric receptor.

[0189] After transduction and/or selection for chimeric receptor bearing cells, the cell populations are preferably expanded *in vitro* until a sufficient number of cells are obtained to provide for at least one infusion into a human subject, typically around 10^4 cells/kg to 10^9 cells/kg. In some alternatives, the transduced cells are cultured in the presence of antigen bearing cells, anti CD3, anti CD28, and IL- 2, IL-7, IL- 15, or IL-21 or combinations thereof.

[0190] Each of the subpopulations of CD4+ and CD8+ cells can be combined with one another. In a specific alternative, modified naïve or central memory CD4+ cells are combined with modified central memory CD8+ T cells to provide a synergistic cytotoxic effect on antigen bearing cells, such as tumor cells.

[0191] The disclosure provides for an adoptive cellular immunotherapy composition comprising a genetically modified T lymphocyte cell preparation, as described herein.

[0192] In some alternatives, the T lymphocyte cell preparation comprises CD4 + T cells that have a chimeric receptor comprising an extracellular antibody variable domain specific for a ligand associated with the disease or disorder, a customizable spacer region, a transmembrane domain, and an intracellular signaling domain of a T cell receptor or other receptors, as described herein. In other alternatives, an adoptive cellular immunotherapy composition further comprises a chimeric receptor modified tumor-specific CD8+ cytotoxic T lymphocyte cell preparation that provides a cellular immune response, wherein the cytotoxic T lymphocyte cell preparation comprises CD8+ T cells that have a chimeric receptor comprising an extracellular single chain antibody specific for a ligand associated with the disease or disorder, a customizable spacer region, a transmembrane domain, and an intracellular signaling domain of a T cell receptor, as described herein.

[0193] In some alternatives, an adoptive cellular immunotherapy composition comprises a chimeric receptor modified tumor-specific CD8+ cytotoxic T lymphocyte cell preparation that provides a cellular immune response, wherein the cytotoxic T lymphocyte cell preparation comprises CD8+ T cells that have a chimeric receptor comprising an extracellular single chain antibody that can target and/or is specific for a ligand associated with the disease or disorder, a customizable spacer region, a transmembrane domain, and an intracellular signaling domain of a T cell receptor, in combination with an antigen-reactive chimeric receptor modified naïve CD4+ T helper cell derived from CD45RO-CD62L+ CD4+ T cells, and a pharmaceutically acceptable carrier.

[0194] In other alternatives, an adoptive cellular immunotherapy composition comprises an antigen specific and/or targeting CD8+ cytotoxic T lymphocyte cell preparation that provides a cellular immune response derived from the patient combined with an antigen-reactive chimeric receptor modified naïve CD4+ T helper cell that augments the CD8+ immune response, wherein the helper T lymphocyte cell preparation comprises CD4 + T cells that have a chimeric receptor comprising an extracellular antibody variable domain that can target and/or is specific for the antigen associated with the disease or disorder, a customizable spacer region, a transmembrane domain, and an intracellular signaling domain of a T cell receptor.

[0195] In a further alternative, an adoptive cellular immunotherapy composition comprises an antigen-reactive chimeric receptor modified naïve CD4+ T helper cell that augments the CD8+ immune response, wherein the helper T lymphocyte cell preparation comprises CD4 + T cells that have a chimeric receptor comprising an extracellular antibody variable domain that can target and/or is specific for a ligand associated with a disease or disorder, a customizable spacer region, a transmembrane domain, and an intracellular signaling domain of a T cell receptor.

[0196] In some alternatives, the CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, or bulk CD4+ T cells. In some alternatives, CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell comprises a CD45RO-, CD45RA+, and/or CD62L+ CD4+ T cell. In some alternatives, the CD8+ T cytotoxic lymphocyte cell is selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells or bulk CD8+ T cells. In

some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell comprises a CD45RO+, CD62L+, and/or CD8+ T cell. In yet other alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell and the CD4+ helper T lymphocyte cell is a naïve or central memory CD4+ T cell.

[0197] The disclosure provides methods of making adoptive immunotherapy compositions and uses or methods of using these compositions for performing cellular immunotherapy in a subject having a disease or disorder. Proliferation and persistence of the chimeric receptor modified T cells can be determined by using an animal model of the disease or disorder and administering the cells and determining persistence and/ or proliferative capacity of the transferred cells. In other alternatives, proliferation and activation can be tested in vitro by going through multiple cycles of activation with antigen bearing cells.

[0198] In some alternatives, a method of manufacturing the compositions comprises obtaining a modified naïve CD4+ T helper cell, wherein the modified helper T lymphocyte cell preparation comprises CD4+ T cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[0199] In another alternative, a method further comprises obtaining a modified CD8+ cytotoxic T cell, wherein the modified cytotoxic T lymphocyte cell preparation comprises CD8+ cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an intracellular signaling domain as described herein.

[0200] In another alternative, a method comprises obtaining a modified CD8+ cytotoxic T cell, wherein the modified cytotoxic T lymphocyte cell preparation comprises CD8+ T cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an intracellular signaling domain, as described herein, and further comprising combining the modified CD8+ cytotoxic T cells with a CD4+ helper cell lymphocyte cell preparation.

[0201] The preparation of the CD4+ and CD8+ cells that are modified with a chimeric receptor has been described above as well as in the examples. Antigen specific or antigen targeting T lymphocytes can be obtained from a patient having the disease or disorder or can be prepared by *in vitro* stimulation of T lymphocytes in the presence of antigen. Subpopulations of CD4+ and CD8+ T lymphocytes that are not selected for antigen specificity or targeting can also be isolated as described herein and combined in the methods of manufacturing. In some alternatives, the combination of cell populations can be evaluated for uniformity of cell surface makers, the ability to proliferate through at least two generations, to have a uniform cell differentiation status. Quality control can be performed by coculturing an cell line expressing the target ligand with chimeric receptor modified T cells to determine if the chimeric receptor modified T cells recognize the cell line using cytotoxicity, proliferation, or cytokine production assays that are known in the field. Cell differentiation status and cell surface markers on the chimeric receptor modified T cells can be determined by flow cytometry. In some alternatives, the markers and cell differentiation status on the CD8+ cells include CD3, CD8, CD62L, CD28, CD27, CD69, CD25, PD-1, CTLA-4, CD45RO, and/or CD45RA. In some alternatives, the markers and the cell differentiation status on the CD4+ cells include CD3, CD4, CD62L, CD28, CD27, CD69, CD25, PD-1, CTLA-4 CD45RO, and/or CD45RA.

[0202] In some alternatives, a method of selecting a spacer region for a chimeric receptor is provided herein. Surprisingly some chimeric receptor constructs, although effective to activate T cells *in vitro*, were not effective *in vivo*. In some alternatives, a method comprises providing a plurality of chimeric receptor nucleic acids, wherein the chimeric receptor nucleic acids differ only in the spacer region; introducing each of the chimeric receptor nucleic acids into a separate T lymphocyte population; expanding each separate lymphocyte population in vitro, and introducing each lymphocyte population into an animal bearing a tumor to determine the anti-tumor efficacy of each of the chimeric receptor modified T cells, and selecting a chimeric receptor that provides anti-tumor efficacy as compared to each of the other separate lymphocyte populations modified with each of the other chimeric receptor modified T cells.

[0203] Animal models of different tumors are known. Anti-tumor efficacy can be measured by identifying a decrease in tumor volume, by determining animal death, persistence of the genetically modified T cells in vivo, activation of genetically modified T

cells (for example, by detecting an increase in expression of CD25 and/CD69), and/or proliferation of genetically modified T cells in vivo. In some alternatives, a chimeric receptor is selected that provides for the best anti-tumor efficacy in vivo as determined by one or more of these parameters. Lack of anti-tumor efficacy can be determined by lack of persistence of the genetically modified lymphocytes *in vivo*, animal death, an increase in apoptosis as measured by an increase in induction of caspase -3, and/or a decrease in proliferation of genetically modified lymphocytes.

[0204] In some alternatives, providing a plurality of chimeric receptor nucleic acids, wherein the chimeric receptor nucleic acids differ only in the spacer region comprises providing a chimeric receptor construct comprising a polynucleotide coding for a ligand binding domain, wherein the ligand is a tumor specific or tumor targeting antigen (e.g.CD171), or any other molecule expressed on a target cell population that is suitable to mediate recognition and elimination by a lymphocyte; a polynucleotide coding for a first polypeptide spacer having a defined restriction site at the 5' and 3' end of the coding sequence for the first polypeptide spacer; a polynucleotide coding for a transmembrane domain; and a polynucleotide coding for an intracellular signaling domain.

[0205] In some alternatives, the present disclosure provides a method of treating or inhibiting cancer, a method of inhibiting or delaying progression and /or metastasis of a cancer, a method of inhibiting or reducing the presence of a tumor or cancer cell, and / or a method of inhibiting or reducing a target population of CD171 expressing cells in a patient in need thereof. Such methods involve administering to a subject or a patient in need thereof a genetically modified cytotoxic T lymphocyte cell preparation that provides a cellular immune response, wherein the cytotoxic T lymphocyte cell preparation comprises CD8+ T cells that have a chimeric receptor comprising a polynucleotide coding for a ligand binding domain, wherein the ligand is a tumor specific or tumor targeting antigen, or any other molecule expressed on a target cell population (e.g. CD171) that is suitable to mediate recognition and elimination by a lymphocyte; a polynucleotide coding for a polypeptide spacer wherein the polypeptide spacer is of a customized length, wherein the spacer provides for enhanced T cell proliferation and/or cytokine production as compared to a reference chimeric receptor; a polynucleotide coding for a transmembrane domain; and a polynucleotide coding for one or more intracellular signaling domains. In some alternatives, the subject is identified or selected as a subject in need of a therapy to

inhibit or treat cancer. Such selection or identification can be made by clinical or diagnostic evaluation.

[0206] The disclosure also provides methods of performing cellular immunotherapy in a subject having a disease or disorder comprising: administering a composition of lymphocytes expressing a chimeric receptor as described herein. In other alternatives, a method comprises administering to the subject a genetically modified cytotoxic T lymphocyte cell preparation that provides a cellular immune response, wherein the cytotoxic T lymphocyte cell preparation comprises CD8 + T cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an intracellular signaling domain as described herein, and a genetically modified helper T lymphocyte cell preparation that elicits direct tumor recognition and augments the genetically modified cytotoxic T lymphocyte cell preparations ability to mediate a cellular immune response, wherein the helper T lymphocyte cell preparation comprises CD4+ T cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an intracellular signaling domain as described herein. In some alternatives, the subject is identified or selected as a subject in need of a therapy to inhibit or treat cancer. Such selection or identification can be made by clinical or diagnostic evaluation.

[0207] While not limiting the scope of the disclosure, it is believed by selecting the chimeric receptor modified T cell population that can persist and proliferate *in vivo* prior to administration may result in the ability to use a lower dose of T cells and provide more uniform therapeutic activity. In some alternatives, the dose of T cells can be reduced at least 10%, 20%, or 30% or greater. Reduction in the dose of T cells may be beneficial to reduce the risk or tumor lysis syndrome and cytokine storm.

[0208] In another alternative, a method of performing cellular immunotherapy in a subject having a disease or disorder comprises: administering to the subject a genetically modified helper T lymphocyte cell preparation, wherein the modified helper T lymphocyte cell preparation comprises CD4+ T cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an

intracellular signaling domain, as described herein. In some alternatives, the method further comprises administering to the subject a genetically modified cytotoxic T lymphocyte cell preparation, wherein the modified cytotoxic T lymphocyte cell preparation comprises CD8+ cells that have a chimeric receptor comprising a ligand binding domain that can target and/or is specific for a tumor cell surface molecule, a customized spacer domain, a transmembrane domain, and an intracellular signaling domain as described herein. In some alternatives, the subject is identified or selected as a subject in need of a therapy to inhibit or treat cancer. Such selection or identification can be made by clinical or diagnostic evaluation.

[0209] Another alternative describes a method of performing cellular immunotherapy in a subject having a disease or disorder comprising: analyzing a biological sample of the subject for the presence of a target molecule (e.g. CD171) associated with the disease or disorder and administering the adoptive immunotherapy compositions described herein, wherein the chimeric receptor specifically binds to and/or targets the target molecule. In some alternatives, the subject is identified or selected as a subject in need of a therapy to inhibit or treat cancer. Such selection or identification can be made by clinical or diagnostic evaluation.

[0210] In some alternatives, the CD4+ T helper lymphocyte cell is selected prior to introduction of the chimeric receptor from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells or bulk CD4+ T cells. In a specific alternative, CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell comprises a CD45RO-, CD45RA+, and/or CD62L+ CD4+ T cell. In yet other alternatives, the CD8+ T cytotoxic lymphocyte cell is selected prior to introduction of the chimeric receptor from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells or bulk CD8+ T cells. In a specific alternative, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell comprises a CD45RO+, CD62L+, and/or CD8+ T cell. In a specific alternative, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell and the CD4+ helper T lymphocyte cell is a naïve CD4+ T cell. In some alternatives, the subject is identified or selected as a subject in need of a therapy to inhibit or treat cancer. Such selection or identification can be made by clinical or diagnostic evaluation.

[0211] In some alternatives, the CD8+ T cell and the CD4+ T cell are both genetically modified with a chimeric receptor comprising an antibody heavy chain domain that specifically binds and/or targets a tumor-specific cell surface molecule. In other alternatives, the intracellular signaling domain of the CD8 cytotoxic T cells is the same as the intracellular signaling domain of the CD4 helper T cells. In yet other alternatives, the intracellular signaling domain of the CD8 cytotoxic T cells is different than the intracellular signaling domain of the CD4 helper T cells.

[0212] Subjects that can be provided the compositions described herein are, in general, human and other primate subjects, such as monkeys and apes for veterinary medicine purposes; however, the technology is also contemplated for use with domestic animals, such as horses, pigs, sheep, cattle, and goats, as well as, companion animals, such as dogs and cats. The subjects can be male or female and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects.

[0213] The methods are useful in the treatment or inhibition of, for example, CD171 bearing cancer or tumor cells. In some alternatives, CD171 cancer or tumor cells include neuroblastoma, melanoma, cervical carcinoma, ovarian cancer, uterine carcinoma, pancreatic cancer, colon carcinoma, renal carcinoma, and glioblastoma.

[0214] Chimeric antigen T cells prepared as described above can be utilized in methods and compositions for adoptive immunotherapy in accordance with known techniques, or variations thereof that will be apparent to those skilled in the art based on the instant disclosure. In some alternatives, the T cells are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a "pharmaceutically acceptable" carrier) in a treatment-effective amount. Suitable infusion medium can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumin, fetal bovine serum or other human serum components.

[0215] A treatment effective amount of cells in the composition is at least 2 cell subsets (for example, 1 CD8+ central memory T cell subset and 1 CD4+ helper T cell subset) or is more typically greater than 10^2 cells, and up to 10^6, up to and including 10^8 or 10^9 cells and can be more than 10^{10} cells. The number of cells will depend upon the

ultimate use for which the composition is intended as will the type of cells included therein. For example, if cells that that can target and/or are specific for a particular antigen are desired, then the population will contain greater than 70%, generally greater than 80%, 85% and 90-95% of such cells. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 mls or less, even 250 mls or 100 mls or less. Hence the density of the desired cells is typically greater than 10^4 cells/m1 and generally is greater than 10^7 cells/ml, generally 10^8 cells/ml or greater. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^6, 10^7, 10^8, 10^8, 10^9, 10^{10} or 10^{11} cells.

[0216] In some alternatives, the lymphocytes of the invention may be used to confer immunity to individuals. By "immunity" is meant a lessening of one or more physical symptoms associated with a response to infection by a pathogen, or to a tumor, to which the lymphocyte response is directed. The amount of cells administered is usually in the range present in normal individuals with immunity to the pathogen. Thus, the cells are usually administered by infusion, with each infusion in a range of from 2 cells, up to at least 10^6 to $3x10^{10}$ cells, preferably in the range of at least 10^7 to 10^9 cells. The T cells may be administered by a single infusion, or by multiple infusions over a range of time. However, since different individuals are expected to vary in responsiveness, the type and amount of cells infused, as well as the number of infusions and the time range over which multiple infusions are given are determined by the attending physician, and can be determined by routine examination. The generation of sufficient levels of T lymphocytes (including cytotoxic T lymphocytes and/or helper T lymphocytes) is readily achievable using the rapid expansion method of the present invention, as exemplified herein. *See, e.g.,* US Patent No. 6,040,177 to Riddell et al. at column 17, which is expressly incorporated by reference in its entirety.

[0217] In some alternatives, the composition as described herein are administered intravenously, intraperitoneally, intratumorly, into the bone marrow, into the lymph node, and /or into cerebrospinal fluid. In some alternatives, the chimeric receptor engineered compositions are delivered to the site of the tumor. Alternatively, the compositions as described herein can be combined with a compound that targets the cells to the tumor or the immune system compartments and avoid sites such as the lung. In some alternatives, the compositions as described herein are administered with chemotherapeutic

agents and/or immunosuppressants. In some alternatives, a patient is first treated with a chemotherapeutic agent that inhibits or destroys other immune cells followed by the compositions described herein. In some cases, chemotherapy may be avoided entirely.

[0218] The present invention is illustrated further in the additional alternatives set forth below.

Alternatives

[0219] The following alternatives are intended to illustrate, but not to limit, the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

Customizing spacer domain length for optimal recognition of CD171 with chimeric receptor modified T cells

[0220] Chimeric receptors that can target and/or is specific for the CD171 molecule that is expressed on a large number of human malignancies including neuroblastoma were constructed. The CD171 chimeric receptors were designed from CD171 specific and/or targeting scFVs that specifically bind to and/or target epitope CE7 on CD171 and contain extracellular spacer domains of different lengths. The sequences for the Ce7scFv-IgG4hinge-CH2-CH3-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (long construct) (SEQ ID NO: 54) are shown in **Figure 5** and **Figure 6**. The sequences for the CE7scFv-IgG4hinge-CH3-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (intermediate) (SEQ ID NO: 55) are shown in **Figures 7** and **8**. The sequences for CE7scFv-IgG4hinge-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (short) (SEQ ID NO: 56) are shown in **Figures 9** and **10**. The sequence for construct CE7scFv-IgG4hinge-CD28tm/cyto-4-1BB-zeta-T2A-EGFRt-epHIV7 with two costimulatory domains (SEQ ID NO: 57) is shown in **Figures 11-12**. The ability of T-cells expressing each CD171 specific and/or targeting chimeric receptor to recognize CD171 neuroblastoma tumors *in vitro,* and to eliminate neuroblastoma tumor cells engrafted into immunodeficient mice was analyzed.

Human subjects

[0221] Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and patients after written informed consent on research protocols.

Cell lines

[0222] The SK-N-BE 2 neuroblastoma cell line (Be2) was obtained from the American Type Culture Collection. EBV-transformed TMLCLs were made from PBMCs as previously described Pelloquin F, Lamelin JP, Lenoir GM. In vitro cell dev biol 1986; 22(12):689-694.

Immunophenotyping

[0223] PBMC and cell lines were stained with the following conjugated mAbs: CD4, CD8, CD28, CD45RA, and CD62L, and matched isotype controls (BD Biosciences). Central memory T cells were isolated from PBMC by isolating CD8+ cells and depleting the CD8+ cell population of CD45RA cells using immunomagnetic beads. The CD8+ cells depleted of CD45RA were enriched for CD62L using immunomagnetic beads as shown in **Figure 1**. Surface expression of CD171chimeric receptor was analyzed using a polyclonal goat anti-mouse-IgG antibody (Fab-specific) (Jackson ImmunoResearch). Flow analyses were done on a FACSCanto®, sort-purifications on a FACSAriaII® (Becton Dickinson) and data analyzed using FlowJo® software (Treestar).

Vector construction and preparation of chimeric receptor encoding lentivirus

[0224] CD171 specific and/or targeting chimeric receptors were constructed using VL and VH chain segments of the CE7 mAb (CD171). (Variable region sequences for CE7 are provided in **Figures 5, 7, 9,** and **11**). Each scFV was linked to a spacer domain derived from IgG4-Fc (Uniprot Database: P01861; **Table 2**) comprising either 'Hinge-CH2-CH3' (long: 229 AA), 'Hinge-CH3' (intermediate:119 AA) or 'Hinge' only (Short:12 AA) sequences (**Table 6**). All spacers contained a S→P substitution within the 'Hinge' domain located at position 108 of the native IgG4-Fc protein, and were linked to the 27 AA transmembrane domain of human CD28 (Uniprot: P10747,**Table 3**) and to a signaling module comprising either (i) the 41 AA cytoplasmic domain of human CD28 with an LL→GG substitution located at positions 186-187 of the native CD28 protein (**Table 3**) linked to the 42 AA cytoplasmic domain of human 4-1BB (Uniprot: Q07011, Table 4) or (ii) the cytoplasmic domain of human 4-1BB alone, each module was linked to the 112 AA cytoplasmic domain of isoform 3 of human CD3ζ (Uniprot: P20963,**Table 5**). The construct encoded a T2A ribosomal skip element (Table 1) and a tEGFR sequence (**Table 1**) downstream of the chimeric receptor. Human codon-optimized nucleotide sequences

encoding each transgene were synthesized (Life Technologies) and cloned into the epHIV7 lentiviral vector

[0225] CD171-chimeric receptor or tEGFR-encoding lentiviruses were produced in 293T cells using the packaging vectors pCHGP-2, pCMV-Rev2 and pCMV-G, and Calphos® transfection reagent (Clontech).

Generation of T-cell lines expressing CD171-chimeric receptors

[0226] $CD8^+$ CD45RA- $CD62L^+$ central memory T-cells (T_{CM}) were sorted from PBMC of normal donors (see **Figure 1**), activated with anti-CD3/CD28 beads (Life Technologies), and transduced on day 3 after activation by centrifugation at 800 g for 45 min at 32°C with lentiviral supernatant (MOI = 3) supplemented with 1 μg/mL polybrene (Millipore). T-cells were expanded in RPMI with 10% human serum, 2 mM L-glutamine (CTL medium), supplemented with recombinant human IL-2 to a final concentration of 50 U/mL. The $tEGFR^+$ subset of each T-cell line was enriched by immunomagnetic selection with biotin-conjugated anti-EGFR mAb (ImClone Systems) and streptavidin-beads (Miltenyi).

Cytotoxicity, and cytokine secretion

[0227] Target cells, either Be2 cells or TML CL, were labeled with ^{51}Cr (PerkinElmer), washed and incubated in triplicate at $1\text{-}2x10^3$ cells/well with effector chimeric receptor modified T-cells at various effector to target (E:T) ratios. Supernatants were harvested for γ-counting after a 4-hour incubation and specific lysis calculated using the standard formula. For analysis of cytokine secretion, $5x10^4$ T-cells were plated in triplicate with target cells at an E:T ratio of 30:1 , 10:1 , 3:1 or 1:1 , and IFN-γ, TNF-α and IL-2 measured by ELISA or multiplex cytokine immunoassay (Luminex) in supernatant removed after 24-h incubation.

Experiments in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice

[0228] Six- to 8-week old female NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ (NSG) mice were obtained from the Jackson Laboratory or bred in-house. Mice were injected with $0.2x10^6$ neuroblastoma tumor cells intracranially and seven days later, received an intracranial injection of 2 $x10^6$ chimeric receptor-modified or control T-cells. For bioluminescence imaging of tumor growth, mice received injections of luciferin substrate (Caliper Life Sciences) resuspended in PBS (15 μg/g body weight). Mice were anesthetized with isoflurane and imaged using an Xenogen IVIS Imaging System (Caliper)

15 minutes after the injection of luciferin in small or medium binning mode at an acquisition time of 1 s to 1 min to obtain unsaturated images. Luciferase activity was analyzed using Living Image Software (Caliper) and the photon flux analyzed within regions of interest that encompassed the entire body.

Statistical analyses

[0229] Statistical analyses were performed using Prism Software (GraphPad®). Student's t-test was performed as a two-sided paired test with a confidence interval of 95% and results with a p-value of $p<0.05$ were considered significant. Statistical analysis of survival were done by log-rank testing and results with a p-value of $p<0.05$ considered significant.

The longer spacer domain of the CD171 chimeric receptor confers superior cytotoxic and cytokine secretion *in vivo*

[0230] The design of a CD171-specific and/or targeting chimeric receptor using the CE7 scFV was known. (Park J et al. Molecular Therapy 2007, April 15(4):825-33). This chimeric receptor conferred specific recognition and/or targeting of CD171 tumors *in vitro*, but it was hypothesized that adjusting the spacer domain would enhance tumor recognition and T-cell signaling. Therefore, chimeric receptors were constructed in which the spacer domain was selected from 'Hinge –CH2-CH3' (229 amino acids, long)'Hinge-CH3' (119 AA, intermediate), and 'Hinge-only' (12 AA, short) variants. Each of the new receptors contained the identical CE7 scFV, and 4-1BB and CD3ζ signaling modules. The transgene cassette included a truncated EGFR (tEGFR) to serve as a transduction, selection and *in vivo* tracking marker for chimeric receptor-modified T-cells.

[0231] Purified $CD8^+$ T_{CM} were transduced with the CD171-chimeric receptors containing different length spacers, and with a tEGFR control vector. Surface expression of each of the chimeric receptors was confirmed by staining with F(ab)-specific antibodies (Figure 2A). Western blot of the transduced cells with antibody specific for the CD3ζ shows expression of the short, medium, and long construct. (**Figure 2B**). Similar expression of F(ab) and EGFRt were found with each of the short, medium, or long spacer domains.

[0232] Analysis of the *in vitro* function of $CD8^+$ T-cells modified to express each of the CD171chimeric receptors demonstrated that each receptor conferred specific lysis of Be2 cells that naturally express CD171, but did not confer recognition of control

TML CL (**Figure 2C**). T-cells expressing the long CD171-chimeric receptor had maximum cytolytic activity, and a hierarchy (long>> intermediate>> short) of tumor lysis was clearly evident against CD171tumor targets (**Figure 2C**).

[0233] Quantitative analysis of cytokine production in response to stimulation with Be2 cells showed production of IFN-γ, TNF-α and IL-2 by T-cells expressing each of the CD171 chimeric receptors. As observed in cytotoxicity assays, the T cells having the long spacer construct was superior in mediating cytokine secretion after tumor recognition (**Figure 2D**).

The CD171 specific and/or targeting CAR requires a short extracellular spacer domain for *in vivo* activity

[0234] It remained uncertain whether the superior *in vitro* activity of T-cells modified with the CD171 chimeric receptor with longer spacer would translate into enhanced or improved anti-tumor activity *in vivo*. To address these questions, cohorts of immunodeficient NSG mice were inoculated with the neuroblastoma cells by intracranial injection, and seven days later the mice were treated with a single intracranial dose of CD171 specific chimeric receptor $CD8^+$ T-cells with a short, intermediate, or long spacer. Control mice were treated with tEGFR T-cells or untreated. Untreated NSG/NB mice developed neuroblastoma necessitating euthanasia approximately 4 weeks after tumor inoculation (**Figure 2E**, right panel).

[0235] Tumor regression and enhanced or improved survival was observed in all mice treated with CD171 chimeric receptor T-cells with the short spacer. Mice treated with CD171 chimeric receptor T-cells with short spacer had a superior anti-tumor response and survival compared to mice treated with CD171 chimeric receptor T-cells with a long spacer (**Figure 2E**, right panel). Measuring total flux from tumor cells loaded with luciferin substrate shows that mice treated with CD8 cells transduced with a construct with a short or intermediate spacer exhibited much lower flux than the untreated mice or mice treated with CD8 cells transduced with a construct having a long spacer. (**Figure 2E**, left panel).

[0236] Tumors from mice treated with long spacer CD171 CAR expressing CD8 Tcm showed higher percentage of CD3+ cells by immunohistochemistry than those from mice treated with mock, short or medium spacer CD171 CAR expressing CD8 Tcm (**Fig. 2F**). No difference of Ki67 was detectable by immunohistochemistry in the different

CD8 Tcm but higher levels of caspase 3 and Granzyme B were found in CD8 Tcm expressing the long spacer CD171 CAR than in CD8 Tcm expressing short or medium spacer after 3 days of T cell injection (**Fig. 2G**).

The CD171 specific and/or targeting CAR having a long extracellular spacer domain showed more activation induced cell death

[0237] In order to determine potential mechanisms underlying the inferior in vivo antitumor activity of T cells that express CD171 chimeric receptors with long spacer domains, the possibility that the T cells were not efficiently activated by tumor cells in vivo or conversely, that they underwent activation induced T cell death *in vivo* was considered.

[0238] CD8 central memory cells transduced with CD171CAR constructs with short, intermediate, or long spacer constructs were exposed to neuroblastoma cells in vitro for 24 hours (round 1). CD8 central memory cells were removed from the culture and characterized phenotypically, and then incubated with the tumor cells for another 24 hours (round 2). CD8 cells were removed from the culture, phenotypically characterized, and then placed in a culture with tumor cells for another 24 hours (round 3). The CD8 cells were then removed and characterized phenotypically.

[0239] The cells from each round were characterized for expression of activation markers CD25 and CD69 using flow cytometry. The percentage of dead cells in cells from each round was determined by Guava Viacount. NB cells were characterized for FasR expression after round I.

[0240] The results show that the short spacer CAR cells exhibited less activation and better viability relative to long spacer CAR-expressing cells following serial tumor cell co-culture challenge (round III: CD25+CD69+ 42%(short) vs 66% (long) (Figure 3A), % dead cells 15% (short) vs 60%(long)(Figure 3B). The long spacer CAR-expressing cells induced FasR expression in NB cells to a greater extent than short spacer CAR cells. (**Figure 3C**).

[0241] Collectively, the data provides evidence that CD171 directed chimeric receptors with long extracellular spacer domain, despite mediating equivalent or superior effector function in vitro, induce a high level of activation induced cell death in vivo and fail to eradicate established neuroblastoma.

[0242] CD171 has attracted interest as a potential target for cancer immunotherapy due to its expression on the surface of many carcinomas. The design and function of CD171 chimeric receptors has been enhanced or improved through modification of the extracellular spacer domain. The results show that central memory T cells transduced with a CD171 directed CAR with a Hinge only short spacer domain performed much better in an in vivo model of neuroblastoma tumor eradication as compared to T cells transduced with a CD171 directed CAR with a hinge-CH3 (intermediate) or hinge-CH2-CH3(long) spacer domain.

Modification of costimulatory domains

[0243] Chimeric receptors that can target or are specific for the CD171 molecule that is expressed on a large number of human malignancies including neuroblastoma were constructed. The CD171 chimeric receptors were designed from CD171 specific and/or targeting scFVs that specifically bind to and/or target epitope CE7 on CD171 and contain a short extracellular spacer domain. One construct contains the costimulatory domain 4-1BB linked to CD3 zeta domain (4-1BB) and the other construct includes a dual costimulatory domain including CD28cyto and 4-1BB linked to CD3 zeta (CD28cyto).The sequence for construct CE7scFv-IgG4hinge-CD28tm/cyto-4-1BB-zeta-T2A-EGFRt-epHIV7 with two costimulatory domains is shown in **Figures 11-12**. The sequence for CE7scFv-IgG4hinge-CD28tm/4-1BB-zeta-T2A-EGFRt-epHIV7 (short) is shown in **Figures 9-10**. The ability of T-cells expressing each CD171 specific chimeric receptor to recognize CD171 neuroblastoma tumors *in vitro,* and to eliminate neuroblastoma tumor cells engrafted into immunodeficient mice was analyzed.

Human subjects

[0244] Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and patients after written informed consent on research protocols.

Cell lines

[0245] The SK-N-BE 2 neuroblastoma cell line (Be2) was obtained from the American Type Culture Collection. EBV-transformed TMLCLs were made from PBMCs as previously described Pelloquin F, Lamelin JP, Lenoir GM. In vitro cell dev biol 1986; 22(12):689-694.

Immunophenotyping

[0246] PBMC and cell lines were stained with the following conjugated mAbs: CD4, CD8, CD28, CD45RA, and CD62L, and matched isotype controls (BD Biosciences). Central memory T cells were isolated from PBMC by isolating CD8+ cells and depleting the CD8+ cell population of CD45RA cells using immuno magnetic beads. The CD8+ cells depleted of CD45RA were enriched for CD62L using immunomagnetic beads as shown in **Figure 1**. Surface expression of CD171chimeric receptor was analyzed using a polyclonal goat anti-mouse-IgG antibody (Fab-specific) (Jackson ImmunoResearch). Flow analyses were done on a FACSCanto®, sort-purifications on a FACSAriaII® (Becton Dickinson) and data analyzed using FlowJo® software (Treestar).

Vector construction and preparation of chimeric receptor encoding lentivirus

[0247] CD171 specific and/or targeting chimeric receptors were constructed using VL and VH chain segments of the CE7 mAb (CD171). (Variable region sequences for CE7 are provided in **Figures 5, 7, 9**, and **11**) Each scFV was linked to a spacer domain derived from IgG4-Fc (Uniprot Database: P01861; **Table 2**) comprising either 'Hinge-CH2-CH3' (long: 229 AA), 'Hinge-CH3' (intermediate:119 AA) or 'Hinge' only (Short:12 AA) sequences (**Table 6**). All spacers contained a S→P substitution within the 'Hinge' domain located at position 108 of the native IgG4-Fc protein, and were linked to the 27 AA transmembrane domain of human CD28 (Uniprot: P10747,**Table 3**) and to a signaling module comprising either (i) the 41 AA cytoplasmic domain of human CD28 with an LL→GG substitution located at positions 186-187 of the native CD28 protein (**Table 3**) linked to the 42 AA cytoplasmic domain of human 4-1BB (Uniprot: Q07011, **Table 4**) or (ii) the cytoplasmic domain of human 4-1BB alone, each module was linked to the 112 AA cytoplasmic domain of isoform 3 of human CD3ζ (Uniprot: P20963,**Table 5**). One construct contained a costimulatory signaling module comprising the 42 AA cytoplasmic domain of human 4-1BB (Uniprot: Q07011, SEQ ID NO:15), which was linked to the 112 AA cytoplasmic domain of isoform 3 of human CD3ζ (Uniprot: P20963, SEQ ID NO:16). The constructs encoded a T2A ribosomal skip element (SEQ ID NO:8) and a tEGFR sequence (SEQ ID NO:9) downstream of the chimeric receptor. Human codon-optimized nucleotide sequences encoding each transgene were synthesized (Life Technologies) and cloned into the epHIV7 lentiviral vector.CD171-chimeric receptor or tEGFR-encoding lentiviruses were produced in 293T cells using the packaging vectors pCHGP-2, pCMV-Rev2 and pCMV-G, and Calphos® transfection reagent (Clontech).

Generation of T-cell lines expressing CD171-chimeric receptors

[0248] $CD8^+$ CD45RA- $CD62L^+$ central memory T-cells (T_{CM}) were sorted from PBMC of normal donors (See **Figure 1**), activated with anti-CD3/CD28 beads (Life Technologies), and transduced on day 3 after activation by centrifugation at 800 g for 45 min at 32°C with lentiviral supernatant (MOI = 3) supplemented with 1 μg/mL polybrene (Millipore). T-cells were expanded in RPMI with 10% human serum, 2 mM L-glutamine (CTL medium), supplemented with recombinant human IL-2 to a final concentration of 50 U/mL and IL-15 to a final concentration of 10ng/μl. The $tEGFR^+$ subset of each T-cell line was enriched by immunomagnetic selection with biotin-conjugated anti-EGFR mAb (ImClone Systems) and streptavidin-beads (Miltenyi).

Cytotoxicity, and cytokine secretion

[0249] Target cells, either Be2 cells or TML CL, were labeled with ^{51}Cr (PerkinElmer), washed and incubated in triplicate at $1\text{-}2x10^3$ cells/well with effector chimeric receptor modified T-cells at various effector to target (E:T) ratios. Supernatants were harvested for γ-counting after a 4-hour incubation and specific lysis calculated using the standard formula. For analysis of cytokine secretion, $5x10^4$ T-cells were plated in triplicate with target cells at an E:T ratio of 30:1 , 10:1 , 3:1 or 1:1 , and IFN-γ, TNF-α and IL-2 measured by ELISA or multiplex cytokine immunoassay (Luminex) in supernatant removed after 24-h incubation.

Experiments in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice

[0250] Six- to 8-week old female NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ (NSG) mice were obtained from the Jackson Laboratory or bred in-house. Mice were injected with $0.2x10^6$ neuroblastoma tumor cells intracranially and received an intracranial injection of 2 $x10^6$ chimeric receptor-modified or control T-cells. For bioluminescence imaging of tumor growth, mice received injections of luciferin substrate (Caliper Life Sciences) resuspended in PBS (15 μg/g body weight). Mice were anesthetized with isoflurane and imaged using an Xenogen IVIS Imaging System (Caliper) 15 minutes after the injection of luciferin in small or medium binning mode at an acquisition time of 1 s to 1 min to obtain unsaturated images. Luciferase activity was analyzed using Living Image Software (Caliper) and the photon flux analyzed within regions of interest that encompassed the entire.

Statistical analyses

[0251] Statistical analyses were performed using Prism Software (GraphPad®). Student's t-test was performed as a two-sided paired test with a confidence interval of 95% and results with a p-value of $p<0.05$ were considered significant. Statistical analysis of survival were done by log-rank testing and results with a p-value of $p<0.05$ considered significant.

The CD171 chimeric receptor with a short spacer region and two costimulatory domains confers superior cytotoxic and cytokine secretion *in vivo*

[0252] We transduced purified $CD8^+$ T_{CM} with the CD171-chimeric receptors containing different costimulatory domains and with a tEGFR control vector. Surface expression of each of the chimeric receptors was confirmed by staining with F(ab)-specific antibodies (**Figure 4A**). Similar expression of F(ab) and EGFRt were found with each of the construct with a single 4-1BB costimulatory domain (**panel 2**) and the construct with CD28cyto/4-1BB costimulatory domains (**panel 3**). Analysis of the *in vitro* function of $CD8^+$ T-cells modified to express each of the CD171chimeric receptors demonstrated that each receptor conferred specific lysis of Be2 cells that naturally express CD171, but did not confer recognition of control TML CL (**Figure 4B**). T-cells expressing the CD171-chimeric receptor with CD28cyto/4-1BB costimulatory domains had higher cytolytic activity. Quantitative analysis of cytokine production in response to stimulation with Be2 cells showed production of IFN-γ by T-cells expressing each of the CD171 chimeric receptors. As observed in cytotoxicity assays, the construct with the CD28cyto/4-1BB costimulatory domains was superior in mediating cytokine secretion after tumor recognition (**Figure 4C**).

The CD171 specific and/or targeting CAR with the CD28cyto/4-1BB costimulatory domains was less efficacious *in vivo*

[0253] It remained uncertain whether the superior *in vitro* activity of T-cells modified with the CD171 chimeric receptor with CD28cyto/4-1BB costimulatory domains would translate into enhanced or improved anti-tumor activity *in vivo*. To address these questions, we inoculated cohorts of immunodeficient NSG mice with the neuroblastoma cells by intracranial injection, and seven days later, treated the mice with a single intracranial dose of CD171 specific and/or targeting chimeric receptor $CD8^+$ T-cells with a short, intermediate, or long spacer. Control mice were treated with tEGFR T-cells or

untreated. Untreated NSG/NB mice developed neuroblastoma necessitating euthanasia approximately 4 weeks after tumor inoculation (**Figure 4D**).

[0254] We observed tumor regression and improved survival in all mice treated with CD171 chimeric receptor T-cells with the construct with a single 4-1BB costimulatory domain. Mice treated with CD171 chimeric receptor T-cells with the construct with a single 4-1BB costimulatory domain had a superior anti-tumor response and survival compared to mice treated with CD171 chimeric receptor T-cells with the CD28cyto/4-1BB costimulatory domains (**Figure 4D**).

The CD171 specific and/or targeting CAR having a long extracellular spacer domain showed more activation induced cell death

[0255] We sought to determine potential mechanisms underlying the inferior in vivo antitumor activity of T cells that express CD171 chimeric receptors with the CD28cyto/4-1BB costimulatory domains. We considered the possibility that the T cells were not efficiently activated by tumor cells in vivo or conversely, that they underwent activation induced T cell death *in vivo*.

[0256] CD8 central memory cells transduced with CD171CAR constructs with the CD28cyto/4-1BB costimulatory domains or the 4-1BB costimulatory domain constructs were exposed to neuroblastoma cells in vitro for 24 hours (round 1). CD8 central memory cells were removed from the culture and characterized phenotypically, and then incubated with the tumor cells for another 24 hours (round 2). CD8 cells were removed from the culture, phenotypically characterized, and then placed in a culture with tumor cells for another 24 hours (round 3). The CD8 cells were then removed and characterized phenotypically. The cells from each round were characterized for expression of activation markers CD25 and CD69 using flow cytometry. The percentage of dead cells in cells from each round was determined by Guava Viacount.

[0257] The results show that the CAR cells with the 4-1BB costimulatory domain alone exhibited less activation and better viability relative to cells expressing CARs with the CD28/cyto/4-1BB co-stimulatory domain following serial tumor cell co-culture challenge (round III: CD25+CD69+ 40% (4-1BB) vs 60% (CD28cyto/4-1BB) (Figure 4E), % dead cells 20% (4-1BB) vs 45% (CD28cyto/4-1BB) (Figure 4F). Collectively, the data provides evidence that CD171 directed chimeric receptors with a dual CD28cyto/ 4-1BB costimulatory domain, despite mediating equivalent or superior

effector function in vitro, induce a high level of activation induced cell death in vivo and had a decreased ability to eradicate established neuroblastoma.

[0258] The design and function of CD171chimeric receptors has been enhanced or improved through modification of the costimulatory domain. The results show that Central memory T cells transduced with a CD171 directed CAR with a single 4-1BB costimulatory domain performed much better in an in vivo model of neuroblastoma tumor eradication than T cells transduced with a CD171 directed CAR with a dual CD28cyto/ 4-1BB costimulatory domain.

Contribution of extracellular spacer length and cytoplasmic signaling domain on the performance of a CAR targeting CD171 *in vitro* and *in vivo*.

[0259] The contribution of extracellular spacer length and cytoplasmic signaling domain on the performance of a CAR targeting CD171 was assessed *in vitro* and *in vivo*. *In vitro*, the focus was on analyzing cytotoxicity, cytokine secretion, activation status and cell death using standard assays and a new CAR T-cell stress test employing multiple sequential cycles of tumor cell exposure. Each study was performed multiple times using different donor T cells and the final analysis included either pooled data or representative experiments with sample replicates of two or more. *In vivo* experiments analyzed anti-tumor activity using live imaging, survival, and immunohistochemistry. All mouse experiments were designed to have at least 2 mice per group. All outliers were included in the data analysis.

CAR construction and lentiviral production

[0260] CD171-specific and/or targeting CARs were constructed using $(G_4S)_3$ peptide linked VL and VH segments of the CE7 (anti-CD171) IgG2 monoclonal antibody. The scFv was codon optimized and subsequently linked to variable spacer length domains based on 12 AA (short spacer (SS)/"hinge-only"), 119 AA (medium spacer (MS)/"hinge-CH3") or 229 AA (long spacer (LS)/"hinge-CH2-CH3") derived from human IgG4-Fc. All spacers contained a S -> P substitution within the "Hinge" domain located at position 108 of the IgG4-Fc protein and were linked to the transmembrane domain of human CD28 and to signaling modules comprising either (i) the cytoplasmic domain of 4-1BB alone (2G CAR) or (ii) of CD28 (mutant) and 4-1BB (3G CAR), with each signaling module being fused on their carboxyl terminus to human CD3-ζ endodomain. The portion of the cytoplasmic domain of CD28 used in the constructs contained an LL -> GG substitution

located at positions 186 to 187 of the native CD28 protein. The cDNA clones encoding CAR variants were linked to a downstream T2A ribosomal skip element and truncated EGF receptor (EGFRt), cloned into the epHIV7 lentiviral vector and CD171-CAR lentiviruses were produced in 293T cells.

Real-time PCR

[0261] Total RNA was extracted from T cells using the RNeasy Minikit according to the manufacturer's instructions (Qiagen). cDNA was synthesized by reverse transcription using the First Strand Kit (Life Technologies). RNA quantitation for specific genes was performed using real-time primers for FasL (IDT) and the CFX96 real-time detection system (Biorad). Actin was used as a house keeping gene. Data was analyzed using the CFX Manager Software version 3.0.

Protein expression

[0262] Western Blot (WB) T-cells were harvested, washed twice in PBS and lysed in protease inhibitor (Millipore). Proteins were analyzed using SDS/PAGE followed by Western blotting using anti-CD247 (CD3-ζ, BD Biosciences), according to manufacturer's instructions. Signals were detected using an Odyssey Infrared Imager and band intensities were quantified using Odyssey v2.0 software (LI-COR).

Flow Cytometry

[0263] Immunophenotyping was conducted with fluorophore-conjugated mAbs: CD4, CD8, CD27, CD28, CD45RA, CD45RO, CD62L, CCR7 (Biolegend). Cell surface expression of L1CAM was analyzed using a fluorophore-conjugated mAb (Clone 014, Sino Biological). EGFRt expression was analyzed using biotinylated cetuximab (Bristol-Myers-Squibb) and a fluorophore conjugated streptavidin secondary reagent. To assess activation and AICD fluorophore conjugated mABs for CD25, CD69, CD137, C178 (Fas Ligand) and CD95 (Fas, all Biolegend) were used. Caspase 3 activity was measured using CaspGlow (eBioscience) following the manufacturer's protocol. Flow analyses were performed on an LSRFortessa (BD Biosciences) and data were analyzed using FlowJo software (Treestar).

Generation of T cell lines expressing CD171 CARs

[0264] Samples of heparinized whole blood were obtained from healthy donors after written informed consent following a research protocol approved by the Institutional Review Board of Seattle Children's Research Institute (SCRI IRB #13795). Peripheral

blood mononuclear cells (PBMC) were isolated by standard protocol using ficoll (GE Healthcare Life Sciences) and $CD8^+CD45RO^+CD62L^+$ central memory T cells (T_{CM}) were isolated by using immunomagnetic microbeads according to the manufacturer's instruction (Miltenyi Biotec). First, $CD8^+CD45RO^+$ cells were obtained by negative selection using a CD8 T cell isolation kit and CD45RA beads, then cells were enriched for CD62L, activated with anti-CD3/CD28 beads at a bead to cell ratio of 3:1 (Life Technologies, Thermo Fisher Scientific) and transduced on day 3 by centrifugation at 800 g for 30 minutes at 32°C with lentiviral supernatant (multiplicity of infection [MOI] = 5) supplemented with 1 mg/mL protamine sulfate (APP Pharmaceuticals). T cells were expanded in RPMI (Cellgro) containing 10% heat-inactivated fetal calf serum (Atlas, Fort Collins, CO), 2 mmol/L L-glutamine (Cellgro), supplemented with a final concentration of 50 U/ml recombinant human interleukin (IL)-2 (Chiron Corporation), and 10ng/µl IL-15 (Miltenyi Biotec). The EGFRt+ subset of each T-cell line was enriched by immunomagnetic selection with biotin-conjugated Erbitux (Bristol-Myers-Squibb) and streptavidin-microbeads (Miltenyi Biotec). CD171-CAR and mock control T cells were expanded using a rapid expansion protocol (T cells used for *in vivo* assays were frozen at $S_1R_2D_{14}$ and thawed on day of injection.

Cell lines

[0265] The NB cell lines Be2 and SK-N-DZ were obtained from the American Type Culture Collection (ATCC). Be2 GFP-ffLuc_epHIV7 and SK-N-DZ GFP-ffLuc_epHIV7 were derived by lentiviral transduction with the *firefly luciferase (ffLuc)* gene and purified by sorting on GFP. Both cell lines were further transduced with CD19t-2A-IL2_pHIV7 in order to generate IL-2 secreting neuroblastoma cell lines purified by sorting on CD19t. All NB cell lines were cultured in DMEM (Cellgro) supplemented with 10% heat-inactivated fetal calf serum and 2mmol/L L-glutamine. EBV-transformed lymphoblastoid cell lines (TMLCL) and TMLCL that expressed membrane tethered CD3 epsilon specific scFvFc derived from OKT3 mAb (TMLCL-OKT3) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 2 mmol/L L-glutamine.

CAR T cell receptor signaling

[0266] After co-culturing 1 x 10^6 effector and target cells for 4 - 8 min, cells were processed to measure Erk/MAP Kinase ½ activity according to the 7-Plex T cell

receptor signaling kit (Millipore). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific).

***In vitro* T cell assays**

[0267] Cytotoxicity measured by Chromium Release Assay. Target cells were labeled with ^{51}Cr (Perkin Elmer), washed and incubated in triplicate at 5 x 10^3 cells per well with T cells ($S_1R_2D_{12-14}$) at various effector to target (E:T) ratios. Supernatants were harvested after a 4-hour incubation for γ-counting using Top Count NTX (Perkin Elmer) and specific lysis was calculated as previously described.

Cytotoxicity measured by Biophotonic Luciferase Assay

[0268] NB cell lines containing GFP-ffLuc_epHIV7 were co-cultured with effector cells at a 5:1 E:T ratio. The effector cells were on their first, second or third round of tumor cell encounter as described above. To assess the amount of viable tumor cells left after T cell encounter, D-Luciferin was added and after 5 minutes the biophotonic signal from the NB cells was measured using an IVIS Spectrum Imaging System (Perkin Elmer).

Cytokine release

[0269] A total of 5 x 10^5 T cells ($S_1R_2D_{12-14}$) were plated with stimulator cells at an E:T ratio of 2:1 for 24 hours. IFN-γ, TNF-a, and IL-2 in the supernatant were measured using Bio-plex cytokine assay and Bioplex-200 system (Bio-rad Laboratories).

Stress Test

[0270] To mimic recursive antigen encounters, a co-culture of adherent target cells and freshly thawed non-adherent effector cells at a 1:1 E:T ratio was started. After 24 (round I) and 48 (round II) hours, T cell viability was assessed using the Guava ViaCount Assay (Millipore) and non-adherent effector cells were moved to a new set of adherent target cells at a 1:1 E:T ratio. After round I, II and III (72 hours) T cells were harvested and treated with a dead cell removal kit (Miltenyi) before further analysis.

Immunohistochemistry

[0271] Mouse brains were harvested post-mortem, fixed for 24 hours in 10% neutral buffered formalin (Thermo), processed, paraffin embedded and cut into 5μm sections. Antigen retrieval was performed using Diva decloaker RTU (Biocare Medical). Primary antibodies were incubated with sections overnight at 4°C and diluted in blocking buffer as follows: rat monoclonal anti human CD3 (Clone CD3-12, AbD Serotec/Bio-rad) 1:100, mouse monoclonal anti human Ki67 (Clone MIB-1, Dako) 1:200, rabbit polyclonal

anti human cleaved caspase-3 (Biocare Medical) 1:100, rabbit polyclonal anti human granzyme B (Covance) 1:200. Secondary antibodies (Life technologies) were incubated with sections for 2 hours at room temperature and diluted 1:500 in PBS with 0.2% BSA.

[0272] Slides were imaged at 40x magnification on an Eclipse Ci upright epifluorescence microscope (Nikon) equipped with a Nuance multispectral imaging system (Perkin Elmer). The imaging data were analyzed by using InForm analysis software (Perkin Elmer).

Experiments in NOD/SCID/γc $^{-/-}$mice

[0273] NSG mouse tumor models were conducted under SCRI IACUC approved protocols.

Intracranial NSG Mouse Human Neuroblastoma Xenograft Model

[0274] Adult male NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ [NOD *scid* gamma(NSG)] mice were obtained from the Jackson Laboratory or bred in-house. Mice were injected intracranially (i.c.) on day 0 with 2 x 10^5 IL-2 secreting, ffLuc expressing Be2 or SK-N-DZ tumor cells 2mm lateral, 0.5mm anterior to the bregma and 2.5mm deep from the dura. Mice received a subsequent intra tumoral injection of 2 x 10^6 CAR-modified $CD8^+T_{E(CM)}$ either seven (therapy response model) or fourteen (stress test model) days later. In the stress test model the mice were euthanized 3 days after T cell injection and brains were harvested for IHC analysis. For bioluminescent imaging of tumor growth, mice received intra peritoneal (i.p.) injections of D-luciferin (Perkin Elmer; 4.29mg/mouse). Mice were anesthetized with isoflurane and imaged using an IVIS Spectrum Imaging System (Perkin Elmer) 15 minutes after D-luciferin injection. Luciferase activity was analyzed using Living Image Software Version 4.3 (Perkin Elmer) and the photon flux analyzed within regions of interest.

Statistical analyses

[0275] Statistical analyses were conducted using Prism Software (GraphPad). Data are presented as means ± SD or SEM as stated in the figure legends. Student *t* test was conducted as a two-sided unpaired test with a confidence interval of 95% and results with a *P* value less than 0.05 were considered significant. Statistical analyses of survival were conducted by log-rank testing and results with a *P* value less than 0.05 were considered significant.

Magnitude of CAR triggered cytolytic and cytokine functional outputs can be incrementally modulated based on CAR extracellular spacer size

[0276] The biophysical synapse between CAR expressing T cell and tumor cell is influenced by the epitope location on the tumor cell surface target molecule relative to the distance from the tumor cell's plasma membrane. It was hypothesized that CAR extracellular spacer size tuning to accommodate a functional signaling synapse is a key attribute to engineering bioactive CARs. In order to assess the impact of CD171-specific and/or targeting CAR extracellular spacer size, a set of spacers were assembled using modular domains of human IgG4 as follows: "long spacer" (LS) IgG4 hinge-CH2-CH3, "medium spacer" (MS) IgG4 hinge-CH3 fusion, and "short spacer" (SS) IgG4 hinge. Each spacer variant was fused to a CD28 transmembrane domain followed by a second generation (2G) 4-1BB:zeta endodomain, that in turn was linked to the cell surface EGFRt tag using a T2A ribosome skip peptide **(Fig. 13A)**. Sets of spacer variant 2G-CAR$^+$/EGFRt$^+$ human CD8$^+$ central memory derived effector T cell lines ($T_{E(CM)}$) from purified CD8$^+$CD45RO$^+$CD62L$^+$ central memory precursors by immunomagnetic selection were generated (**Figs. 19A and B**). Following expansion of lentivirally transduced $T_{E(CM)}$, lines were further enriched for homogeneous levels of EGFRt expression by cetuximab immunomagnetic positive selection (*18*). Similar surface expression levels of each of the CAR spacer variants by anti-murine F(ab)- and EGFR-specific flow cytometric staining and protein expression quantified by western blot for CD3ζ of each T cell line was confirmed **(Figs. 13B and C).**

[0277] Following the results it was to be determined if the magnitude of 2G-CAR triggered *in vitro* activation of CD8$^+$$T_{E(CM)}$ is influenced by spacer domain size. Following activation by CD171+ human neuroblastoma (NB) tumor cells, CD171-specific and/or targeting 2G-CAR(LS) CD8+ T E(CM) exhibited 3.1-fold higher levels of phospho-ERK ($p=0.003$), and 5.7-fold higher percentage of cells expressing the activation marker CD137 ($p=0.015$) as compared to their CD171-CAR(SS) counterparts (**Figs. 13D and E**). 2G-CAR(MS) exhibited intermediate levels of phospho-ERK and CD137 induction as compared to LS and SS 2G-CARs. Next, it was determined if spacer size also modulated the magnitude of anti-tumor cytolytic activity in a LS>MS>SS pattern. Using 4-hour chromium release assays, lysis of CD171+ NB target cells was observed following the same potency gradient of LS>MS>SS against both CD171 high Be2 and CD171 low SK-

N-DZ NB cell lines **(Fig. 13F and Fig. 20A)**. Further, activation for cytokine secretion followed the same incremental output hierarchy such that 2G-CAR(LS) produced 8.4-fold higher amount of IFN-γ ($p=0.003$), 6.3-fold more IL-2 ($p<0.0001$) and 6.1-fold higher levels of TNF-α ($p=0.005$; **Fig. 13G**) as compared to 2G-CAR(SS), with 2G-CAR(MS) falling between the two extremes. These data demonstrate that the biophysical synapse created by CARs can be tuned by spacer size such that incremental levels of activation and functional outputs are achieved. Based on standard CAR development criteria typically utilized in the field, the 2G-CAR(LS) spacer variant would be a lead candidate for further development for clinical applications.

Inverse correlation of spacer modulated CAR redirected CTL functional potency in vitro with *in vivo* anti-tumor activity

[0278] In order to delineate the relationship of the observed potency of CAR signaling based on *in vitro* assays to therapeutic activity *in vivo*, adoptive transfer experiments in NSG mice with established human NB xenografts stereotactically implanted in the cerebral hemisphere were performed **(Fig. 14A)**. Surprisingly, Be2 tumor engrafted mice treated with intratumoral injection of 2G-CAR(LS) exhibited no therapeutic activity necessitating animal euthanasia approximately 3 weeks after tumor inoculation **(Figs. 14B and C)**. In comparison, biophotonic tumor signal was reduced and survival enhanced in mice treated with 2G-CAR(SS) CAR CD8+ $T_{E(CM)}$ and to an intermediate extent in mice treated with the 2G-CAR(MS) variant ($p=0.001$; median survival of the different groups: LS=20d, mock=21d, MS=59.5d, SS=76d). SK-N-DZ tumor engrafted mice exhibited a SS>MS>>LS hierarchy of tumor responses with uniform tumor clearance and 100% survival of animals treated with 2G-CAR(SS) CD8+ $T_{E(CM)}$ **(Figs. 20C and D)**. Failure of 2G-CAR(LS) redirected CTLs, which are injected directly into engrafted tumors, could not be attributed to their failure to survive adoptive transfer and be activated *in situ*, as equivalent intratumor densities of transferred T cells which expressed granzyme B and Ki67 were observed by IHC early after adoptive transfer (day 3) **(Figs. 14D-G)**. While not statistically significant ($p=0.34$), 2G-CAR(LS) CD8+ $T_{E(CM)}$ in which activated caspase 3 was detected were 12.1-fold more frequent than their 2G-CAR(SS) counterparts. These data reveal an unexpected discordance between the *in vitro* anti-tumor potency of CAR redirected T cells dictated by extracellular spacer size, and their *in vivo* anti-tumor therapeutic activity.

Augmented Activation Induced Cell Death Accompanies Hyperactive Signaling Outputs of Long Spacer Formatted Second Generation CAR Upon Recursive Antigen Exposure

[0279] It was hypothesized that whereas *in vitro* activation for cytolysis in a 4-hour CRA is the consequence of a limited duration of CAR mediated signaling, the *in vivo* tumor model requires recursive rounds of activation to achieve tumor eradication. Thus, the signaling performance of a particular CAR format that dictates superior metrics *in vitro* may fail to reveal the consequences of the signaling amplitude *in vivo*. In order to reproduce recursive serial stimulation *in vitro*, a CAR T cell-tumor cell co-culture "stress test" assay was devised whereby every 24 hours, CAR T cells are harvested and recursively transferred to culture dishes seeded with tumor cells adjusting for a constant viable T cell:tumor cell ratio of 1:1 **(Fig. 15A)**. Be2 modified to express firefly luciferase was utilized to concurrently track tumor cell killing upon each of three rounds of serial transfer. The recursive activation of the 2G-CAR spacer variant lines resulted in equivalent loss of anti-tumor activity by round III **(Fig. 15B)**. Additionally, analysis of each spacer variant expressing effector cells after each round by flow cytometric measurement revealed that 2G-CAR(LS) CD8+$T_{E(CM)}$ displayed higher frequencies of cells expressing activation markers CD25 and CD69, as compared to their 2G-CAR(SS) counterparts (round I 79.4 vs 46.8%, p=0.007; round II 74.0 vs 47.6%; round III 65.7 vs 42.1%, p=0.037) **(Fig. 15C)**.

[0280] In contrast to the LS>MS>SS pattern of upregulation of activation markers in round I that mimicked earlier *in vitro* analysis, it was observed that a LS/MS>SS loss of T cell viability that was most substantial in round III (round III percent dead cells LS 58.7%, MS 62.6% vs SS 21.1%, LS vs SS p=0.024 and MS vs SS p=0.007) **(Fig. 15D)**. To substantiate that the asymmetric loss of T cell viability by 2G-CAR(LS) CTLs occurring with recursive activation was the result of exaggerated AICD, the mechanism of cell death was assessed, focusing on FasL-Fas mediated T cell fratricide. It was observed that tumor-induced CAR activation dependent upregulation of FasL followed a LS>MS>SS hierarchy as 2G-CAR(LS) $CD8^{+}T_{E(CM)}$ displayed 4.8- and 2.5-fold higher FasL surface expression, and, 5.5- and 3.3-fold higher FasL mRNA abundance than the short or medium spacer CAR T cells, respectively (long vs short: p<0.0001 and p=0.002; long vs medium: p<0.0001 and p=0.016) **(Figs. 15E and F)**. To link FasL expression with increased Fas mediated apoptosis, caspase 3 activity was analyzed and

13.2-fold higher levels of cleaved caspase 3 in 2G-CAR(LS) CD8+ $T_{E(CM)}$ as compared to their SS counterpart ($p<0.0001$) was observed **(Fig. 15G)**. Lastly, 2G-CAR(LS) CD8+ $T_{E(CM)}$ were subjected to siRNA knockdown of Fas or FasL, then exposed to tumor and a 1.4-fold (Fas) ($p=0.005$) and 1.6-fold (FasL) ($p=0.0001$) increase in T cell viability after round III, was observed, respectively **(Fig. 15H)**. To verify that the siRNA knock-down led to a reduction of Fas/FasL, their surface expression on the 2G-CAR(LS) CD8+ $T_{E(CM)}$ was assessed and a 91.3% reduction in Fas+ ($p<0.0001$) and 80.1% reduction in FasL+ CTLs ($p<0.0001$) was observed than 2G-CAR(LS) CD8+$T_{E(CM)}$ treated with scrambled siRNA **(Figs. 21A and B)**. In aggregate, these data demonstrate that tuning of CAR spacer size can modulate downstream signaling events that result not only in differential magnitudes of anti-tumor functional outputs but coordinated increases in susceptibility to AICD. The balance between these two processes for optimal *in vivo* anti-tumor activity may not always be achieved by spacer tuning to achieve the highest levels of CAR signaling outputs as exemplified by these comparisons of 2G-CAR(LS) and 2G-CAR(SS) structural variants.

Augmentation of CAR Cytoplasmic Endodomain Composition Reverts Short Spacer CD171-CAR to AICD Prone Variant Upon Recursive Tumor Encounter

[0281] Third generation CARs contain two co-stimulatory endodomain modules in series with the CD3-ζ activation module and have been reported to augment the magnitude of cytolysis and cytokine production levels over their second generation counterparts. CD171-specific and/or targeting 3G-CAR were assembled through the addition of a CD28 endodomain to the 2G 4-1BB:zeta endodomain **(Fig. 16A)**. CD8+$T_{E(CM)}$ expressing comparable levels of 2G-CAR(SS) and 3G-CAR(SS) were derived from purified TCM precursors by immunomagnetic selection **(Figs. 16B and C)**. 3G-CAR(SS) CD8+$T_{E(CM)}$ demonstrated an 8.4-fold higher induction of CD137 expression upon tumor contact than their second generation counterparts ($p<0.0001$) **(Fig. 16D)**, a 1.3-fold increase in cytolytic activity against Be2 targets (effector to target ratio 1:10, $p=0.0001$) **(Fig. 16E)** and 5.1-fold more IL-2 and 2.5-fold more TNF-α secretion ($p<0.0001$ and $p=0.003$) **(Fig. 16F)**.

[0282] Next it was assessed whether heightened T cell activation through an augmented CAR mediated by the 3G endodomain, in the context of an extracellular short spacer, could selectively enhance anti-tumor activity *in vivo* without exacerbation of

AICD. Surprising, Be2 **(Fig. 17A)** and SK-N-DZ **(Fig. 17B)** was inferior, though not to a statistically significant degree to their 2G-CAR(SS) counterparts. These findings could not be attributable to differences in short term persistence of CAR T cells within tumors based on similar densities of human $CD3^+$ T cells detected 3 days after adoptive transfer **(Fig. 17C)**. Despite the finding of higher frequencies of granzyme B^+ 3G-CAR(SS) T cells compared to 2G-CAR(SS) intratumoral T cells, augmented numbers of third generation T cells with activated caspase 3 was again observed, suggesting that the augmented costimulation through a combined effect of CD28 and 4-1BB in the third generation CAR was capable of hyperstimulation resulting in heightened AICD, despite the context of a short spacer extracellular domain **(Fig. 17D)**. This was confirmed by comparing their performance using the *in vitro* stress test assay. Following each round of tumor stimulation, higher frequencies of CD25+CD69+ T cells in the 3G-CAR(SS) T cell population was observed **(Fig. 18A)** accompanied by increased frequencies of dead T cells through successive rounds of activation **(Fig. 18B)**. Augmented AICD was again associated with heightened levels of FasL expression by surface staining and mRNA content, that in turn coincided with increased levels of activated caspase 3 **(Figs. 18C-E)**. These data demonstrate that over tuning of CAR signaling outputs based on intracellular signaling domain composition negatively impacted on a tuned short spacer dimension in a combinatorial manner by enhancing FasL-mediated T cell AICD.

[0283] CARs are capable of mediating multiplexed signaling outputs that trigger redirected anti-tumor T cell effector function. Despite the irrefutable therapeutic potency of CAR T cells redirected to CD19 in patients with acute lymphoblastic leukemia, the biophysical structure-function attributes of this class of synthetic receptor remain incompletely understood. While it stands to reason that the tuning of CARs for effective T cell anti-tumor activity will be more stringent in solid tumor applications, empiric designs of CARs based on limited understanding of the impact of their composition on *in vivo* anti-tumor function will only hamper progress in human clinical applications. Here, CAR structure-function in human central memory derived $CD8^+$ effector CTL's focusing on the combinatorial effect(s) of extracellular spacer dimension in the context of cytoplasmic signaling module composition was systematically interrogated. By surveying CAR signaling strength using *in vitro* assays, a potency hierarchy of CAR structural variants was identified. These analyses have revealed a range of CAR signaling outputs that is

permissive for *in vivo* anti-tumor activity above which *in vivo* potency is attenuated by heightened AICD.

[0284] The evolution of CAR design has proceeded to date via a largely empiric process, and has focused predominantly on the augmentation of signaling outputs through combinatorial modules of costimulatory receptor cytoplasmic domains fused in series to ITAM containing activation domains, such as the zeta chain of the CD3 complex. Comparisons of the function of CTL's expressing first, second, or third generation CARs have typically been made in the context of a "stock" extracellular spacer domain preferred by a particular laboratory, ranging from full length IgGs to relatively short CD8α hinges or membrane proximal portions of CD28 Many have studied the impact of spacer dimension on CAR signaling and functional activity. Unlike a TcR contact with peptide loaded HLA Class I or II, which defines a scripted biophysical gap between T cell plasma membrane and target cell plasma membrane that is permissive for assembly of a supramolecular activation complex (SMAC), CARs do not conform to this dimensional relationship as a consequence of the target molecule's structural dimensions, the scFv's epitope location on the target molecule, and the CAR's spacer size. The molecular assembly of a SMAC via CARs is relatively unknown, but analysis to date suggests that it does not replicate the orderly architecture of a TcR SMAC. While the first two dimensions are unique to each selected antigen and antibody binding domain choice, the CAR spacer is size tunable and can compensate to some extent in normalizing the orthogonal synapse distance between CAR T cell and target cell. This topography of the immunologic synapse between T cell and target cell also defines distances that cannot be functionally bridged by a CAR due to a membrane distal epitope on a cell surface target molecule that, even with a short spacer CAR, cannot bring the synapse distance in to an approximation for signaling. Likewise, membrane proximal CAR target antigen epitopes have been described for which signaling outputs are only observed in the context of a long spacer CAR.

[0285] Using a CD171-specific and/or targeting scFv binding domain derived from the CE7 mAb, the impact of extracellular spacer size on signaling outputs from a 4-1BB:zeta second generation CAR was first assessed. It was observed that incremental gains of function in signaling outputs based on *in vitro* assays as spacer size increased from the short IgG4 hinge spacer, to an intermediate hinge:CH3, to the full length IgG4 hinge:Fc spacer. Unexpectedly, upon *in vivo* testing against established stereotactically implanted

intraparenchymal brain neuroblastoma xenografts in NSG mice, the anti-tumor potency of intratumorally injected CAR CD8+ CTL was inversely correlated to spacer size (i.e. SS>MS>>LS) and *in vitro* functional potency. The direct intratumoral route of T cell administration was used to eliminate potential spacer effects on T cell migration or interactions with mouse Fc+ cells that could affect survival. Given these findings, it was hypothesized that commonly employed *in vitro* assays that assess CAR T cell function (i.e. tumor cell cytolysis, stimulation of cytokine secretion, and proliferation), upon a single limited duration tumor cell encounter fail to detect the subsequent fate of CAR T cells upon recursive tumor exposure, as would be predicted to occur within solid tumors *in vivo*. To better assess this possibility an *in vitro* assay was devised in which CAR T cells are recursively exposed to equal numbers of biophotonic reporter gene expressing tumor cells. Tumor cell killing can thereby be quantitated biophotonically and retrieved CAR T cells can be interrogated for activation status, viability, and caspase activity after each round of tumor co-culture. It was observed that upon three recursive tumor encounters *in vitro,* disproportionate increases in the frequency of T cells undergoing apoptosis among 2G-CAR(LS) T cells as compared to 2G-CAR(SS) T cells. The exaggerated AICD correlated with heightened LS CAR induced expression of FasL and activated caspase 3 relative to SS CAR. AICD in LS CAR T cells was reduced by siRNA knockdown of FAS or FasL prior to exposure to tumor cells. These *in vitro* findings correlated with limited intratumor persistence of LS CAR T cells within tumor xenografts as compared with SS CAR T cells. In aggregate these data demonstrate that the non-signaling extracellular spacer is a major tunable CAR design element that impacts not only on signaling activity but persistence of CAR T cells in solid tumors.

[0286] Given the relation between spacer dimension and *in vivo* survival in the context of a 4-1BBzeta second generation (2G) CAR, endeavors to determine if the short spacer dimension would be generically optimal in the context of the augmenting signaling outputs of a third generation (3G) CD28:4-1BB:zeta CAR endodomain format were performed. Consistent with observations made by multiple other groups, the CD171-specific and/or targeting 3G-CAR(SS) stimulated heightened levels of cytolytic activity and cytokine synthesis compared to the 2G-CAR(SS) upon *in vitro* tumor stimulation. However, the augmented signaling outputs of the 3G-CAR in the context of its short spacer also increased FasL expression, exacerbated apoptosis as indicated by increased

levels of activated caspase 3 and resulted in higher frequencies of cell death. Correspondingly, impaired *in vivo* anti-tumor efficacy of the 3G-CAR(SS) T cells, as compared to the 2G-CAR(SS) due to attenuated *in vivo* intratumoral survival was observed. While CD28 costimulates T cells upon initial antigen activation and enhances T cell viability by deflecting AICD through NFAT regulated increases in cFLIPshort, published studies have also revealed that recursive CD28 costimulation of previously activated T cells can reduce their subsequent survival via augmented FasL expression and consequently, increased AICD. It is interesting therefore, to speculate if recursive CD28 signaling mediated by anti-CD19 CD28:zeta CAR T cells is responsible for the relatively short persistence duration in treated ALL patients, as compared to the often prolonged persistence of anti-CD19 4-1BB:zeta treated patients in reported clinical trials (*2, 35*). In aggregate, these data demonstrate that *in vivo* potency of CAR redirected T cells is dependent, in part, on identifying permissive combinations of size optimized extracellular spacer domains in the context of a particular cytoplasmic signaling domain composition. Further, i*n vitro* assay for assessing the proclivity of a CAR construct to induce AICD in primary human $CD8^+$ CTL upon recursive activation events was described. These studies reveal a caveat of "overtuning" of CARs based on the combinatorial net effect of spacer dimension and cytoplasmic signaling module selection.

[0287] There is as yet no predictive structural model that can reliably direct *a priori* how CARs should be built based on target molecule epitope location relative to the plasma membrane of the tumor cell. Moreover, commonly used surrogate *in vitro* bioassays may instruct away from a definitive choice of CAR composition that results in the greatest differential between high-level functional anti-tumor CAR T cell outputs and low-level AICD. The work here demonstrates that a CAR structural library screen technique using the *in vitro* stress test assay may be a valuable additional parameter to integrate into CAR engineering. It is conceivable that genetic strategies might limit susceptibility of hyperactive CAR constructs to undergo AICD, such as forced over expression of cFLIP or Toso, or, vector directed synthesis of siRNAs that knock down FasL or Fas. Manipulation of T cell susceptibility to undergo apoptosis will require commensurately stringent safety features, such as inclusion of inducible suicide constructs, or, transgene expression control rheostats under clinician control, such as small molecule regulated transcriptional or translational control systems. Additional secondary

consequences of CAR overtuning also require interrogation, such as predilection of hyperactive CARs to trigger augmented expression of inhibitory receptors, such as PD-1, capable of enforcing an exhausted T cell functional status within PD-L1. The data demonstrate that: 1.) CAR structure-function *in vitro* testing using commonly employed functional assays can misdirect selection of candidate constructs as common practice is to focus on those constructs that display the highest functional activity, and; 2.) potency tuning of CAR redirected effector CTLs has an upper limit above which gains in the magnitude of effector outputs are negated by augmentation in AICD upon recursive triggering through the CAR. These results have guided the selection of a CD171-specific and/or targeting short spacer CAR for a Phase I study in children with relapsed/refractory neuroblastoma.

Additional Alternatives

[0288] In some alternatives, a chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is can target and/or is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0289] In some alternatives, a chimeric receptor polypeptide is provided. In some alternatives, the chimeric receptor polypeptide is coded for by a chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a)a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence.

[0290] In some alternatives, an expression vector comprising an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino

acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence.

[0291] In some alternatives a host cell is provided. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and

bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO.

[0292] In some alternatives, a composition is provided. In some alternatives, the composition comprises a host cell in a pharmaceutically acceptable excipient. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell, wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host

cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the composition comprises a host cell, wherein the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells or wherein the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+ and another host cell, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or wherein the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor.

[0293] In some alternatives, an *in vitro* method for preparing a host cell is provided. In some alternatives, the *in vitro* method for preparing a host cell comprises: a) providing a library of nucleic acids coding for a chimeric receptor wherein each of the plurality of nucleic acids code for a chimeric receptor that differs in length, b) introducing each of the plurality of the nucleic acids into a separate isolated T lymphocyte population and expanding each T lymphocyte population in vitro, c) administering each genetically modified T lymphocyte population into an animal model bearing a tumor and determining whether a genetically modified T lymphocyte population has anti-tumor efficacy and d) selecting a nucleic acid coding for the chimeric receptor that provides for anti-tumor efficacy in vitro and/or in an animal model. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an

intracellular signaling domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and negative for CD45RO. In some alternatives, the nucleic acids coding for a chimeric receptor comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric

receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the method further comprises introducing the selected nucleic acid coding for the chimeric receptor into a host cell.

[0294] In some alternatives, an *in vitro* method for preparing a host cell is provided. In some alternatives, the method comprises a) introducing a chimeric receptor nucleic acid or an expression vector into a lymphocyte population that has a CD45RA-, CD45RO+, and/or CD62L+ phenotype and b) culturing the cells in the presence of anti-CD3 and/or anti CD28, and at least one homeostatic cytokine until the cells expand sufficiently for use as a cell infusion. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a

hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of

CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less. In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the lymphocyte is CD8+ or CD4+.

[0295] In some alternatives, a use of a host cell or composition in the treatment of cancer or a solid tumor expressing CD171 is provided. In some alternatives, the composition comprises a host cell in a pharmaceutically acceptable excipient. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling

domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the composition comprises a host cell, wherein the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells or wherein the CD8+ cytotoxic T lymphocyte cell is a central memory T cell, wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+ and another host cell, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or wherein the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the

host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the cancer is a neuroblastoma. In some alternatives, the solid tumor is selected from the group consisting of a colon cancer, renal cancer, pancreatic cancer, and ovarian cancer.

[0296] In some alternatives, a method of performing cellular immunotherapy in a subject having cancer or tumor is provided. In some alternatives, the method comprises administering a composition or a host cell to the subject. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand as compared to a reference chimeric receptor. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some

alternatives, the composition comprises a host cell in a pharmaceutically acceptable excipient. In some alternatives, the host cell comprises an expression vector. In some alternatives, the expression vector comprises an isolated chimeric receptor nucleic acid is provided. In some alternatives, the chimeric receptor nucleic acid comprises: a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or targets CD171, b) a polynucleotide coding for a polypeptide spacer of a length that is specific for the ligand, wherein the spacer is optimized, c) a polynucleotide coding for a transmembrane domain and d) a polynucleotide coding for an intracellular signaling domain. In some alternatives, the ligand binding domain is an antibody fragment. In some alternatives, the ligand binding domain is single chain variable fragment. In some alternatives, the spacer is 15 amino acids or less (but not less than 1 or 2 amino acids). In some alternatives, the spacer comprises an amino acid sequence of X_1PPX_2P. In some alternatives, the spacer region comprises a portion of a hinge region of a human antibody. In some alternatives, the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof. In some alternatives, the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB. In some alternatives, the chimeric receptor nucleic acid further comprises a nucleic acid that codes for a marker sequence. In some alternatives, the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells. In some alternatives, the CD8+ cytotoxic T lymphocyte cell is a central memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+. In some alternatives, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells. In some alternatives, the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the composition comprises a host cell, wherein the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells or wherein the CD8+ cytotoxic T lymphocyte cell is a central

memory T cell wherein the central memory T cell is positive for CD45RO+, CD62L+, and/or CD8+ and another host cell, wherein the host cell is a CD4+ T helper lymphocyte cell is selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells or wherein the CD4+ helper lymphocyte cell is a naïve CD4+ T cell, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and/or CD4+ and/or negative for CD45RO. In some alternatives, the cancer is neuroblastoma. In some alternatives, the tumor is selected from the group consisting of a colon cancer, renal cancer, pancreatic cancer, and ovarian cancer.

Table 1 Exemplary sequences

IgG4hinge

DNA:GAGAGCAAGTACGGA
AA: E S K Y G

CD28tm

DNA:CCGCCCTGCCCCCCTTGCCCT:ATGTTCTGGGTGCTGGTGGTGGTCGGAGGC
AA: P P C P P C P M F W V L V V V G G

DNA: GTGCTGGCCTGCTACAGCCTGCTGGTCACCGTGGCCTTCATCATCTTTTGG
AA: V L A C Y S L L V T V A F I I F W

41BB

DNA: GTG:AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATG
AA: V K R G R K K L L Y I F K Q P F M

DNA: AGACCAGTACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCA
AA: R P V Q T T Q E E D G C S C R F P

CD3**Zeta**

DNA: GAAGAAGAAGAAGGAGGATGTGAACTGCGGGTGAAG:TTCAGCAGAAGCGCC
AA: E E E E G G C E L R V K F S R S A

DNA: GACGCCCCTGCCTACCAGCAGGGCCAGAATCAGCTGTACAACGAGCTGAAC
AA: D A P A Y Q Q G Q N Q L Y N E L N

DNA: CTGGGCAGAAGGGAAGAGTACGACGTCCTGGATAAGCGGAGAGGCCGGGAC
AA: L G R R E E Y D V L D K R R G R D

DNA: CCTGAGATGGGCGGCAAGCCTCGGCGGAAGAACCCCCAGGAAGGCCTGTAT
AA: P E M G G K P R R K N P Q E G L Y

DNA: AACGAACTGCAGAAAGACAAGATGGCCGAGGCCTACAGCGAGATCGGCATG
AA: N E L Q K D K M A E A Y S E I G M

DNA: AAGGGCGAGCGGAGGCGGGGCAAGGGCCACGACGGCCTGTATCAGGGCCTG
AA: K G E R R R G K G H D G L Y Q G L

DNA: TCCACCGCCACCAAGGATACCTACGACGCCCTGCACATGCAGGCCCTGCCC
AA: S T A T K D T Y D A L H M Q A L P

T2A
DNA: CCAAGG:CTCGAGGGCGGCGGAGAGGGCAGAGGAAGTCTTCTAACATGCGGT
AA: P R L E G G G E G R G S L L T C G

EGFRt

DNA:GACGTGGAGGAGAATCCCGGCCCTAGG:**ATGCTTCTCCTGGTGACAAGCCTT**
AA: D V E E N P G P R M L L L V T S L

DNA: **CTGCTCTGTGAGTTACCACACCCAGCATTCCTCCTGATCCCACGCAAAGTG**
AA: L L C E L P H P A F L L I P R K V

DNA: **TGTAACGGAATAGGTATTGGTGAATTTAAAGACTCACTCTCCATAAATGCT**
AA: C N G I G I G E F K D S L S I N A

DNA: **ACGAATATTAAACACTTCAAAAACTGCACCTCCATCAGTGGCGATCTCCAC**
AA: T N I K H F K N C T S I S G D L H

DNA: **ATCCTGCCGGTGGCATTTAGGGGTGACTCCTTCACACATACTCCTCCTCTG**
AA: I L P V A F R G D S F T H T P P L

DNA: **GATCCACAGGAACTGGATATTCTGAAAACCGTAAAGGAAATCACAGGGTTT**
AA: D P Q E L D I L K T V K E I T G F

DNA: **TTGCTGATTCAGGCTTGGCCTGAAAACAGGACGGACCTCCATGCCTTTGAG**
AA: L L I Q A W P E N R T D L H A F E

DNA: **AACCTAGAAATCATACGCGGCAGGACCAAGCAACATGGTCAGTTTTCTCTT**
AA: N L E I I R G R T K Q H G Q F S L

DNA: **GCAGTCGTCAGCCTGAACATAACATCCTTGGGATTACGCTCCCTCAAGGAG**
AA: A V V S L N I T S L G L R S L K E

DNA: **ATAAGTGATGGAGATGTGATAATTTCAGGAAACAAAAATTTGTGCTATGCA**
AA: I S D G D V I I S G N K N L C Y A

DNA: **AATACAATAAACTGGAAAAAACTGTTTGGGACCTCCGGTCAGAAAACCAAA**
AA: N T I N W K K L F G T S G Q K T K

DNA: **ATTATAAGCAACAGAGGTGAAAACAGCTGCAAGGCCACAGGCCAGGTCTGC**
AA: I I S N R G E N S C K A T G Q V C

DNA: **CATGCCTTGTGCTCCCCCGAGGGCTGCTGGGGCCCGGAGCCCAGGGACTGC**
AA: H A L C S P E G C W G P E P R D C

DNA: **GTCTCTTGCCGGAATGTCAGCCGAGGCAGGGAATGCGTGGACAAGTGCAAC**
AA: V S C R N V S R G R E C V D K C N

DNA: **CTTCTGGAGGGTGAGCCAAGGGAGTTTGTGGAGAACTCTGAGTGCATACAG**
AA: L L E G E P R E F V E N S E C I Q

DNA: **TGCCACCCAGAGTGCCTGCCTCAGGCCATGAACATCACCTGCACAGGACGG**
AA: C H P E C L P Q A M N I T C T G R

DNA: **GGACCAGACAACTGTATCCAGTGTGCCCACTACATTGACGGCCCCCACTGC**
AA: G P D N C I Q C A H Y I D G P H C

DNA: **GTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACACCCTGGTCTGG**
AA: V K T C P A G V M G E N N T L V W

DNA: **AAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACC**
AA: K Y A D A G H V C H L C H P N C T

DNA: **TACGGATGCACTGGGCCAGGTCTTGAAGGCTGTCCAACGAATGGGCCTAAG**
AA: Y G C T G P G L E G C P T N G P K

DNA: **ATCCCGTCCATCGCCACTGGGATGGTGGGGGCCCTCCTCTTGCTGCTGGTG**
AA: I P S I A T G M V G A L L L L L V

DNA: **GTGGCCCTGGGGATCGGCCTCTTCATG*TGA*** (SEQ ID NO:10)
AA: V A L G I G L F M * (SEQ ID NO:11)

Table 2
Exemplary Sequence Uniprot P0861 IgG4-Fc (SEQ ID NO:13)

```
    10         20         30         40         50         60
ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS

       70         80         90        100        110        120
GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPSCP APEFLGGPSV

      130        140        150        160        170        180
FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY

      190        200        210        220        230        240
RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK

      250        260        270        280        290        300
NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSRL TVDKSRWQEG
```

```
       310        320
NVFSCSVMHE ALHNHYTQKS LSLSLGK
```

1-98 CH1

99-110 Hinge

111-220 CH2

221-327 CH3

Position 108 S→P

Table 3

Exemplary sequence/Uniprot P10747 CD28(SEQ ID NO:14)

```
10         20         30         40         50         60
MLRLLLALNL FPSIQVTGNK ILVKQSPMLV AYDNAVNLSC KYSYNLFSRE FRASLHKGLD

        70         80         90        100        110        120
SAVEVCVVYG NYSQQLQVYS KTGFNCDGKL GNESVTFYLQ NLYVNQTDIY FCKIEVMYPP

       130        140        150        160        170        180
PYLDNEKSNG TIIHVKGKHL CPSPLFPGPS KPFWVLVVVG GVLACYSLLV TVAFIIFWVR

       190        200        210        220
SKRSRLLHSD YMNMTPRRPG PTRKHYQPYA PPRDFAAYRS
```

1-18 signal peptide

19-152 extracellular domain

153-179 transmembrane domain

180-220 intracellular domain

Position 186-187 LL→GG

Table 4

Exemplary Sequence/Uniprot Q07011 4-1BB(SEQ ID NO:15)

```
     10         20         30         40         50         60
MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR

        70         80         90        100        110        120
TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC

       130        140        150        160        170        180
CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE

       190        200        210        220        230        240
PGHSPQIISF FLALTSTALL FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG

       250
CSCRFPEEEE GGCEL
```

1-23 signal peptide

24-186 extracellular domain

187-213 transmembrane domain

214-255 intracellular domain

Table 5

Exemplary sequence/Uniprot P20963 human CD3ζ isoform 3 (SEQ ID NO:16)

```
10         20         30         40         50         60
MKWKALFTAA ILQAQLPITE AQSFGLLDPK LCYLLDGILF IYGVILTALF LRVKFSRSAD

        70         80         90        100        110        120
APAYQQGQNQ LYNELNLGRR EEYDVLDKRR GRDPEMGGKP QRRKNPQEGL YNELQKDKMA

       130        140        150        160
EAYSEIGMKG ERRRGKGHDG LYQGLSTATK DTYDALHMQA LPPR
```

1-21 signal peptide

22-30 extracellular

31-51 transmembrane

52-164 intracellular domain

61-89 ITAM1

100-128 ITAM2

131-159 ITAM3

Table 6 Exemplary Hinge region Sequences

Human IgG1 EPKSCDKTHTCPPCP (SEQ ID NO:17)

Human IgG2 ERKCCVECPPCP (SEQ ID NO:18)

Human IgG3 ELKTPLGDTHTCPRCP $(EPKSCDTPPPCPRCP)_3$ (SEQ ID NO:19)

Human IgG4 ESKYGPPCPSCP (SEQ ID NO:20)

Modified Human IgG4 ESKYGPPCPPCP (SEQ ID NO:21)

Modified Human IgG4 YGPPCPPCP (SEQ ID NO:51)

Modified Human IgG4 KYGPPCPPCP (SEQ ID NO:52)

Modified Human IgG4 EVVKYGPPCPPCP (SEQ ID NO:53)

Table 7

Medium spacer IgG4hinge-CH3 (SEQ ID NO: 37)

IgG4 spacer

TA CGGACCGCCCTGCCCCCCTTGCCCT

CH3

GGCCAGCCTCGCGAGCCCCAGGTGTACACCCTGCCTCCCTCCCAGGAAGAGATG
ACCAAGAACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACCCCAGCGAC
ATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCTGAGAACAACTACAAGACCAC
CCCTCCCGTGCTGGACAGCGACGGCAGCTTCTTCCTGTACAGCCGGCTGACCGTG
GACAAGAGCCGGTGGCAGGAAGGCAACGTCTTTAGCTGCAGCGTGATGCACGAG
GCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTGTCCCTGGGCAAG

Long spacer IgG4hinge-CH2-CH3 (SEQ ID NO: 58)

IgG4 spacer

TA CGGACCGCCCTGCCCCCCTTGCCCT

CH2

GCCCCCGAGTTCCTGGGCGGACCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGG
ACACCCTGATGATCAGCCGGACCCCCGAGGTGACCTGCGTGGTGGTGGACGTGA
GCCAGGAAGATCCCGAGGTCCAGTTCAATTGGTACGTGGACGGCGTGGAAGTGC
ACAACGCCAAGACCAAGCCCAGAGAGGAACAGTTCAACAGCACCTACCGGGTG
GTGTCTGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAAGAATACAAG
TGCAAGGTGTCCAACAAGGGCCTGCCCAGCAGCATCGAAAAGACCATCAGCAAG
GCCAAG

CH3

GGCCAGCCTCGCGAGCCCCAGGTGTACACCCTGCCTCCCTCCCAGGAAGAGATG
ACCAAGAACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACCCCAGCGAC
ATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCTGAGAACAACTACAAGACCAC
CCCTCCCGTGCTGGACAGCGACGGCAGCTTCTTCCTGTACAGCCGGCTGACCGTG
GACAAGAGCCGGTGGCAGGAAGGCAACGTCTTTAGCTGCAGCGTGATGCACGAG
GCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTGTCCCTGGGCAAG

Table 8

Short spacer (SEQ ID NO: 21)

Hinge Spacer

ESKYGPPCPPCP

Medium spacer (SEQ ID NO: 59)

Hinge Spacer

ESKYGPPCPPCP

CH3

GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV

LDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

Long spacer (SEQ ID NO: 60)

Hinge Spacer

ESKYGPPCPPCP

CH2

APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA

KTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK

CH3

GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV

LDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

WHAT IS CLAIMED IS:

1. A chimeric receptor nucleic acid comprising:

a) a polynucleotide coding for a ligand binding domain, wherein the ligand binding domain binds to and/or interacts with CD171;

b) a polynucleotide coding for a polypeptide spacer of a length, wherein the spacer is optimized;

c) a polynucleotide coding for a transmembrane domain; and

d) a polynucleotide coding for an intracellular signaling domain.

2. The chimeric receptor nucleic acid of claim 1, wherein the ligand binding domain is an antibody fragment.

3. The chimeric receptor nucleic acid of claim 2, wherein the ligand binding domain is single chain variable fragment.

4. The chimeric receptor nucleic acid of any one of claims 1-4, wherein the spacer is 15 amino acids or less but not less than 1 or 2 amino acids.

5. The chimeric receptor nucleic acid of any one of claims 1-4, wherein the spacer comprises an amino acid sequence of X_1PPX_2P.

6. The chimeric receptor nucleic acid of claim 5, wherein the spacer region comprises a portion of a hinge region of a human antibody.

7. The chimeric receptor nucleic acid of any one of claims 1 to 6, wherein the intracellular signaling domain comprises all of a portion of CD3 zeta in combination with a costimulatory domain selected from the group consisting of CD27, CD28, 4-1BB, OX-40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, NKG2C, and B7-H3 or combinations thereof.

8. The chimeric receptor nucleic acid of claim 7, wherein the intracellular signaling domain comprises a portion of CD3 zeta and a portion of 4-1BB.

9. The chimeric receptor nucleic acid of any one of claims 1-8, further comprising a nucleic acid that encodes a marker sequence.

10. A chimeric receptor polypeptide coded for by a chimeric receptor nucleic acid of any one of claims 1 to 9.

11. An expression vector comprising an isolated chimeric receptor nucleic acid of any one of claims 1-9.

12. A host cell comprising an expression vector of claim 11.

13. The host cell of claim 12, wherein the host cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells and bulk CD8+ T cells.

14. The host cell of claim 13, wherein the CD8+ cytotoxic T lymphocyte cell is a central memory T cell and, wherein the central memory T cell is positive for CD45RO+, CD62L+, and CD8+.

15. The host cell according to claim 12, wherein the host cell is a CD4+ T helper lymphocyte cell selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells.

16. The host cell of claim 15, wherein the CD4+ helper lymphocyte cell is a naïve CD4+ T cell and, wherein the naïve CD4+ T cell is positive for CD45RA+, CD62L+ and CD4+ and negative for CD45RO.

17. A composition comprising a host cell of any one of claims 12-16 or 31-31 in a pharmaceutically acceptable excipient.

18. A composition of comprising a host cell of claims 13 or 14 and a host cell of claims 15 or 16.

19. A method for preparing a host cell of any one of claims 12-16 or 30-31 comprising:

a) providing a library of nucleic acids coding for a chimeric receptor of any one of claims 1-9 or 11, wherein each of the plurality of nucleic acids code for a chimeric receptor that differs in length;

b) introducing each of the plurality of the nucleic acids into a separate isolated T lymphocyte population and expanding each T lymphocyte population *in vitro*;

c) administering each genetically modified T lymphocyte population into an animal model bearing a tumor and determining whether a genetically modified T lymphocyte population has an anti-tumor response; and

d) selecting a nucleic acid coding for the chimeric receptor that provides for the anti-tumor response.

20. The method of claim 19, further comprising introducing the selected nucleic acid coding for the chimeric receptor into a host cell.

21. A method for preparing a host cell of any one of claims 12-16 or 30-31 comprising:

a) introducing a nucleic acid of any one of claims 1-9 or an expression vector of claim 11 into a lymphocyte population that has a CD45RA-, CD45RO+, and CD62L+ phenotype; and

b) culturing the cells in the presence of anti-CD3 and/or anti CD28, and at least one homeostatic cytokine until the cells expand sufficiently for use as a cell infusion.

22. The method of any one of claims 19-21, wherein the lymphocyte is CD8+ or CD4+.

23. A use of the host cell of any one of claims 12-16 or 30-31 or the composition of claims 17 or18 in the treatment or inhibition of cancer or a solid tumor expressing CD171.

24. The use of claim 23, wherein the cancer is a neuroblastoma.

25. The use of claim 23, wherein the solid tumor is selected from the group consisting of a breast cancer, brain cancer, colon cancer, renal cancer, pancreatic cancer, and ovarian cancer.

26. A method of performing cellular immunotherapy in a subject having cancer or a tumor comprising: administering the composition of any one of claims 17 to 18 or a host cell of claims 12-16 to the subject.

27. The method of claim 26, wherein the cancer is neuroblastoma.

28. The method of claim 26, wherein the tumor is selected from the group consisting of a breast cancer, brain cancer, colon cancer, renal cancer, pancreatic cancer, and ovarian cancer.

29. The method of claim 1, wherein the spacer is optimized for increased T cell proliferation and/or cytokine production in response to the ligand, as compared to a reference chimeric receptor.

30. The host cell of claim 12, wherein the host cell is a precursor T cell.

31. The host cell of claim 12, wherein the host cell is a hematopoietic stem cell.

FIG. 1

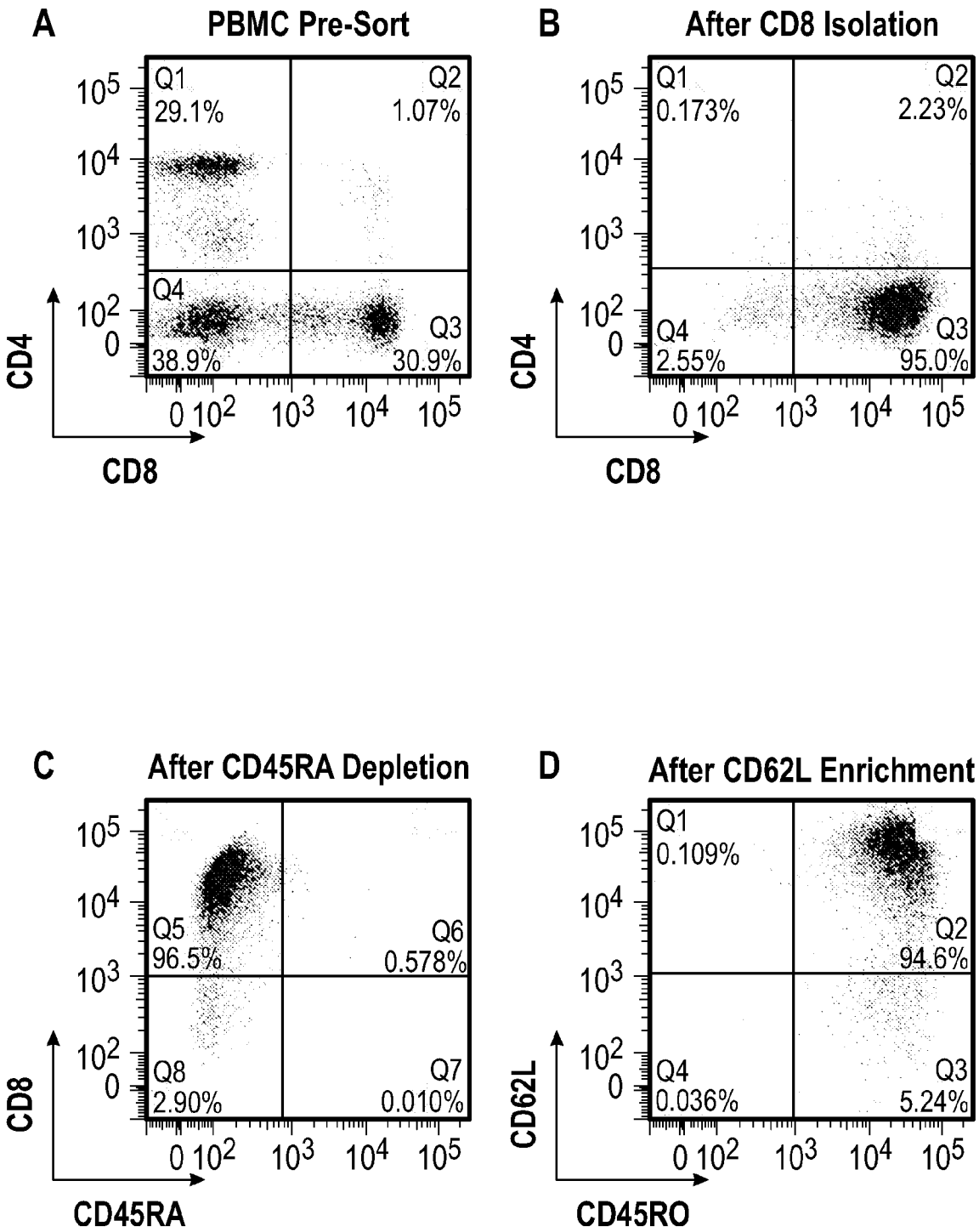
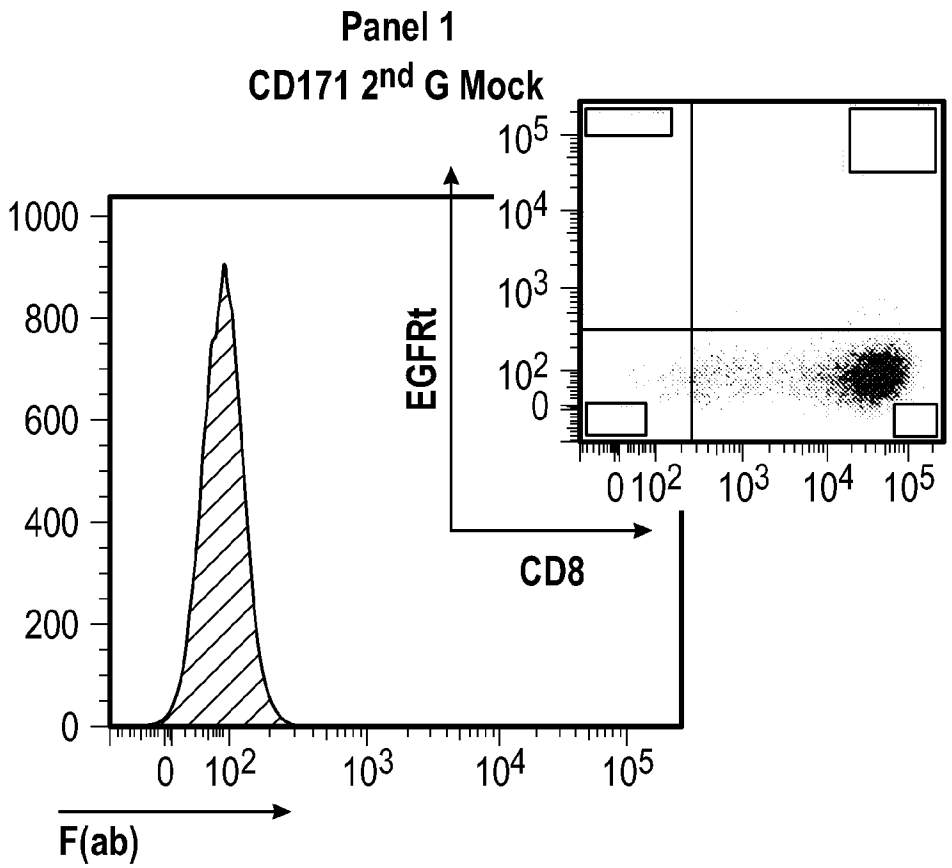

A
PBMC Pre-Sort
Q1
29.1%
Q2
1.07%
Q4
38.9%
Q3
30.9%
CD4
CD8
B
After CD8 Isolation
Q1
0.173%
Q2
2.23%
Q4
2.55%
Q3
95.0%
CD4
CD8
C
After CD45RA Depletion
Q5
96.5%
Q6
0.578%
Q8
2.90%
Q7
0.010%
CD8
CD45RA
D
After CD62L Enrichment
Q1
0.109%
Q2
94.6%
Q4
0.036%
Q3
5.24%
CD62L
CD45RO

FIG. 2A


Panel 1
CD171 2nd G Mock
1000
800
600
400
200
0
0 10^2 10^3 10^4 10^5
F(ab)
EGFRt
CD8
10^5
10^4
10^3
10^2
0
0 10^2 10^3 10^4 10^5

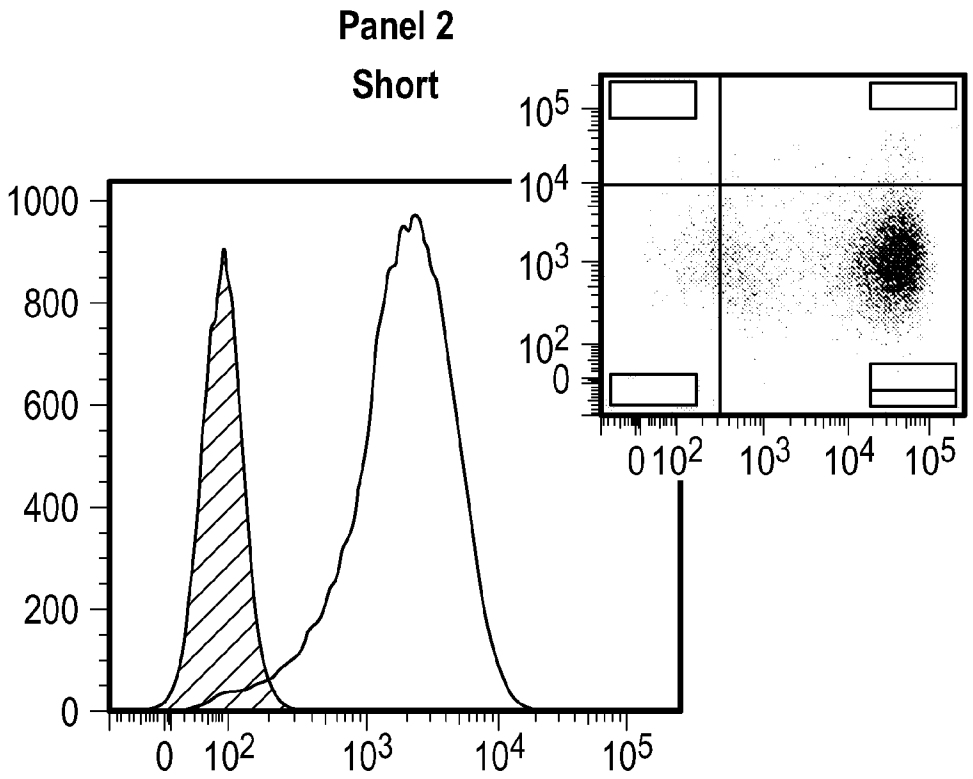
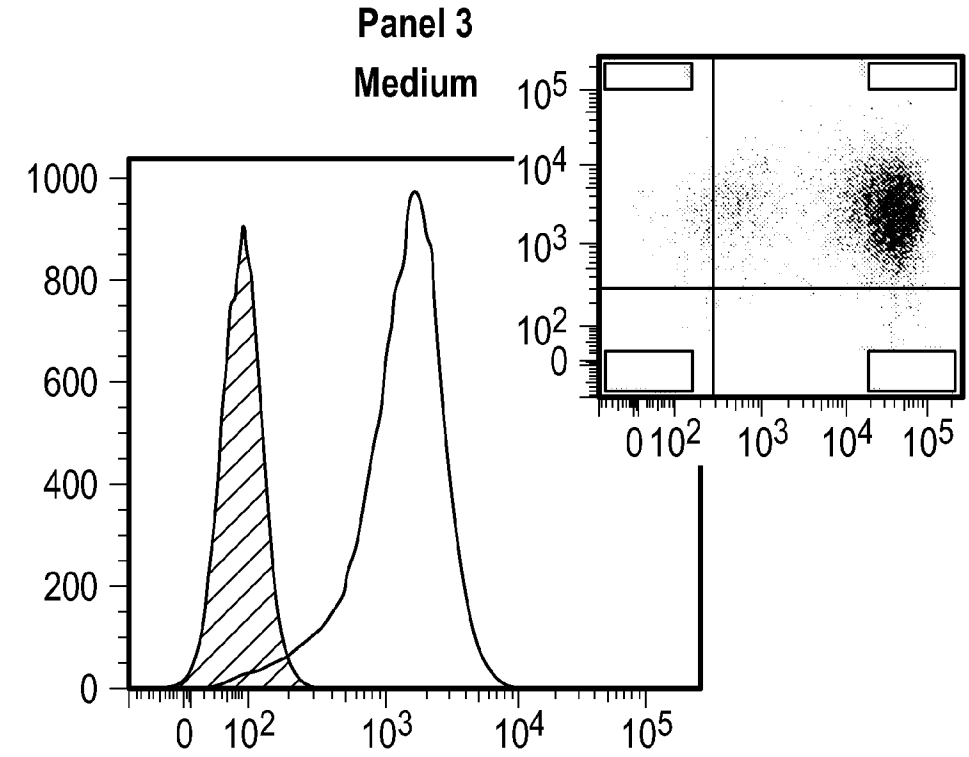
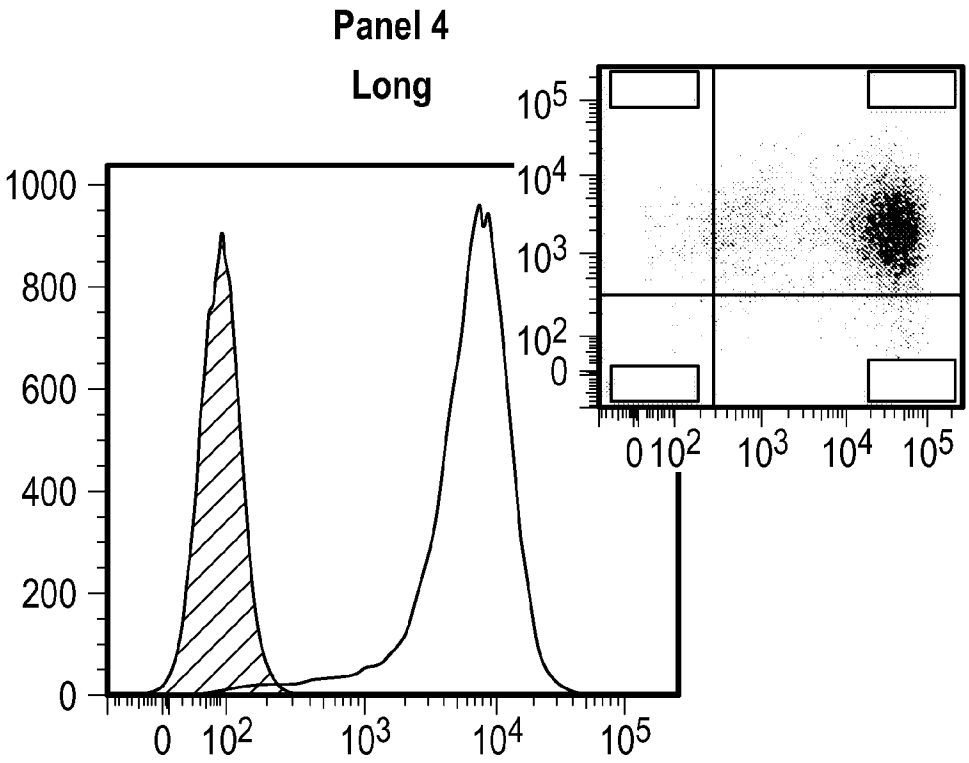

Panel 2
Short
1000
800
600
400
200
0
0 10^2 10^3 10^4 10^5
10^5
10^4
10^3
10^2
0
0 10^2 10^3 10^4 10^5

FIG. 2A (Continued)





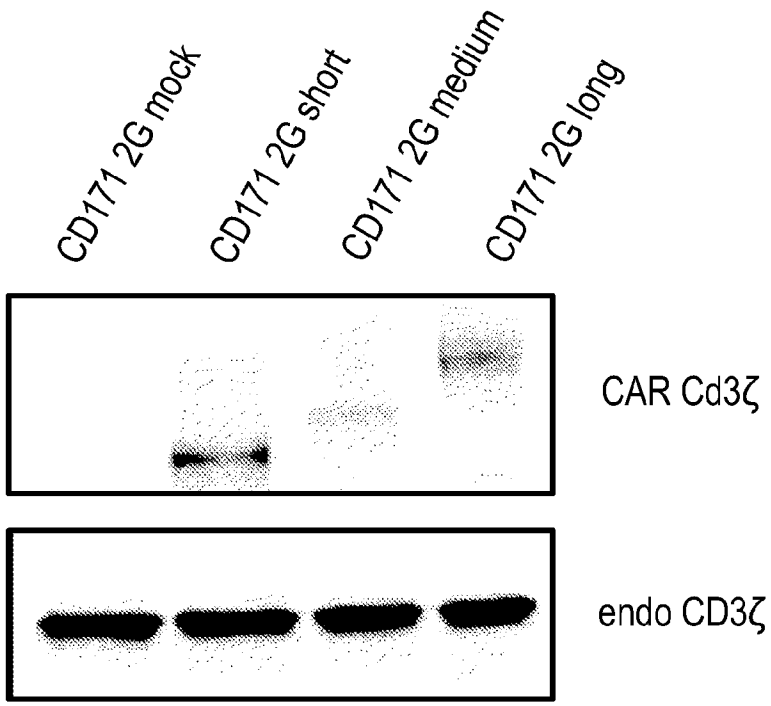

CD171 2G mock
CD171 2G short
CD171 2G medium
CD171 2G long
CAR Cd3ζ
endo CD3ζ

FIG. 2B

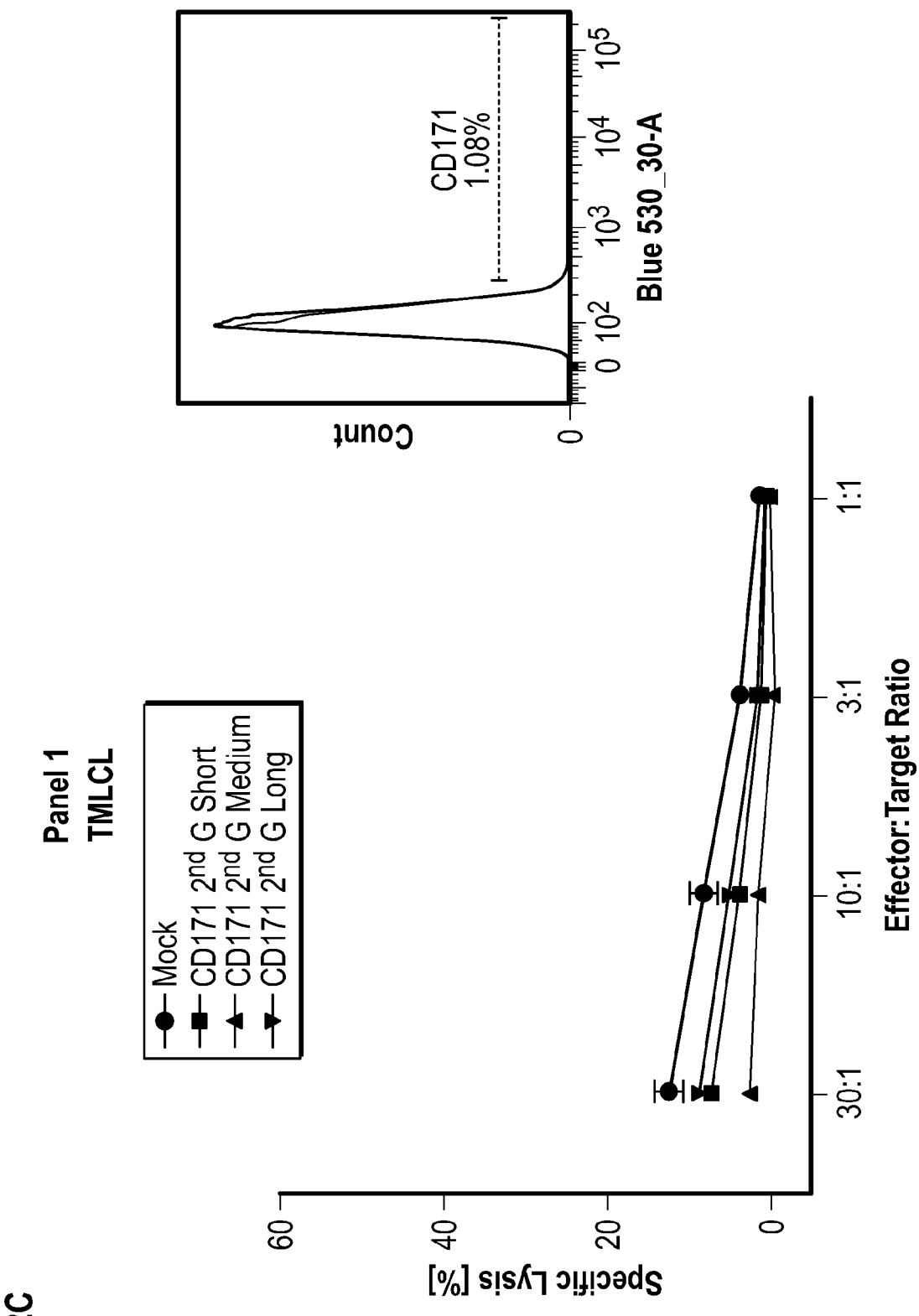

Panel 1
TMLCL
Mock
CD171 2nd G Short
CD171 2nd G Medium
CD171 2nd G Long
Specific Lysis [%]
0
20
40
60
30:1
10:1
3:1
1:1
Effector:Target Ratio
Count
0
0 10^2 10^3 10^4 10^5
Blue 530_30-A
CD171
1.08%

FIG. 2C

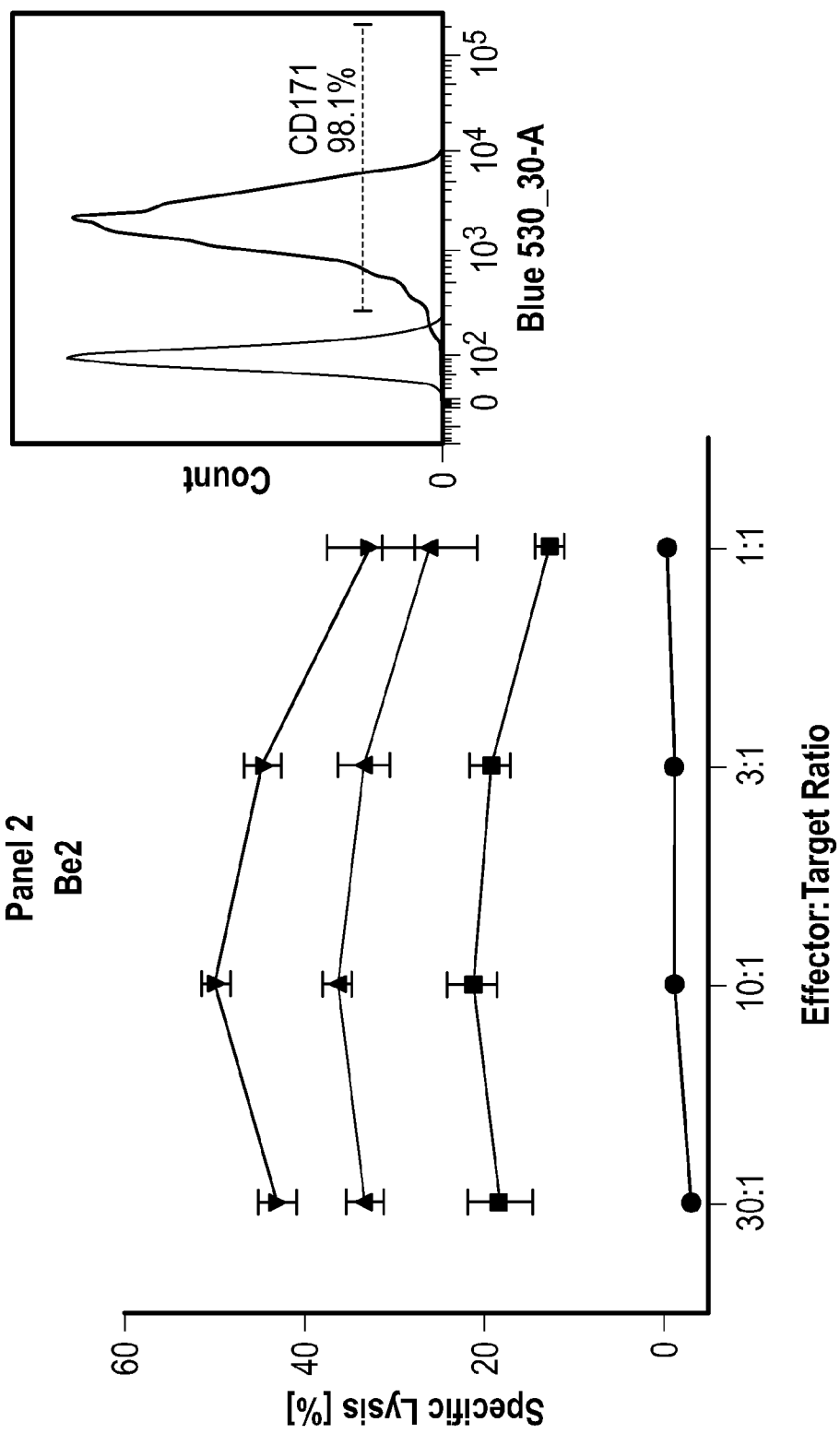


FIG. 2C (Continued)

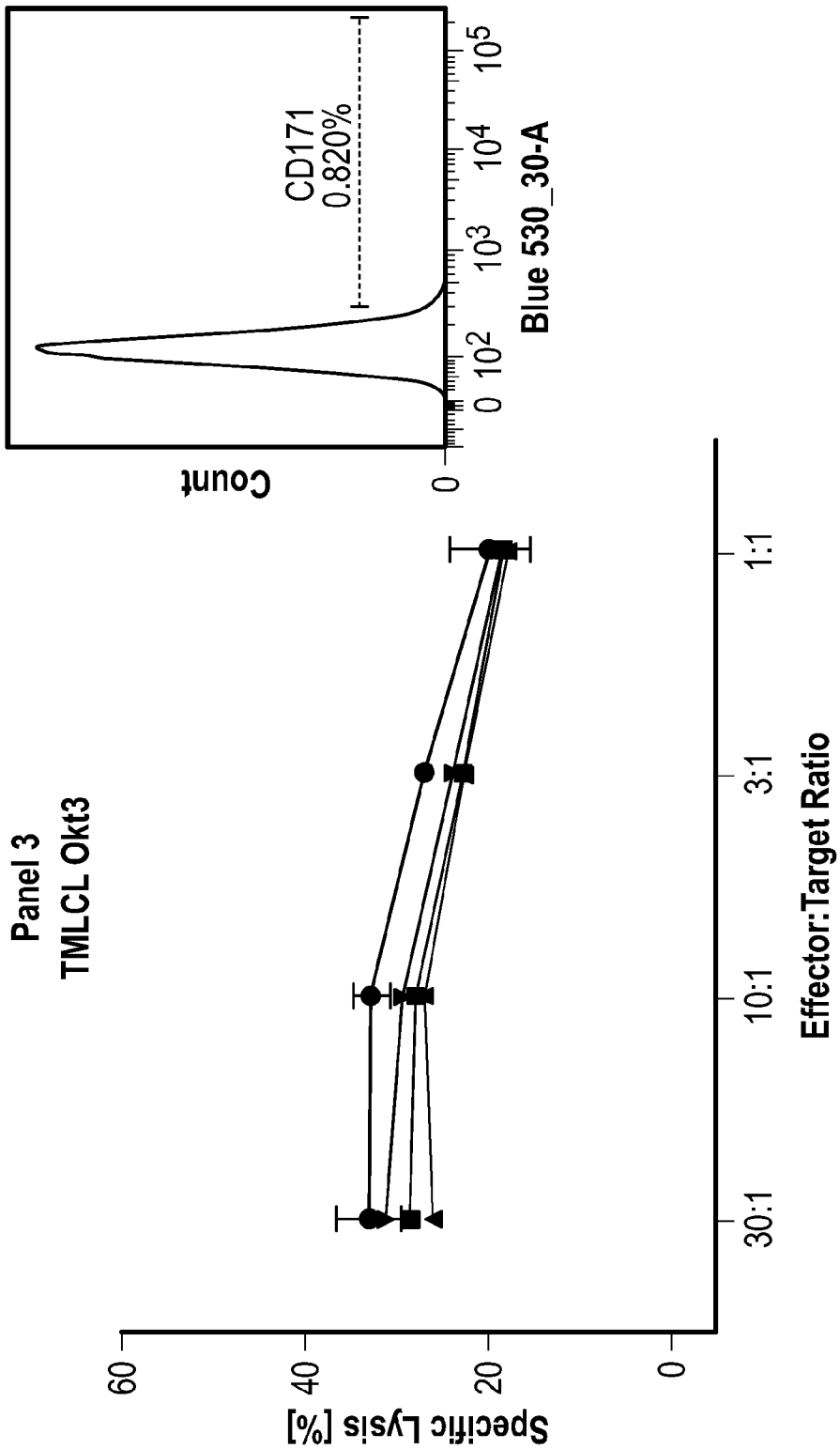
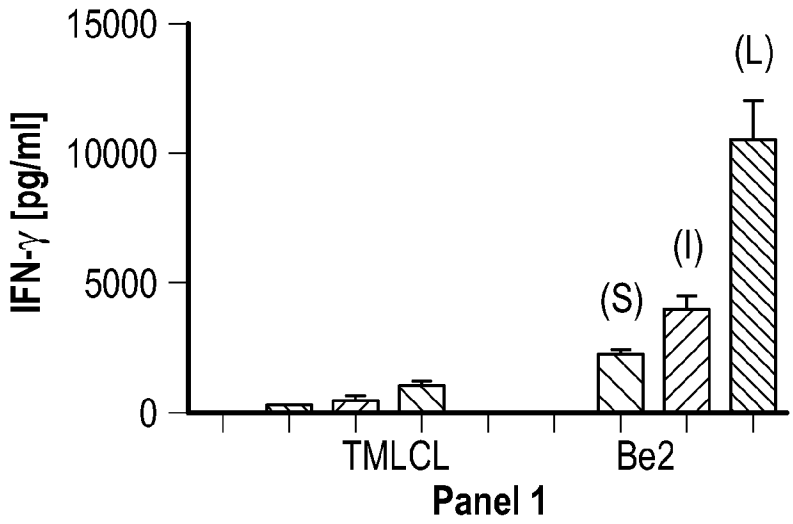
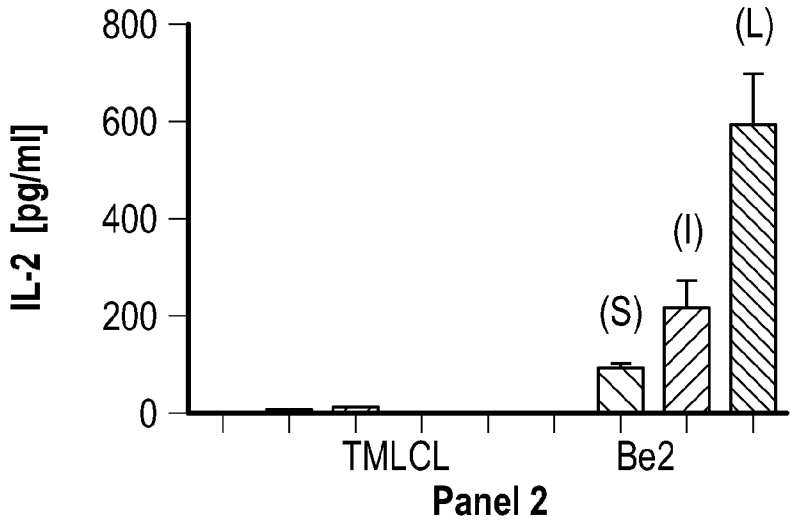


FIG. 2C (Continued)

FIG. 2D


15000
10000
5000
0
IFN-γ [pg/ml]
(L)
(I)
(S)
TMLCL
Be2
Panel 1


800
600
400
200
0
IL-2 [pg/ml]
(L)
(I)
(S)
TMLCL
Be2
Panel 2

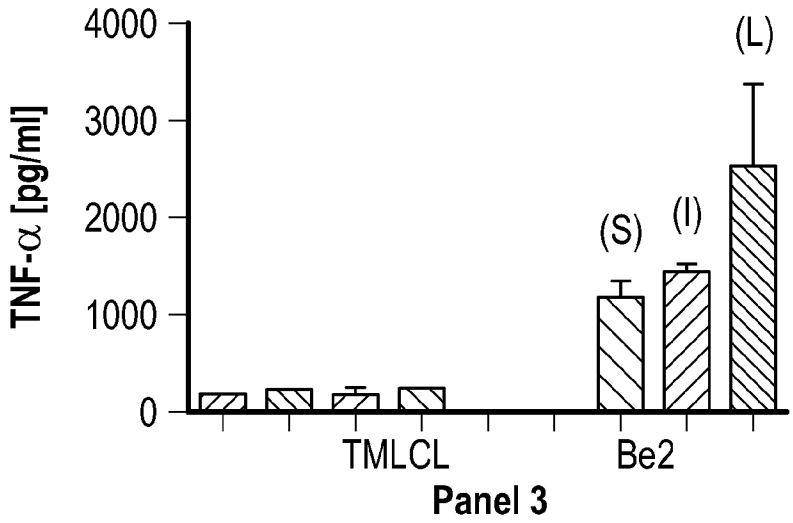
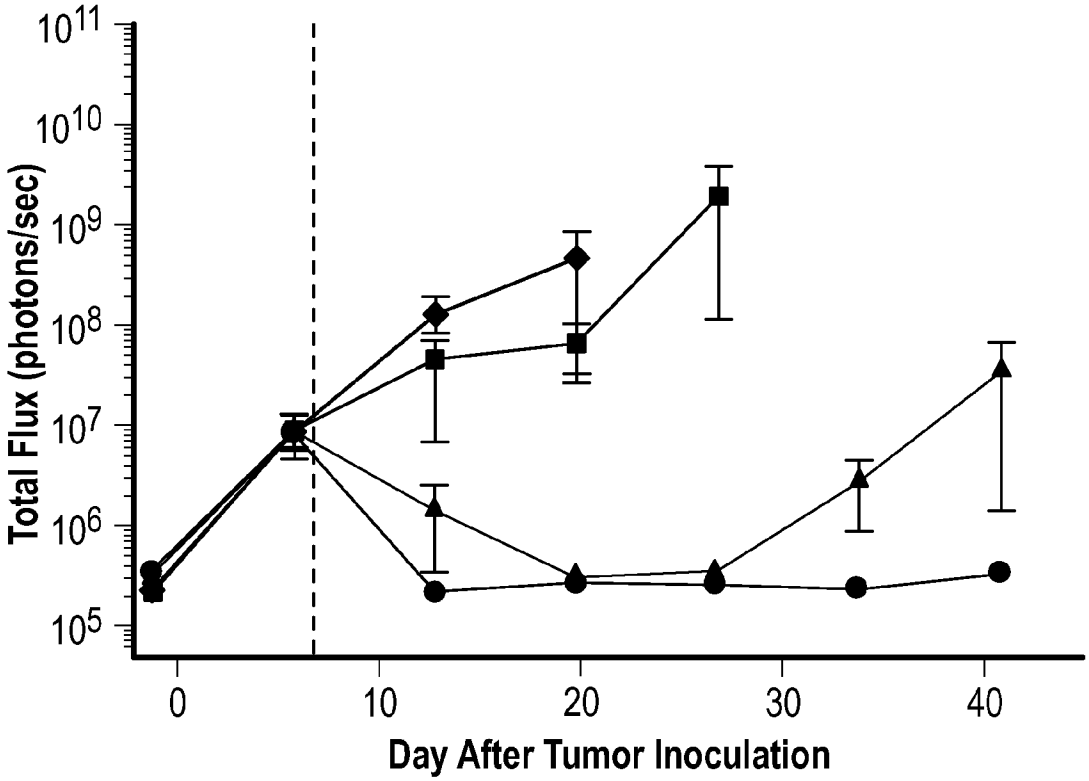
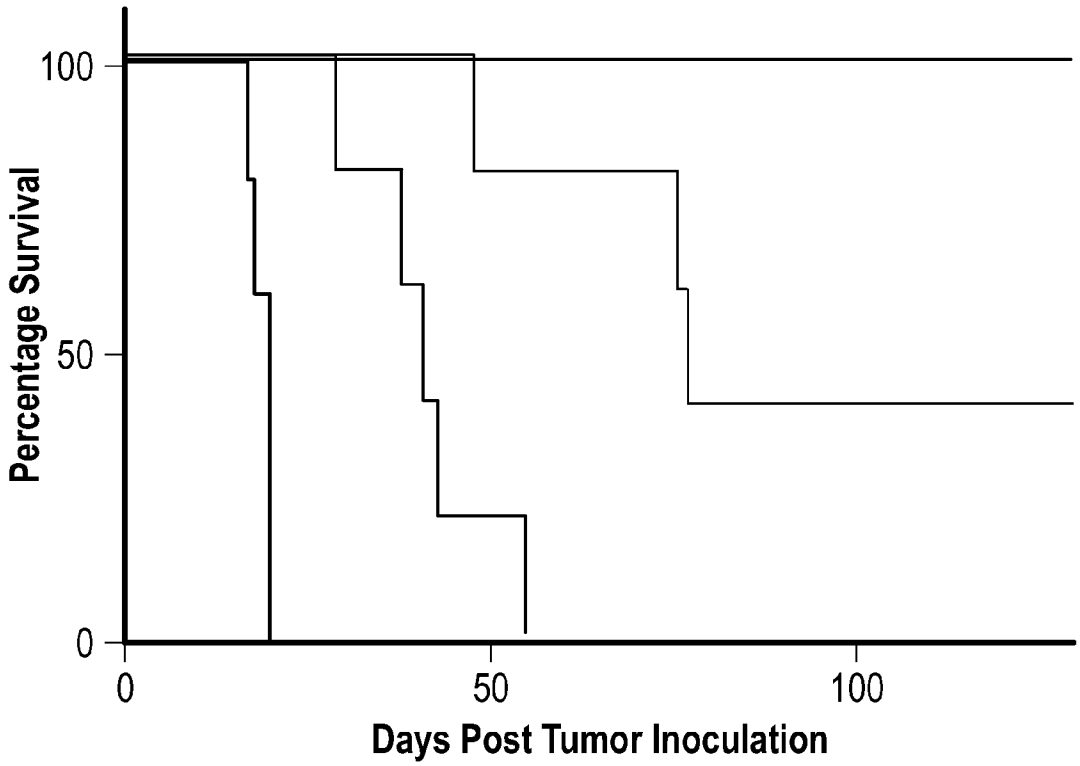

4000
3000
2000
1000
0
TNF-α [pg/ml]
(L)
(I)
(S)
TMLCL
Be2
Panel 3

FIG. 2E


Total Flux (photons/sec)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
10
20
30
40
Day After Tumor Inoculation


Percentage Survival
100
50
0
0
50
100
Days Post Tumor Inoculation

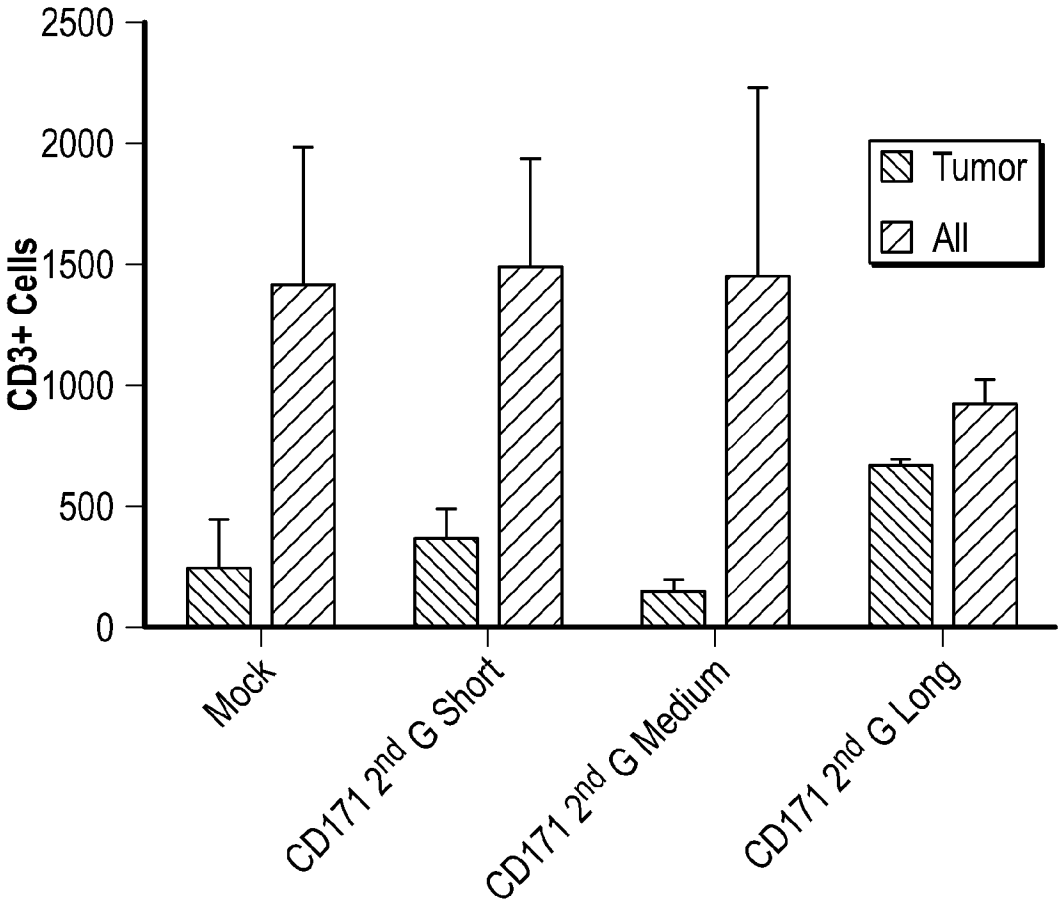

2500
2000
1500
1000
500
0
CD3+ Cells
Tumor
All
Mock
CD171 2nd G Short
CD171 2nd G Medium
CD171 2nd G Long

FIG. 2F

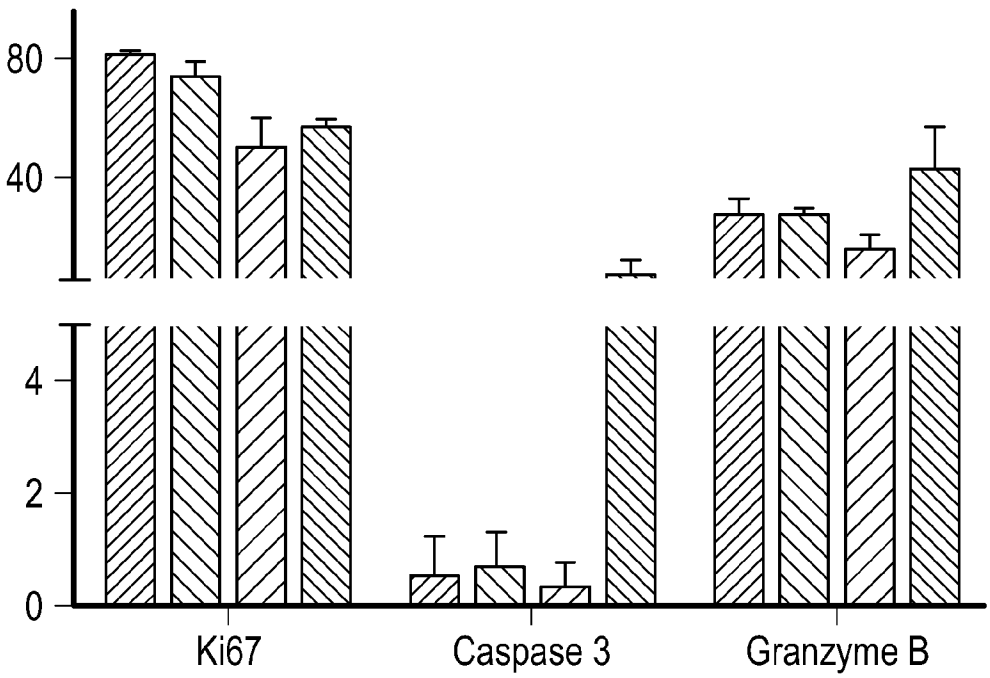

80
40
4
2
0
Ki67
Caspase 3
Granzyme B

FIG. 2G

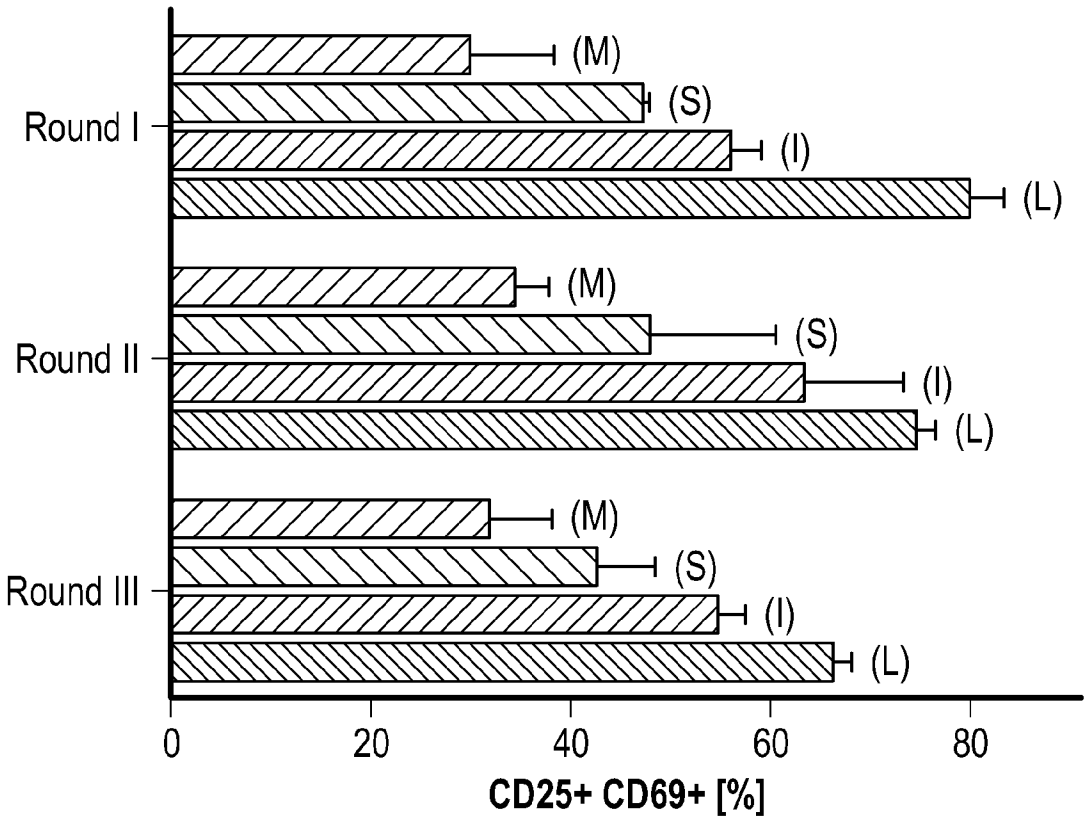

Round I
(M)
(S)
(I)
(L)
Round II
(M)
(S)
(I)
(L)
Round III
(M)
(S)
(I)
(L)
0
20
40
60
80
CD25+ CD69+ [%]

FIG. 3A

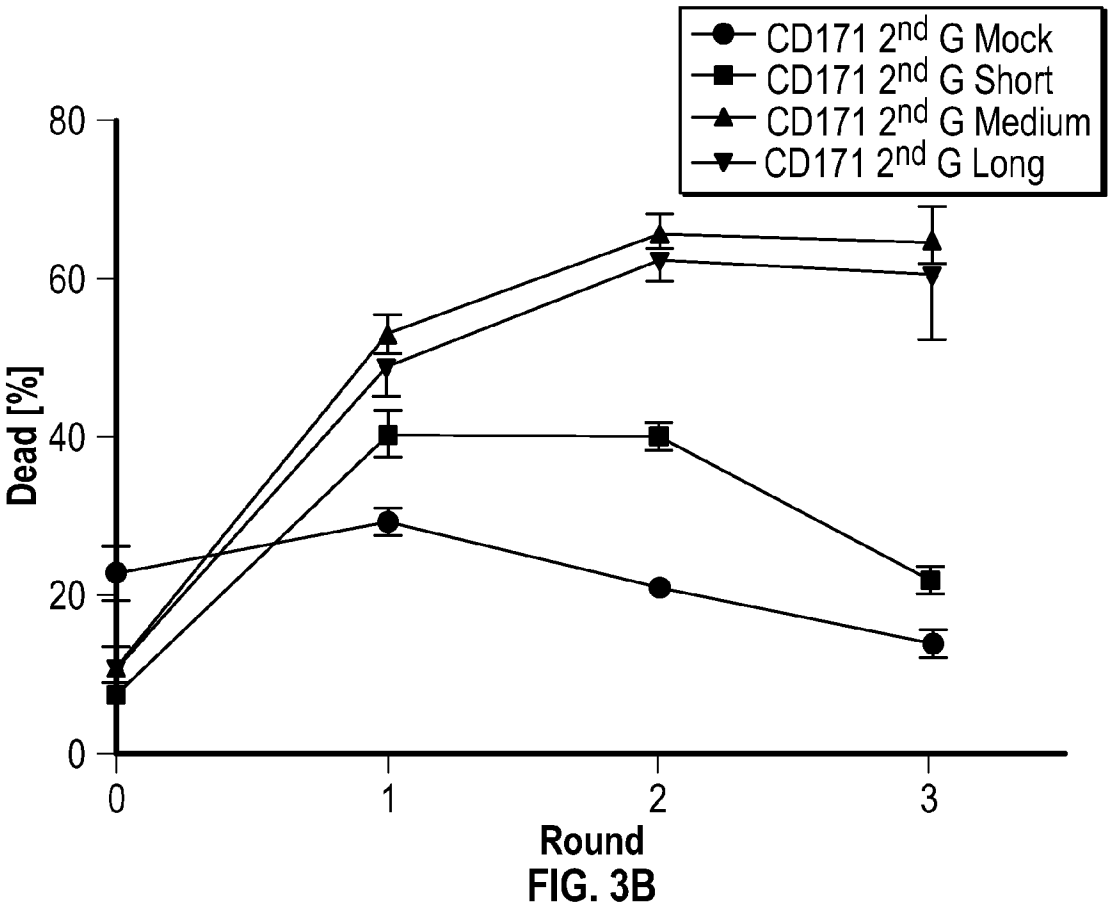

CD171 2nd G Mock
CD171 2nd G Short
CD171 2nd G Medium
CD171 2nd G Long
Dead [%]
0
20
40
60
80
0
1
2
3
Round

FIG. 3B

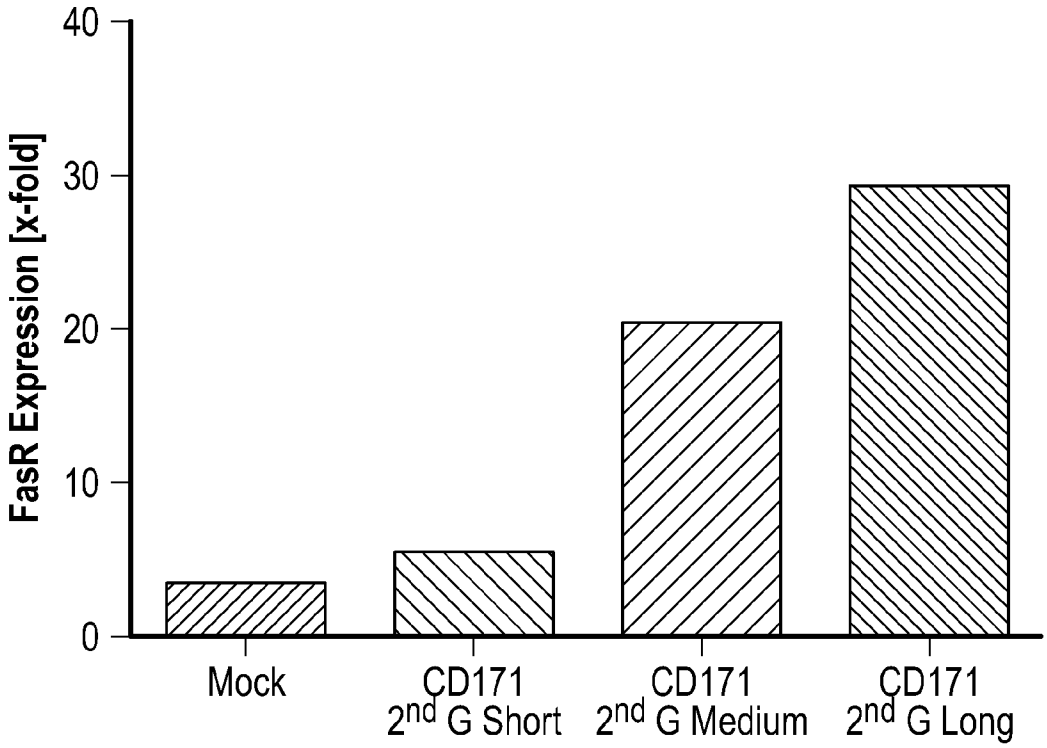
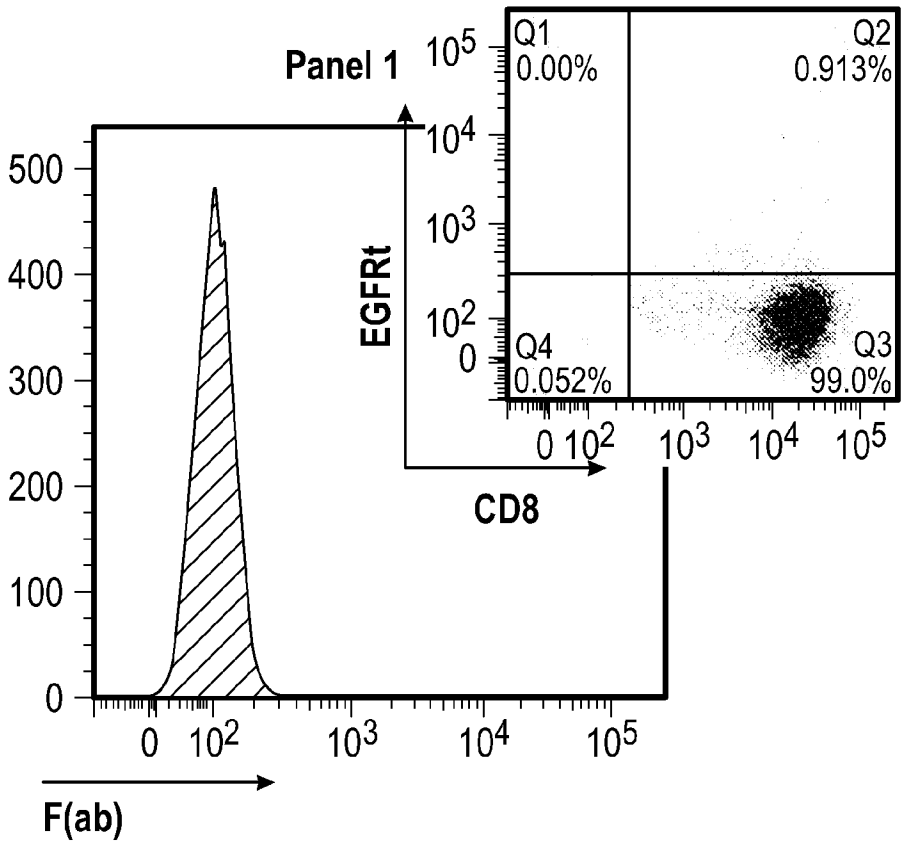
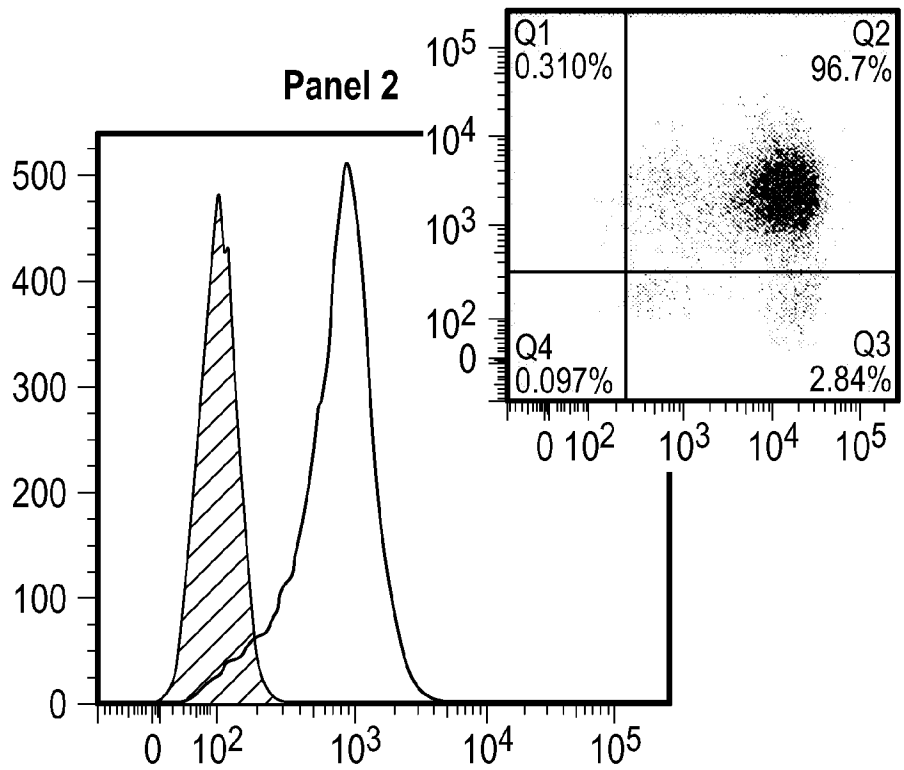

FasR Expression [x-fold]
40
30
20
10
0
Mock
CD171 2nd G Short
CD171 2nd G Medium
CD171 2nd G Long

FIG. 3C

FIG. 4A


Panel 1
Q1
0.00%
Q2
0.913%
Q4
0.052%
Q3
99.0%
EGFRt
CD8
F(ab)
0
100
200
300
400
500
0 10^2 10^3 10^4 10^5


Panel 2
Q1
0.310%
Q2
96.7%
Q4
0.097%
Q3
2.84%
0
100
200
300
400
500
0 10^2 10^3 10^4 10^5

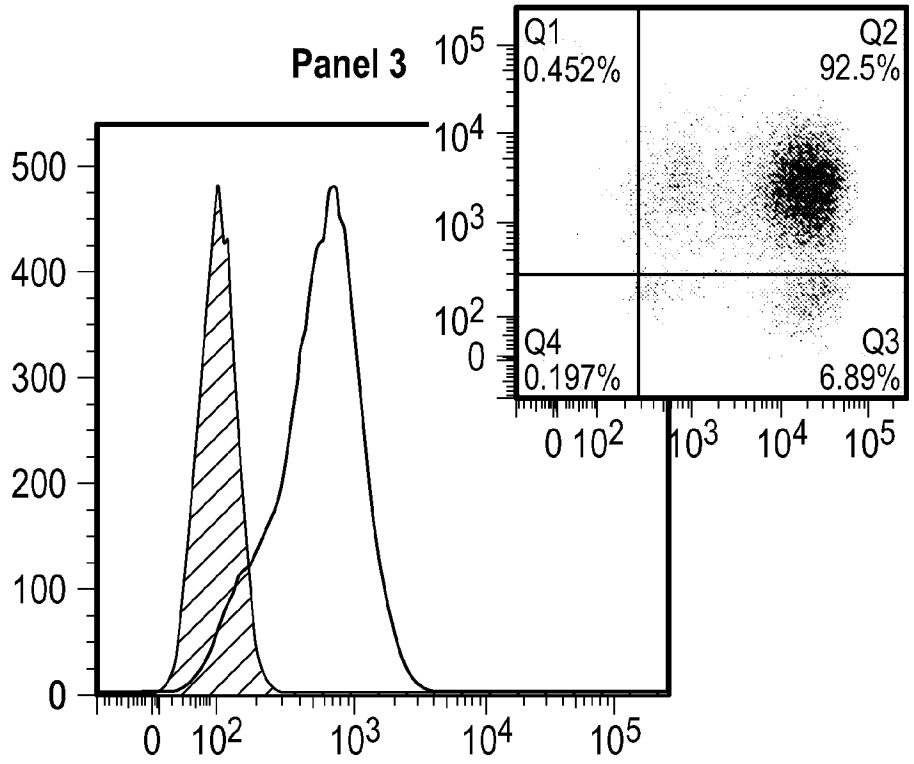


FIG. 4A (Continued)

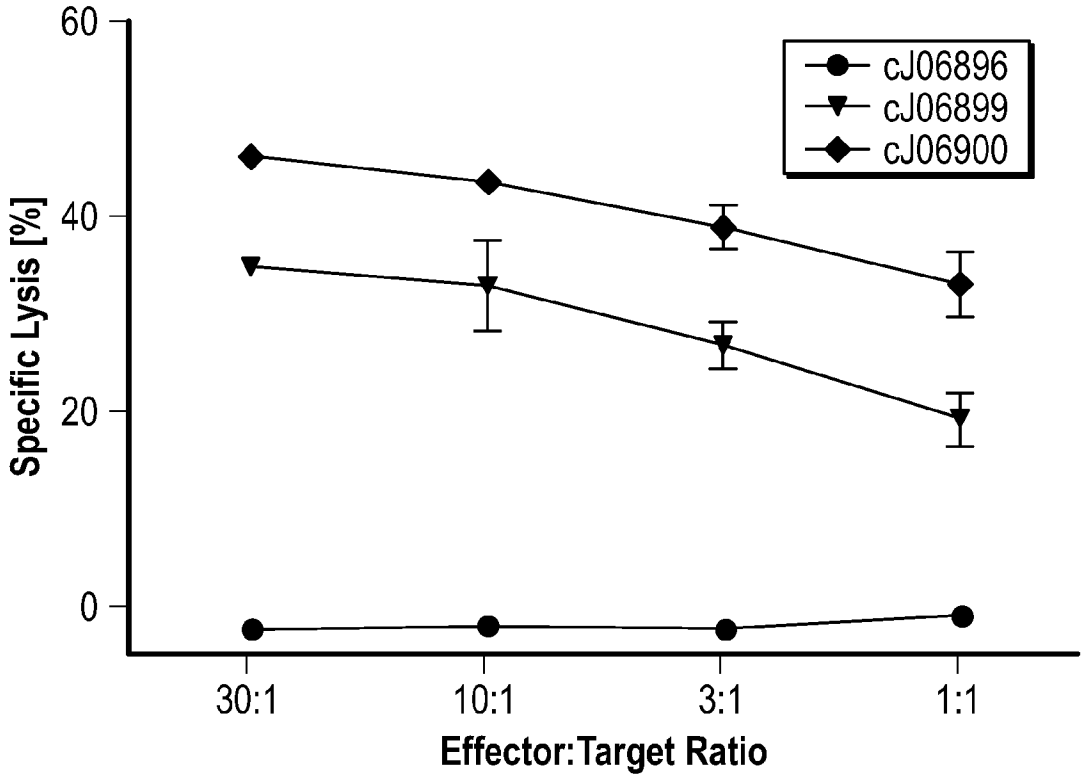


FIG. 4B

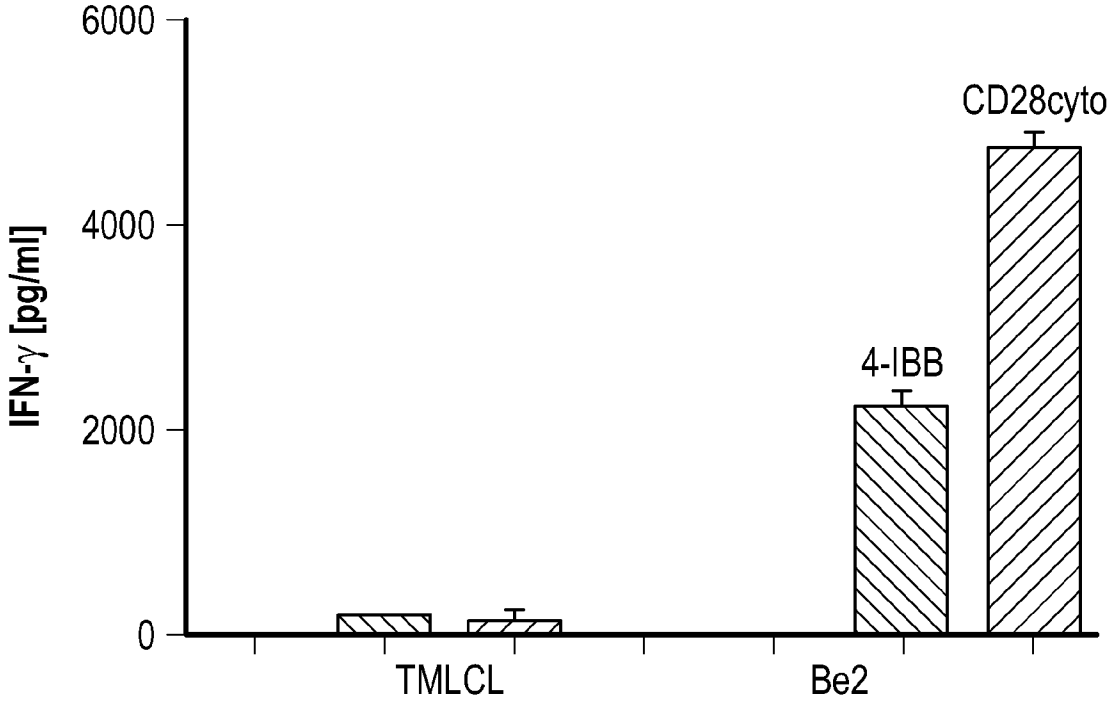

6000
4000
2000
0
IFN-γ [pg/ml]
CD28cyto
4-IBB
TMLCL
Be2

FIG. 4C

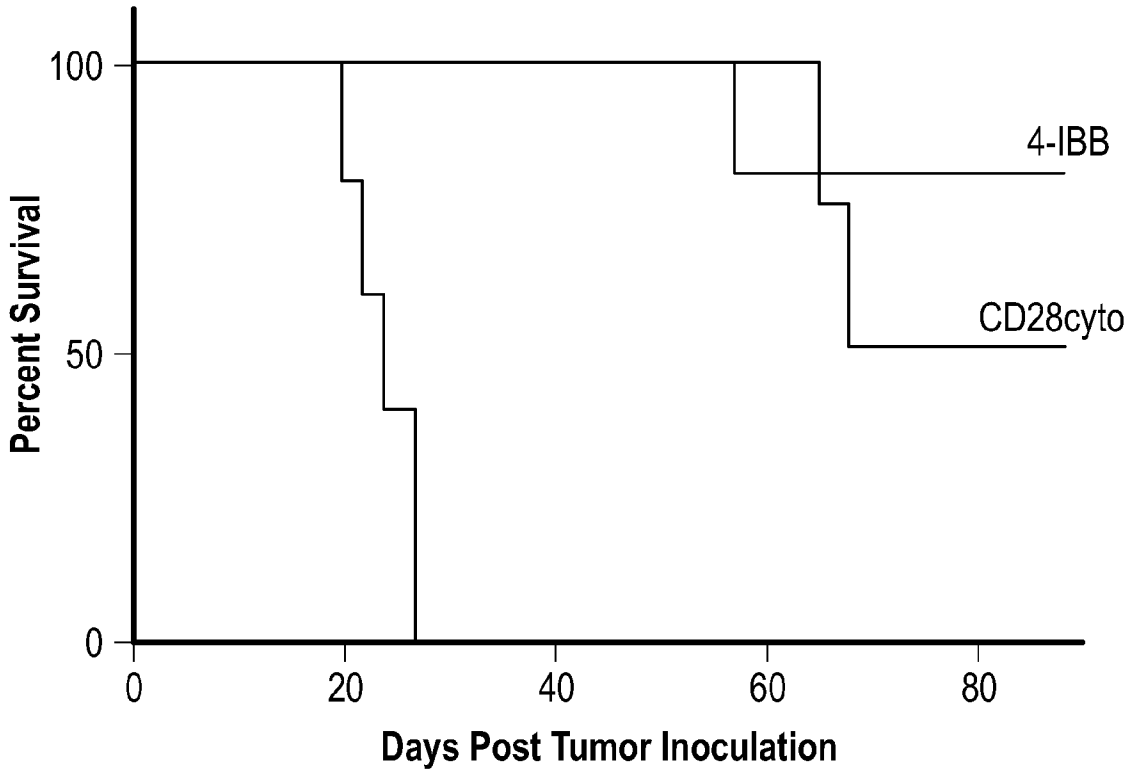

100
50
0
Percent Survival
4-IBB
CD28cyto
0
20
40
60
80
Days Post Tumor Inoculation

FIG. 4D

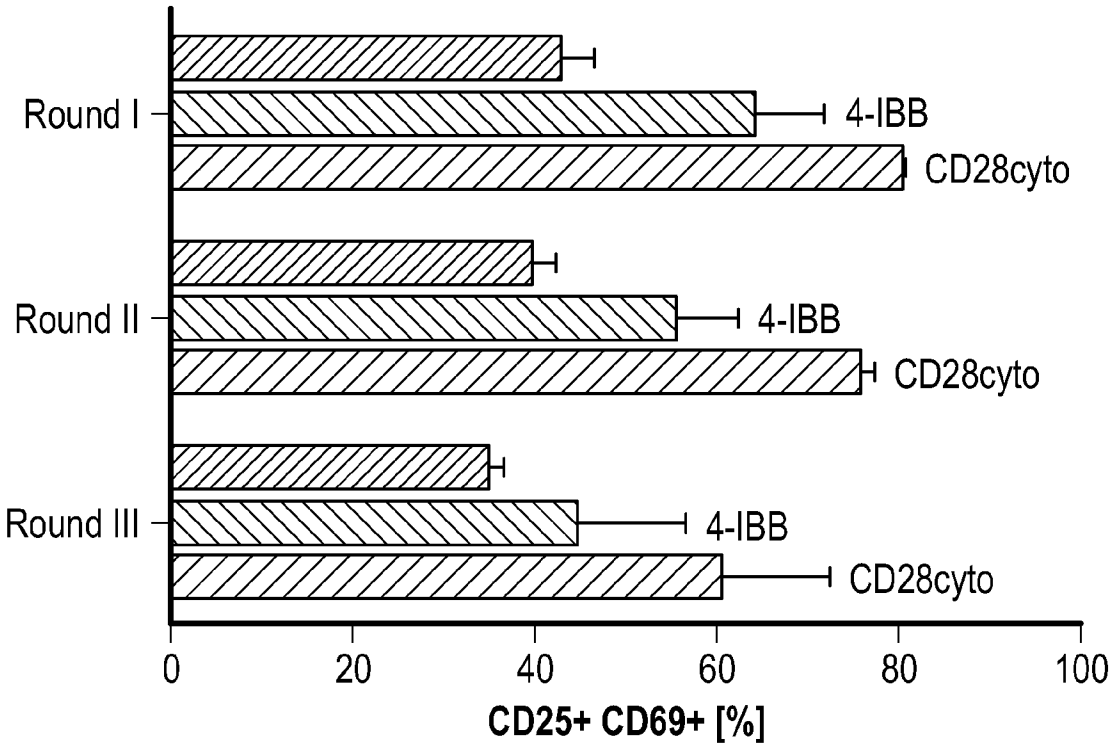

Round I
4-IBB
CD28cyto
Round II
4-IBB
CD28cyto
Round III
4-IBB
CD28cyto
0
20
40
60
80
100
CD25+ CD69+ [%]

FIG. 4E

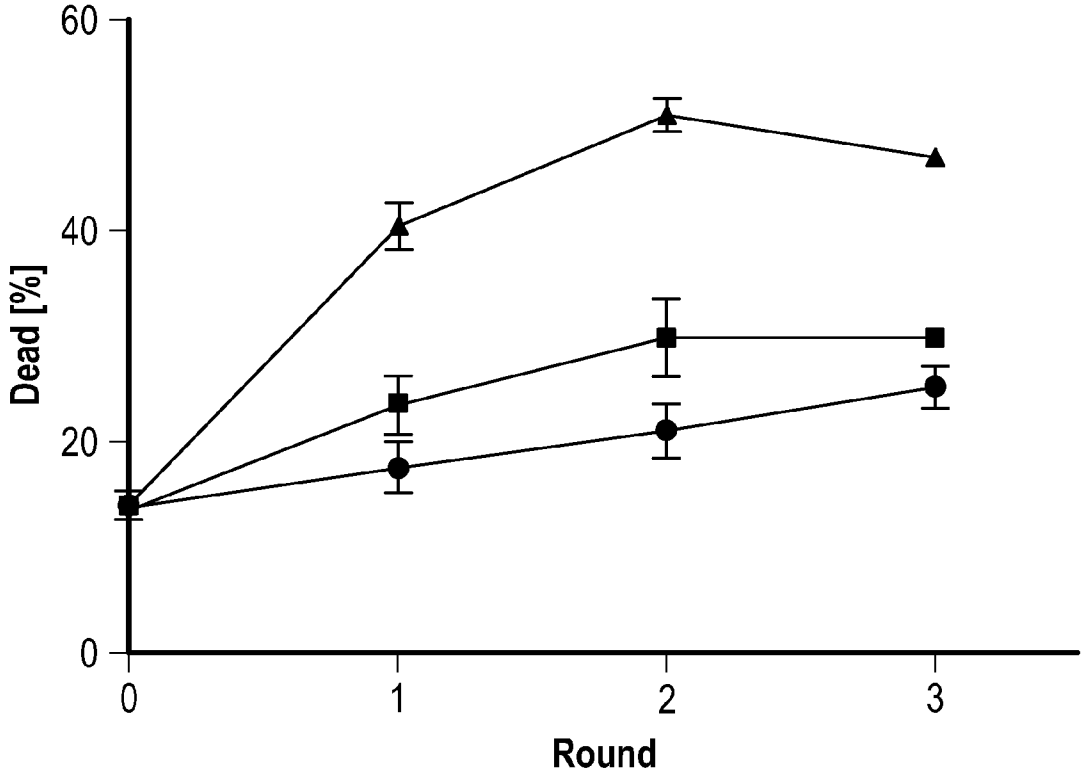

60
40
20
0
Dead [%]
0
1
2
3
Round

FIG. 4F

```
   1 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC
     CAATCTGGTC TAGACTCGGA CCCTCGAGAG ACCGATTGAT CCCTTGGGTG ACGAATTCGG AGTTATTTCG AACGGAACTC ACGAAGTTCA TCACACACGG
 101 CGTCTGTTGT GTGACTCTGG TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG AACAGGGACT TGAAAGCGAA
     GCAGACAACA CACTGAGACC ATTGATCTCT AGGGAGTCTG GGAAAATCAG TCACACCTTT TAGAGATCGT CACCGCGGGC TTGTCCCTGA ACTTTCGCTT
                                                                        psi
 201 AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
     TCCCTTTGGT CTCCTCGAGA GAGCTGCGTC CTGAGCCGAA CGACTTCGCG CGTGCCGTTC TCCGCTCCCC GCCGCTGACC ACTCATGCGG TTTTTAAAAC
                          psi
 301 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA ATTAGATCGA TGGGAAAAAA TTCGGTTAAG GCCAGGGGGA
     TGATCGCCTC CGATCTTCCT CTCTCTACCC ACGCTCTCGC AGTCATAATT CGCCCCCTCT TAATCTAGCT ACCCTTTTTT AAGCCAATTC CGGTCCCCCT
 401 AAGAAAAAAT ATAAATTAAA ACATATAGTA TGGGCAAGCA GGGAGCTAGA ACGATTCGCA GTTAATCCTG GCCTGTTAGA AACATCAGAA GGCTGTAGAC
     TTCTTTTTTA TATTTAATTT TGTATATCAT ACCCGTTCGT CCCTCGATCT TGCTAAGCGT CAATTAGGAC CGGACAATCT TTGTAGTCTT CCGACATCTG
 501 AAATACTGGG ACAGCTACAA CCATCCCTTC AGACAGGATC AGAAGAACTT AGATCATTAT ATAATACAGT AGCAACCCTC TATTGTGTGC ATCAAAGGAT
     TTTATGACCC TGTCGATGTT GGTAGGGAAG TCTGTCCTAG TCTTCTTGAA TCTAGTAATA TATTATGTCA TCGTTGGGAG ATAACACACG TAGTTTCCTA
 601 AGAGATAAAA GACACCAAGG AAGCTTTAGA CAAGATAGAG GAAGAGCAAA ACAAAAGTAA GAAAAAAGCA CAGCAAGCAG CAGCTGACAC AGGACACAGC
     TCTCTATTTT CTGTGGTTCC TTCGAAATCT GTTCTATCTC CTTCTCGTTT TGTTTTCATT CTTTTTTCGT GTCGTTCGTC GTCGACTGTG TCCTGTGTCG
 701 AATCAGGTCA GCCAAAATTA CCCTATAGTG CAGAACATCC AGGGGCAAAT GGTACATCAG GCCATATCAC CTAGAACTTT AAATGCATGG GTAAAAGTAG
     TTAGTCCAGT CGGTTTTAAT GGGATATCAC GTCTTGTAGG TCCCCGTTTA CCATGTAGTC CGGTATAGTG GATCTTGAAA TTTACGTACC CATTTTCATC
 801 TAGAAGAGAA GGCTTTCAGC CCAGAAGTGA TACCCATGTT TTCAGCATTA TCAGAAGGAG CCACCCCACA AGATTTAAAC ACCATGCTAA ACACAGTGGG
     ATCTTCTCTT CCGAAAGTCG GGTCTTCACT ATGGGTACAA AAGTCGTAAT AGTCTTCCTC GGTGGGGTGT TCTAAATTTG TGGTACGATT TGTGTCACCC
                                                                                          RRE
 901 GGGACATCAA GCAGCCATGC AAATGTTAAA AGAGACCATC AATGAGGAAG CTGCAGGCAA AGAGAAGAGT GGTGCAGAGA GAAAAAAGAG CAGTGGGAAT
     CCCTGTAGTT CGTCGGTACG TTTACAATTT TCTCTGGTAG TTACTCCTTC GACGTCCGTT TCTCTTCTCA CCACGTCTCT CTTTTTTCTC GTCACCCTTA
                                                     RRE
1001 AGGAGCTTTG TTCCTTGGGT TCTTGGGAGC AGCAGGAAGC ACTATGGGCG CAGCGTCAAT GACGCTGACG GTACAGGCCA GACAATTATT GTCTGGTATA
     TCCTCGAAAC AAGGAACCCA AGAACCCTCG TCGTCCTTCG TGATACCCGC GTCGCAGTTA CTGCGACTGC CATGTCCGGT CTGTTAATAA CAGACCATAT
                                                     RRE
1101 GTGCAGCAGC AGAACAATTT GCTGAGGGCT ATTGAGGCGC AACAGCATCT GTTGCAACTC ACAGTCTGGG GCATCAAGCA GCTCCAGGCA AGAATCCTGG
     CACGTCGTCG TCTTGTTAAA CGACTCCCGA TAACTCCGCG TTGTCGTAGA CAACGTTGAG TGTCAGACCC CGTAGTTCGT CGAGGTCCGT TCTTAGGACC
                                                                                                      flap
                                             RRE
1201 CTGTGGAAAG ATACCTAAAG GATCAACAGC TCCTGGGGAT TTGGGGTTGC TCTGGAAAAC TCATTTGCAC CACTGCTGTG CCTTGGATCT ACAAATGGCA
     GACACCTTTC TATGGATTTC CTAGTTGTCG AGGACCCCTA AACCCCAACG AGACCTTTTG AGTAAACGTG GTGACGACAC GGAACCTAGA TGTTTACCGT
```

FIG. 5

FIG. 5 (Continued)

```
     GCCGGACTGG TCGCTCCTGT CGCGGCACAT GAAGACGCGG TCCCTGATGA TGCCGTGGTC GATGTTGAAG CTGATGACCC CGGTCCCGTG GTGTGACTGG
                                                  Ce7scFv
2501 GTGAGCAGCG GCGGAGGGGG CTCTGGCGGC GGAGGATCTG GGGGAGGGGG CAGCGACATC CAGATGACCC AGAGCAGCAG CAGCTTCAGC GTGAGCCTGG
     CACTCGTCGC CGCCTCCCCC GAGACCGCCG CCTCCTAGAC CCCCTCCCCC GTCGCTGTAG GTCTACTGGG TCTCGTCGTC GTCGAAGTCG CACTCGGACC
                                                  Ce7scFv
2601 GCGACCGGGT GACCATCACC TGTAAGGCCA ACGAGGACAT CAACAACCGG CTGGCCTGGT ATCAGCAGAC CCCCGGCAAC AGCCCCAGGC TGCTGATCAG
     CGCTGGCCCA CTGGTAGTGG ACATTCCGGT TGCTCCTGTA GTTGTTGGCC GACCGGACCA TAGTCGTCTG GGGGCCGTTG TCGGGGTCCG ACGACTAGTC
                                                  Ce7scFv
2701 CGGCGCCACC AACCTGGTGA CCGGCGTGCC CAGCCGGTTT AGCGGCAGCG GCTCCGGCAA GGACTACACC CTGACCATCA CAAGCCTGCA GGCCGAGGAC
     GCCGCGGTGG TTGGACCACT GGCCGCACGG GTCGGCCAAA TCGCCGTCGC CGAGGCCGTT CCTGATGTGG GACTGGTAGT GTTCGGACGT CCGGCTCCTG
                                        Ce7scFv                                         IgG4 Hinge
2801 TTCGCCACCT ACTACTGCCA GCAGTACTGG TCCACCCCCT TCACCTTCGG CAGCGGCACC GAGCTGGAAA TCAAAGAGAG CAAGTACGGA CCGCCCTGCC
     AAGCGGTGGA TGATGACGGT CGTCATGACC AGGTGGGGGA AGTGGAAGCC GTCGCCGTGG CTCGACCTTT AGTTTCTCTC GTTCATGCCT GGCGGGACGG
     IgG4 Hinge                                              IgG4 CH2
2901 CCCCTTGCCC TGCCCCCGAG TTCCTGGGCG GACCCAGCGT GTTCCTGTTC CCCCCCAAGC CCAAGGACAC CCTGATGATC AGCCGGACCC CCGAGGTGAC
     GGGGAACGGG ACGGGGGCTC AAGGACCCGC CTGGGTCGCA CAAGGACAAG GGGGGGTTCG GGTTCCTGTG GGACTACTAG TCGGCCTGGG GGCTCCACTG
                                                IgG4 CH2
3001 CTGCGTGGTG GTGGACGTGA GCCAGGAAGA TCCCGAGGTC CAGTTCAATT GGTACGTGGA CGGCGTGGAA GTGCACAACG CCAAGACCAA GCCCAGAGAG
     GACGCACCAC CACCTGCACT CGGTCCTTCT AGGGCTCCAG GTCAAGTTAA CCATGCACCT GCCGCACCTT CACGTGTTGC GGTTCTGGTT CGGGTCTCTC
                                                IgG4 CH2
3101 GAACAGTTCA ACAGCACCTA CCGGGTGGTG TCTGTGCTGA CCGTGCTGCA CCAGGACTGG CTGAACGGCA AAGAATACAA GTGCAAGGTG TCCAACAAGG
     CTTGTCAAGT TGTCGTGGAT GGCCCACCAC AGACACGACT GGCACGACGT GGTCCTGACC GACTTGCCGT TTCTTATGTT CACGTTCCAC AGGTTGTTCC
                   IgG4 CH2                                                     IgG4 CH3
3201 GCCTGCCCAG CAGCATCGAA AAGACCATCA GCAAGGCCAA GGGCCAGCCT CGCGAGCCCC AGGTGTACAC CCTGCCTCCC TCCCAGGAAG AGATGACCAA
     CGGACGGGTC GTCGTAGCTT TTCTGGTAGT CGTTCCGGTT CCCGGTCGGA GCGCTCGGGG TCCACATGTG GGACGGAGGG AGGGTCCTTC TCTACTGGTT
                                                IgG4 CH3
3301 GAACCAGGTG TCCCTGACCT GCCTGGTGAA GGGCTTCTAC CCCAGCGACA TCGCCGTGGA GTGGGAGAGC AACGGCCAGC CTGAGAACAA CTACAAGACC
     CTTGGTCCAC AGGGACTGGA CGGACCACTT CCCGAAGATG GGGTCGCTGT AGCGGCACCT CACCCTCTCG TTGCCGGTCG GACTCTTGTT GATGTTCTGG
                                                IgG4 CH3
3401 ACCCCTCCCG TGCTGGACAG CGACGGCAGC TTCTTCCTGT ACAGCCGGCT GACCGTGGAC AAGAGCCGGT GGCAGGAAGG CAACGTCTTT AGCTGCAGCG
     TGGGGAGGGC ACGACCTGTC GCTGCCGTCG AAGAAGGACA TGTCGGCCGA CTGGCACCTG TTCTCGGCCA CCGTCCTTCC GTTGCAGAAA TCGACGTCGC
```

FIG. 5 (Continued)

FIG. 5 (Continued)

```
     GAGGAGACCT AGGTGTCCTT GACCTATAAG ACTTTTGGCA TTTCCTTTAG TGTCCCAAAA ACGACTAAGT CCGAACCGGA CTTTTGTCCT GCCTGGAGGT
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4501 TGCCTTTGAG AACCTAGAAA TCATACGCGG CAGGACCAAG CAACATGGTC AGTTTTCTCT TGCAGTCGTC AGCCTGAACA TAACATCCTT GGGATTACGC
     ACGGAAACTC TTGGATCTTT AGTATGCGCC GTCCTGGTTC GTTGTACCAG TCAAAAGAGA ACGTCAGCAG TCGGACTTGT ATTGTAGGAA CCCTAATGCG
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4601 TCCCTCAAGG AGATAAGTGA TGGAGATGTG ATAATTTCAG GAAACAAAAA TTTGTGCTAT GCAAATACAA TAAACTGGAA AAAACTGTTT GGGACCTCCG
     AGGGAGTTCC TCTATTCACT ACCTCTACAC TATTAAAGTC CTTTGTTTTT AAACACGATA CGTTTATGTT ATTTGACCTT TTTTGACAAA CCCTGGAGGC
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4701 GTCAGAAAAC CAAAATTATA AGCAACAGAG GTGAAAACAG CTGCAAGGCC ACAGGCCAGG TCTGCCATGC CTTGTGCTCC CCCGAGGGCT GCTGGGGCCC
     CAGTCTTTTG GTTTTAATAT TCGTTGTCTC CACTTTTGTC GACGTTCCGG TGTCCGGTCC AGACGGTACG GAACACGAGG GGGCTCCCGA CGACCCCGGG
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4801 GGAGCCCAGG GACTGCGTCT CTTGCCGGAA TGTCAGCCGA GGCAGGGAAT GCGTGGACAA GTGCAACCTT CTGGAGGGTG AGCCAAGGGA GTTTGTGGAG
     CCTCGGGTCC CTGACGCAGA GAACGGCCTT ACAGTCGGCT CCGTCCCTTA CGCACCTGTT CACGTTGGAA GACCTCCCAC TCGGTTCCCT CAAACACCTC
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4901 AACTCTGAGT GCATACAGTG CCACCCAGAG TGCCTGCCTC AGGCCATGAA CATCACCTGC ACAGGACGGG GACCAGACAA CTGTATCCAG TGTGCCCACT
     TTGAGACTCA CGTATGTCAC GGTGGGTCTC ACGGACGGAG TCCGGTACTT GTAGTGGACG TGTCCTGCCC CTGGTCTGTT GACATAGGTC ACACGGGTGA
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5001 ACATTGACGG CCCCCACTGC GTCAAGACCT GCCCGGCAGG AGTCATGGGA GAAAACAACA CCCTGGTCTG GAAGTACGCA GACGCCGGCC ATGTGTGCCA
     TGTAACTGCC GGGGGTGACG CAGTTCTGGA CGGGCCGTCC TCAGTACCCT CTTTTGTTGT GGGACCAGAC CTTCATGCGT CTGCGGCCGG TACACACGGT
                                                     EGFRt
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5101 CCTGTGCCAT CCAAACTGCA CCTACGGATG CACTGGGCCA GGTCTTGAAG GCTGTCCAAC GAATGGGCCT AAGATCCCGT CCATCGCCAC TGGGATGGTG
     GGACACGGTA GGTTTGACGT GGATGCCTAC GTGACCCGGT CCAGAACTTC CGACAGGTTG CTTACCCGGA TTCTAGGGCA GGTAGCGGTG ACCCTACCAC
                                  EGFRt                                                                             WPRE
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~                                                      ~~
5201 GGGCCCCTCC TCTTGCTGCT GGTGGTGGCC CTGGGGATCG GCCTCTTCAT GTGAGCGGCC GCTCTAGACC CGGGCTGCAG GAATTCGATA TCAAGCTTAT
     CCCCGGGAGG AGAACGACGA CCACCACCGG GACCCCTAGC CGGAGAAGTA CACTCGCCGG CGAGATCTGG GCCCGACGTC CTTAAGCTAT AGTTCGAATA
                                                     WPRE
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5301 CGATAATCAA CCTCTGGATT ACAAAATTTG TGAAAGATTG ACTGGTATTC TTAACTATGT TGCTCCTTTT ACGCTATGTG GATACGCTGC TTTAATGCCT
     GCTATTAGTT GGAGACCTAA TGTTTTAAAC ACTTTCTAAC TGACCATAAG AATTGATACA ACGAGGAAAA TGCGATACAC CTATGCGACG AAATTACGGA
                                                     WPRE
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5401 TTGTATCATG CTATTGCTTC CCGTATGGCT TTCATTTTCT CCTCCTTGTA TAAATCCTGG TTGCTGTCTC TTTATGAGGA GTTGTGGCCC GTTGTCAGGC
     AACATAGTAC GATAACGAAG GGCATACCGA AAGTAAAAGA GGAGGAACAT ATTTAGGACC AACGACAGAG AAATACTCCT CAACACCGGG CAACAGTCCG
                                                     WPRE
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5501 AACGTGGCGT GGTGTGCACT GTGTTTGCTG ACGCAACCCC CACTGGTTGG GGCATTGCCA CCACCTGTCA GCTCCTTTCC GGGACTTTCG CTTTCCCCCT
     TTGCACCGCA CCACACGTGA CACAAACGAC TGCGTTGGGG GTGACCAACC CCGTAACGGT GGTGGACAGT CGAGGAAAGG CCCTGAAAGC GAAAGGGGGA
                                                     WPRE
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

FIG. 5 (Continued)

FIG. 5 (Continued)

FIG. 5 (Continued)

```
                                                    CoE1 ori
8301 TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC
     AGGACAATGG TCACCGACGA CGGTCACCGC TATTCAGCAC AGAATGGCCC AACCTGAGTT CTGCTATCAA TGGCCTATTC CGCGTCGCCA GCCCGACTTG
                                                    CoE1 ori
8401 GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG
     CCCCCCAAGC ACGTGTGTCG GGTCGAACCT CGCTTGCTGG ATGTGGCTTG ACTCTATGGA TGTCGCACTC GATACTCTTT CGCGGTGCGA AGGGCTTCCC
                                                    CoE1 ori
8501 AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG
     TCTTTCCGCC TGTCCATAGG CCATTCGCCG TCCCAGCCTT GTCCTCTCGC GTGCTCCCTC GAAGGTCCCC CTTTGCGGAC CATAGAAATA TCAGGACAGC
                                                    CoE1 ori
8601 GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT
     CCAAAGCGGT GGAGACTGAA CTCGCAGCTA AAAACACTAC GAGCAGTCCC CCCGCCTCGG ATACCTTTTT GCGGTCGTTG CGCCGGAAAA ATGCCAAGGA
                                                    CoE1 ori
8701 GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC
     CCGGAAAACG ACCGGAAAAC GAGTGTACAA GAAAGGACGC AATAGGGGAC TAAGACACCT ATTGGCATAA TGGCGGAAAC TCACTCGACT ATGGCGAGCG
                            CoE1 ori
8801 CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCATTAAT
     GCGTCGGCTT GCTGGCTCGC GTCGCTCAGT CACTCGCTCC TTCGCCTTCT CGCGGGTTAT GCGTTTGGCG GAGAGGGGCG CGCAACCGGC TAAGTAATTA
8901 GCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC GCAATTAATG TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT
     CGTCGACCGT GCTGTCCAAA GGGCTGACCT TTCGCCCGTC ACTCGCGTTG CGTTAATTAC ACTCAATCGA GTGAGTAATC CGTGGGGTCC GAAATGTGAA
9001 TATGCTTCCG GCTCGTATGT TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG CCAAGCTCGA AATTAACCCT
     ATACGAAGGC CGAGCATACA ACACACCTTA ACACTCGCCT ATTGTTAAAG TGTGTCCTTT GTCGATACTG GTACTAATGC GGTTCGAGCT TTAATTGGGA
                                                                            SV40
9101 CACTAAAGGG AACAAAAGCT GGAGCTCCAC CGCGGTGGCG GCCTCGAGGT CGAGATCCGG TCGACCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC
     GTGATTTCCC TTGTTTTCGA CCTCGAGGTG GCGCCACCGC CGGAGCTCCA GCTCTAGGCC AGCTGGTCGT TGGTATCAGG GCGGGGATTG AGGCGGGTAG
                                                      SV40
9201 CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG AGGCCGAGGC CGCCTCGGCC TCTGAGCTAT
     GGCGGGGATT GAGGCGGGTC AAGGCGGGTA AGAGGCGGGG TACCGACTGA TTAAAAAAAA TAAATACGTC TCCGGCTCCG GCGGAGCCGG AGACTCGATA
                          SV40                                                              CMV
9301 TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG GCCTAGGCTT TTGCAAAAAG CTTCGACGGT ATCGATTGGC TCATGTCCAA CATTACCGCC ATGTTGACAT
     AGGTCTTCAT CACTCCTCCG AAAAAACCTC CGGATCCGAA AACGTTTTTC GAAGCTGCCA TAGCTAACCG AGTACAGGTT GTAATGGCGG TACAACTGTA
                                                      CMV
9401 TGATTATTGA CTAGTTATTA ATAGTAATCA ATTACGGGGT CATTAGTTCA TAGCCCATAT ATGGAGTTCC GCGTTACATA ACTTACGGTA AATGGCCCGC
     ACTAATAACT GATCAATAAT TATCATTAGT TAATGCCCCA GTAATCAAGT ATCGGGTATA TACCTCAAGG CGCAATGTAT TGAATGCCAT TTACCGGGCG
                                                      CMV
9501 CTGGCTGACC GCCCAACGAC CCCCGCCCAT TGACGTCAAT AATGACGTAT GTTCCCATAG TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA
```

FIG. 5 (Continued)

```
    GACCGACTGG CGGGTTGCTG GGGGCGGGTA ACTGCAGTTA TTACTGCATA CAAGGGTATC ATTGCGGTTA TCCCTGAAAG GTAACTGCAG TTACCCACCT
                                                CMV
    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 9601 GTATTTACGG TAAACTGCCC ACTTGGCAGT ACATCAAGTG TATCATATGC CAAGTACGCC CCCTATTGAC GTCAATGACG GTAAATGGCC CGCCTGGCAT
      CATAAATGCC ATTTGACGGG TGAACCGTCA TGTAGTTCAC ATAGTATACG GTTCATGCGG GGGATAACTG CAGTTACTGC CATTTACCGG GCGGACCGTA
                                                CMV
    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 9701 TATGCCCAGT ACATGACCTT ATGGGACTTT CCTACTTGGC AGTACATCTA CGTATTAGTC ATCGCTATTA CCATGGTGAT GCGGTTTTGG CAGTACATCA
      ATACGGGTCA TGTACTGGAA TACCCTGAAA GGATGAACCG TCATGTAGAT GCATAATCAG TAGCGATAAT GGTACCACTA CGCCAAAACC GTCATGTAGT
                                                CMV
    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 9801 ATGGGCGTGG ATAGCGGTTT GACTCACGGG GATTTCCAAG TCTCCACCCC ATTGACGTCA ATGGGAGTTT GTTTTGGCAC CAAAATCAAC GGGACTTTCC
      TACCCGCACC TATCGCCAAA CTGAGTGCCC CTAAAGGTTC AGAGGTGGGG TAACTGCAGT TACCCTCAAA CAAAACCGTG GTTTTAGTTG CCCTGAAAGG
                                                CMV
    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
 9901 AAAATGTCGT AACAACTCCG CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGAATTC GGAGTGGCGA GCCCTCAGAT CCTGCATATA AGCAGCTGCT
      TTTTACAGCA TTGTTGAGGC GGGGTAACTG CGTTTACCCG CCATCCGCAC ATGCCTTAAG CCTCACCGCT CGGGAGTCTA GGACGTATAT TCGTCGACGA
10001 TTTTGCCTGT ACTGGGTCTC TCTG
      AAAACGGACA TGACCCAGAG AGAC
```

FIG. 5 (Continued)

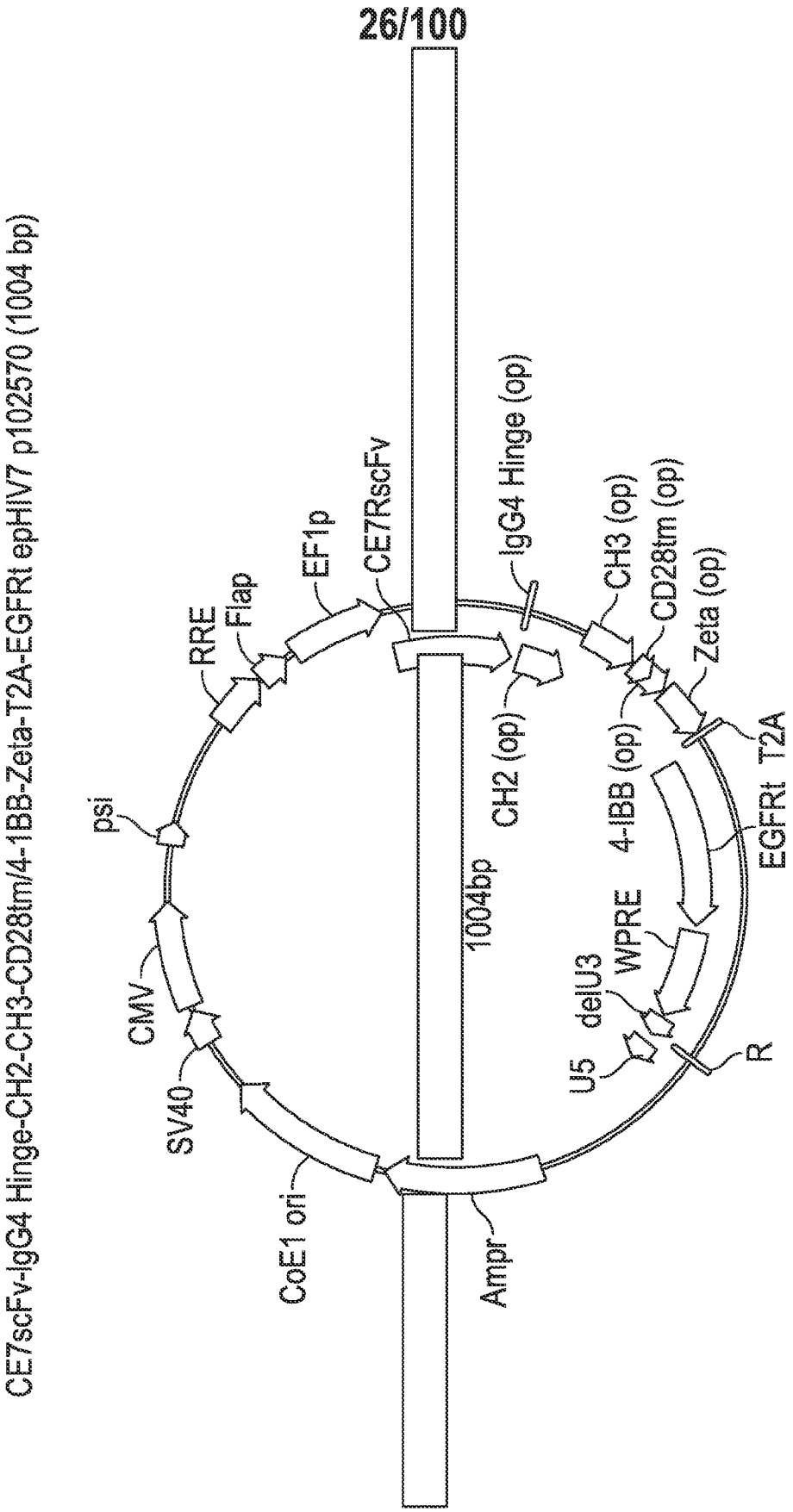

CE7scFv-IgG4 Hinge-CH2-CH3-CD28tm/4-1BB-Zeta-T2A-EGFRt epHIV7 p102570 (1004 bp)
1004bp
psi
CMV
SV40
CoE1 ori
Ampr
RRE
Flap
EF1p
CE7RscFv
IgG4 Hinge (op)
CH2 (op)
CH3 (op)
CD28tm (op)
4-1BB (op)
Zeta (op)
T2A
EGFRt
WPRE
delU3
U5
R

FIG. 6

FIG. 7

```
1301 GTATTCATCC ACAATTTTAA AAGAAAAGGG GGATTGGGG GGTACAGTGC AGGGGAAAGA ATAGTAGACA TAATAGCAAC AGACATACAA ACTAAAGAAT
     CATAAGTAGG TGTTAAAATT TTCTTTTCCC CCCTAACCCC CCATGTCACG TCCCCTTTCT TATCATCTGT ATTATCGTTG TCTGTATGTT TGATTTCTTA
                                  flap

1401 TACAAAAACA AATTACAAAA ATTCAAAATT TTCGGGTTTA TTACAGGGAC AGCAGAGATC CAGTTTGGGG ATCAATTGCA TGAAGAATCT GCTTAGGGTT
     ATGTTTTTGT TTAATGTTTT TAAGTTTTTAA AAGCCCAAAT AATGTCCCTG TCGTCTCTAG GTCAAACCCC TAGTTAACGT ACTTCTTAGA CGAATCCCAA
                                                  EF1p

1501 AGGCGTTTTG CGCTGCTTCG CGATCGCTCC GGTGCCCGTC AGTGGGCAGA GCGCACATCG CCCACAGTCC CCGAGAAGTT GGGGGAGGG
     TCCGCAAAAC GCGACGAAGC GCTAGCGAGG CCACGGGCAG TCACCCGTCT CGCGTGTAGC GGGTGTCAGG GGCTCTTCAA CCCCCCTCCC
                                  EF1p

1601 GTCGGCAATT GAACCGGTGC CTAGAGAAGG TGGCGCGGGG TAAACTGGGA AAGTGATGTC GTGTACTGGC TCCGCCTTTT TCCCGAGGGT GGGGGAGAAC
     CAGCCGTTAA CTTGGCCACG GATCTCTTCC ACCGCGCCCC ATTTGACCCT TTCACTACAG CACATGACCG AGGCGGAAAA AGGGCTCCCA CCCCCTCTTG
                                  EF1p

1701 CGTATATAAG TGCAGTAGTC GCCGTGAACG TTCTTTTTCG CAACGGGTTT GCCGCCAGAA CACAGCTGAA GCTTCGAGGG GCTCGCATCT CTCCTTCACG
     GCATATATTC ACGTCATCAG CGGCACTTGC AAGAAAAAGC GTTGCCCAAA CGGCGGTCTT GTGTCGACTT CGAAGCTCCC CGAGCGTAGA GAGGAAGTGC
                                  EF1p

1801 CGCCCGCCGC CCTACCTGAG GCCGCCATCC ACGCCGGTTG AGTCGCGTTC TGCCGCCTCC CGCCTGTGGT GCCTCCTGAA CTGCGGTCCGC CGTCTAGGTA
     GCGGGCGGCG GGATGGACTC CGGCGGTAGG TGCGGCCAAC TCAGCGCAAG ACGGCGGAGG GCGGACACCA CGGAGGACTT GACGCAGGCG GCAGATCCAT
                                  EF1p

1901 AGTTTAAAGC TCAGGTCGAG ACCGGGCCTT TGTCCGGCGC TCCCTTGGAG CCTACCTAGA CTCAGCCGGC TCTCCACGCT TTGCCCTGACC CTGCTTGCTC
     TCAAATTTCG AGTCCAGCTC TGGCCCGGAA ACAGGCCGCG AGGGAACCTC GGATGGATCT GAGTCGGCCG AGAGGTGCGA AACGGACTGG GACGAACGAG
                                  EF1p                                                  CE7R scFv

2001 AACTCTACGT CTTTGTTTCG TTTTCTGTTC TGCGCCGTTA CAGATCCAAG CTGTGACCGG CGCCTACGGC TAGCGCCGCC ACCATGCTGC TGCTGGTGAC
     TTGAGATGCA GAAACAAAGC AAAAGACAAG ACGCGGCAAT GTCTAGGTTC GACACTGGCC GCGGATGCCG ATCGCGGCGG TGGTACGACG ACGACCACTG
                                                  CE7R scFv

2101 CAGCCTGCTG CTGTGCGAGC TGCCCCACCC CGCCTTTCTG CTGATCCCCC AGGTGCAGCT GCAGCAGCCT GGCGCCGAGC TGGTGAAGCC AGGCGCCAGC
     GTCGGACGAC GACACGCTCG ACGGGGTGGG GCGGAAAGAC GACTAGGGGG TCCACGTCGA CGTCGTCGGA CCGCGGCTCG ACCACTTCGG TCCGCGGTCG
                                                  CE7R scFv

2201 GTGAAGCTGT CCTGCAAGGC CAGCGGCTAC ACCTTCACCG GCTACTGGGT GAACTGGGTG AAGCAGAGAC CCGGCCACGG CCTGGAATGG ATCGGCGAGA
     CACTTCGACA GGACGTTCCG GTCGCCGATG TGGAAGTGGC CGATGACCTA CGTGACCCAC TTCGTCTCTG GGCCGGTGCC GGACCTTACC TAGCCGCTCT
                                                  CE7R scFv

2301 TCAACCCCAG CAACGGCCGG ACCAACTACA ACGAGCGGTT CAAGAGCAAG GCCACCCTGA CCGTGGACAA GAGCAGCACC ACCGCCTTCA TGCAGCTGTC
     AGTTGGGGTC GTTGCCGGCC TGGTTGATGT TGCTCGCCAA GTTCTCGTTC CGGTGGGACT GGCACCTGTT CTCGTCGTGG TGGCGGAAGT ACGTCGACAG
                                                  CE7R scFv

2401 CGGCCTGACC AGCGAGGACA GCGCCGTGTA CTTCTGCGCC AGGGCACTACT ACGGCACCAG CTACAACTTC GACTACTGGG GCCAGGGCAC CACACTGACC
     GCCGGACTGG TCGCTCCTGT CGCGGCACAT GAAGACGCGG TCCCGTGGTC GATGTTGAAG CTGATGACCC CGGTCCCGTG GTGTGACTGG
                                                  CE7R scFv
```

FIG. 7 (Continued)

```
2501 GTGAGCAGCG GCGGAGGGGG CTCTGGCGGC GGAGGATCTG GGGGAGGGGG CAGCGACATC CAGATGACCC AGAGCAGCAG CAGCTTCAGC GTGAGCCTGG
     CACTCGTCGC CGCCTCCCCC GAGACCGCCG CCTCCTAGAC CCCCTCCCCC GTCGCTGTAG GTCTACTGGG TCTCGTCGTC GTCGAAGTCG CACTCGGACC
                                        CE7R scFv

2601 GCGACCGGGT GACCATCACC TGTAAGGCCA ACGAGGACAT CAACAACCGG CTGGCCTGGT ATCAGCAGAC CCCCGGCAAC AGCCCCAGGC TGCTGATCAG
     CGCTGGCCCA CTGGTAGTGG ACATTCCGGT TGCTCCTGTA GTTGTTGGCC GACCGGACCA TAGTCGTCTG GGGGCCGTTG TCGGGGTCCG ACGACTAGTC
                                        CE7R scFv

2701 CGGCGCCACC AACCTGGTGA CCGGCGTGCC CAGCCGGTTT AGCGGCAGCG GCTCCGGCAA GGACTACACC CTGACCATCA CAAGCCTGCA GGCCGAGGAC
     GCCGCGGTGG TTGGACCACT GGCCGCACGG GTCGGCCAAA TCGCCGTCGC CGAGGCCGTT CCTGATGTGG GACTGGTAGT GTTCGGACGT CCGGCTCCTG
                                        CE7R scFv
                                                                                                  IgG4 Hinge

2801 TTCGCCACCT ACTACTGCCA GCAGTACTGG TCCACCCCCT TCACCTTCGG CAGCGGCACC GAGCTGGAAA TCAAAGAATC TAAGTACGGA CCGCCCTGCC
     AAGCGGTGGA TGATGACGGT CGTCATGACC AGGTGGGGGA AGTGGAAGCC GTCGCCGTGG CTCGACCTTT AGTTCTTAG ATTCATGCCT GGCGGGACGG
                                                  IgG4 CH3
     IgG4 Hinge

2901 CCCCTTGCCC TGGCCAGCCT AGAGAACCCC AGGTGTACAC CCTGCCTCCC AGCCAGGAAG AGATGACCAA GAACCAGGTG TCCCTGACCT GCCTGGTCAA
     GGGGAACGGG ACCGGTCGGA TCTCTTGGGG TCCACATGTG GGACGGAGGG TCGGTCCTTC TCTACTGGTT CTTGGTCCAC AGGGACTGGA CGGACCAGTT
                                                  IgG4 CH3

3001 AGGCTTCTAC CCCAGCGATA TCGCCGTGGA ATGGGAGAGC AACGGCCAGC CCGAGAACAA CTACAAGACC ACCCCCCCTG TGCTGGACAG CGACGGCAGC
     TCCGAAGATG GGGTCGCTAT AGCGGCACCT TACCCTCTCG TTGCCGGTCG GGCTCTTGTT GATGTTCTGG TGGGGGGGAC ACGACCTGTC GCTGCCGTCG
                                                  IgG4 CH3

3101 TTCTTCCTGT ACTCCCGGCT GACCGTGGAC AAGAGCCGGT GGCAGGAAGG CAACGTCTTC AGCTGCAGCG TGATGCACGA GGCCCTGCAC AACCACTACA
     AAGAAGGACA TGAGGGCCGA CTGGCACCTG TTCTCGGCCA CCGTCCTTCC GTTGCAGAAG TCGACGTCGC ACTACGTGCT CCGGGACGTG TTGGTGATGT
                                                                 CD28tm

     IgG4 CH3

3201 CCCAGAAGTC CCTGAGCCTG AGCCTGGGCA AGATGTTCTG GGTGCTGGTG GTGGTCGGAG GCGTGCTGGC CTGCTACAGC CTGCTGGTCA CCGTGGCCTT
     GGGTCTTCAG GGACTCGGAC TCGGACCCGT TCTACAAGAC CCACGACCAC CACCAGCCTC CGCACGACCG GACGATGTCG GACGACCAGT GGCACCGGAA
       CD28tm
                                        4-1BB

3301 CATCATCTTT TGGGTGAAAC GGGGCAGAAA GAAACTCCTG TATATATTCA AACAACCATT TATGAGACCA GTACAAACTA CTCAAGAGGA AGATGGCTGT
     GTAGTAGAAA ACCCACTTTG CCCCGTCTTT CTTTGAGGAC ATATATAAGT TTGTTGGTAA ATACTCTGGT CATGTTTGAT GAGTTCTCCT TCTACCGACA
                                                                 Zeta
                                        4-1BB
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FIG. 7 (Continued)

```
3401 AGCTGCCGAT TTCCAGAAGA AGAAGAAGGA GGATGTGAAC TGCGGGTGAA GTTCAGCAGA AGCGCCGACG CCCCTGCCTA CCAGCAGGGC CAGAATCAGC
     TCGACGGCTA AAGGTCTTCT TCTTCTTCCT CCTACACTTG ACGCCCACTT CAAGTCGTCT TCGCGGCTGC GGGGACCGAT GGTCGTCCCG GTCTTAGTCG
                                                  Zeta
3501 TGTACAACGA GCTGAACCTG GGCAGAAGGG AAGAGTACGA CGTCCTGGAT AAGCGGAGAG GCCGGGACCC TGAGATGGGC GGCAAGCCTC GGCGGAAGAA
     ACATGTTGCT CGACTTGGAC CCGTCTTCCC TTCTCATGCT GCAGGACCTA TTCGCCTCTC CGGCCCTGGG ACTCTACCCG CCGTTCGGAG CCGCCTTCTT
                                                  Zeta
3601 CCCCCAGGAA GGCCTGTATA ACGAACTGCA GAAAGACAAG ATGGCCGAGG CCTACAGCGA GATCGGCATG AAGGGCGAGC GGAGGCGGGG CAAGGGCCAC
     GGGGGTCCTT CCGGACATAT TGCTTGACGT CTTTCTGTTC TACCGGCTCC GGATGTCGCT CTAGCCGTAC TTCCCGCTCG CCTCCCCCCC GTTCCCGGTG
                                                                                  T2A
3701 GACGGCCTGT ATCAGGGCCT GTCCACCGCC ACCAAGGATA CCTACGACGC CCTGCACATG CAGGCCCTGC CCCCAAGGCT CGAGGGCGGC GGAGAGGGCA
     CTGCCGGACA TAGTCCCGGA CAGGTGGCGG TGGTTCCTAT GGATGCTGCG GGACGTGTAC GTCCGGGACG GGGGTTCCGA GCTCCCGCCG CCTCTCCCGT
                                                  T2A                                                 EGFRt
3801 GAGGAAGTCT TCTAACATGC GGTGACGTGG AGGAGAATCC CGGCCCTAGG ATGCTTCTCC TGGTGACAAG CCTTCTGCTC TGTGAGTTAC CACACCCAGC
     CTCCTTCAGA AGATTGTACG CCACTGCACC TCCTCTTAGG GCCGGGATCC TACGAAGAGG ACCACTGTTC GGAAGACGAG ACACTCAATG GTGTGGGTCG
                                                  EGFRt
3901 ATTCCTCCTG ATCCCACGCA AAGTGTGTAA CGGAATAGGT ATTGGTGAAT TTAAAGACTC ACTCTCCATA AATGCTACGA ATATTAAACA CTTCAAAAAC
     TAAGGAGGAC TAGGGTGCGT TTCACACATT GCCTTATCCA TAACCACTTA AATTTCTGAG TGAGAGGTAT TTACGATGCT TATAATTTGT GAAGTTTTTG
                                                  EGFRt
4001 TGCACCTCCA TCAGTGGCGA TCTCCACATC CTGCCCGTGG CATTTAGGGG TGACTCCTTC ACACATACTC CTCCTCTGGA TCCACAGGAA CTGGATATTC
     ACGTGGAGGT AGTCACCGCT AGAGGTGTAG GACGGCCACC GTAAATCCCC ACTGAGGAAG TGTGTATGAG GAGGAGACCT AGGTGTCCTT GACCTATAAG
                                                  EGFRt
4101 TGAAAACCGT AAAGGAAATC ACAGGGTTTT TGCTGATTCA GGCTTGGCCT GAAAACAGGA CGGACCTCCA TGCCTTTGAG AACCTAGAAA TCATACGCGG
     ACTTTTGGCA TTTCCTTTAG TGTCCCAAAA ACGACTAAGT CCGAACCGGA CTTTTGTCCT GCCTGGAGGT ACGGAAACTC TTGGATCTTT AGTATGCGCC
                                                  EGFRt
4201 CAGGACCAAG CAACATGGTC AGTTTTCTCT TGCAGTCGTC AGCCTGAACA TAACATCCTT GGGATTACGC TCCCTCAAGG AGATAAGTGA TGGAGATGTG
     GTCCTGGTTC GTTGTACCAG TCAAAAGAGA ACGTCAGCAG TCGGACTTGT ATTGTAGGAA CCCTAATGCG AGGGAGTTCC TCTATTCACT ACCTCTACAC
                                                  EGFRt
4301 ATAATTTCAG GAAACAAAAA TTTGTGCTAT GCAAATACAA TAAACTGCAA AAAACTGTTT GGGACCTCCG GTCAGAAAAC CAAAATTATA AGCAACAGAG
     TATTAAAGTC CTTTGTTTTT AAACACGATA CGTTTATGTT ATTTGACGTT TTTTGACAAA CCCTGGAGGC CAGTCTTTTG GTTTTAATAT TCGTTGTCTC
                                                  EGFRt
4401 GTGAAAACAG CTGCAAGGCC ACAGGCCAGG TCTGCCATGC CTTGTGCTCC CCCGAGGGCT GCTGGGGCCC GGAGCCCAGG GACTGCGTCT CTTGCCGGAA
     CACTTTTGTC GACGTTCCGG TGTCCGGTCC AGACGGTACG GAACACGAGG GGGCTCCCGA CGACCCCGGG CCTCGGGTCC CTGACGCAGA GAACGGCCTT
                                                  EGFRt
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FIG. 7 (Continued)

FIG. 7 (Continued)

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5601 GACTTACAAG GCAGCTGTAG ATCTTAGCCA CTTTTTAAAA GAAAAGGGGG GACTGGAAGG GCTAATTCAC TCCCAAAGAA GACAAGATCT GCTTTTTGCC
     CTGAATGTTC CGTCGACATC TAGAATCGGT GAAAAATTTT CTTTTCCCCC CTGACCTTCC CGATTAAGTG AGGGTTTCTT CTGTTCTAGA CGAAAAACGG
                                R                                                                U5
5701 TGTACTGGGT CTCTCTGGTT AGACCAGATC TGAGCCTGGG AGCTCTCTGG CTAACTAGGG AACCCACTGC TTAAGCCTCA ATAAAGCTTG CCTTGAGTGC
     ACATGACCCA GAGAGACCAA TCTGGTCTAG ACTCGGACCC TCGAGAGACC GATTGATCCC TTGGGTGACG AATTCGGAGT TATTTCGAAC GGAACTCACG
                                   U5
5801 TTCAAGTAGT GTGTGCCCGT CTGTTGTGTG ACTCTGGTAA CTAGAGATCC CTCAGACCCT TTTAGTCAGT GTGGAAAATC TCTAGCAGAA TTCGATATCA
     AAGTTCATCA CACAGGGCA GACAACACAC TGAGACCATT GATCTCTAGG GAGTCTGGGA AAATCAGTCA CACCTTTTAG AGATCGTCTT AAGCTATAGT
5901 AGCTTATCGA TACCGTCGAC CTCGAGGGGG GGCCCGGTAC CCAATTCGCC CTATAGTGAG TCGTATTACA ATTCACTGGC CGTCGTTTTA CAACGTCGTG
     TCGAATAGCT ATGGCAGCTG GAGCTCCCCC CGGGCCATG GGTTAAGCGG GATATCACTC AGCATAATGT TAAGTGACCG GCAGCAAAAT GTTGCAGCAC
6001 ACTGGGAAAA CCCTGGCGTT ACCCAACTTA ATCGCCTTGC AGCACATCCC CCTTTCGCCA GCTGGCGTAA TAGCGAAGAG GCCCGCACCG ATCGCCCTTC
     TGACCCTTTT GGGACCGCAA TGGGTTGAAT TAGCGGAACG TCGTGTAGGG GCAAAGCGGT CGACCGCATT ATCGCTTCTC CGGGCGTGGC TAGCGGGAAG
6101 CCAACAGTTG CGCAGCCTGA ATGGCGAATG GCGTTAATAT TTTGTTAAAA TTCGCGTTAA AATTTTGTTA AATCAGCTCA TTTTTTTAACC
     GGTTGTCAAC GCGTCGGACT TACCGCTTAC CTTTAACATT CGCAATTATA AAACAATTTT AAGCGCAATT TAAAAACAAT TTAGTCGAGT AAAAAATTGG
6201 AATAGGCCGA AATCGGCAAA ATCCCTTATA AATCAAAAGA ATAGACCGAG ATAGGGTTGA GTGTTGTTCC AGTTTGGAAC AAGAGTCCAC TATTAAAGAA
     TTATCCGGCT TTAGCCGTTT TAGGGAATAT TTAGTTTTCT TATCTGGCTC TATCCCAACT CACAACAAGG TCAAACCTTG TTCTCAGGTG ATAATTTCTT
6301 CGTGGACTCC AACGTCAAAG GGCCGAAAAC CGTCTATCAG GGCGATGGCC CACTACGTGA ACCATCACCC TAATCAAGTT TTTTGGCGTC GAGGTGCCGT
     GCACCTGAGG TTGCAGTTTC CCGCTTTTTG GCAGATAGTC CCGCTACCGG GTGATGCACT TGGTAGTGGG ATTAGTTCAA AAAACCCCAG CTCCACGGCA
6401 AAAGCACTAA ATCGGAACCC TAAAGGGAGC CCCCGATTTA GAGCTTGACG GGGAAAGCCG GCGAACGTGG CGAGAAAGGA AGGGAAGGAG GCGAAAGGAG
     TTTCGTGATT TAGCCTTGGG ATTTCCCTCG GGGGCTAAAT CTCGAACTGC CCCTTTCGGC CGCTTGCACC GCTCTTTCCT TCCCTTCTTT CGCTTTCCTC
6501 CGGGCGCTAG GGCGCTGGCA AGTGTAGCGG TCACGCTGCG CGTAACCACC ACACCCGCCG CGCTTAATGC GCCGCTACAG GGCGGCGTCAG GTGGCACTTT
     GCCCGCGATC CCGCGACCGT TCACATCGCC AGTGCGACGC GCATTGGTGG TGTGGGCGGC GCGAATTACG CGGCGATGTC CCGCCGCAGTC CACCGTGAAA
6601 TCGGGGAAAT GTGCGCGGAA CCCCTATTTG TTTTATTTTC TAAATACATT CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAT GCTTCAATAA
     AGCCCCTTTA CACGCGCCTT GGGGATAAAC AAATAAAAAG ATTTATGTAA GTTTATACAT AGGCGAGTAC TCTGTTATTG GGACTATTTA CGAAGTTATT
                                                                Ampr
6701 TATTGAAAAA GGAAGAGTAT GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT GCCTTCCTGT TTTTGCTCAC CCAGAAACGC
     ATAACTTTTT CCTTCTCATA CTCATAAGTT GTAAAGGCAC AGCGGGAATA AGGGAAAAAA CGCCGTAAAA CGGAAGGACA AAAACGAGTG GGTCTTTGCG
                                                   Ampr
6801 TGGTGAAAGT AAAAGATGCT GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT TTCGCCCCGA
     ACCACTTTCA TTTTCTACGA CTTCTAGTCA ACCCACGTGC TCACCCAATG TAGCTTGACC TAGAGTTGTC GCCATTCTAG GAACTCTCAA AAGCGGGCT
                                                      Ampr
6901 AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTCG CCGCATACAC
     TCTTGCAAAA GGTTACTACT CGTGAAAATT TCAAGACGAT ACACCGCGCC ATAATAGGGC ATAACTGCGG CCCGTTCTCG TTGAGCCAGC GGCGTATGTG
                                                         Ampr
7001 TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA GAGAATTATG CAGTGCTGCC ATAACCATGA
     ATAAGAGTCT TACTGAACCA ACTCATGAGT GGTCAGTGTC TTTTCGTAGA ATGCCTACGG TACTGTCATT CTCTTAATAC GTCACGACGG TATTGGTACT
                                                            Ampr
7101 GTGATAACAC TGCGGCCAAC TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG GATCATGTAA CTCGCCTTGA
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FIG. 7 (Continued)

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      CACTATTGTG ACGCCGGTTG AATGAAGACT GTTGCTAGCC TCCTGGCTTC CTCGATTGGC GAAAAAACGT GTTGTACCCC CTAGTACATT GAGCGGAACT
                                                  Ampr
7201  TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGTAGCAATG GCAACAACGT TGCGCAAACT ATTAACTGGC
      AGCAACCCTT GGCCTCGACT TACTTCGGTA TGGTTTGCTG CTCGCACTGT GGTGCTACGG ACATCGTTAC CGTTGTTGCA ACGCGTTTGA TAATTGACCG
                                                  Ampr
7301  GAACTACTTA CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT
      CTTGATGAAT GAGATCGAAG GGCCGTTGTT AATTATCTGA CCTACCTCCG CCTATTTCAA CGTCCTGGTG AAGACGCGAG CCGGGAAGGC CGACCGACCA
                                                  Ampr
7401  TTATTGCTGA TAAATCTGGA GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG TTATCTACAC
      AATAACGACT ATTTAGACCT CGGCCACTCG CACCCAGAGC GCCATAGTAA CGTCGTGACC CCGGTCTACC ATTCGGGAGG GCATAGCATC AATAGATGTG
                                                  Ampr
7501  GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA
      CTGCCCCTCA GTCCGTTGAT ACCTACTTGC TTTATCTGTC TAGCGACTCT ATCCACGGAG TGACTAATTC GTAACCATTG ACAGTCTGGT TCAAATGAGT
                                                  CoE1 ori
7601  TATATACTTT AGATTGATTT AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA ATCTCATGAC CAAAATCCCT TAACGTGAGT
      ATATATGAAA TCTAACTAAA TTTTCGAAGTA AAAATTAAAT TTTCCTAGAT CCACTTCTAG GAAAAACTAT TAGAGTACTG GTTTTAGGGA ATTGCACTCA
                                                  CoE1 ori
7701  TTTCGTTCCA CTGAGCGTCA GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG CGTAATCTGC TGCTTGCAAA CAAAAAAACC
      AAAGCAAGGT GACTCGCAGT CTGGGGCATC TTTTCTAGTT TCCTAGAAGA ACTCTAGGAA AAAAAGACGC GCATTAGACG ACGAACGTTT GTTTTTTTGG
                                                  CoE1 ori
7801  ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTTCTT
      TGGCGATGGT CGCCACCAAA CAAACGGCCT AGTTCTCGAT GGTTGAGAAA AAGGCTTCCA TTGACCGAAG TCGTCTCGCG TCTATGGTTT ATGACAAGAA
                                                  CoE1 ori
7901  CTAGTGTAGC CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA TCCTGTTACC AGTGGCTGCT GCCAGTGGCG
      GATCACATCG GCATCAATCC GGTGGTGAAG TTCTTGAGAC ATCGTGGCGG ATGTATGGAG CGAGACGATT AGGACAATGG TCACCGACGA CGGTCACCGC
                                                  CoE1 ori
8001  ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG TGCACACAGC CCAGCTTGGA
      TATTCAGCAC AGAATGGCCC AACCTGAGTT CTGCTATCAA TGGCCTAATC CGCGTCGCCA GCCCGACTTG CCCCCCAAGC ACGTGTGTCG GGTCGAACCT
                                                  CoE1 ori
8101  GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC
      CGCTTGCTGG ATGTGGCTTG ACTCTATGGA TGTCGGCACTC GATACTCTTT CGCGGTGCGA AGGGCTTCCC TCTTTCCGCC TGTCCATAGG CCATTCGCCG
                                                  CoE1 ori
8201  AGGGTCGGAA CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG GGTTTCGCCA CCTCTGACTT GAGCGTCGAT
      TCCCAGCCTT GTCCTCTCGC GTGCTCCCTC GAAGGTCCCC CTTTGCGGAC CATAGAAATA TCAGGACAGC CCAAAGCGGT GGAGACTGAA CTCGCAGCTA
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FIG. 7 (Continued)

```
8301  TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG CTCACATGTT
      AAAACACTAC GAGCAGTCCC CCCGCCTCGG ATACCTTTTT GCGGTCGTTG CGCCGGAAAA ATGCCAAGGA CCGGAAAACG ACCGGAAAAC GAGTGTACAA
                                        CoE1 ori

8401  CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA
      GAAAGGACGC AATAGGGGAC TAAGACACCT ATTGGCATAA TGGCGGAAAC TCACTCGACT ATGGCGAGCG GCGTCGGCTT GCTGGCTCGC GTCGCTCAGT
           CoE1 ori

8501  GTGAGCGAGG AAGCGGAAGA GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCATTAAT GCAGCTGGCA CGACAGGTTT CCCGACTGGA
      CACTCGCTCC TTCGCCTTCT CGCGGGTTAT GCGTTTGGCG GAGAGGGGCG CGCAACCGGC TAAGTAATTA CGTCGACCGT GCTGTCCAAA GGGCTGACCT

8601  AAGCGGGCAG TGAGCGCAAC GCAATTAATG TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT TGTGTGGAAT
      TTCGCCCGTC ACTCGCGTTG CGTTAATTAC ACTCAATCGA GTGAGTAATC CGTGGGGTCC GAAATGTGAA ATACGAAGGC CGAGCATACA ACACACCTTA

8701  TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG CCAAGCTCGA AATTAACCCT CACTAAAGGG AACAAAAGCT GGAGCTCCAC
      ACACTCGCCT ATTGTTAAAG TGTGTCCTTT GTCGATACTG GTACTAATGC GGTTCGAGCT TTAATTGGGA GTGATTTCCC TTGTTTTCGA CCTCGAGGTG
                                                  SV40

8801  CGCGGTGGCG GCCTCGAGGT CGAGATCCGG TCGACCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT
      GCGCCACCGC CGGAGCTCCA GCTCTAGGCC AGCTGGTCGT TGGTATCAGG GCGGGGATTG AGGCGGGTAG GGCGGGGATT GAGGCGGGTC AAGGCGGGTA
                                        SV40

8901  TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG AGGCCGAGGC CGCCTCGGCC TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG
      AGAGGCGGGG TACCGACTGA TTAAAAAAAA TAAATACGTC TCCGGCTCCG GCGGAGCCGG AGACTCGATA AGGTCTTCAT CACTCCTCCG AAAAAACCTC
           SV40                                                 CMV

9001  GCCTAGGCTT TTGCAAAAAG CTTCGACGGT ATCGATTGGC TCATGTCCAA CATTACCGCC ATGTTGACAT TGATTATTGA CTAGTTATTA ATAGTAATCA
      CGGATCCGAA AACGTTTTTC GAAGCTGCCA TAGCTAACCG AGTACAGGTT GTAATGGCGG TACAACTGTA ACTAATAACT GATCAATAAT TATCATTAGT
                                        CMV

9101  ATTACGGGGT CATTAGTTCA TAGCCCATAT ATGGAGTTCC GCGTTACATA ACTTACGGTA AATGGCCCGC CTGGCTGACC GCCCAACGAC CCCCGCCCAT
      TAATGCCCCA GTAATCAAGT ATCGGGTATA TACCTCAAGG CGCAATGTAT TGAATGCCAT TTACCGGGCG GACCGACTGG CGGGTTGCTG GGGGCGGGTA
                                        CMV

9201  TGACGTCAAT AATGACGTAT GTTCCCATAG TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA GTATTTACGG TAAACTGCCC ACTTGGCAGT
      ACTGCAGTTA TTACTGCATA CAAGGGTATC ATTGCGGTTA TCCCTGAAAG GTAACTGCAG TTACCCACCT CATAAATGCC ATTTGACGGG TGAACCGTCA
                                        CMV

9301  ACATCAAGTG TATCATATGC CAAGTACGCC CCCTATTGAC GTCAATGACG GTAAATGGCC CGCCTGGCAT TATGCCCAGT ACATGACCTT ATGGGACTTT
      TGTAGTTCAC ATAGTATACG GTTCATGCGG GGGATAACTG CAGTTACTGC CATTTACCGG GCGGACCGTA ATACGGGTCA TGTACTGGAA TACCCTGAAA
                                        CMV

9401  CCTACTTGGC AGTACATCTA CGTATTAGTC ATCGCTATTA CCATGGTGAT GCGGTTTTGG CAGTACATCA ATGGGCGTGG ATAGCGGTTT GACTCACGGG
      GGATGAACCG TCATGTAGAT GCATAATCAG TAGCGATAAT GGTACCACTA CGCCAAACCC GTCATGTAGT TACCCGCACC TATCGCCAAA CTGAGTGCCC
                                        CMV

9501  GATTTCCAAG TCTCCACCCC ATTGACGTCA ATGGGAGTTT GTTTTGGCAC CAAAATCAAC GGGACTTTCC AAAATGTCGT AACAACTCCG CCCCATTGAC
      CTAAAGGTTC AGAGGTGGGG TAACTGCAGT TACCCTCAAA CAAAACCGTG CTTTTAGTTG CCCTGAAAGG TTTTACAGCA TTGTTGAGGC GGGGTAACTG
                CMV
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FIG. 7 (Continued)

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     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
9601 GCAAATGGGC GGTAGGCGTG TACGGAATTC GGAGTGGCGA GCCCTCAGAT CCTGCATATA AGCAGCTGCT TTTTGCCTGT ACTGGGTCTC TCTG
     CGTTTACCCG CCATCCGCAC ATGCCTTAAG CCTCACCGCT CGGGAGTCTA GGACGTATAT TCGTCGACGA AAAACGGACA TGACCCAGAG AGAC
```

FIG. 7 (Continued)

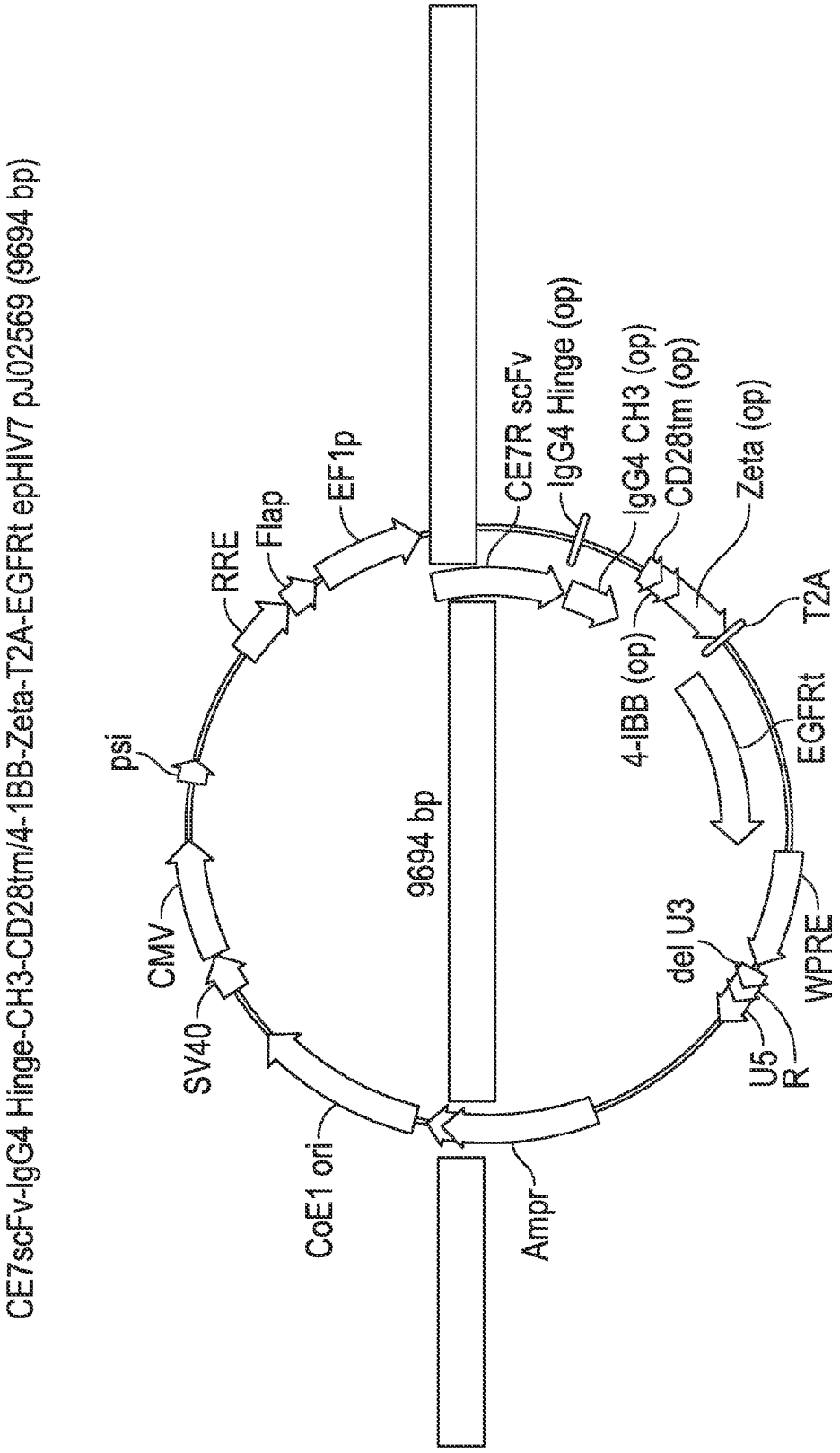
CE7scFv-IgG4 Hinge-CH3-CD28tm/4-1BB-Zeta-T2A-EGFRt epHIV7 pJ02569 (9694 bp)
9694 bp
psi
RRE
Flap
EF1p
CE7R scFv
IgG4 Hinge (op)
IgG4 CH3 (op)
CD28tm (op)
Zeta (op)
T2A
EGFRt
4-IBB (op)
WPRE
del U3
U5
R
CMV
SV40
CoE1 ori
Ampr

FIG. 8

FIG. 9

FIG. 9 (Continued)

FIG. 9 (Continued)

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     AGGTGGCGGT GGTTCCTATG GATGCTGCGG GACGTGTACG TCCGGGACGG GGGTTCCGAG CTCCCGCCGC CTCTCCCGTC TCCTTCAGAA GATTGTACGC
                                                                  EGFRt
            T2A
3501 GTGACGTGGA GGAGAATCCC GGCCCTAGGA TGCTTCTCCT GGTGACAAGC CTTCTGCTCT GTGAGTTACC ACACCCAGCA TTCCTCCTGA TCCCACGCAA
     CACTGCACCT CCTCTTAGGG CCGGGATCCT ACGAAGAGGA CCACTGTTCG GAAGACGAGA CACTCAATGG TGTGGGTCGT AAGGAGGACT AGGGTGCGTT
                                                     EGFRt
3601 AGTGTGTAAC GGAATAGGTA TTGGTGAATT TAAAGACTCA CTCTCCATAA ATGCTACGAA TATTAAACAC TTCAAAAACT GCACCTCCAT CAGTGGCGAT
     TCACACATTG CCTTATCCAT AACCACTTAA ATTTCTGAGT GAGAGGTATT TACGATGCTT ATAATTTGTG AAGTTTTTGA CGTGGAGGTA GTCACCGCTA
                                                     EGFRt
3701 CTCCACATCC TGCCGGTGGC ATTTAGGGGT GACTCCTTCA CACATACTCC TCCTCTGGAT CCACAGGAAC TGGATATTCT GAAAACCGTA AAGGAAATCA
     GAGGTGTAGG ACGGCCACCG TAAATCCCCA CTGAGGAAGT GTGTATGAGG AGGAGACCTA GGTGTCCTTG ACCTATAAGA CTTTTGGCAT TTCCTTTAGT
                                                     EGFRt
3801 CAGGGTTTTT GCTGATTCAG GCTTGGCCTG AAAACAGGAC GGACCTCCAT GCCTTTGAGA ACCTAGAAAT CATACGCGGC AGGACCAAGC AACATGGTCA
     GTCCCAAAAA CGACTAAGTC CGAACCGGAC TTTTGTCCTG CCTGGAGGTA CGGAAACTCT TGGATCTTTA GTATGCGCCG TCCTGGTTCG TTGTACCAGT
                                                     EGFRt
3901 GTTTTCTCTT GCAGTCGTCA GCCTGAACAT AACATCCTTG GGATTACGCT CCCTCAAGGA GATAAGTGAT GGAGATGTGA TAATTTCAGG AAACAAAAAT
     CAAAAGAGAA CGTCAGCAGT CGGACTTGTA TTGTAGGAAC CCTAATGCGA GGGAGTTCCT CTATTCACTA CCTCTACACT ATTAAAGTCC TTTGTTTTTA
                                                     EGFRt
4001 TTGTGCTATG CAAATACAAT AAACTGGAAA AAACTGTTTG GGACCTCCGG TCAGAAAACC AAAATTATAA GCAACAGAGG TGAAAACAGC TGCAAGGCCA
     AACACGATAC GTTTATGTTA TTTGACCTTT TTTGACAAAC CCTGGAGGCC AGTCTTTTGG TTTTAATATT CGTTGTCTCC ACTTTTGTCG ACGTTCCGGT
                                                     EGFRt
4101 CAGGCCAGGT CTGCCATGCC TTGTGCTCCC CCGAGGGCTG CTGGGGCCCG GAGCCCAGGG ACTGCGTCTC TTGCCGGAAT GTCAGCCGAG GCAGGGAATG
     GTCCGGTCCA GACGGTACGG AACACGAGGG GGCTCCCGAC GACCCCGGGC CTCGGGTCCC TGACGCAGAG AACGGCCTTA CAGTCGGCTC CGTCCCTTAC
                                                     EGFRt
4201 CGTGGACAAG TGCAACCTTC TGGAGGGTGA GCCAAGGGAG TTTGTGGAGA ACTCTGAGTG CATACAGTGC CACCCAGAGT GCCTGCCTCA GGCCATGAAC
     GCACCTGTTC ACGTTGGAAG ACCTCCCACT CGGTTCCCTC AAACACCTCT TGAGACTCAC GTATGTCACG GTGGGTCTCA CGGACGGAGT CCGGTACTTG
                                                     EGFRt
4301 ATCACCTGCA CAGGACGGGG ACCAGACAAC TGTATCCAGT GTGCCCACTA CATTGACGGC CCCCACTGCG TCAAGACCTG CCCGGCAGGA GTCATGGGAG
     TAGTGGACGT GTCCTGCCCC TGGTCTGTTG ACATAGGTCA CACGGGTGAT GTAACTGCCG GGGGTGACGC AGTTCTGGAC GGGCCGTCCT CAGTACCCTC
                                                     EGFRt
4401 AAAACAACAC CCTGGTCTGG AAGTACGCAG ACGCCGGCCA TGTGTGCCAC CTGTGCCATC CAAACTGCAC CTACGGATGC ACTGGGCCAG GTCTTGAAGG
     TTTTGTTGTG GGACCAGACC TTCATGCGTC TGCGGCCGGT ACACACGGTG GACACGGTAG GTTTGACGTG GATGCCTACG TGACCCGGTC CAGAACTTCC
                                                     EGFRt
4501 CTGTCCAACG AATGGGCCTA AGATCCCGTC CATCGCCACT GGGATGGTGG GGGCCCTCCT CTTGCTGCTG GTGGTGGCCC TGGGGATCGG CCTCTTCATG
     GACAGGTTGC TTACCCGGAT TCTAGGGCAG GTAGCGGTGA CCCTACCACC CCCGGGAGGA GAACGACGAC CACCACCGGG ACCCCTAGCC GGAGAAGTAC
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FIG. 9 (Continued)

```
     EGFRt                                                                                 WPRE
4601 TGAGCGGCCG CTCTAGACCC GGGCTGCAGG AATTCGATAT CAAGCTTATC GATAATCAAC CTCTGGATTA CAAAATTTGT GAAAGATTGA CTGGTATTCT
     ACTCGCCGGC GAGATCTGGG CCCGACGTCC TTAAGCTATA GTTCGAATAG CTATTAGTTG GAGACCTAAT GTTTTAAACA CTTTCTAACT GACCATAAGA
                                                       WPRE
4701 TAACTATGTT GCTCCTTTTA CGCTATGTGG ATACGCTGCT TTAATGCCTT TGTATCATGC TATTGCTTCC CGTATGGCTT TCATTTTCTC CTCCTTGTAT
     ATTGATACAA CGAGGAAAAT GCGATACACC TATGCGACGA AATTACGGAA ACATAGTACG ATAACGAAGG GCATACCGAA AGTAAAAGAG GAGGAACATA
                                                       WPRE
4801 AAATCCTGGT TGCTGTCTCT TTATGAGGAG TTGTGGCCCG TTGTCAGGCA ACGTGGCGTG GTGTGCACTG TGTTTGCTGA CGCAACCCCC ACTGGTTGGG
     TTTAGGACCA ACGACAGAGA AATACTCCTC AACACCGGGC AACAGTCCGT TGCACCGCAC CACACGTGAC ACAAACGACT GCGTTGGGGG TGACCAACCC
                                                       WPRE
4901 GCATTGCCAC CACCTGTCAG CTCCTTTCCG GGACTTTCGC TTTCCCCCTC CCTATTGCCA CGGCGGAACT CATCGCCGCC TGCCTTGCCC GCTGCTGGAC
     CGTAACGGTG GTGGACAGTC GAGGAAAGGC CCTGAAAGCG AAAGGGGGAG GGATAACGGT GCCGCCTTGA GTAGCGGCGG ACGGAACGGG CGACGACCTG
                                                       WPRE
5001 AGGGGCTCGG CTGTTGGGCA CTGACAATTC CGTGGTGTTG TCGGGGAAAT CATCGTCCTT TCCTTGGCTG CTCGCCTGTG TTGCCACCTG GATTCTGCGC
     TCCCCGAGCC GACAACCCGT GACTGTTAAG GCACCACAAC AGCCCCTTTA GTAGCAGGAA AGGAACCGAC GAGCGGACAC AACGGTGGAC CTAAGACGCG
                                                       WPRE
5101 GGGACGTCCT TCTGCTACGT CCCTTCGGCC CTCAATCCAG CGGACCTTCC TTCCCGCGGC CTGCTGCCGG CTCTGCGGCC TCTTCCGCGT CTTCGCCTTC
     CCCTGCAGGA AGACGATGCA GGGAAGCCGG GAGTTAGGTC GCCTGGAAGG AAGGGCGCCG GACGACGGCC GAGACGCCGG AGAAGGCGCA GAAGCGGAAG
                WPRE                                                                                 del U3
5201 GCCCTCAGAC GAGTCGGATC TCCCTTTGGG CCGCCTCCCC GCATCGATAC CGTCGACTAG CCGTACCTTT AAGACCAATG ACTTACAAGG CAGCTGTAGA
     CGGGAGTCTG CTCAGCCTAG AGGGAAACCC GGCGGAGGGG CGTAGCTATG GCAGCTGATC GGCATGGAAA TTCTGGTTAC TGAATGTTCC GTCGACATCT
                          del U3
                                                                                                 R
5301 TCTTAGCCAC TTTTTAAAAG AAAAGGGGGG ACTGGAAGGG CTAATTCACT CCCAAAGAAG ACAAGATCTG CTTTTTGCCT GTACTGGGTC TCTCTGGTTA
     AGAATCGGTG AAAAATTTTC TTTTCCCCCC TGACCTTCCC GATTAAGTGA GGGTTTCTTC TGTTCTAGAC GAAAAACGGA CATGACCCAG AGAGACCAAT
                                                                                      US
                R
5401 GACCAGATCT GAGCCTGGGA GCTCTCTGGC TAACTAGGGA ACCCACTGCT TAAGCCTCAA TAAAGCTTGC CTTGAGTGCT TCAAGTAGTG TGTGCCCGTC
     CTGGTCTAGA CTCGGACCCT CGAGAGACCG ATTGATCCCT TGGGTGACGA ATTCGGAGTT ATTTCGAACG GAACTCACGA AGTTCATCAC ACACGGGCAG
                          US
5501 TGTTGTGTGA CTCTGGTAAC TAGAGATCCC TCAGACCCTT TTAGTCAGTG TGGAAAATCT CTAGCAGAAT TCGATATCAA GCTTATCGAT ACCGTCGACC
     ACAACACACT GAGACCATTG ATCTCTAGGG AGTCTGGGAA AATCAGTCAC ACCTTTTAGA GATCGTCTTA AGCTATAGTT CGAATAGCTA TGGCAGCTGG
5601 TCGAGGGGGG GCCCGGTACC CAATTCGCCC TATAGTGAGT CGTATTACAA TTCACTGGCC GTCGTTTTAC AACGTCGTGA CTGGGAAAAC CCTGGCGTTA
     AGCTCCCCCC CGGGCCATGG GTTAAGCGGG ATATCACTCA GCATAATGTT AAGTGACCGG CAGCAAAATG TTGCAGCACT GACCCTTTTG GGACCGCAAT
5701 CCCAACTTAA TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA TCGCCCTTCC CAACAGTTGC GCAGCCTGAA
     GGGTTGAATT AGCGGAACGT CGTGTAGGGG GAAAGCGGTC GACCGCATTA TCGCTTCTCC GGGCGTGGCT AGCGGGAAGG GTTGTCAACG CGTCGGACTT
```

FIG. 9 (Continued)

```
5801 TGGCGAATGG AAATTGTAAG CGTTAATATT TTGTTAAAAT TCGCGTTAAA TTTTTGTTAA ATCAGCTCAT TTTTTAACCA ATAGGCCGAA ATCGGCAAAA
     ACCGCTTACC TTTAACATTC GCAATTATAA AACAATTTTA AGCGCAATTT AAAAACAATT TAGTCGAGTA AAAAATTGGT TATCCGGCTT TAGCCGTTTT
5901 TCCCTTATAA ATCAAAAGAA TAGACCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA AGAGTCCACT ATTAAAGAAC GTGGACTCCA ACGTCAAAGG
     AGGGAATATT TAGTTTTCTT ATCTGGCTCT ATCCCAACTC ACAACAAGGT CAAACCTTGT TCTCAGGTGA TAATTTCTTG CACCTGAGGT TGCAGTTTCC
6001 GCGAAAAACC GTCTATCAGG GCGATGGCCC ACTACGTGAA CCATCACCCT AATCAAGTTT TTTGGGGTCG AGGTGCCGTA AAGCACTAAA TCGGAACCCT
     CGCTTTTTGG CAGATAGTCC CGCTACCGGG TGATGCACTT GGTAGTGGGA TTAGTTCAAA AAACCCCAGC TCCACGGCAT TTCGTGATTT AGCCTTGGGA
6101 AAAGGGAGCC CCCGATTTAG AGCTTGACGG GGAAAGCCGG CGAACGTGGC GAGAAAGGAA GGGAAGAAAG CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA
     TTTCCCTCGG GGGCTAAATC TCGAACTGCC CCTTTCGGCC GCTTGCACCG CTCTTTCCTT CCCTTCTTTC GCTTTCCTCG CCCGACCGTT
6201 GTGTAGCGGT CACGCTGCGC GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG GCGCGTCAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC
     CACATCGCCA GTGCGACGCG CATTGGTGGT GTGGGCGGCG CGAATTACGC GGCGATGTCC CGCGCAGTCC ACCGTGAAAA GCCCCTTTAC ACGCGCCTTG
                                                                                                          Ampr
6301 CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG CTTCAATAAT ATTGAAAAAG GAAGAGTATG
     GGGATAAACA AATAAAAAGA TTTATACATA GGCGAGTACT TCTGTTATTG GACTATTTAC GAAGTTATTA TAACTTTTTC CTTCTCATAC
                                        Ampr
6401 AGTATTCAAC ATTTCCGTGT CGCCCTTTTT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT GGTGAAAGTA AAAGATGCTG
     TCATAAGTTG TAAAGGCACA GCGGGAATAA GGGAAAAAAC GCCGTAAAAC GGAAGGACAA AAACGAGTGG GTCTTTGCGA CCACTTTCAT TTTCTACGAC
                                        Ampr
6501 AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA GAACGTTTTC CAATGATGAG
     TTCTAGTCAA CCCACGTGCT CACCCAATGT AGCTTGACCT AGAGTTGTCG CCATTCTAGG AACTCTCAAA AGCGGGGCTT CTTTGCAAAAG GTTACTACTC
                                        Ampr
6601 CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT
     GTGAAAATTT CAAGACGATA CACCGCGCCA TAATAGGGCA TAACTGCGGC CCGTTCTCGT TGAGCCAGCG GCGTATGTGA TAAGAGTCTT ACTGAACCAA
                                        Ampr
6701 GAGTACTCAC CAGTCACAGA AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG TGATAACACT GCGGCCAACT
     CTCATGAGTG GTCAGTGTCT TTTCGTAGAA TGCCTACCGT ACTGTCATTC TCTTAATACG TCACGACGGT ATTGGTACTC ACTATTGTGA CGCCGGTTGA
                                        Ampr
6801 TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC CGGAGCTGAA
     ATGAAGACTG TTGCTAGCCT CCTGGCTTCC TCGATTGGCG AAAAACGTTG TTGTACCCCC TAGTACATTG AGCCGAACTA GCAACCCTTG GCCTCGACTT
                                        Ampr
6901 TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCT GTAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC TCTAGCTTCC
     ACTTCGGTAT GGTTTGCTGC TCGCACTGTG GTGCTACGGA CATCGTTACC GTTGTTGCAA CGCGTTTGAT AATTGACCGC TTGATGAATG AGATCGAAGG
7001 CGGCAACAAT TAATAGACTG GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT TATTGCTGAT AAATCTGGAG
     GCCGTTGTTA ATTATCTGAC CTACCTCCGC CTATTTCAAC GTCCTGGTGA AGACGCCGAG CGGGAAGGCC GACCGACCAA ATAACGACTA TTTAGACCTC
                                        Ampr
7101 CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG ACGGGGAGTC AGGCAACTAT
     GGCCACTCGC ACCCAGAGCG CCATAGTAAC GTCGTGACCC CGGTCTACCA TTCGGGAGGG CATAGCATCA ATAGATGTGC TGCCCCTCAG TCCGTTGATA
```

FIG. 9 (Continued)

```
                  Ampr
7201 GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT ATATACTTTA GATTGATTTA
     CCTACTTGCT TTATCTGTCT AGCGACTCTA TCCACGGAGT GACTAATTCG TAACCATTGA CAGTCTGGTT CAAATGAGTA TATATGAAAT CTAACTAAAT
                                                                CoE1 ori
7301 AAACTTCATT TTTAATTTAA AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC TGAGCGTCAG
     TTTGAAGTAA AAATTAAATT TTCCTAGATC CACTTCTAGG AAAAACTATT AGAGTACTGG TTTTAGGGAA TTGCACTCAA AAGCAAGGTG ACTCGCAGTC
                                                     CoE1 ori
7401 ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG CGGTGGTTTG
     TGGGGCATCT TTTCTAGTTT CCTAGAAGAA CTCTAGGAAA AAAAGACGCG CATTAGACGA CGAACGTTTG TTTTTTTGGT GGCGATGGTC GCCACCAAAC
                                                     CoE1 ori
7501 TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTTCTTC TAGTGTAGCC GTAGTTAGGC
     AAACGGCCTA GTTCTCGATG GTTGAGAAAA AGGCTTCCAT TGACCGAAGT CGTCTCGCGT CTATGGTTTA TGACAAGAAG ATCACATCGG CATCAATCCG
                                                     CoE1 ori
7601 CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT CTTACCGGGT
     GTGGTGAAGT TCTTGAGACA TCGTGGCGGA TGTATGGAGC GAGACGATTA GGACAATGGT CACCGACGAC GGTCACCGCT ATTCAGCACA GAATGGCCCA
                                                     CoE1 ori
7701 TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT
     ACCTGAGTTC TGCTATCAAT GGCCTATTCC GCGTCGCCAG CCCGACTTGC CCCCCAAGCA CGTGTGTCGG GTCGAACCTC GCTTGCTGGA TGTGGCTTGA
                                                     CoE1 ori
7801 GAGATACCTA CAGCGTGAGC TATGAGAAAG CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC
     CTCTATGGAT GTCGCACTCG ATACTCTTTC GCGGTGCGAA GGGCTTCCCT CTTTCCGCCT GTCCATAGGC CATTCGCCGT CCCAGCCTTG TCCTCTCGCG
                                                     CoE1 ori
7901 ACGAGGGAGC TTCCAGGGGG AAACGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT TTTGTGATGC TCGTCAGGGG
     TGCTCCCTCG AAGGTCCCCC TTTGCGGACC ATAGAAATAT CAGGACAGCC CAAAGCGGTG GAGACTGAAC TCGCAGCTAA AAACACTACG AGCAGTCCCC
                                                     CoE1 ori
8001 GGCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT ACGGTTCCTG GCCTTTTGCT GGCCTTTTGC TCACATGTTC TTTCCTGCGT TATCCCCTGA
     CCGCCTCGGA TACCTTTTTG CGGTCGTTGC GCCGGAAAAA TGCCAAGGAC CGGAAAACGA CCGGAAAACG AGTGTACAAG AAAGGACGCA ATAGGGGACT
                                                     CoE1 ori
8101 TTCTGTGGAT AACCGTATTA CCGCCTTTGA GTGAGCTGAT ACCGCTCGCC GCAGCCGAAC GACCGAGCGC AGCGAGTCAG TGAGCGAGGA AGCGGAAGAG
     AAGACACCTA TTGGCATAAT GGCGGAAACT CACTCGACTA TGGCGAGCGG CGTCGGCTTG CTGGCTCGCG TCGCTCAGTC ACTCGCTCCT TCGCCTTCTC
8201 CGCCCAATAC GCAAACCGCC TCTCCCCGCG CGTTGGCCGA TTCATTAATG CAGCTGGCAC GACAGGTTTC CCGACTGGAA AGCGGGCAGT GAGCGCAACG
     GCGGGTTATG CGTTTGGCGG AGAGGGGCGC GCAACCGGCT AAGTAATTAC GTCGACCGTG CTGTCCAAAG GGCTGACCTT TCGCCCGTCA CTCGCGTTGC
8301 CAATTAATGT GAGTTAGCTC ACTCATTAGG CACCCCAGGC TTTACACTTT ATGCTTCCGG CTCGTATGTT GTGTGGAATT GTGAGCGGAT AACAATTTCA
     GTTAATTACA CTCAATCGAG TGAGTAATCC GTGGGGTCCG AAATGTGAAA TACGAAGGCC GAGCATACAA CACACCTTAA CACTCGCCTA TTGTTAAAGT
                                                                                                              SV40
8401 CACAGGAAAC AGCTATGACC ATGATTACGC CAAGCTCGAA ATTAACCCTC ACTAAAGGGA ACAAAAGCTG GAGCTCCACC GCGGTGGCGG CCTCGAGGTC
```

FIG. 9 (Continued)

```
GTGTCCTTTG TCGATACTGG TACTAATGCG GTTCGAGCTT TAATTGGGAG TGATTTCCCT TGTTTTCGAC CTCGAGGTGG CGCCACCGCC GGAGCTCCAG
                                                Sv40
8501 GAGATCCGGT CGACCAGCAA CCATAGTCCC GCCCCTAACT CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCGAGCTATT GTCCGCCCCA TGGCTGACTA
     CTCTAGGCCA GCTGGTCGTT GGTATCAGGG CGGGGATTGA GGCGGGGATTG AGGCGGGTCA AGGCGGGTAA GAGGCGGGGT ACCGACTGAT
                                          Sv40
8601 ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG CCTAGGCTTT TGCAAAAAGC
     TAAAAAAAAT AAATACGTCT CCGGCTCCGG CGGAGCCGGA GACTCGATAA GGTCTTCATC ACTCCTCCGA AAAAACCTCC GGATCCGAAA ACGTTTTTCG
                                                CMV
8701 TTCGACGGTA TCGATTGGCT CATGTCCAAC ATTACCGCCA TGTTGACATT GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT
     AAGCTGCCAT AGCTAACCGA GTACAGGTTG TAATGGCGGT ACAACTGTAA CTAATAACTG ATCAATAATT ATCATTAGTT AATGCCCCAG TAATCAAGTA
                                                CMV
8801 AGCCCATATA TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG
     TCGGGTATAT ACCTCAAGGC GCAATGTATT GAATGCCATT TACCGGGCGG ACCGACTGGC GGGTTGCTGG GGGCGGGTAA CTGCAGTTAT TACTGCATAC
                                                CMV
8901 TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
     AAGGGTATCA TTGCGGTTAT CCCTGAAAGG TAACTGCAGT TACCCACCTC ATAAATGCCA TTTGACGGGT GAACCGTCAT GTAGTTCACA TAGTATACGG
                                                CMV
9001 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC
     TTCATGCGGG GGATAACTGC AGTTACTGCC ATTTACCGGG CGGACCGTAA TACGGGTCAT GTACTGGAAT ACCCTGAAAG GATGAACCGT CATGTAGATG
                                                CMV
9101 GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA
     CATAATCAGT AGCGATAATG GTACCACTAC GCCAAAAACCG TCATGTAGTT ACCCGCACCT ATCGCCAAAC TGAGTGCCCC TAAAGGTTCA GAGGTGGGGT
                                                CMV
9201 TTGACGTCAA TGGGAGTTTG TTTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT
     AACTGCAGTT ACCCTCAAAC AAAACCGTGG TTTTAGTTGC CCTGAAAGGT TTTACAGCAT TGTTGAGGCG GGGTAACTGC GTTTACCCGC CATCCGCACA
          CMV
9301 ACGGAATTCG GACTGGCGAG CCCTCAGATC CTGCATATAA GCAGCTGCTT TTTGCCTGTA CTGGGTCTCT CTG
     TGCCTTAAGC CTCACCGCTC GGGAGTCTAG GACGTATATT CGTCGACGAA AAACGGACAT GACCCAGAGA GAC
```

FIG. 9 (Continued)

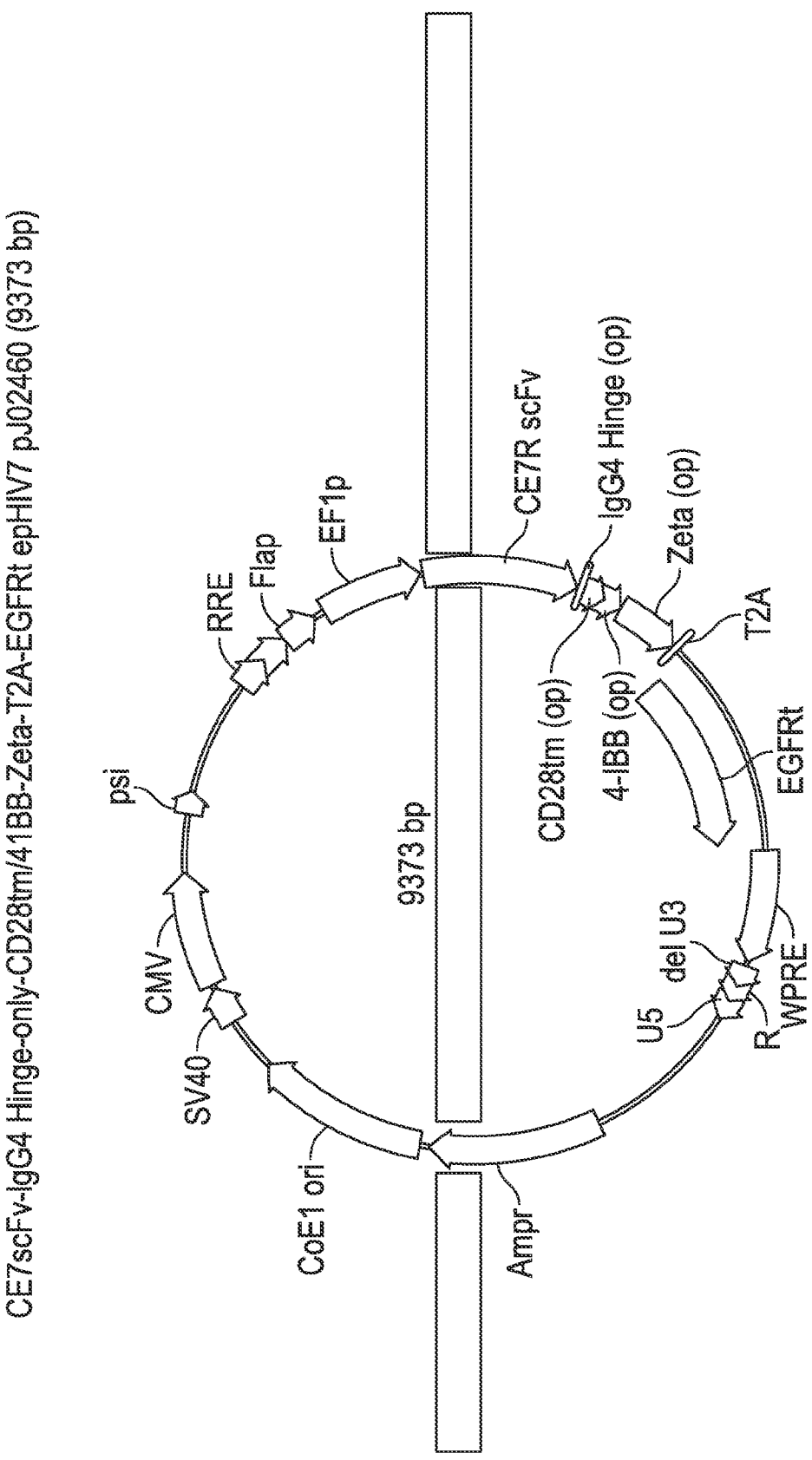

CE7scFv-IgG4 Hinge-only-CD28tm/41BB-Zeta-T2A-EGFRt epHIV7 pJ02460 (9373 bp)
9373 bp
psi
RRE
Flap
EF1p
CE7R scFv
IgG4 Hinge (op)
Zeta (op)
T2A
CD28tm (op)
4-IBB (op)
EGFRt
del U3
U5
R
WPRE
CMV
SV40
CoE1 ori
Ampr

FIG. 10

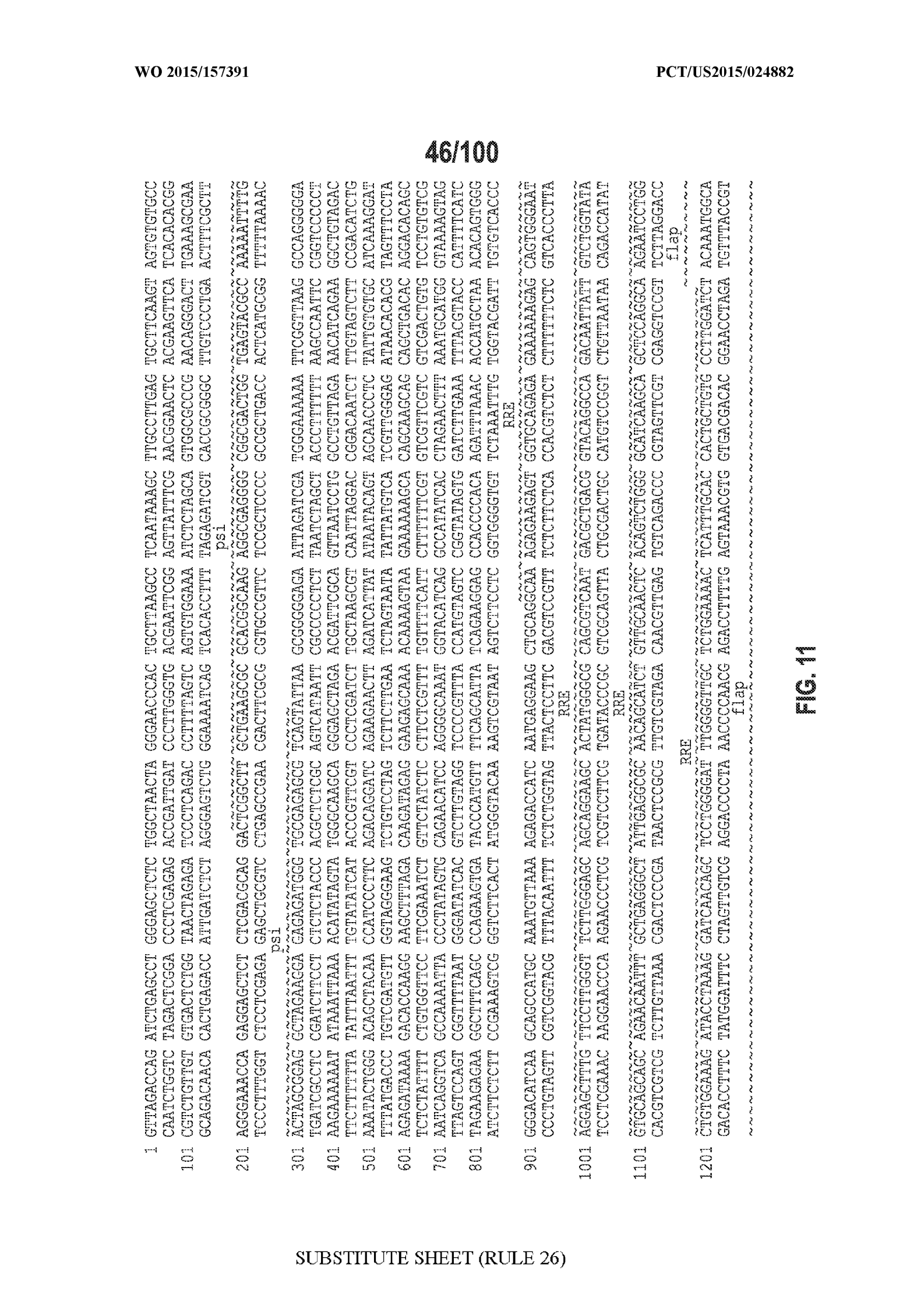


FIG. 11

```
1301 GTATTCATCC ACAATTTTAA AAGAAAAGGG GGGATTGGGG GGTACAGTGC AGGGGAAAGA ATAGTAGACA TAATAGCAAC AGACATACAA ACTAAAGAAT
     CATAAGTAGG TGTTAAAATT TTCTTTTCCC CCCTAACCCC CCATGTCACG TCCCCTTTCT TATCATCTGT ATTATCGTTG TCTGTATGTT TGATTTCTTA
                                  flap

1401 TACAAAAACA AATTACAAAA ATTCAAAATT TTCGGGTTTA TTACAGGGAC AGCAGAGATC CAGTTTGGGG ATCAATTGCA TGAAGAATCT GCTTAGGGTT
     ATGTTTTTGT TTAATGTTTT TAAGTTTTAA AAGCCCAAAT AATGTCCCTG TCGTCTCTAG GTCAAACCCC TAGTTAACGT ACTTCTTAGA CGAATCCCAA
                                                  EF1p

1501 AGGCGTTTTG CGCTGCTTCG CGATGCATCTG CGATCGCTCC GGTGCCCGTC AGTGGGCAGA GCGCACATCG CCCACAGTCC CCGAGAAGTT GGGGGAGGGG
     TCCGCAAAAC GCGACGAAGC GCTACTAGAC GCTAGCGAGG CCACGGGCAG TCACCCGTCT CGCGTGTAGC GGGTGTCAGG GGCTCTTCAA CCCCCCTCCC
                                  EF1p

1601 GTCGGCAATT GAACCGGTGC CTAGAGAAGG TGGCGCGGGG TAAACTGGGA AAGTGATGTC GTGTACTGGC TCCGCCTTTT TCCCGAGGGT GGGGGAGAAC
     CAGCCGTTAA CTTGGCCACG GATCTCTTCC ACCGCGCCCC ATTTGACCCT TTCACTACAG CACATGACCG AGGCGGAAAA AGGGCTCCCA CCCCCTCTTG
                                  EF1p

1701 CGTATATAAG TGCAGTAGTC GCCGTGAACG TTCTTTTTCG CAACGGGTTT GCCGCCAGAA CACAGCTGAA GCTTCGAGGG GCTCGCATCT CTCCTTCACG
     GCATATATTC ACGTCATCAG CGGCACTTGC AAGAAAAAGC GTTGCCCAAA CGGCGGTCTT GTGTCGACTT CGAAGCTCCC CGAGCGTAGA GAGGAAGTGC
                                  EF1p

1801 CGCCCGCCGC CCTACCTGAG GCCGCCATCC ACGCCGGTTG AGTCGCGTTC TGCCGCCTCC CGCCTGTGGT GCCTCCTGAA CTGCGTCCGC CGTCTAGGTA
     GCGGGCGGCG GGATGGACTC CGGCGGTAGG TGCGGCCAAC TCAGCGCAAG ACGGCGGAGG GCGGACACCA CGGAGGACTT GACGCAGGCG GCAGATCCAT
                                  EF1p

1901 AGTTTAAAGC TCAGGTCGAG ACCGGGCCTT TGTCCGGCGC TCCCTTGGAG CCTACCTAGA CTCAGCCGGC TCTCCACGCT TTGCCTGACC CTGCTTGCTC
     TCAAATTTCG AGTCCAGCTC TGGCCCGGAA ACAGGGCCGCG AGGGAACCTC GGATGGATCT GAGTCGGCCG AGAGGTGCGA AACGGACTGG GACGAACGAG
                                  EF1p                                                  CE7R scFv

2001 AACTCTACGT CTTTGTTTCG TTTTCTGTTC TGCGCCGTTA CAGATCCAAG CTGTGACCGG CGCCTACGGC TAGCGCCGCC ACCATGCTGC TGCTGGTGAC
     TTGAGATGCA GAAACAAAGC AAAAGACAAG ACGCGGCAAT GTCTAGGTTC GACACTGGCC GCGGATGCCG ATCGCGGCGG TGGTACGACG ACGACCACTG
                                                  CE7R scFv

2101 CAGCCTGCTG CTGTGCGAGC TGCCCCACCC CGCCTTTCTG CTGATCCCCC AGGTGCAGCT GCAGCAGCCT GGCGCCGAGC TGGTGAAGCC AGGCGCCAGC
     GTCGGACGAC GACACGCTCG ACGGGGTGGG GCGGAAAGAC GACTAGGGGG TCCACGTCGA CGTCGTCGGA CCGCGGCTCG ACCACTTCGG TCCGCGGTCG
                                  CE7R scFv

2201 GTGAAGCTGT CCTGCAAGGC CAGCGGCTAC ACCTTCACCG GCTACTGGAT GCACTGGGTG AAGCAGAGAC CCGGCCACGG CCTGGAATGG ATCGGCGAGA
     CACTTCGACA GGACGTTCCG GTCGCCGATG TGGAAGTGGC CGATGACCTA CGTGACCCAC TTCGTCTCTG GGCCGGTGCC GGACCTTACC TAGCCGCTCT
                                  CE7R scFv

2301 TCAACCCCAG CAACGGCCGG ACCAACTACA ACGAGCGGTT CAAGAGCAAG GCCACCCTGA CCGTGGACAA GAGCAGCACC ACCGCCTTCA TGCAGCTGTC
     AGTTGGGGTC GTTGCCGGCC TGGTTGATGT TGCTCGCCAA GTTCTCGTTC CGGTGGGACT GGCACCTGTT CTCGTCGTGG TGGCGGAAGT ACGTCGACAG
                                  CE7R scFv

2401 CGGCCTGACC AGCGAGGACA GCGCCGTGTA CTTCTGCGCC AGGGACTACT ACGGCACCAG CTACAACTTC GACTACTGGG GCCAGGGCAC CACACTGACC
     GCCGGACTGG TCGCTCCTGT CGCGGCACAT GAAGACGCGG TCCCTGATGA TGCCGTGGTC GATGTTGAAG CTGATGACCC CGGTCCCGTG GTGTGACTGG
                                  CE7R scFv
```

FIG. 11 (Continued)

```
2501 GTCAGCAGCG GCGGAGGGGG CTCTGGCGGC GGAGGATCTG GGGGAGGGGG CAGCGACATC CAGATGACCC AGAGCAGCAG CAGCTTCAGC GTGAGCCTGG
     CACTCGTCGC CGCCTCCCCC GAGACCGCCG CCTCCTAGAC CCCCTCCCCC GTCGCTGTAG GTCTACTGGG TCTCGTCGTC GTCGAAGTCG CACTCGGACC
                                        CE7R scFv

2601 GCGACCGGGT GACCATCACC TGTAAGGCCA ACGAGGACAT CAACAACCGG CTGGCCTGGT ATCAGCAGAC CCCCGGCAAC AGCCCCAGGC TGCTGATCAG
     CGCTGGCCCA CTGGTAGTGG ACATTCCGGT TGCTCCTGTA GTTGTTGGCC GACCGGACCA TAGTCGTCTG GGGGCCGTTG TCGGGGTCCG ACGACTAGTC
                                        CE7R scFv

2701 CGGCGCCACC AACCTGGTGA CCGGCGTGCC CAGCCGGTTT AGCGGCACGG GCTCCGGCAA GGACTACACC CTGACCATCA CAAGCCTGCA GGCCGAGGAC
     GCCGCGGTGG TTGGACCACT GGCCGCACGG GTCGGCCAAA TCGCCGTCGC CGAGGCCGTT CCTGATGTGG GACTGGTAGT GTTCGGACGT CCGGCTCCTG
                                        CE7R scFv                                                   IgG4 Hinge

2801 TTCGCCACCT ACTACTGCCA GCAGTACTGG TCCACCCCCT TCACCTTCGG CAGCGGCACC GAGCTGGAAA TCAAAGAATC TAAGTACGGA CCGCCCTGCC
     AAGCGGTGGA TGATGACGGT CGTCATGACC AGGTGGGGGA AGTGGAAGCC GTCGCCGTGG CTCGACCTTT AGTTCTTAG ATTCATGCCT GGCGGGACGG
                                                  CD28tm                                                    CD28gg
     IgG4 Hinge
2901 CCCCTTGCCC TATGTTCTGG GTGCTGGTGG TGGTCGGAGG CGTGCTGGCC TGCTACAGCC TGCTGGTCAC CGTGGCCTTC ATCATCTTTT GGGTCCGCAG
     GGGGAACGGG ATACAAGACC CACGACCACC ACCAGCCTCC GCACGACCGG ACGATGTCGG ACGACCAGTG GCACCGGAAG TAGTAGAAAA CCCAGGCGTC
                                        CD28gg

3001 CAAGCGGAGC AGAGGCGGCC ACAGCGACTA CATGAACATG ACCCCTAGAC GGCCTGGCCC CACCAGAAAG CACTACCAGC CCTACGCCCC TCCCCGGGAC
     GTTCGCCTCG TCTCCGCCGG TGTCGCTGAT GTACTTGTAC TGGGGATCTG CCGGACCGGG GTGGTCTTTC GTGATGGTCG GGATGCGGGG AGGGGCCCTG
       CD28gg

3101 TTTGCCGCCT ACAGAAGCAA ACGGGGCAGA AAGAACTCCT TGTATATATT CAAACAACCA TTTATGAGAC CAGTACAAAC TACTCAAGAG GAAGATGGCT
     AAACGGCGGA TGTCTTCGTT TGCCCCGTCT TTCTTTGAGG ACATATATAA GTTTGTTGGT AAATACTCTG GTCATGTTTG ATGAGTTCTC CTTCTACCGA
                                                                                  Zeta

     4-1BB
3201 GTAGCTGCCG ATTTCCAGAA GAAGAAGAAG GAGGATGTGA ACTGCGGGTG AAGTTCAGCA GAAGCGCCGA CGCCCCTGCC TACCAGCAGG GCCAGAATCA
     CATCGACGGC TAAAGGTCTT CTTCTTCTTC CTCCTACACT TGACGCCCAC TTCAAGTCGT CTTCGCGGCT GCGGGGACGG ATGGTCGTCC CGGTCTTAGT
                                        Zeta

3301 GCTGTACAAC GAGCTGAACC TGGGCAGAAG GGAAGAGTAC GACGTCCTGG ATAAGCGGAG AGGCCGGGAC CCTGAGATGG GCGGCAAGCC TCGGCGAAG
     CGACATGTTG CTCGACTTGG ACCCGTCTTC CCTTCTCATG CTGCAGGACC TATTCGCCTC TCCGGCCCTG GGACTCTACC CGCCGTTCGG AGCCGCTTC
                                        Zeta

3401 AACCCCCAGG AAGGCCTGTA TAACGAACTG CAGAAAGACA AGATGGCCGA GGCCTACAGC GAGATCGGCA TGAAGGGCGA GCGGAGGCGG GGCAAGGGCC
     TTGGGGGTCC TTCCGGACAT ATTGCTTGAC GTCTTTCTGT TCTACCGGCT CCGGATGTCG CTCTAGCCGT ACTTCCCGCT CGCCTCCGCC CCGTTCCCGG
                                                                                                T2A
```

FIG. 11 (Continued)

```
                                         Zeta
3501 ACGACGGCCT GTATCAGGGC CTGTCCACCG CCACCAAGGA TACCTACGAC GCCCTGCACA TGCAGGCCCT GCCCCCAAGG CTCGAGGGCG GCGGAGAGGG
     TGCTGCCGGA CATAGTCCCG GACAGGTGGC GGTGGTTCCT ATGGATGCTG CGGGACGTGT ACGTCCGGGA CGGGGGTTCC GAGCTCCCGC CGCCTCTCCC
                                                                                   EGFRt
                               T2A
3601 CAGAGGAAGT CTTCTAACAT GCGGTGACGT GGAGGAGAAT CCCGGCCCTA GGATGCTTCT CCTGGTGACA AGCCTTCTGC TCTGTGAGTT ACCACACCCA
     GTCTCCTTCA GAAGATTGTA CGCCACTGCA CCTCCTCTTA GGGCCGGGAT CCTACGAAGA GGACCACTGT TCGGAAGACG AGACACTCAA TGGTGTGGGT
                                                       EGFRt
3701 GCATTCCTCC TGATCCCACG CAAAGTGTGT AACGGAATAG GTATTGGTGA ATTTAAAGAC TCACTCTCCA TAAATGCTAC GAATATTAAA CACTTCAAAA
     CGTAAGGAGG ACTAGGGTGC GTTTCACACA TTGCCTTATC CATAACCACT TAAATTTCTG AGTGAGAGGT ATTTACGATG CTTATAATTT GTGAAGTTTT
                                                       EGFRt
3801 ACTGCACCTC CATCAGTGGC GATCTCCACA TCCTGCCGGT GGCATTTAGG GGTGACTCCT TCACACATAC TCCTCCTCTG GATCCACAGG AACTGGATAT
     TGACGTGGAG GTAGTCACCG CTAGAGGTGT AGGACGGCCA CCGTAAATCC CCACTGAGGA AGTGTGTATG AGGAGGAGAC CTAGGTGTCC TTGACCTATA
                                                       EGFRt
3901 TCTGAAAACC GTAAAGGAAA TCACAGGGTT TTTGCTGATT CAGGCTTGGC CTGAAAACAG GACGGACCTC CATGCCTTTG AGAACCTAGA AATCATACGC
     AGACTTTTGG CATTTCCTTT AGTGTCCCAA AAACGACTAA GTCCGAACCG GACTTTTGTC CTGCCTGGAG GTACGGAAAC TCTTGGATCT TTAGTATGCG
                                                       EGFRt
4001 GGCAGGACCA AGCAACATGG TCAGTTTTCT CTTGCAGTCG TCAGCCTGAA CATAACATCC TTGGGATTAC GCTCCCTCAA GGAGATAAGT GATGGAGATG
     CCGTCCTGGT TCGTTGTACC AGTCAAAAGA GAACGTCAGC AGTCGGACTT GTATTGTAGG AACCCTAATG CGAGGGAGTT CCTCTATTCA CTACCTCTAC
                                                       EGFRt
4101 TGATAATTTC AGGAAACAAA AATTTGTGCT ATGCAAATAC AATAAACTGG AAAAAACTGT TTGGGACCTC CGGTCAGAAA ACCAAAATTA TAAGCAACAG
     ACTATTAAAG TCCTTTGTTT TTAAACACGA TACGTTTATG TTATTTGACC TTTTTTGACA AACCCTGGAG GCCAGTCTTT TGGTTTTAAT ATTCGTTGTC
                                                       EGFRt
4201 AGGTGAAAAC AGCTGCAAGG CCACAGGCCA GGTCTGCCAT GCCTTGTGCT CCCCCGAGGG CTGCTGGGGC CCGGAGCCCA GGGACTGCGT CTCTTGCCGG
     TCCACTTTTG TCGACGTTCC GGTGTCCGGT CCAGACGGTA CGGAACACGA GGGGGCTCCC GACGACCCCG GGCCTCGGGT CCCTGACGCA GAGAACGGCC
                                                       EGFRt
4301 AATGTCAGCC GAGGCAGGGA ATGCGTGGAC AAGTGCAACC TTCTGGAGGG TGAGCCAAGG GAGTTTGTGG AGAACTCTGA GTGCATACAG TGCCACCCAG
     TTACAGTCGG CTCCGTCCCT TACGCACCTG TTCACGTTGG AAGACCTCCC ACTCGGTTCC CTCAAACACC TCTTGAGACT CACGTATGTC ACGGTGGGTC
                                                       EGFRt
4401 AGTGCCTGCC TCAGGCCATG AACATCACCT GCACAGGACG GGGACCAGAC AACTGTATCC AGTGTGCCCA CTACATTGAC GGCCCCCACT GCGTCAAGAC
     TCACGGACGG AGTCCGGTAC TTGTAGTGGA CGTGTCCTGC CCCTGGTCTG TTGACATAGG TCACACGGGT GATGTAACTG CCGGGGGTGA CGCAGTTCTG
                                                       EGFRt
4501 CTGCCCGGCA GGAGTCATGG GAGAAAACAA CACCCTGGTC TGGAAGTACG CAGACGCCGG CCATGTGTGC CACCTGTGCC ATCCAAACTG CACCTACGGA
     GACGGGCCGT CCTCAGTACC CTCTTTTGTT GTGGGACCAG ACCTTCATGC GTCTGCGGCC GGTACACACG GTGGACACGG TAGGTTTGAC GTGGATGCCT
                                                       EGFRt
```

FIG. 11 (Continued)

```
4601 TGCACTGGGC CAGGTCTTGA AGGCTGTCCA ACGAATGGGC CTAAGATCCC GTCCATCGCC ACTGGGATGG TGGGGGCCCT CCTCTTGCTG CTGGTGGTGG
     ACGTGACCCG GTCCAGAACT TCCGACAGGT TGCTTACCCG GATTCTAGGG CAGGTAGCGG TGACCCTACC ACCCCCGGGA GGAGAACGAC GACCACCACC
                EGFRt

4701 CCCTGGGGAT CGGCCTCTTC ATGTGAGCGG CCGCTCTAGA CCCGGGCTGC AGGAATTCGA TATCAAGCTT ATCGATAATC AACCTCTGGA TTACAAAATT
     GGGACCCCTA GCCGGAGAAG TACACTCGCC GGCGAGATCT GGGCCCGACG TCCTTAAGCT ATAGTTCGAA TAGCTATTAG TTGGAGACCT AATGTTTTAA
                                          WPRE

4801 TGTGAAAGAT TGACTGGTAT TCTTAACTAT GTTGCTCCTT TTACGCTATG TGGATACGCT GCTTTAATGC CTTTGTATCA TGCTATTGCT TCCCGTATGG
     ACACTTTCTA ACTGACCATA AGAATTGATA CAACGAGGAA AATGCGATAC ACCTATGCGA CGAAATTACG GAAACATAGT ACGATAACGA AGGGCATACC
                                          WPRE

4901 CTTTCATTTT CTCCTCCTTG TATAAATCCT GGTTGCTGTC TCTTTATGAG GAGTTGTGGC CCGTTGTCAG GCAACGTGGC GTGGTGTGCA CTGTGTTTGC
     GAAAGTAAAA GAGGAGGAAC ATATTTAGGA CCAACGACAG AGAAATACTC CTCAACACCG GGCAACAGTC CGTTGCACCG CACCACACGT GACACAAACG
                                          WPRE

5001 TGACGCAACC CCCACTGGTT GGGGCATTGC CACCACCTGT CAGCTCCTTT CCGGGACTTT CGCTTTCCCC CTCCCTATTG CCACGGCGGA ACTCATCGCC
     ACTGCGTTGG GGGTGACCAA CCCCGTAACG GTGGTGGACA GTCGAGGAAA GGCCCTGAAA GCGAAAGGGG GAGGGATAAC GGTGCCGCCT TGAGTAGCGG
                                          WPRE

5101 GCCTGCCTTG CCCGCTGCTG GACAGGGGCT CGGCTGTTGG GCACTGACAA TTCCGTGGTG TTGTCGGGGA AATCATCGTC CTTTCCTTGG CTGCTCGCCT
     CGGACGGAAC GGGCGACGAC CTGTCCCCGA GCCGACAACC CGTGACTGTT AAGGCACCAC AACAGCCCCT TTAGTAGCAG GAAAGGAACC GACGAGCGGA
                                          WPRE

5201 GTGTTGCCAC CTGGATTCTG CGCGGGACGT CCTTCTGCTA CGTCCCTTCG GCCCTCAATC CAGCGGACCT TCCTTCCCGC GGCCTGCTGC CGGCTCTGCG
     CACAACGGTG GACCTAAGAC GCGCCCTGCA GGAAGACGAT GCAGGGAAGC CGGGAGTTAG GTCGCCTGGA AGGAAGGGCG CCGGACGACG GCCGAGACGC
                                          WPRE                                                  del U3

5301 GCCTCTTCCG CGTCTTCGCC TTCGCCCTCA GACGAGTCGG ATCTCCCTTT GGGCCGCCTC CCCGCATCGA TACCGTCGAC TAGCCGTACC TTTAAGACCA
     CGGAGAAGGC GCAGAAGCGG AAGCGGGAGT CTGCTCAGCC TAGAGGGAAA CCCGGCGGAG GGGCGTAGCT ATGGCAGCTG ATCGGCATGG AAATTCTGGT
                                    del U3

                                                                                                                R

5401 ATGACTTACA AGGCAGCTGT AGATCTTAGC CACTTTTTAA AAGAAAAGGG GGGACTGGAA GGGCTAATTC ACTCCCAAAG AAGACAAGAT CTGCTTTTTG
     TACTGAATGT TCCGTCGACA TCTAGAATCG GTGAAAAATT TTCTTTTCCC CCCTGACCTT CCCGATTAAG TGAGGGTTTC TTCTGTTCTA GACGAAAAAC
                                                                                    U5

                                                R

5501 CCTGTACTGG GTCTCTCTGG TTAGACCAGA TCTGAGCCTG GGAGCTCTCT GGCTAACTAG GGAACCCACT GCTTAAGCCT CAATAAAGCT TGCCTTGAGT
     GGACATGACC CAGAGAGACC AATCTGGTCT AGACTCGGAC CCTCGAGAGA CCGATTGATC CCTTGGGTGA CGAATTCGGA GTTATTTCGA ACGGAACTCA
                                    U5

5601 GCTTCAAGTA GTGTGTGCCC GTCTGTTGTG TGACTCTGGT AACTAGAGAT CCCTCAGACC CTTTTAGTCA GTGTGGAAAA TCTCTAGCAG AATTCGATAT
     CGAAGTTCAT CACACAGGGG CAGACAACAC ACTGAGACCA TTGATCTCTA GGGAGTCTGG GAAAATCAGT CACACCTTTT AGAGATCGTC TTAAGCTATA
```

FIG. 11 (Continued)

```
5701 CAAGCTTATC GATACCGTCG ACCTCGAGGG GGGGCCCGGT ACCCAATTCG CCCTATAGTG AGTCGTATTA CAATTCACTG GCCGTCGTTT TACAACGTCG
     GTTCGAATAG CTATGGCAGC TGGAGCTCCC CCCCGGGCCA TGGGTTAAGC GGGATATCAC TCAGCATAAT GTTAAGTGAC CGGCAGCAAA ATGTTGCAGC
5801 TGACTGGGAA AACCCTGGCG TTACCCAACT TAATCGCCTT GCAGCACATC CCCCTTTCGC CAGCTGGCGT AATAGCGAAG AGGCCCGCAC CGATCGCCCT
     ACTGACCCTT TTGCGACCGC AATGGGTTGA ATTAGCGGAA CGTCGTGTAG GGGGAAAGCG GTCGACCGCA TTATCGCTTC TCCGGGCGTG GCTAGCGGGA
5901 TCCCAACAGT TGCGCAGCCT GAATGGCGAA TGGAAATTGT AAGCGTTAAT ATTTTGTTAA AATTCGCGTT AAATTTTTGT TAAATCAGCT CATTTTTTAA
     AGGGTTGTCA ACGCGTCGGA CTTACCGCTT ACCTTTAACA TTCGCAATTA TAAAACAATT GAATAGACCG AGATAGGGTT GAGTGTTGTT CCAGTTTGGA ACAAGAGTCC ACTATTAAAG
6001 CCAATAGGCC GAAATCGGCA AAATCCCTTA TAAATCAAAA GAATAGACCG AGATAGGGTT GAGTGTTGTT CCAGTTTGGA ACAAGAGTCC ACTATTAAAG
     GGTTATCCGG CTTTAGCCGT TTTAGGGAAT ATTTAGTTTT CTTATCTGGC TCTATCCCAA CTCACAACAA GGTCAAACCT TGTTCTCAGG TGATAATTTC
6101 AACGTGGACT CCAACGTCAA AGGGCGAAAA ACCGTCTATC AGGGCGATGG CCCACTACGT GAACCATCAC CCTAATCAAG TTTTTTGGGG TCGAGGTGCC
     TTGCACCTGA GGTTGCAGTT TCCCGCTTTT TGGCAGATAG TCCCGCTACC GGGTGATGCA CTTGGTAGTG GGATTAGTTC AAAAAACCCC AGCTCCACGG
6201 GTAAAGCACT AAATCGGAAC CCTAAAGGGA GCCCCCCGAT TAGAGCTTGA CGGGGAAAGC CGGCGAACGT GGCGAGAAAG GAAGGGAAGA AAGCGAAAGG
     CATTTCGTGA TTTAGCCTTG GGATTTCCCT CGGGGGCTAA ATCTCGAACT GCCCCTTTCG GCCGCTTGCA CCGCTCTTTC CTTCCCTTCT TTCGCTTTCC
6301 AGCGGGCGCT AGGGCGCTGG CAAGTGTAGC GGTCACGCTG CGCGTAACCA CCACACCCGC CGCGCTTAAT GCGCCGCTAC AGGGCGCGTC AGGTGGCACT
     TCGCCCGCGA TCCCGCGACC GTTCACATCG CCAGTGCGAC GCGCATTGGT GGTGTGGGCG GCGCGAATTA CGCGGCGATG TCCCGCGCAG TCCACCGTGA
6401 TTTCGGGGAA ATGTGCGCGG AACCCCTATT TGTTTATTTT TCTAAATACA TTCAAATATG TATCCGCTCA TGAGACAATA ACCCTGATAA ATGCTTCAAT
     AAAGCCCCTT TACACGCGCC TTGGGGATAA ACAAATAAAA AGATTTATAC AAGTTTATAC ATAGGCGAGT ACTCTGTTAT TGGGACTATT TACGAAGTTA
                                                    Ampr
6501 AATATTGAAA AAGGAAGAGT ATGAGTATTC AACATTTCCG TGTCGCCCTT ATTCCCTTTT TTGCGGCATT TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC
     TTATAACTTT TTCCTTCTCA TACTCATAAG TTGTAAAGGC ACAGCGGGAA TAAGGGAAAA AACGCCGTAA AACGGAAGGA CAAAAACGAG TGGGTCTTTG
                                                    Ampr
6601 GCTGGTGAAA GTAAAAGATG CTGAAGATCA GTTGGGTGCA CGAGTGGGTT ACATCGAACT GGATCTCAAC AGCGGTAAGA TCCTTGAGAG TTTTCGCCCC
     CGACCACTTT CATTTTCTAC GACTTCTAGT CAACCCACGT GCTCACCCAA TGTAGCTTGA CCTAGAGTTG TCGCCATTCT AGGAACTCTC AAAAGCGGGG
                                                    Ampr
6701 GAAGAACGTT TTCCAATGAT GAGCACTTTT AAAGTTCTGC TATGTGGCGC GGTATTATCC CGTATTGACG CCGGGCAAGA GCAACTCGGT CGCCGCATAC
     CTTCTTGCAA AAGGTTACTA CTCGTGAAAA TTTCAAGACG ATACACCGCG CCATAATAGG GCATAACTGC GGCCCGTTCT CGTTGAGCCA GCGGCGTATG
                                                    Ampr
6801 ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC AGAAAAGCAT CTTACGGATG GCATGACAGT AAGAGAATTA TGCAGTGCTG CCATAACCAT
     TGATAAGAGT CTTACTGAAC CAACTCATGA GTGGTCAGTG TCTTTTCCGTA GAATGCCTAC CGTACTGTCA TTCTCTTAAT ACGTCACGAC GGTATTGGTA
                                                    Ampr
6901 GAGTGATAAC ACTGCGGCCA ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC CGCTTTTTTG CACAACATGG GGGATCATGT AACTCGCCTT
     CTCACTATTG TGACGCCGGT TGAATGAAGA CTGTTGCTAG CCTCCTGGCT TCCTCGATTG GCGAAAAAAC GTGTTGTACC CCCTAGTACA TTGAGCGGAA
                                                    Ampr
7001 GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAAACG ACGAGCGTGA CACCACGATG CCTGTAGCAA TGGCAACAAC GTTGCGCAAA CTATTAACTG
     CTAGCAACCC TTGGCCTCGA CTTACTTCGG TATGGTTTGC TGCTCGCACT GTGGTGCTAC GGACATCGTT ACCGTTGTTG CAACGCGTTT GATAATTGAC
                                                    Ampr
7101 GCGAACTACT TACTCTAGCT TCCCGGCAAC AATTAATAGA CTGGATGGAG GCGGATAAAG TTGCAGGACC ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG
     CGCTTGATGA ATGAGATCGA AGGGCCGTTG TTAATTATCT GACCTACCTC CGCCTATTTC AACGTCCTGG TGAAGACGCG AGCCGGGAAG GCCGACCGAC
```

FIG. 11 (Continued)

FIG. 11 (Continued)

```
8501 ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACCATGATTA CGCCAAGCTC GAAATTAACC CTCACTAAAG GGAACAAAAG CTGGAGCTCC
     TAACACTCGC CTATTGTTAA AGTGTGTCCT TTGTCGATAC TGGTACTAAT GCGGTTCGAG CTTTAATTGG GAGTGATTTC CCTTGTTTTC GACCTCGAGG
                                                                SV40

8601 ACCGCGGTGG CGGCCTCGAG GTCGACGATC CGTCGACCAG CAACCATAGT CCCGCCCCTA ACTCCGCCCA TCCCGCCCCT AACTCCGCCC AGTTCCGCCC
     TGGCGCCACC GCCGGAGCTC CAGCTCTAGG CCAGCTGGTC GTTGGTATCA GGGCGGGGAT TGAGGCGGGA AGGGCGGGGA TTGAGGCGGG TCAAGGCGGG
                                                SV40

8701 ATTCTCCGCC CCATGGCTGA CTAATTTTTT TTATTTATGC AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG TAGTGAGGAG GCTTTTTTGG
     TAAGAGGCGG GGTACCGACT GATTAAAAAA AATAAATACG TCTCCGGCTC CGGCGGAGCC GGAGACTCGA TAAGGTCTTC ATCACTCCTC CGAAAAACC
                                                          CMV
           SV40

8801 AGGCCTAGGC TTTTGCAAAA AGCTTCGACG GTATCGATTG GCTCATGTCC AACATTACCG CCATGTTGAC ATTGATTATT GACTAGTTAT TAATAGTAAT
     TCCGGATCCG AAAACGTTTT TCGAAGCTGC CATAGCTAAC CGAGTACAGG TTGTAATGGC GGTACAACTG TAACTAATAA CTGATCAATA ATTATCATTA
                                                CMV

8901 CAATTACGGG GTCATTAGTT CATAGCCCAT ATATGGAGTT CCGCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA CCGCCCAACG ACCCCGCCC
     GTTAATGCCC CAGTAATCAA GTATCGGGTA TATACCTCAA GGCGCAATGT ATTGAATGCC ATTTACCGGG CGGACCGACT GGCGGGTTGC TGGGGGCGGG
                                                CMV

9001 ATTGACGTCA ATAATGACGT ATGTTCCCAT AGTAACGCCA ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC GGTAAACTGC CCACTTGGCA
     TAACTGCAGT TATTACTGCA TACAAGGGTA TCATTGCGGT TATCCCTGAA AGGTAACTGC AGTTACCCAC CTCATAAATG CCATTTGACG GGTGAACCGT
                                                CMV

9101 GTACATCAAG TGTATCATAT GCCAAGTACG CCCCCTATTG ACGTCAATGA CGGTAAATGG CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT
     CATGTAGTTC ACATAGTATA CGGTTCATGC GGGGGATAAC TGCAGTTACT GCCATTTACC GGGCGGACCG TAATACGGGT CATGTACTGG AATACCCTGA
                                                CMV

9201 TTCCTACTTG GCAGTACATC TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT GGATAGCGGT TTGACTCACG
     AAGGATGAAC CGTCATGTAG ATGCATAATC AGTAGCGATA ATGGTACCAC TACGCCAAAA CCGTCATGTA GTTACCCGCA CCTATCGCCA AACTGAGTGC
                                                CMV

9301 GGGATTTCCA AGTCTCCACC CCATTGACGT CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAAATGTC GTAACAACTC CGCCCCATTG
     CCCTAAAGGT TCAGAGGTGG GGTAACTGCA GTTACCCTCA AACAAAACCG TGGTTTTAGT TGCCCTGAAA GGTTTTACAG CATTGTTGAG GCGGGGTAAC
                CMV

9401 ACGCAAATGG GCGGTAGGCG TGTACGGAAT TCGGAGTGGC GAGCCCTCAG ATCCTGCATA TAAGCAGCTG CTTTTTGCCT GTACTGGGTC TCTCTG
     TGCGTTTACC CGCCATCCGC ACATGCCTTA AGCCTCACCG CTCGGGAGTC TAGGACGTAT ATTCGTCGAC GAAAAACGGA CATGACCCAG AGAGAC
```

FIG. 11 (Continued)

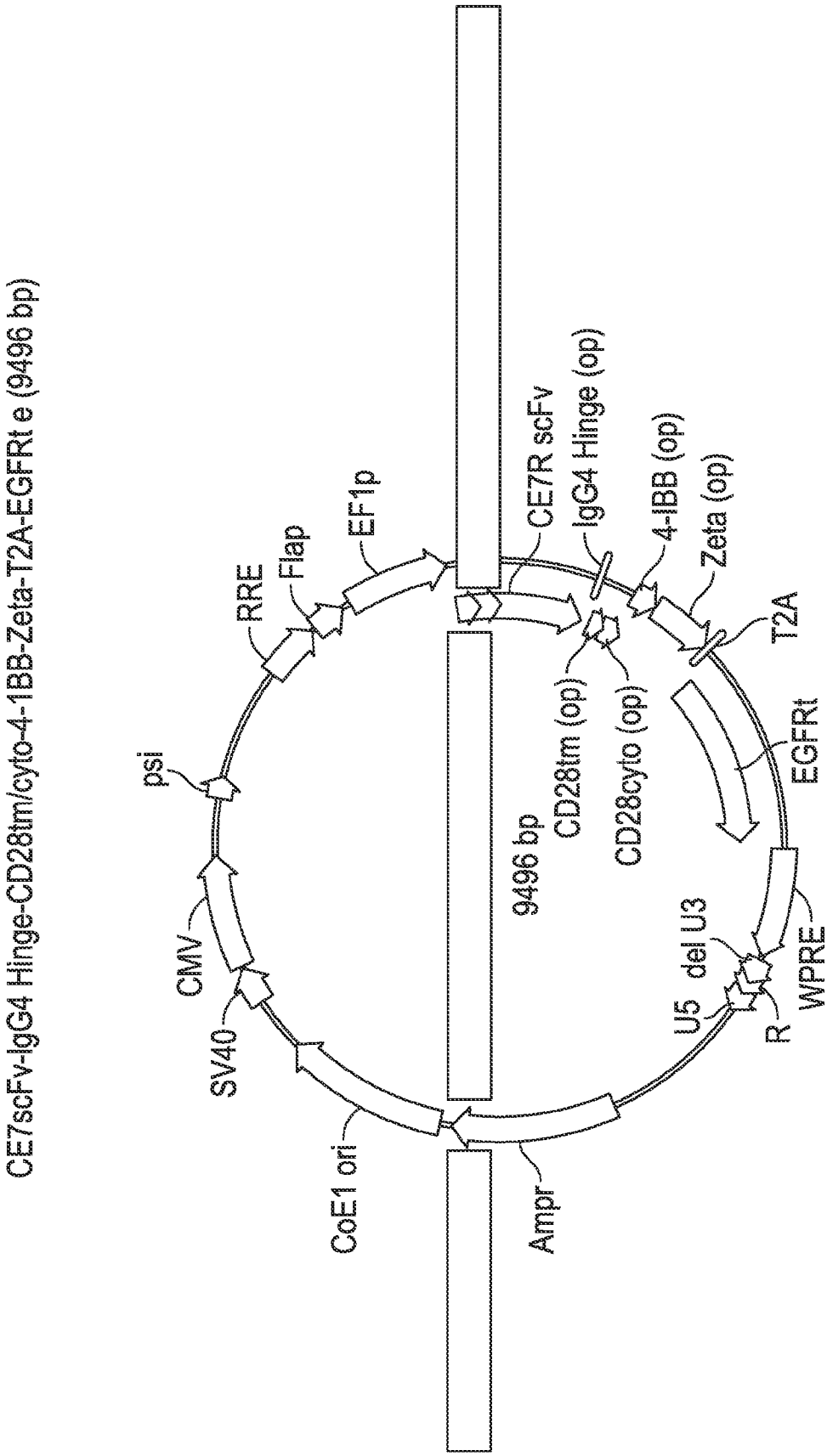

CE7scFv-IgG4 Hinge-CD28tm/cyto-4-1BB-Zeta-T2A-EGFRt e (9496 bp)
9496 bp
psi
CMV
SV40
CoE1 ori
Ampr
RRE
Flap
EF1p
CE7R scFv
IgG4 Hinge (op)
4-IBB (op)
Zeta (op)
T2A
EGFRt
CD28tm (op)
CD28cyto (op)
U5
del U3
R
WPRE

FIG. 12

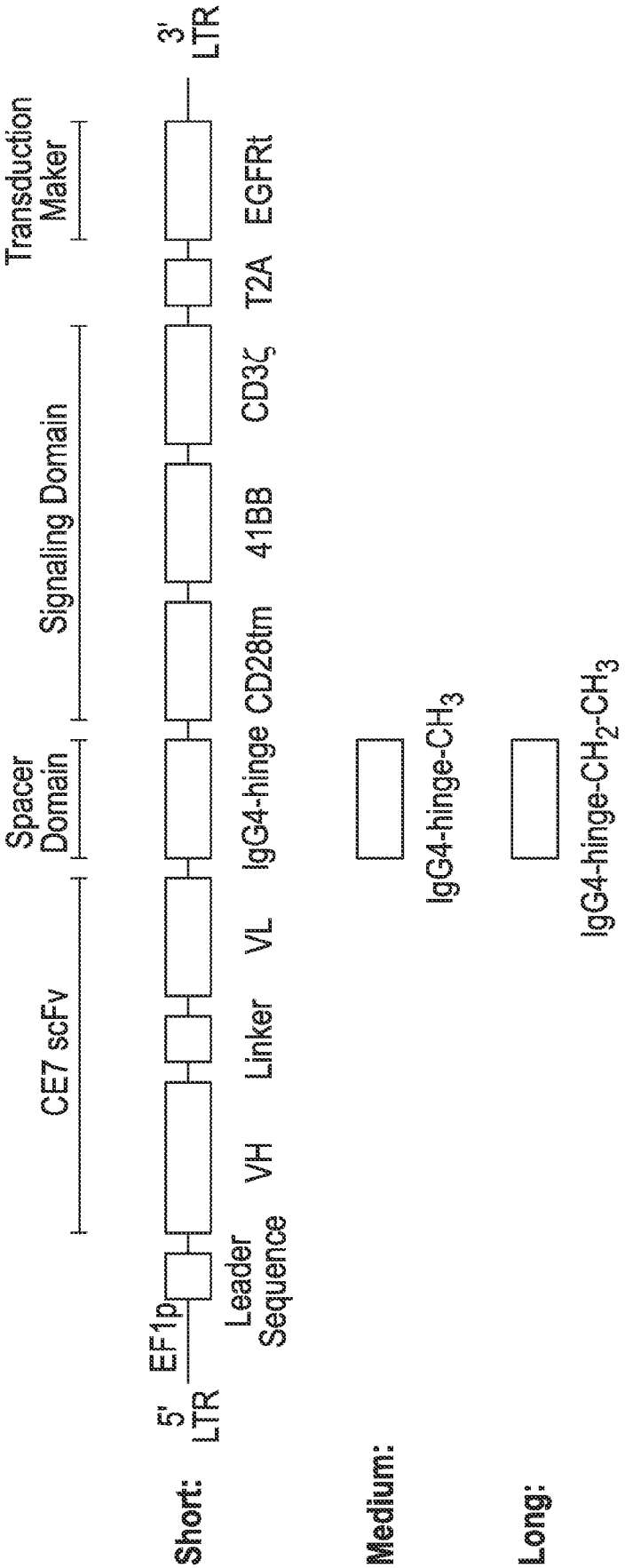
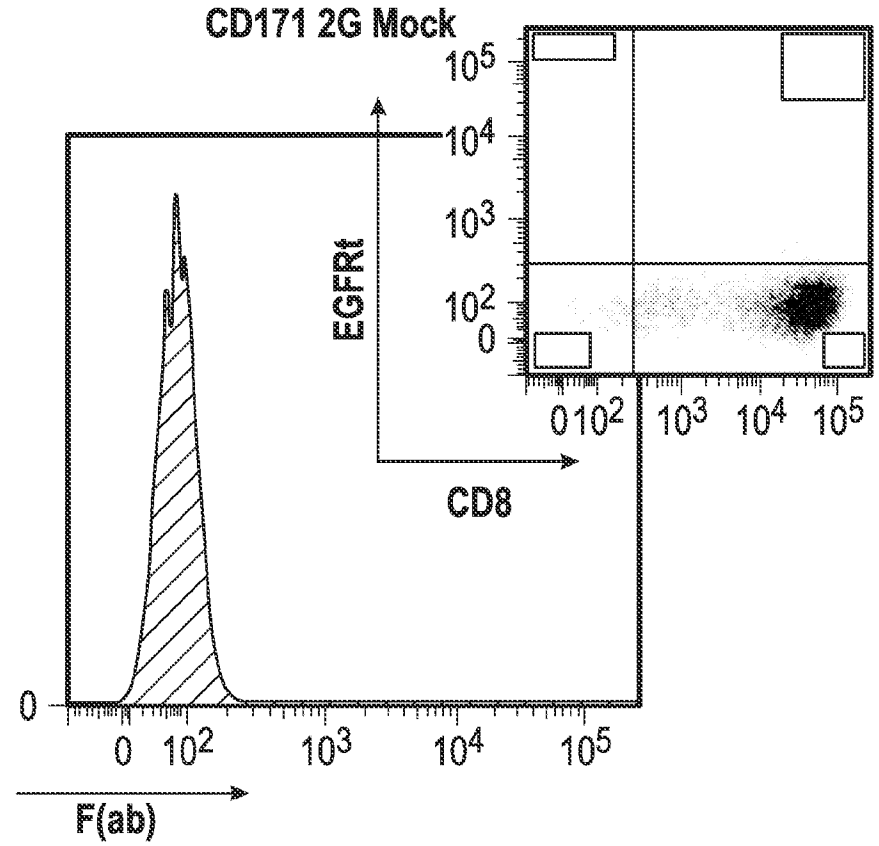

Short:
Medium:
Long:
5' LTR
EF1p
Leader Sequence
VH
Linker
VL
IgG4-hinge
CD28tm
41BB
CD3ζ
T2A
EGFRt
3' LTR
CE7 scFv
Spacer Domain
Signaling Domain
Transduction Maker
IgG4-hinge-CH3
IgG4-hinge-CH2-CH3

FIG. 13A

FIG. 13B


CD171 2G Mock
EGFRt
CD8
F(ab)
0
0 10^2 10^3 10^4 10^5

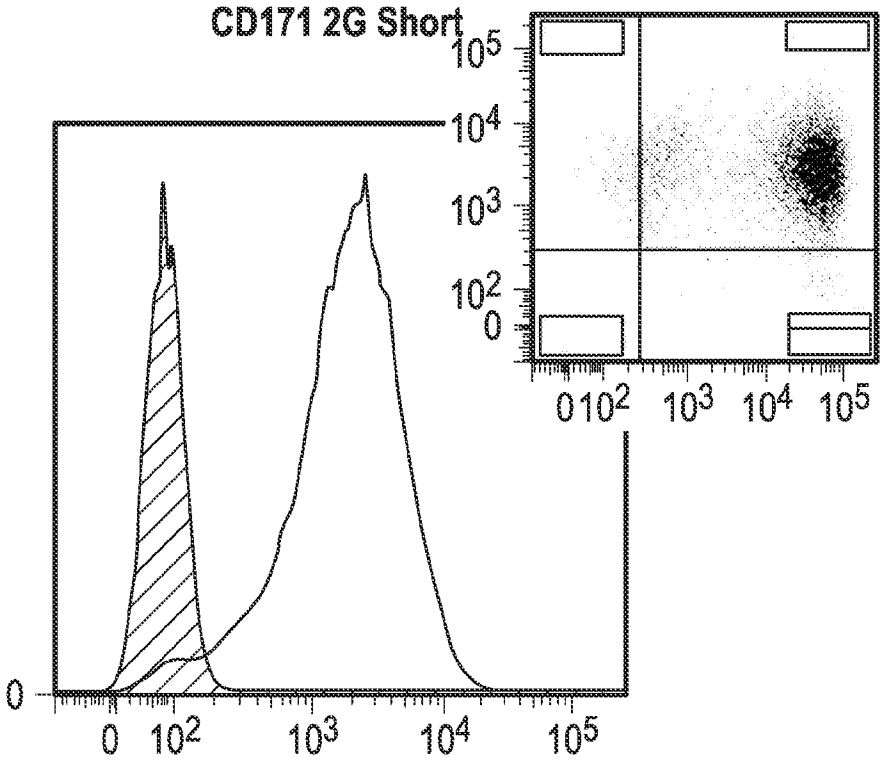
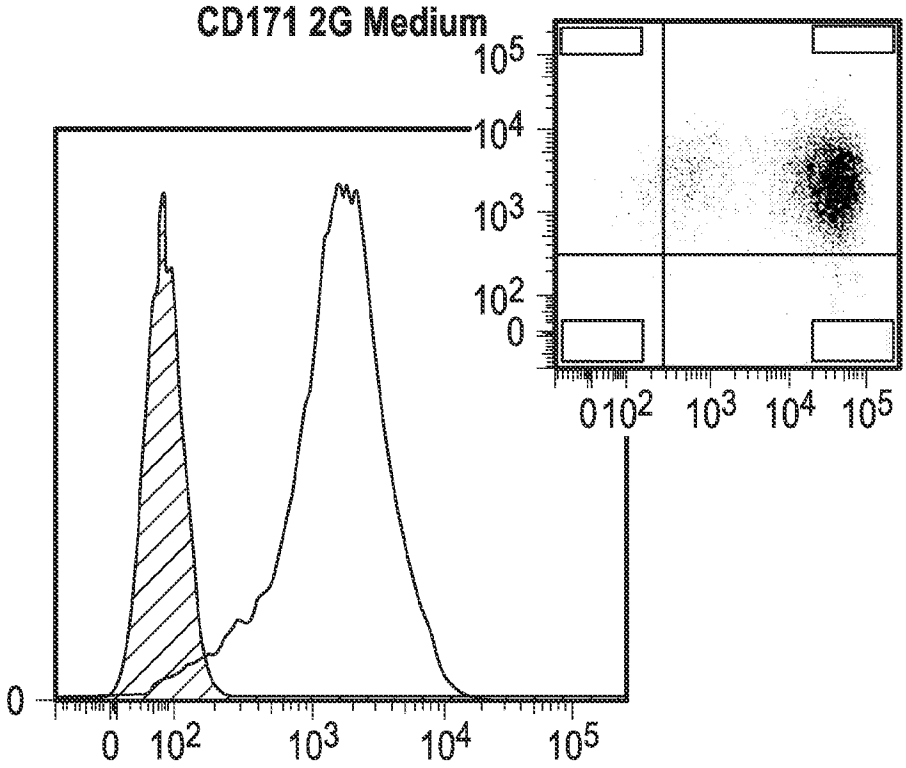
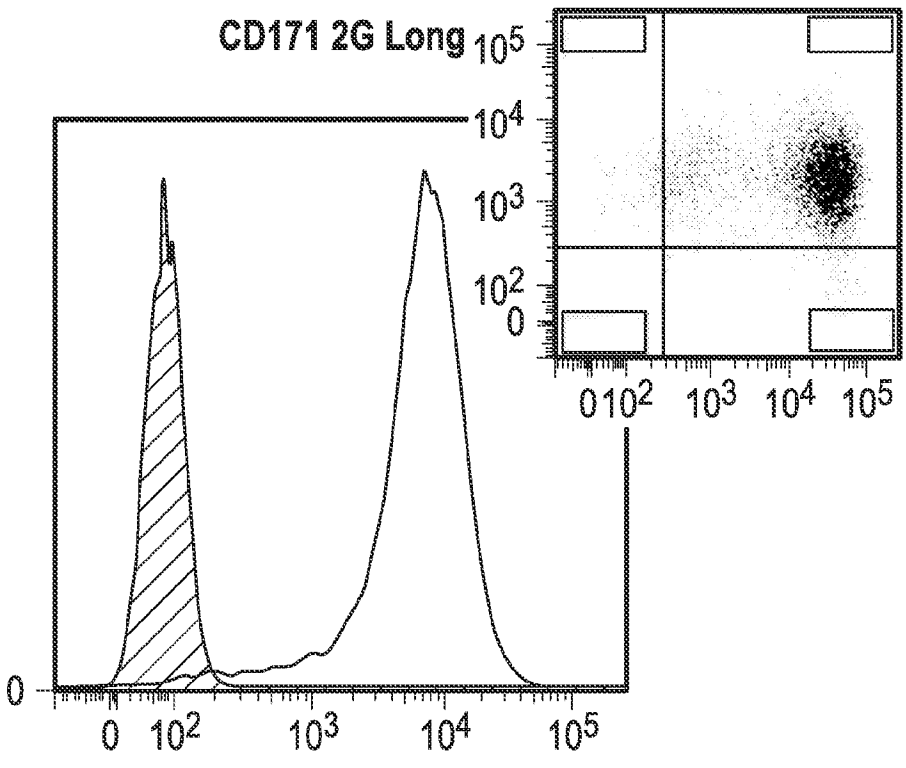

CD171 2G Short
0
0 10^2 10^3 10^4 10^5

FIG. 13B (Continued)





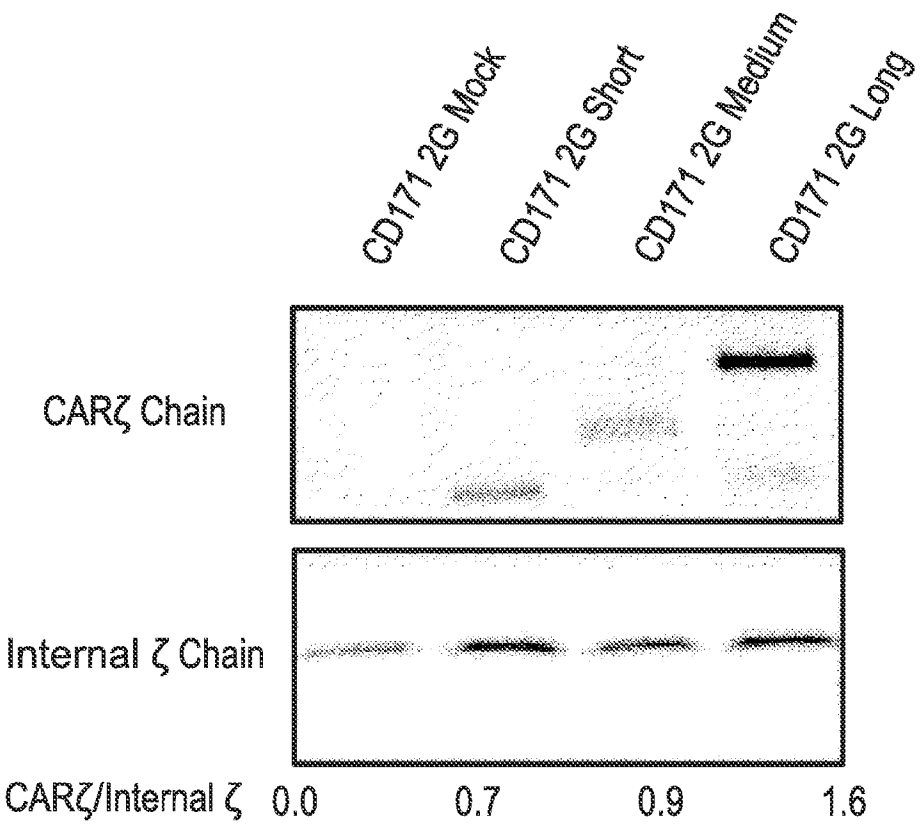

CD171 2G Mock
CD171 2G Short
CD171 2G Medium
CD171 2G Long
CARζ Chain
Internal ζ Chain
CARζ/Internal ζ
0.0
0.7
0.9
1.6

FIG. 13C

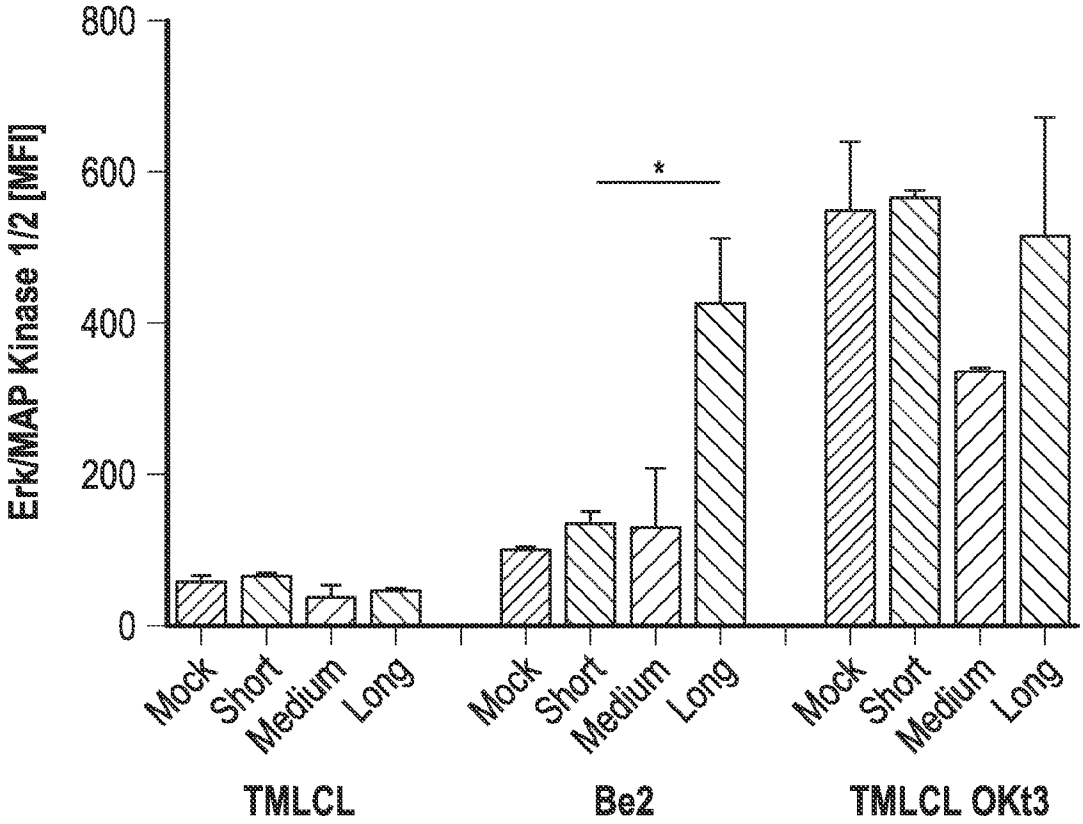


FIG. 13D

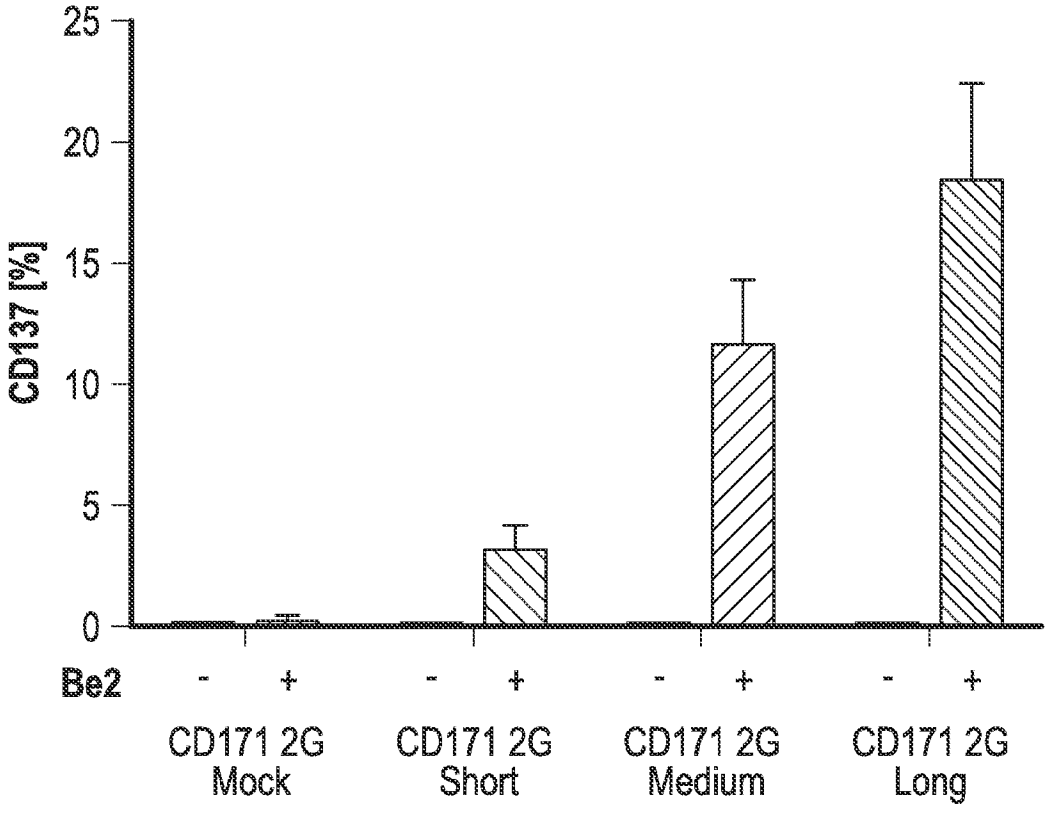


FIG. 13E

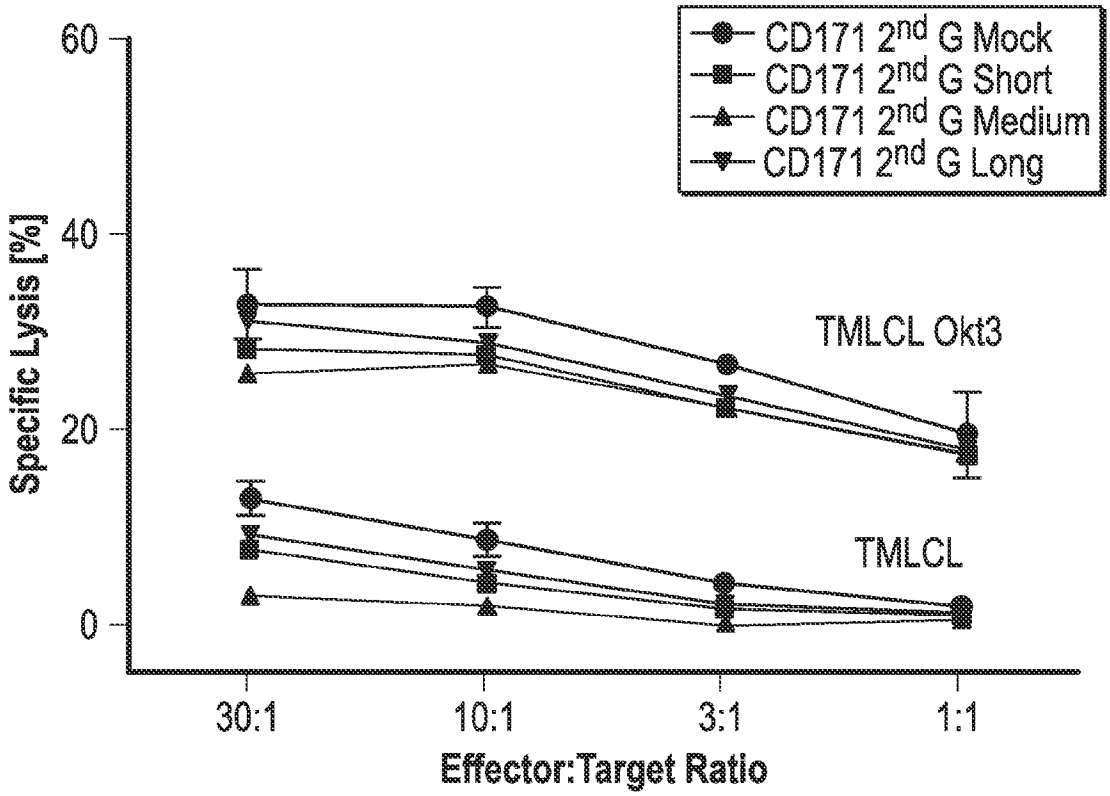

CD171 2nd G Mock
CD171 2nd G Short
CD171 2nd G Medium
CD171 2nd G Long
TMLCL Okt3
TMLCL
Specific Lysis [%]
60
40
20
0
30:1
10:1
3:1
1:1
Effector:Target Ratio

FIG. 13F

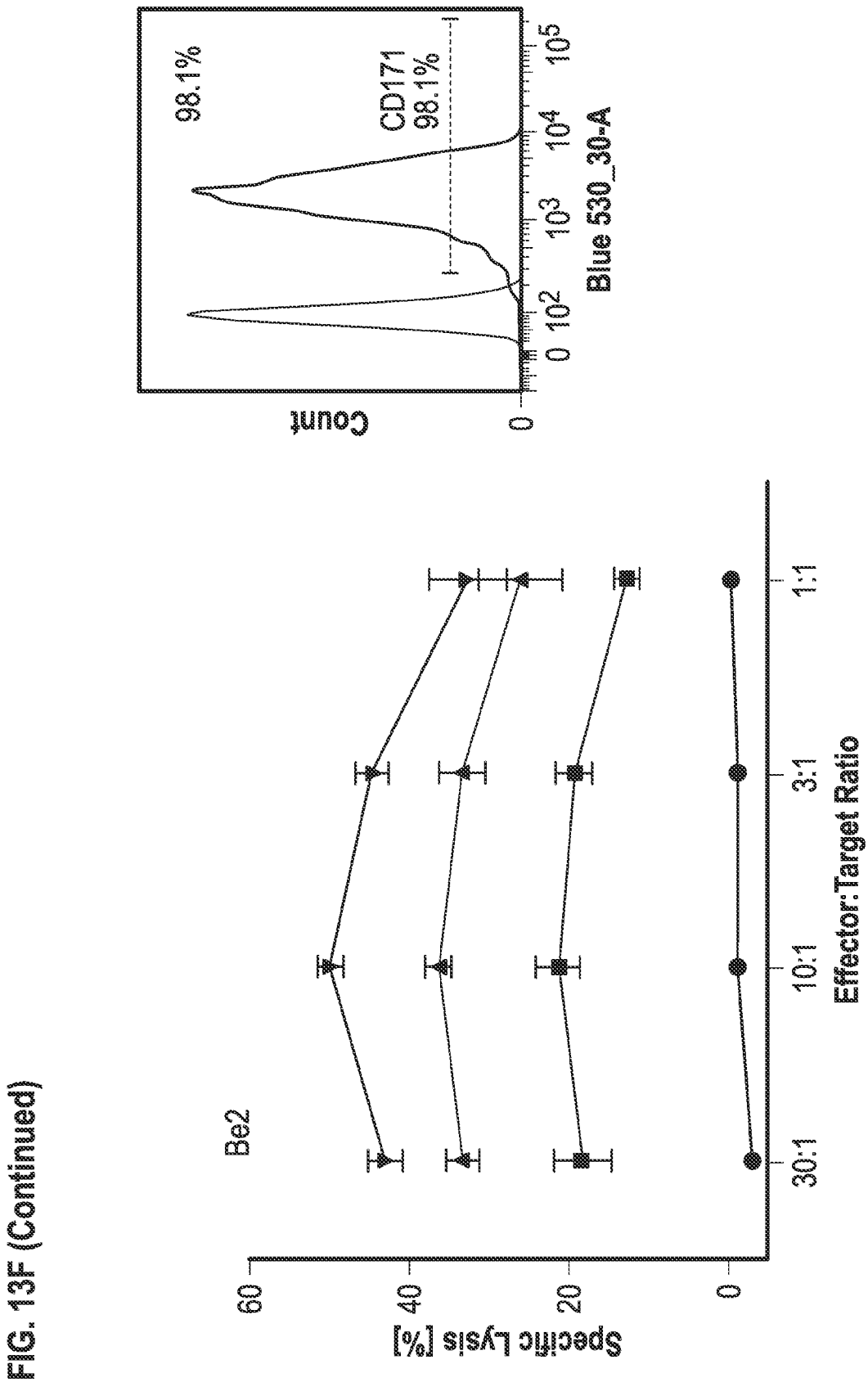


FIG. 13F (Continued)

FIG. 13F (Continued)

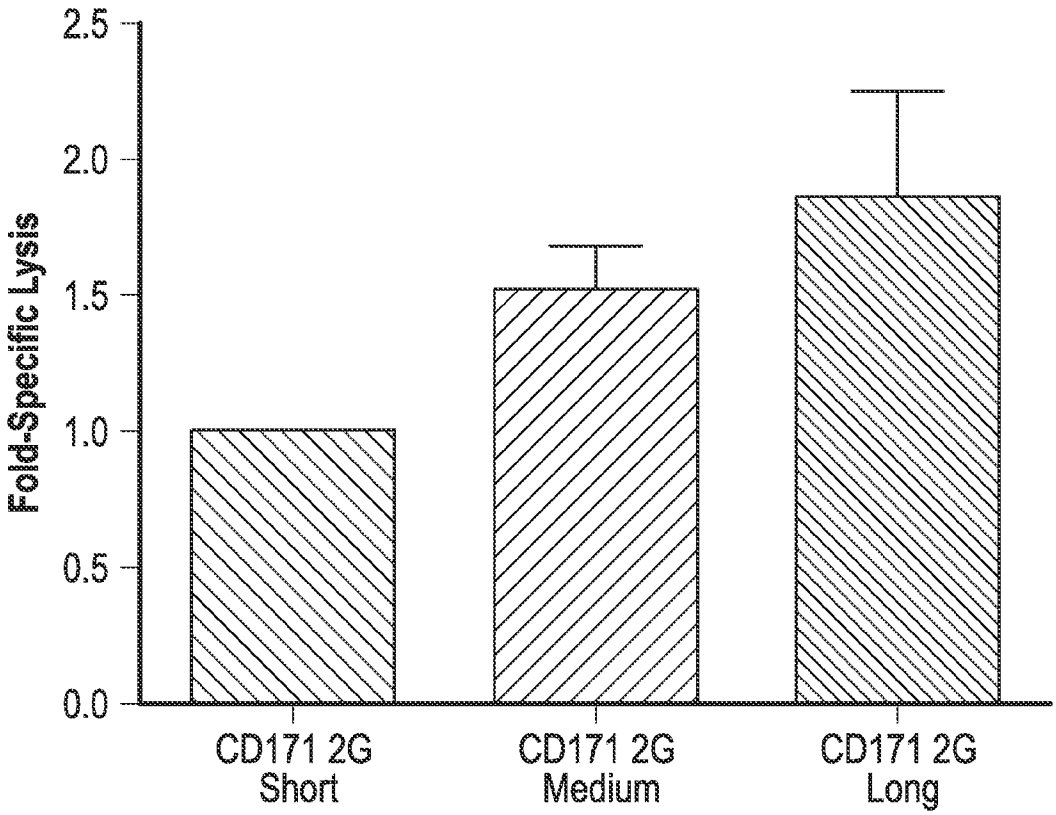


FIG. 13G

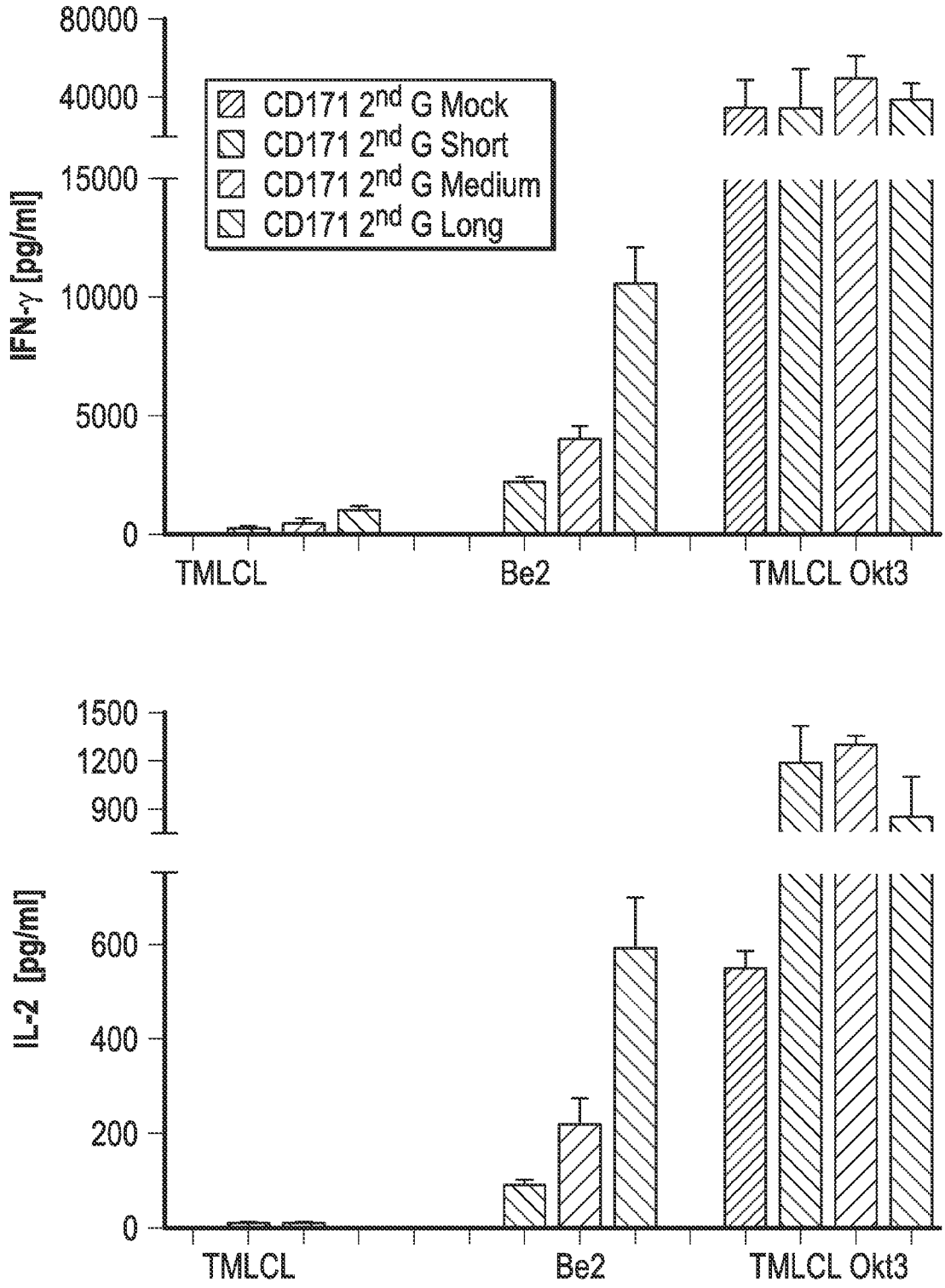

80000
40000
15000
10000
5000
0
IFN-γ [pg/ml]
CD171 2nd G Mock
CD171 2nd G Short
CD171 2nd G Medium
CD171 2nd G Long
TMLCL
Be2
TMLCL Okt3
1500
1200
900
600
400
200
0
IL-2 [pg/ml]
TMLCL
Be2
TMLCL Okt3

FIG. 13G (Continued)

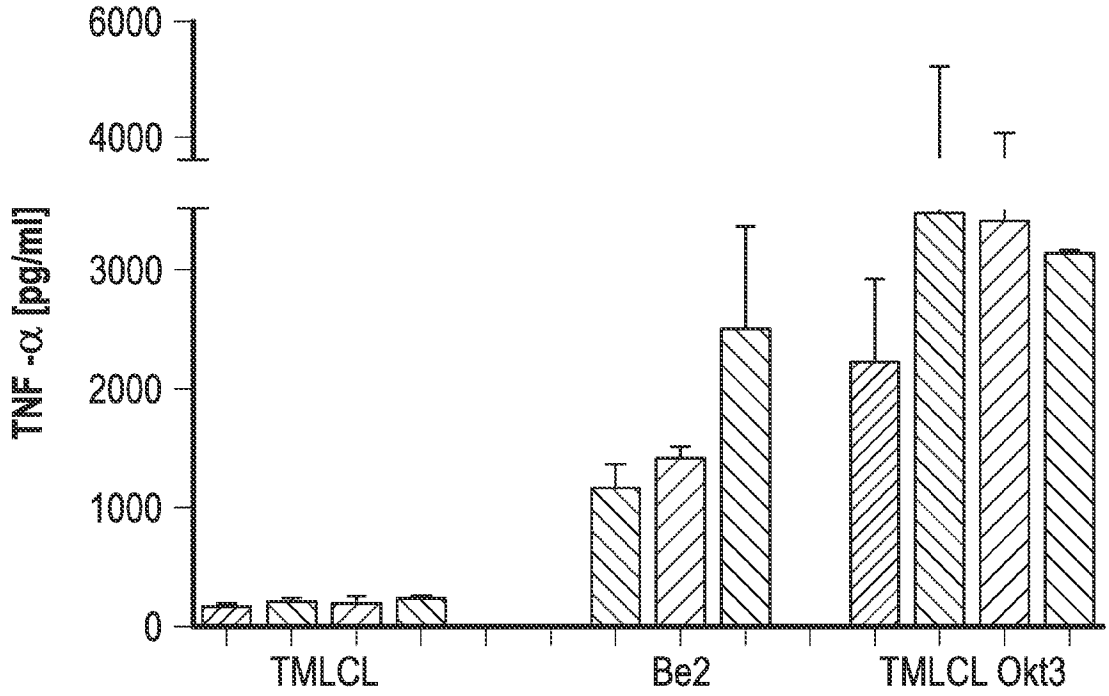


FIG. 13H

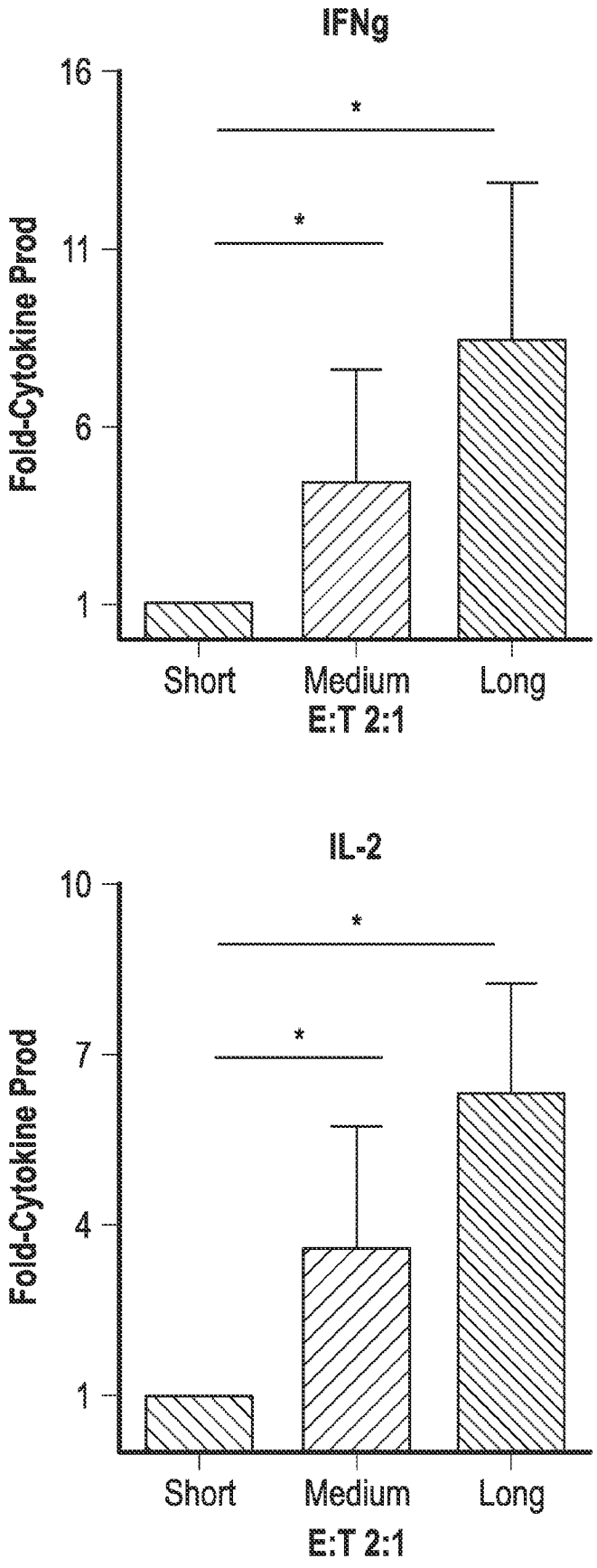
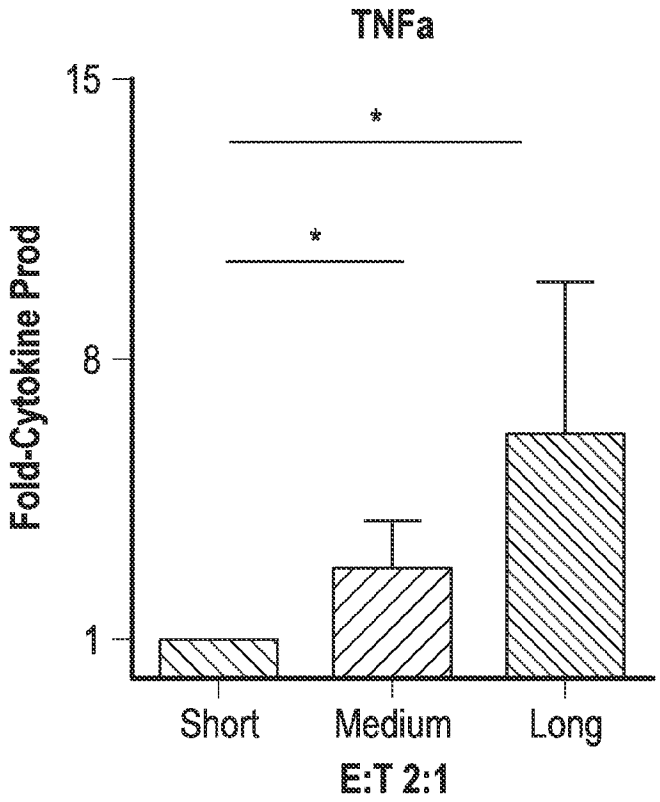

IFNg
16
11
6
1
Fold-Cytokine Prod
*
*
Short
Medium
Long
E:T 2:1
IL-2
10
7
4
1
Fold-Cytokine Prod
*
*
Short
Medium
Long
E:T 2:1

FIG. 13H (Continued)



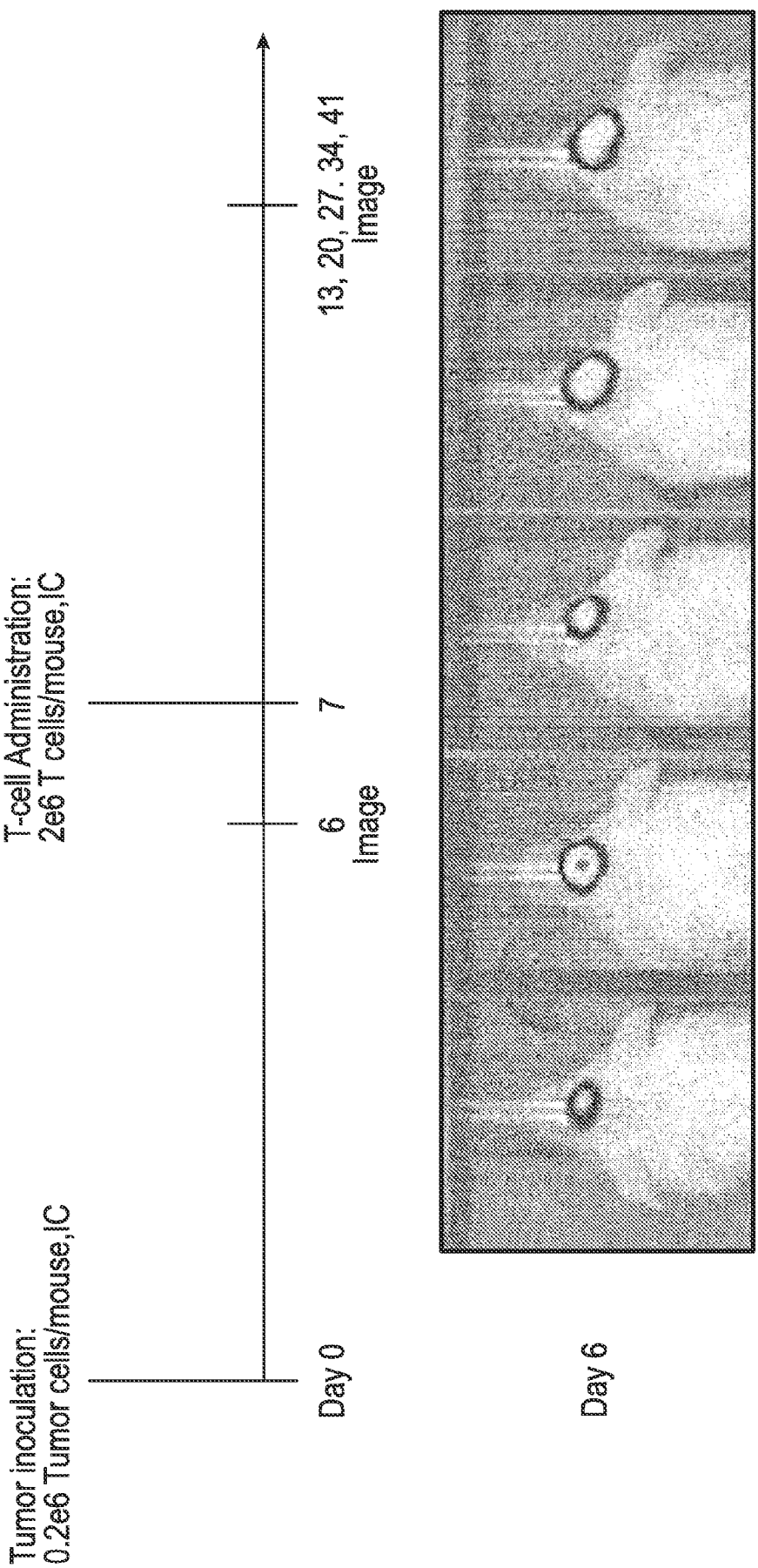

Tumor inoculation:
0.2e6 Tumor cells/mouse,IC
Day 0
T-cell Administration:
2e6 T cells/mouse,IC
7
6
Image
13, 20, 27, 34, 41
Image
Day 6

FIG. 14A

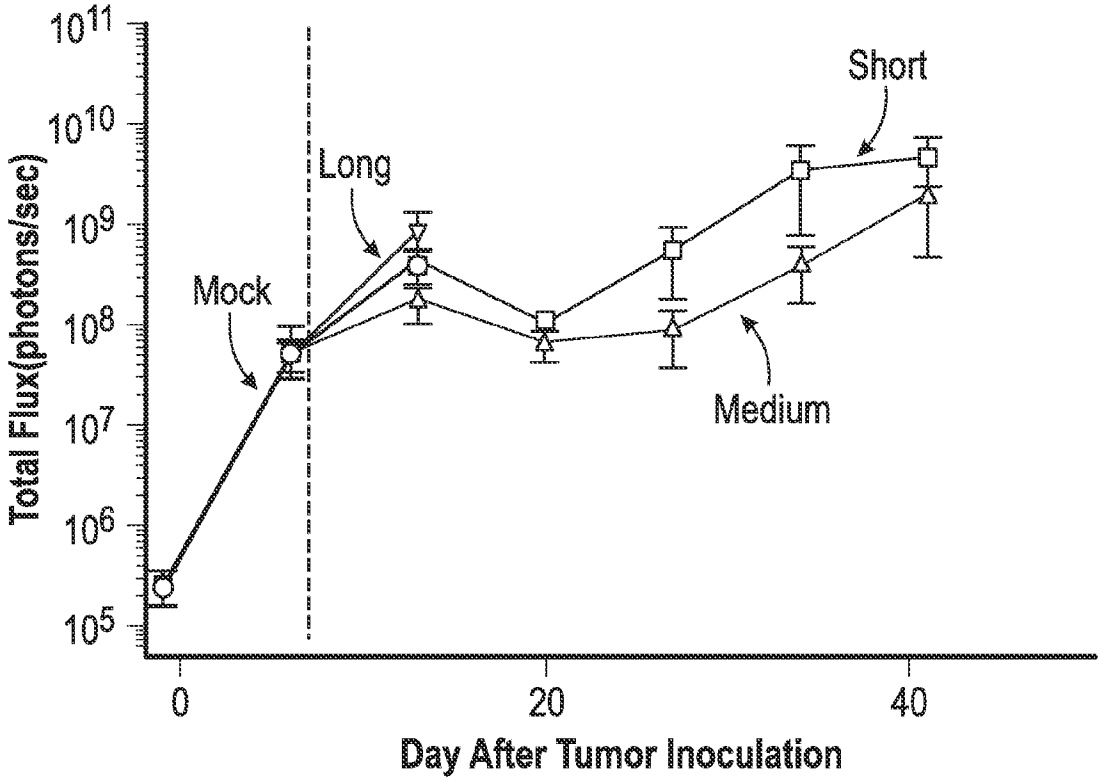


FIG. 14B

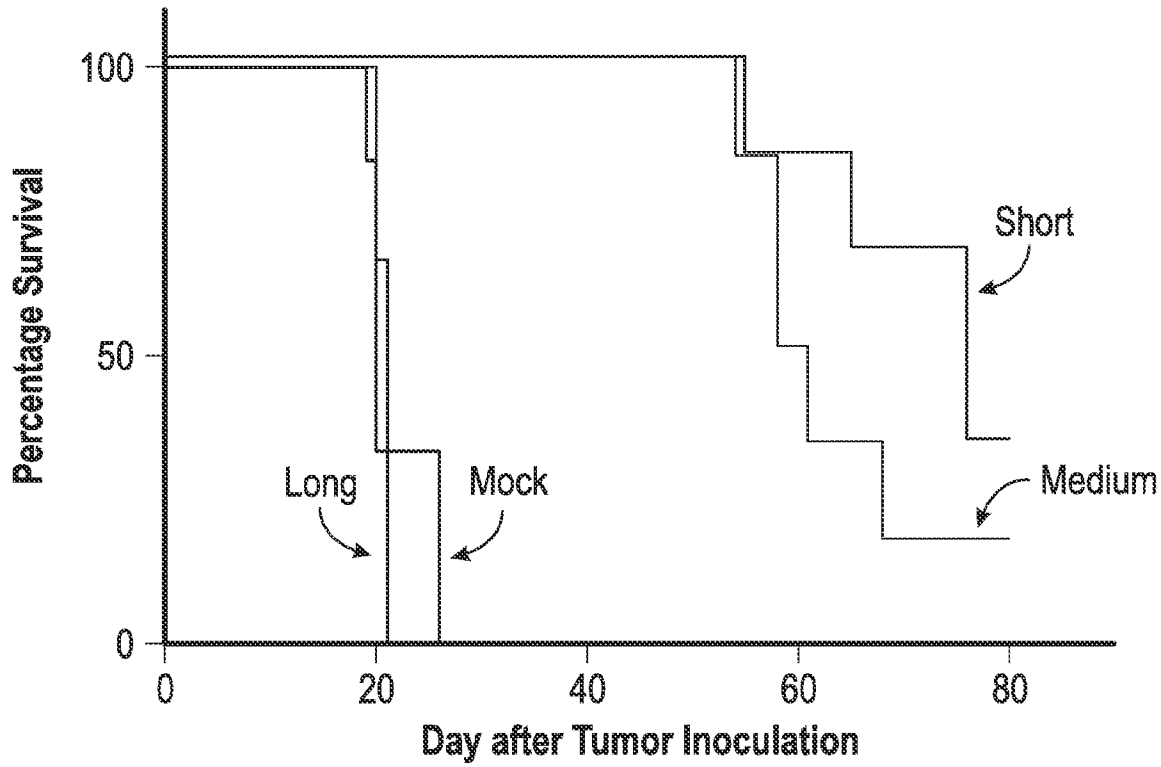


FIG. 14C

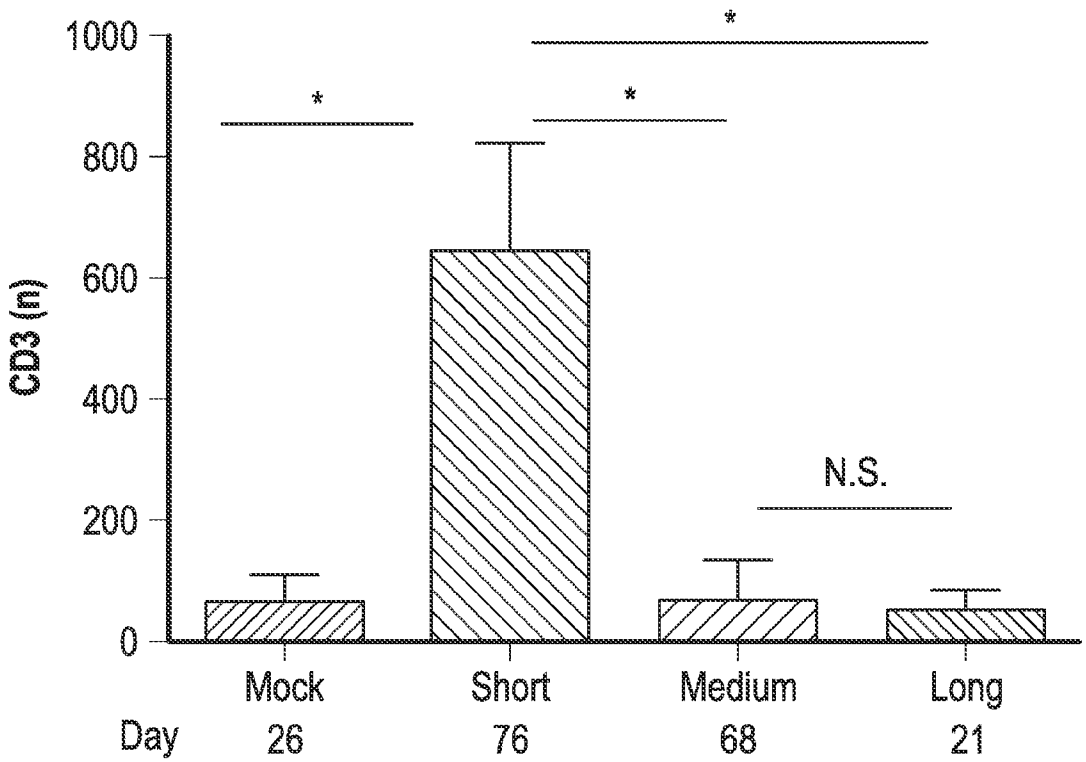

1000
800
600
400
200
0
CD3 (n)
*
*
*
N.S.
Mock
Short
Medium
Long
Day
26
76
68
21

FIG. 14D

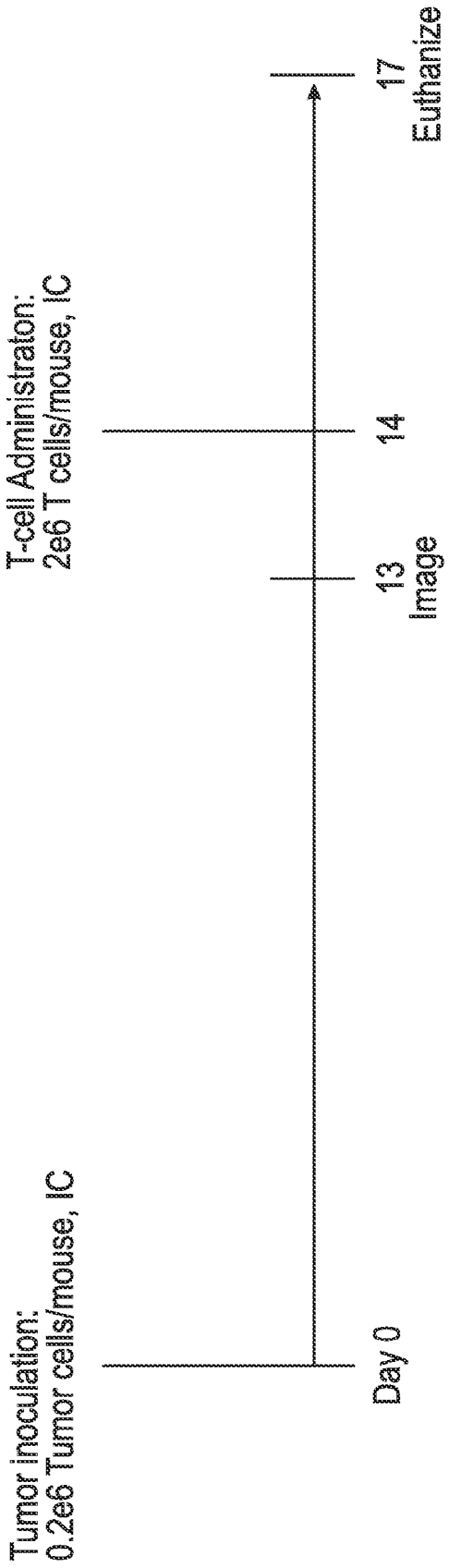
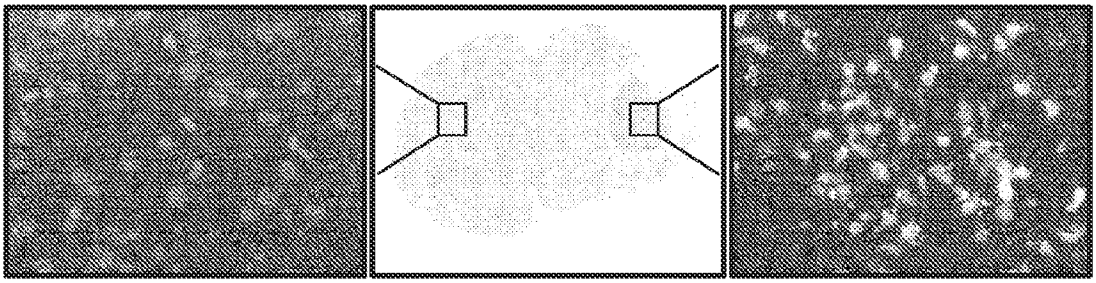
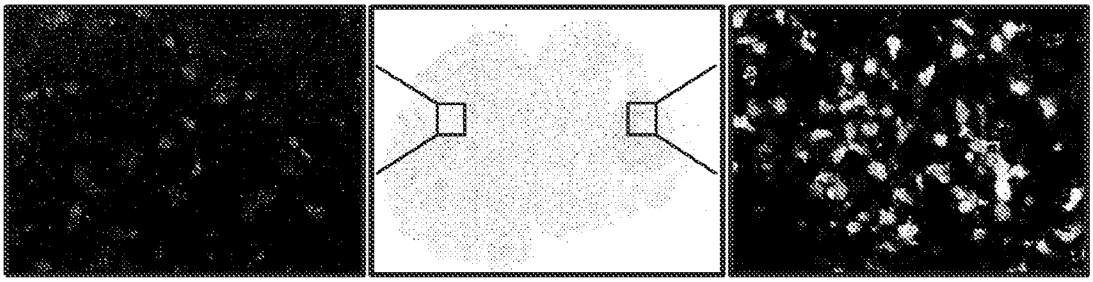

Tumor inoculation:
0.2e6 Tumor cells/mouse, IC
Day 0
T-cell Administraton:
2e6 T cells/mouse, IC
13
Image
14
17
Euthanize

FIG. 14E



CD3,Ki67,Caspase 3,Hoechst

FIG. 14F1



Cd3, Ki67, Caspase 3, Hoechst

FIG. 14F2

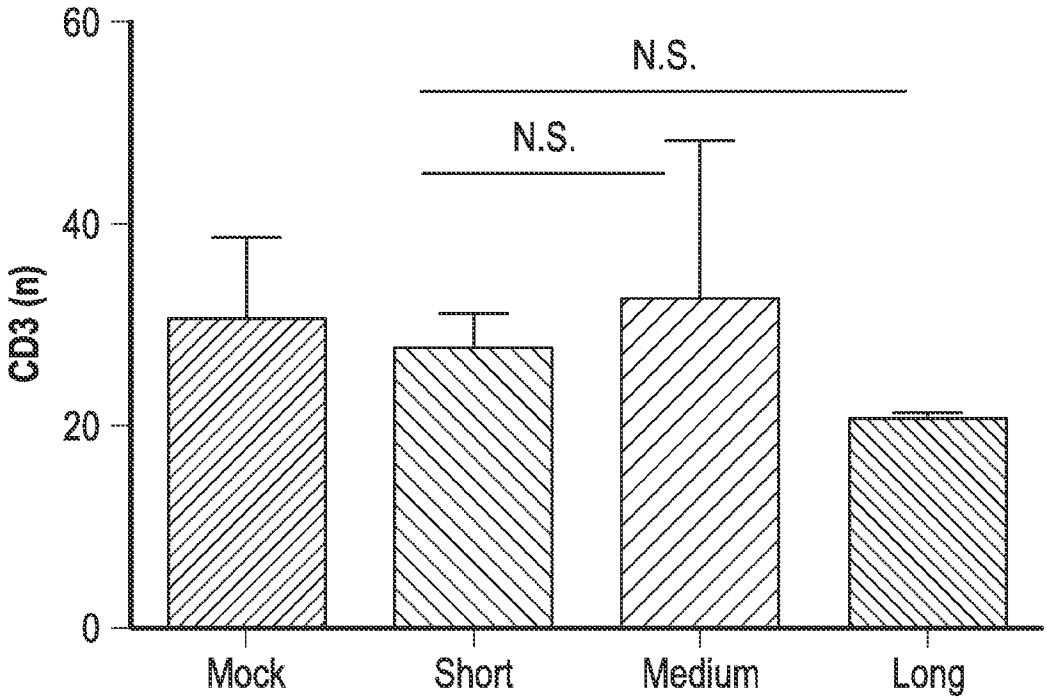
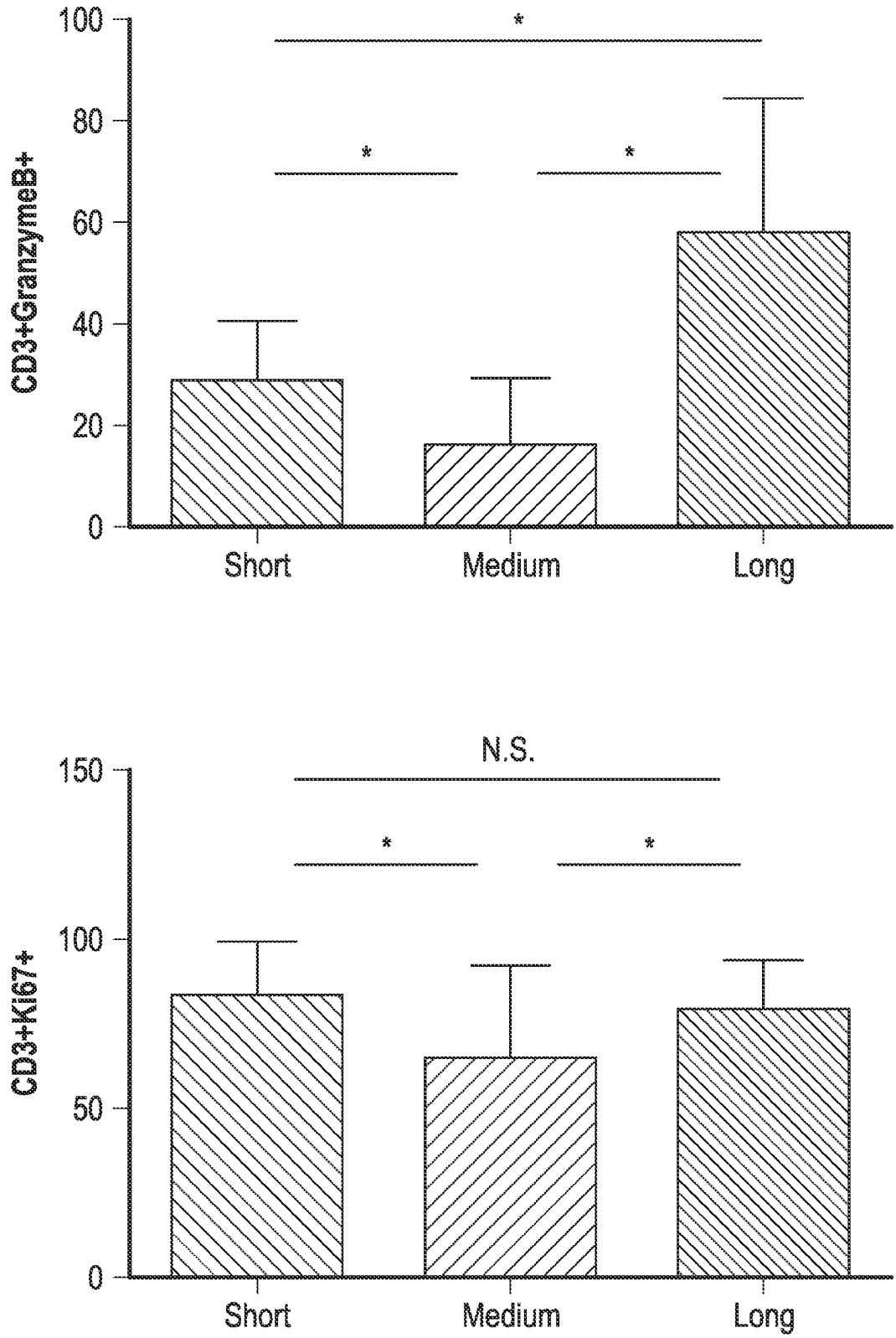


FIG. 14G

FIG. 14H


CD3+GranzymeB+
100
80
60
40
20
0
*
*
*
Short
Medium
Long
CD3+Ki67+
150
100
50
0
N.S.
*
*
Short
Medium
Long

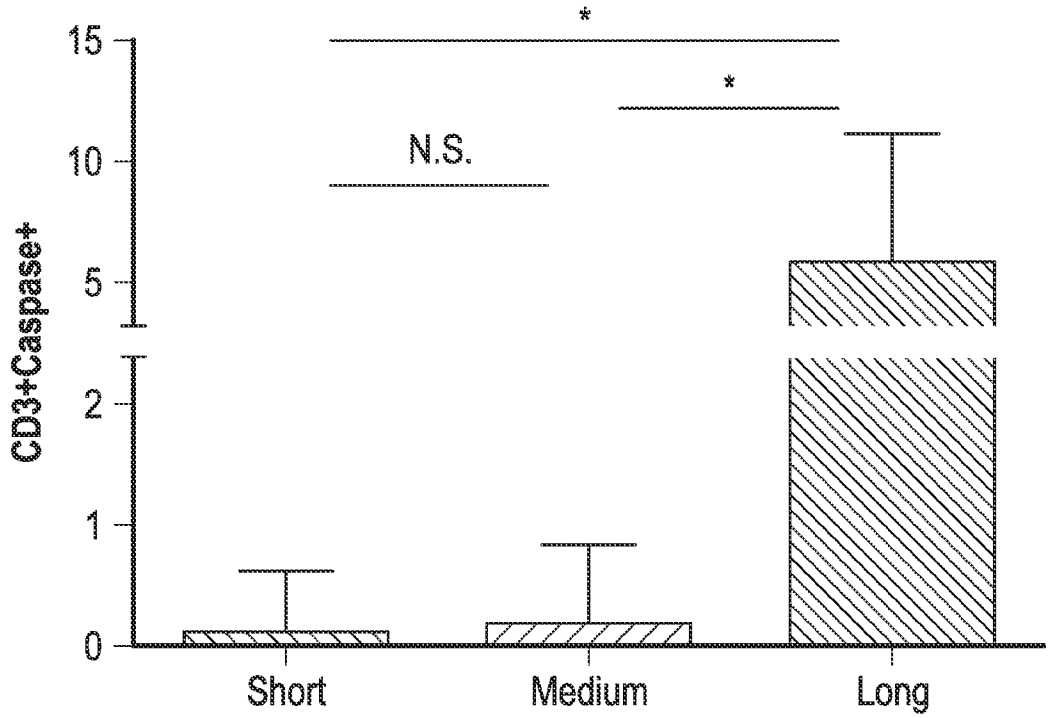


FIG. 14H (Continued)

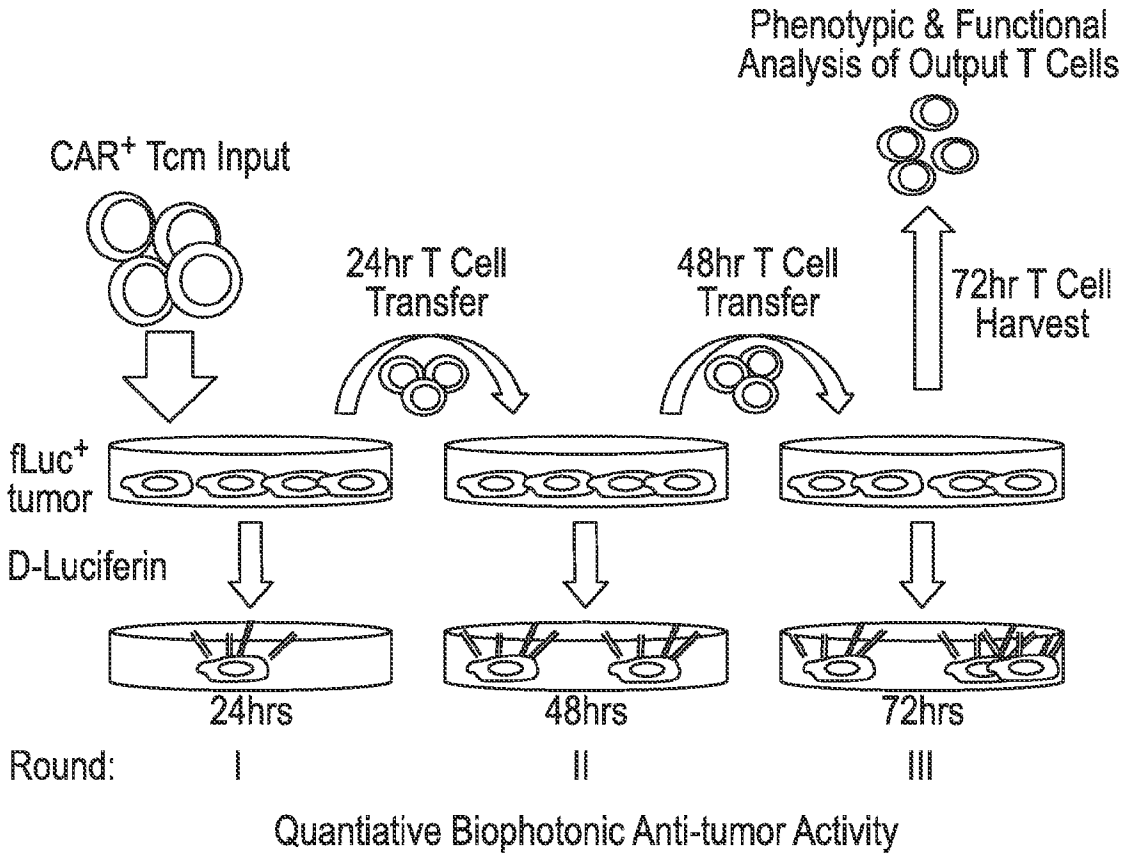


FIG. 15A

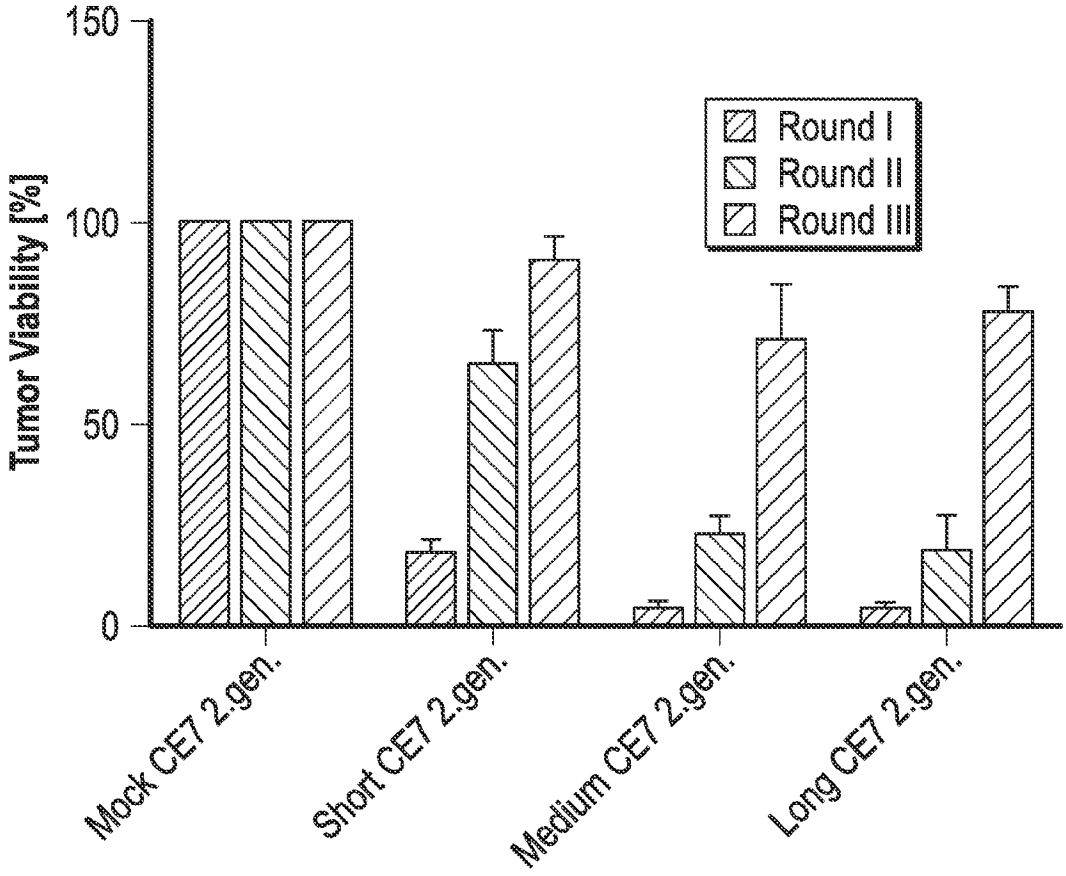


FIG. 15B

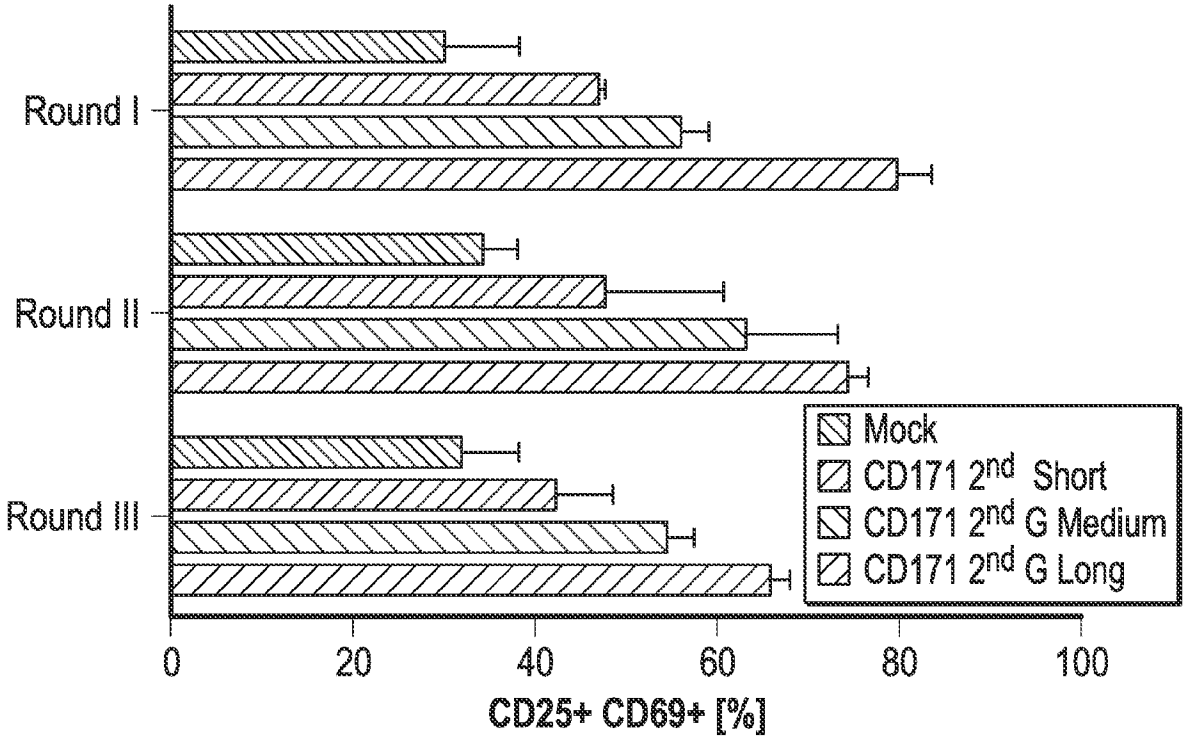


FIG. 15C

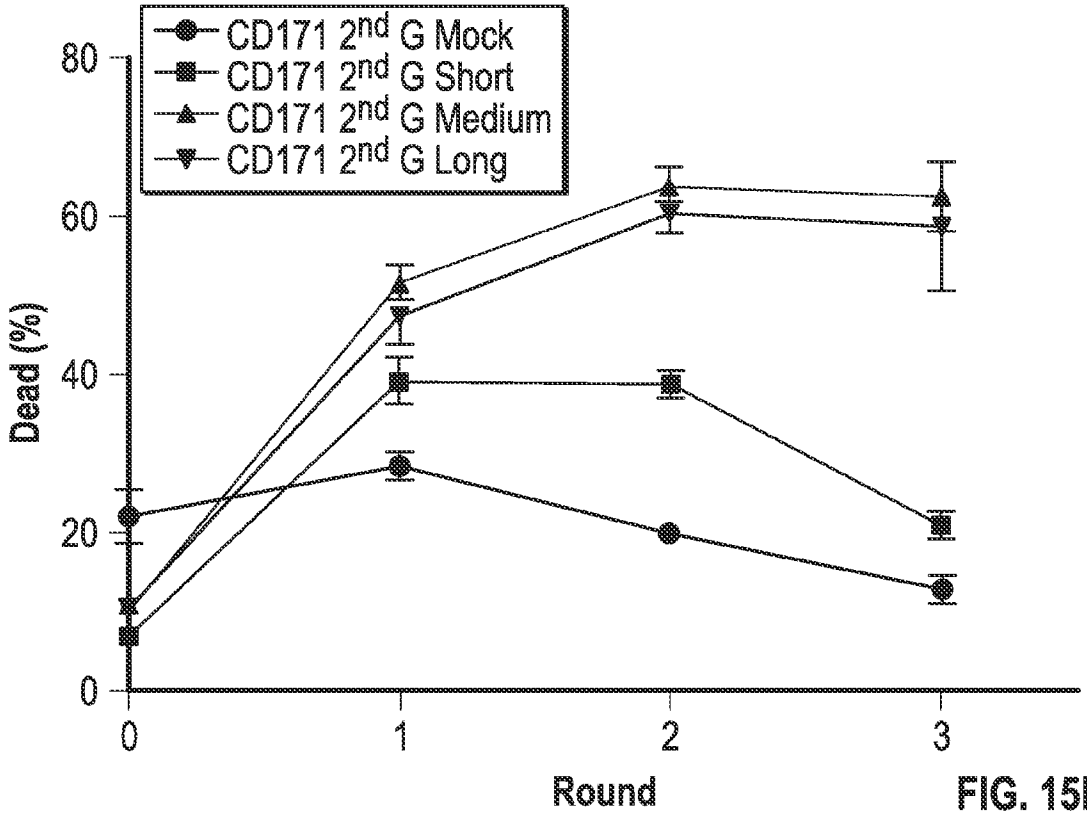


FIG. 15D

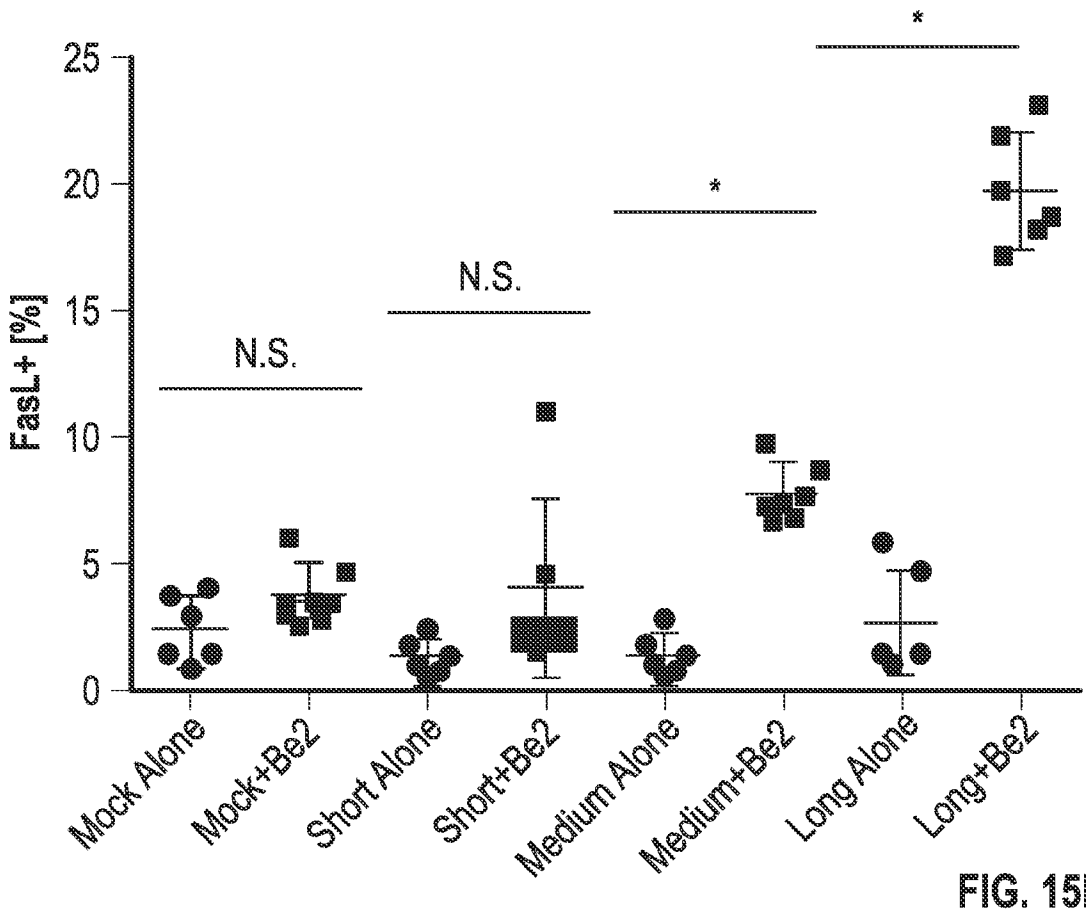


FIG. 15E

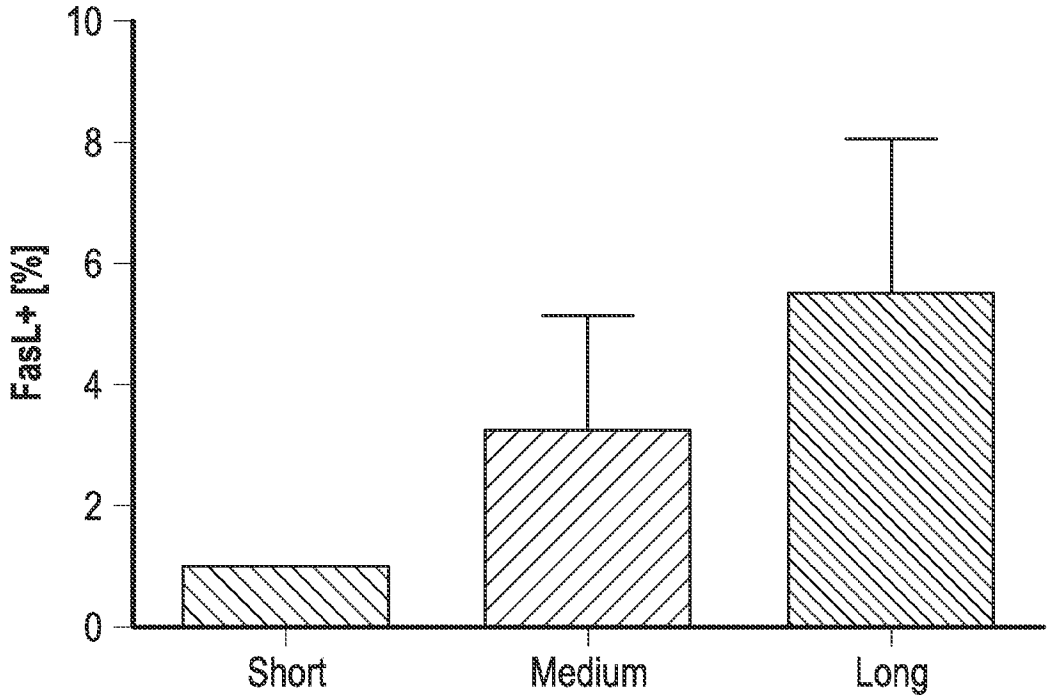


FIG. 15F

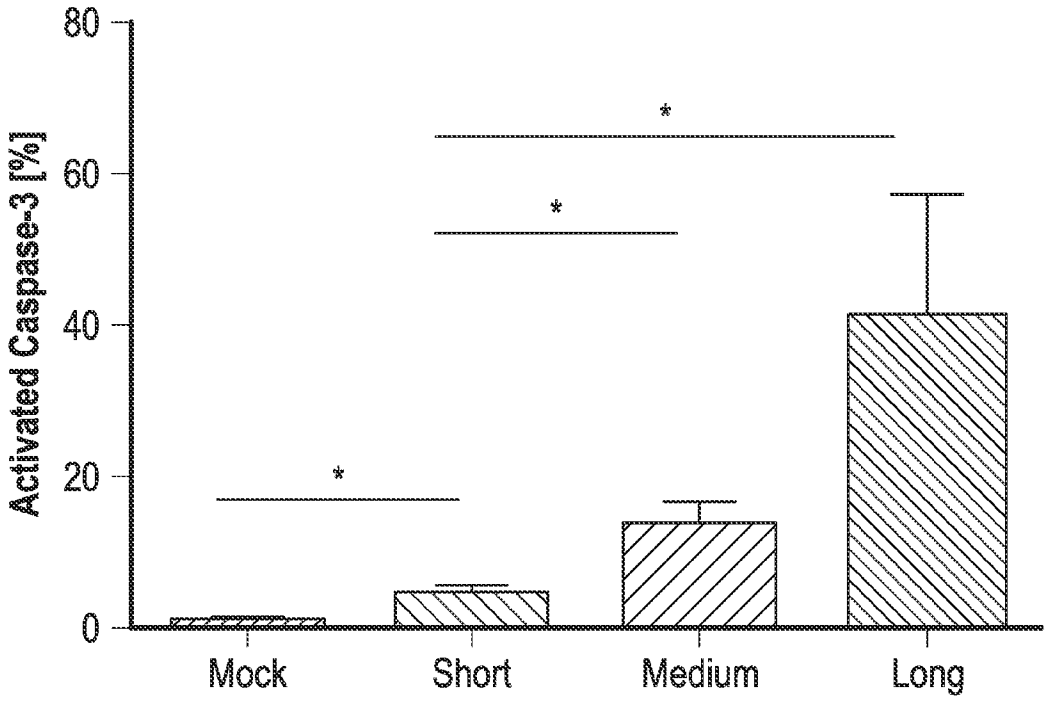


FIG. 15G

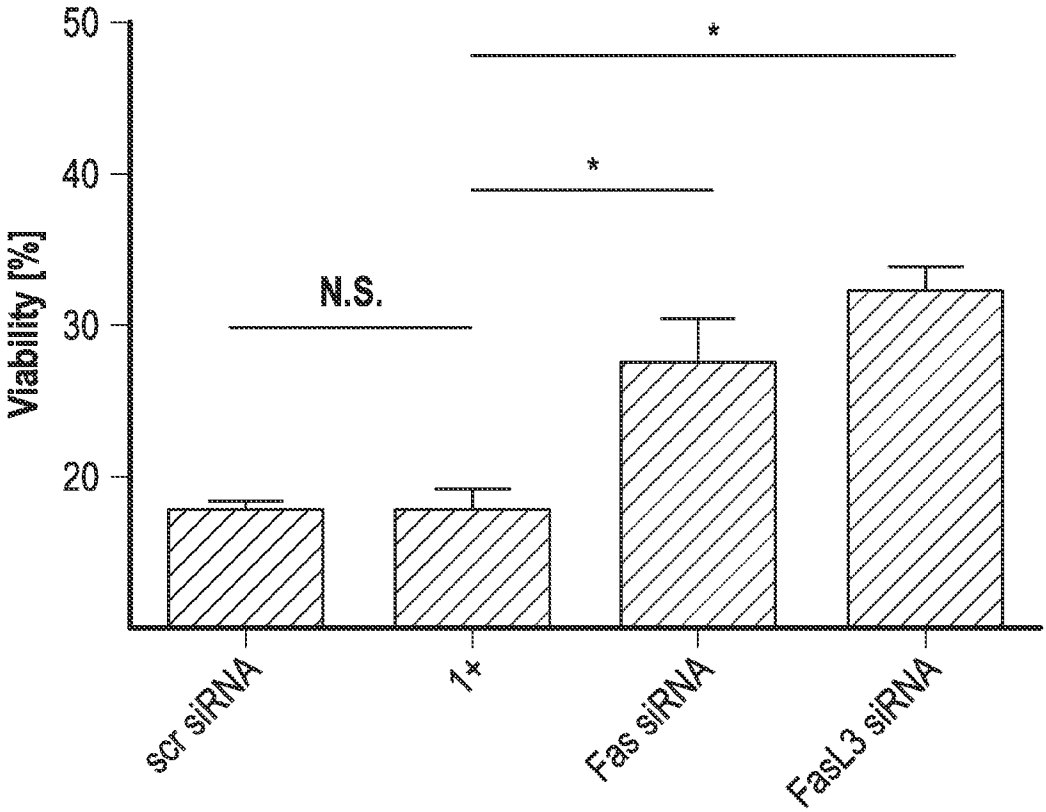


FIG. 15H

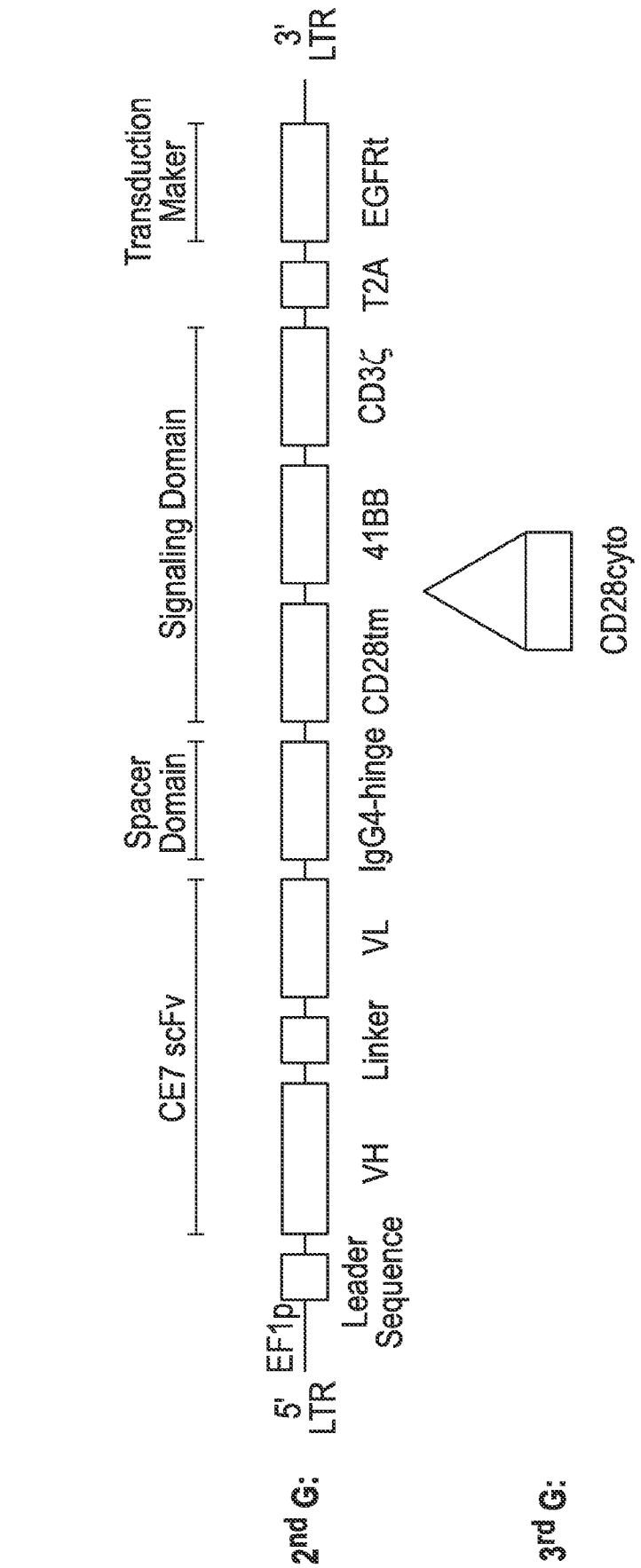

2nd G:
5' LTR
EF1p
Leader Sequence
VH
Linker
VL
IgG4-hinge
CD28tm
41BB
CD3ζ
T2A
EGFRt
3' LTR
CE7 scFv
Spacer Domain
Signaling Domain
Transduction Maker
3rd G:
CD28cyto

FIG. 16A

FIG. 16B

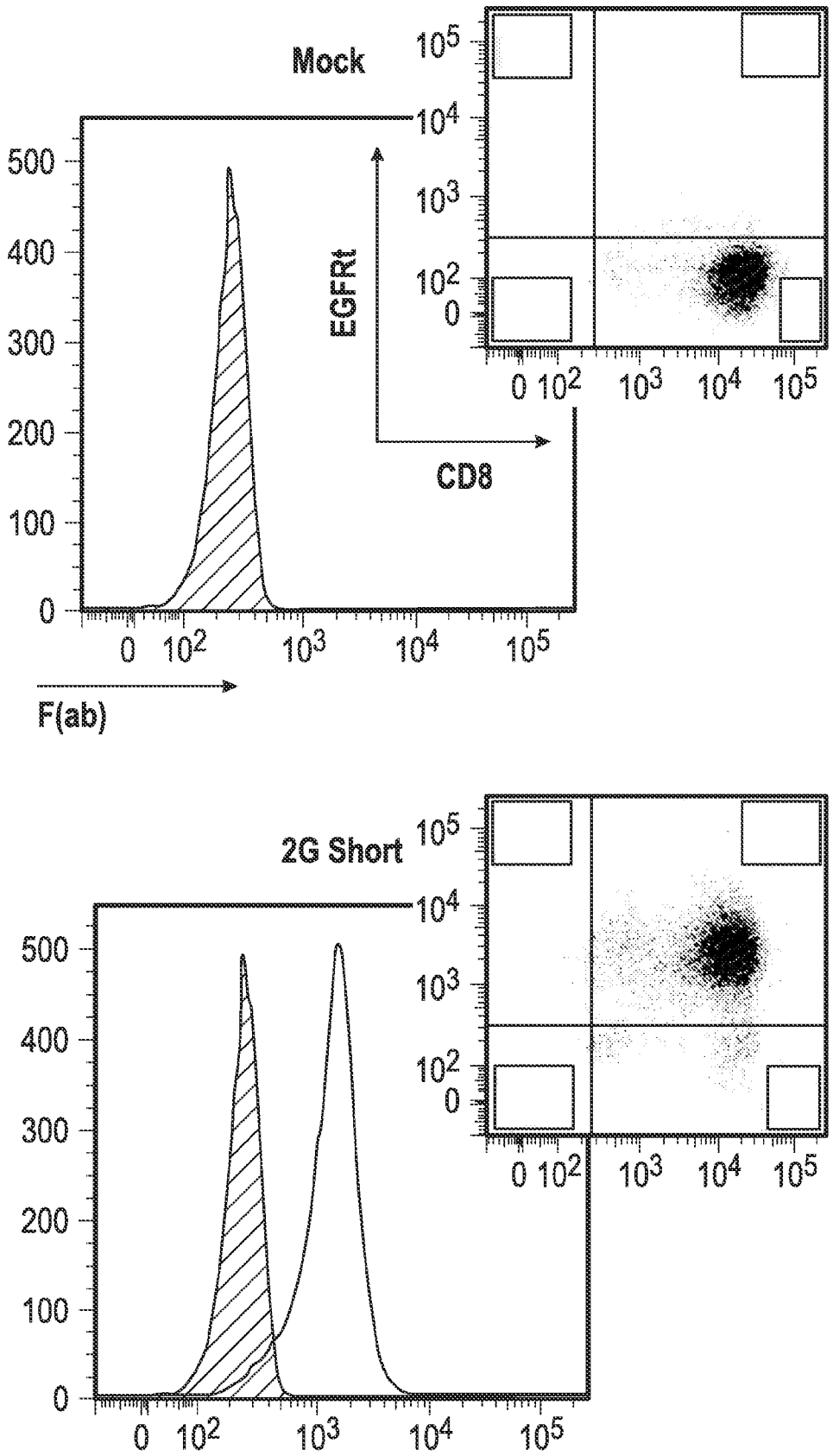
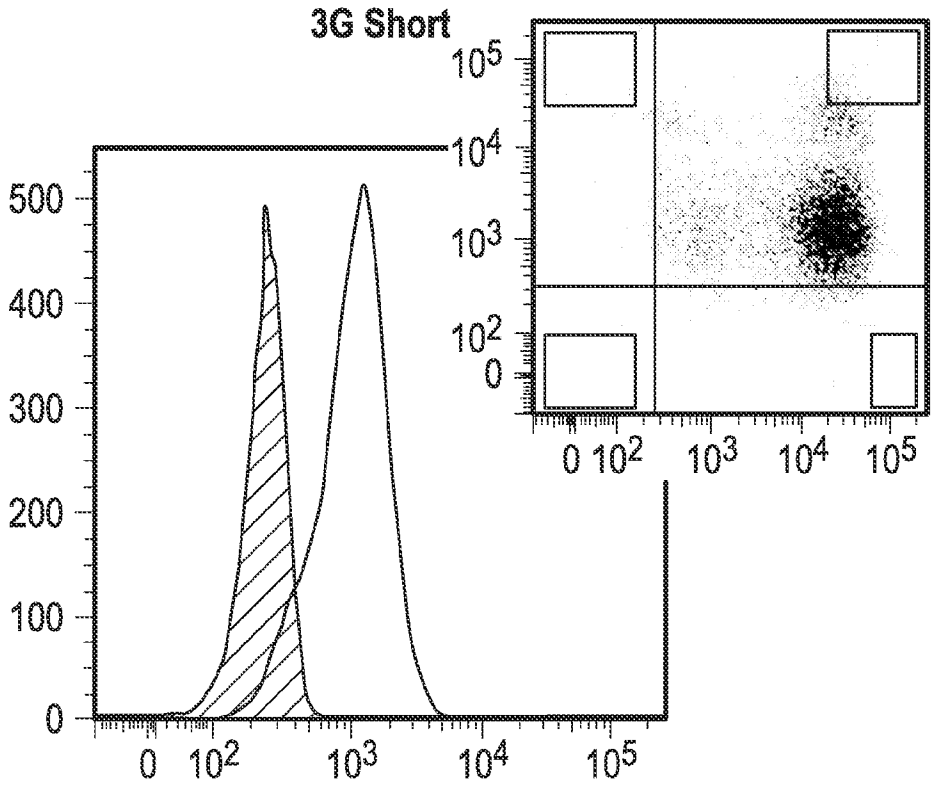

Mock
500
400
300
200
100
0
0 10^2 10^3 10^4 10^5
F(ab)
10^5
10^4
10^3
10^2
0
EGFRt
CD8
0 10^2 10^3 10^4 10^5
2G Short
500
400
300
200
100
0
0 10^2 10^3 10^4 10^5
10^5
10^4
10^3
10^2
0
0 10^2 10^3 10^4 10^5

FIG. 16B (Continued)



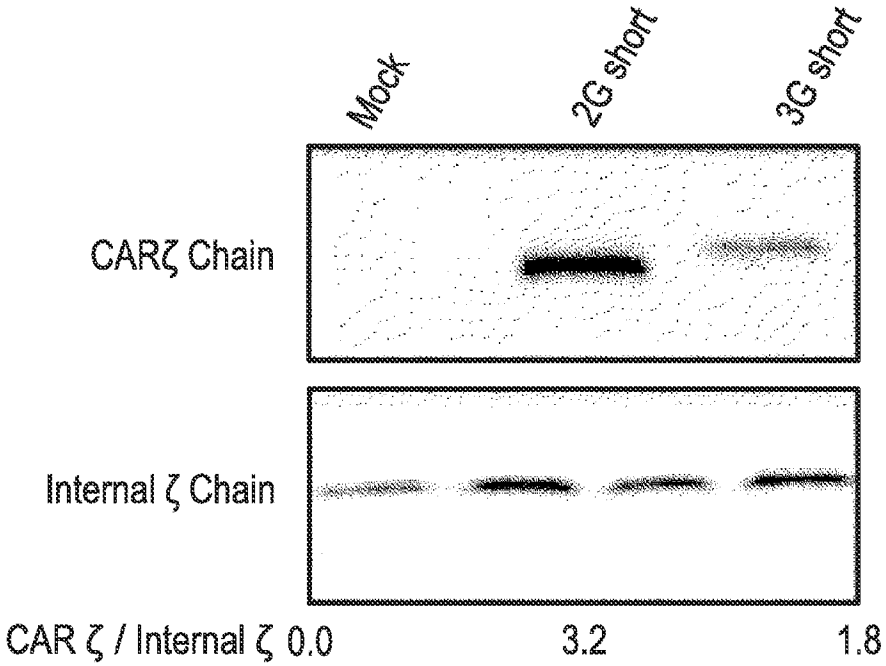


FIG. 16C

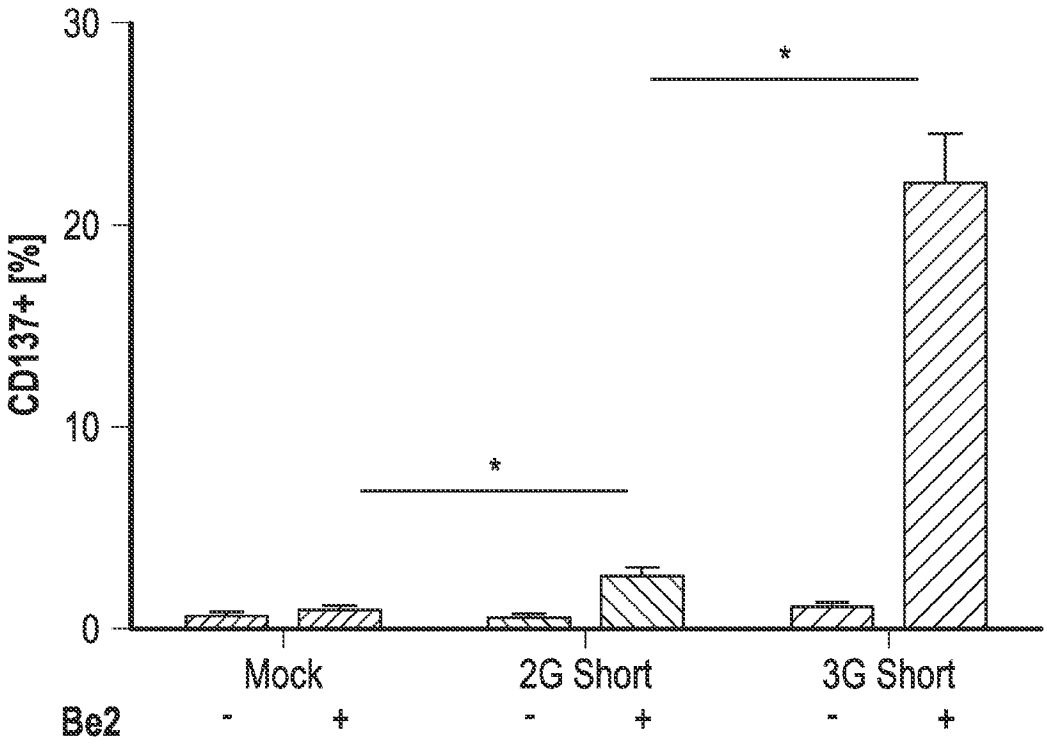
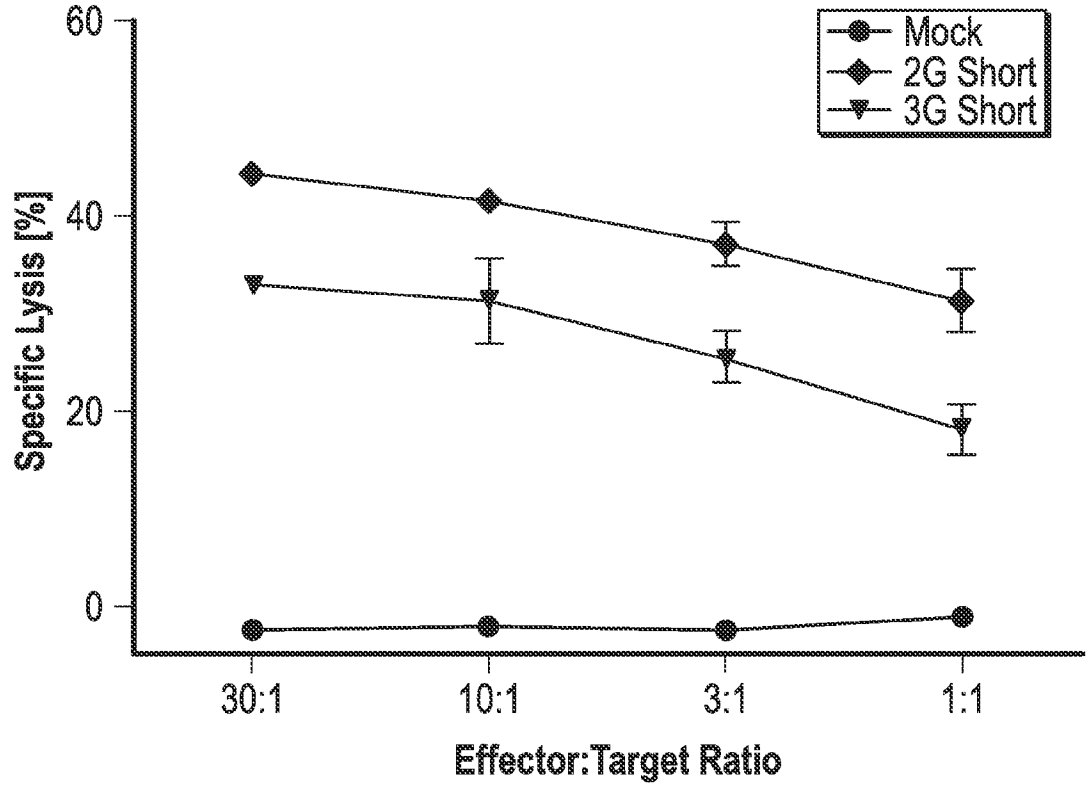


FIG. 16D

FIG. 16E


60
40
20
0
Specific Lysis [%]
Mock
2G Short
3G Short
30:1
10:1
3:1
1:1
Effector:Target Ratio

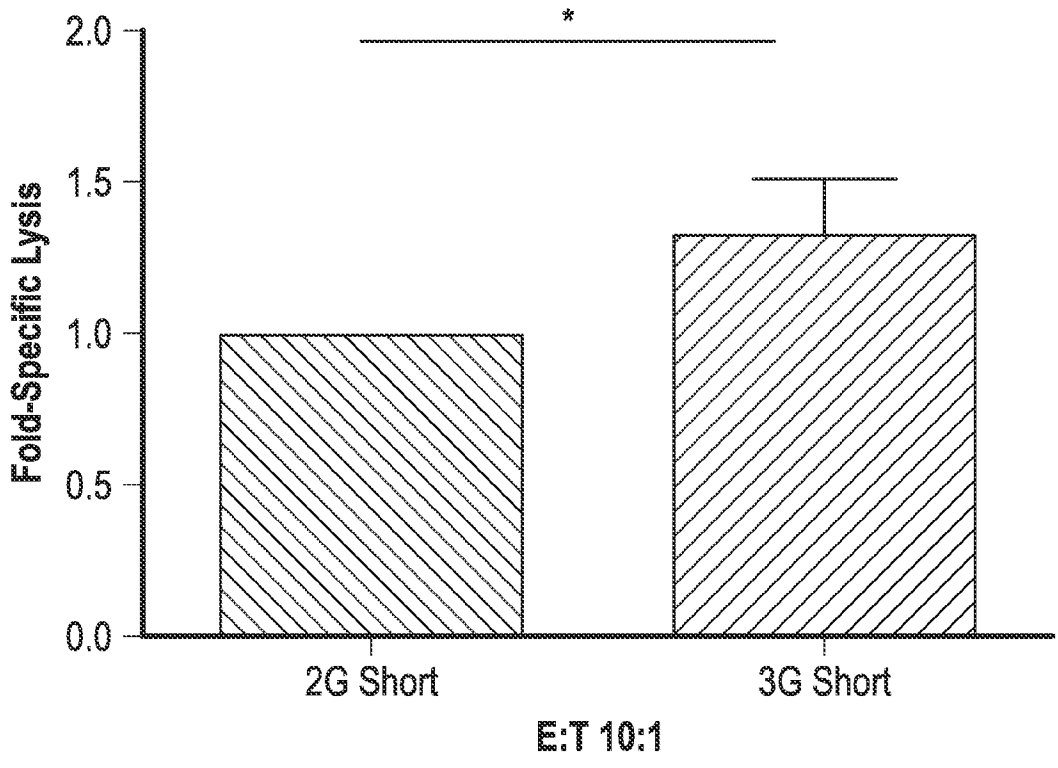

*
2.0
1.5
1.0
0.5
0.0
Fold-Specific Lysis
2G Short
3G Short
E:T 10:1

FIG. 16F

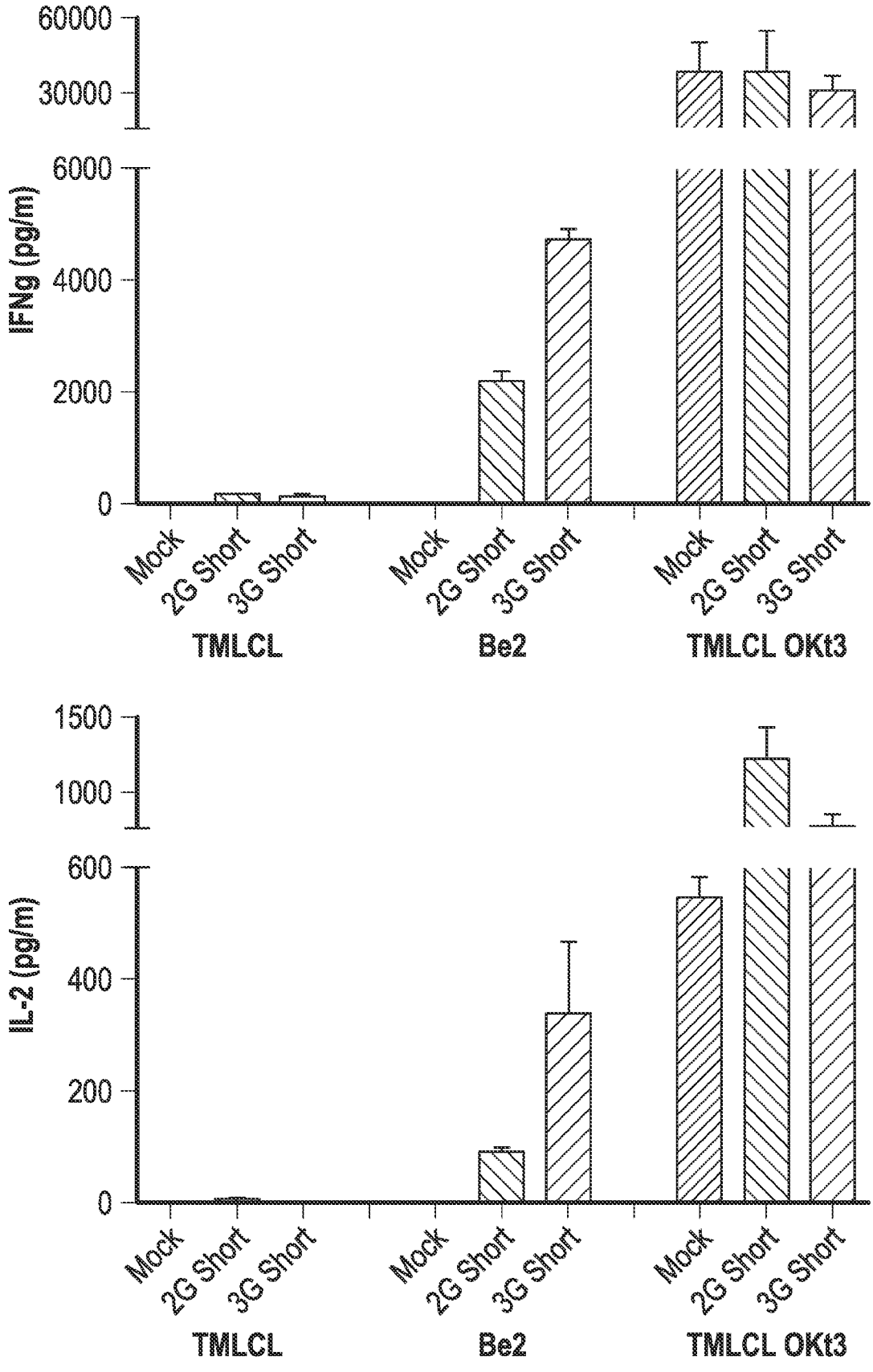

60000
30000
6000
4000
2000
0
IFNg (pg/m)
Mock
2G Short
3G Short
TMLCL
Be2
TMLCL OKt3
1500
1000
600
400
200
0
IL-2 (pg/m)

FIG. 16F (Continued)

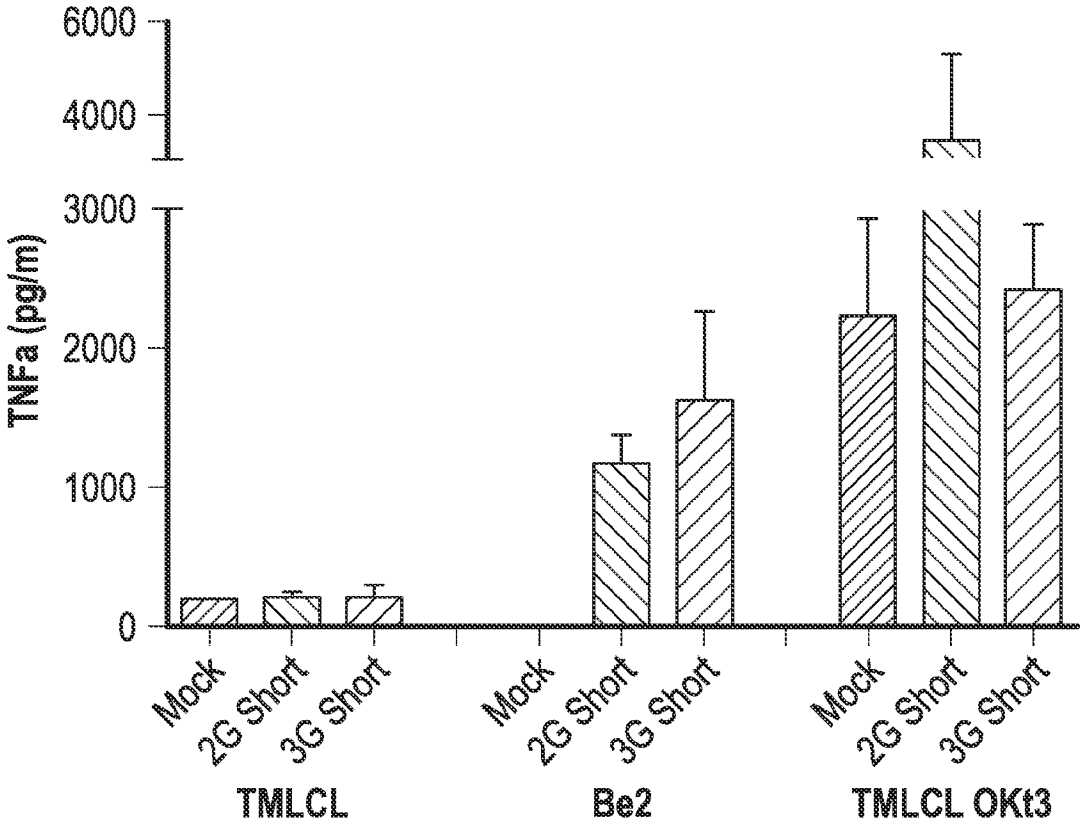
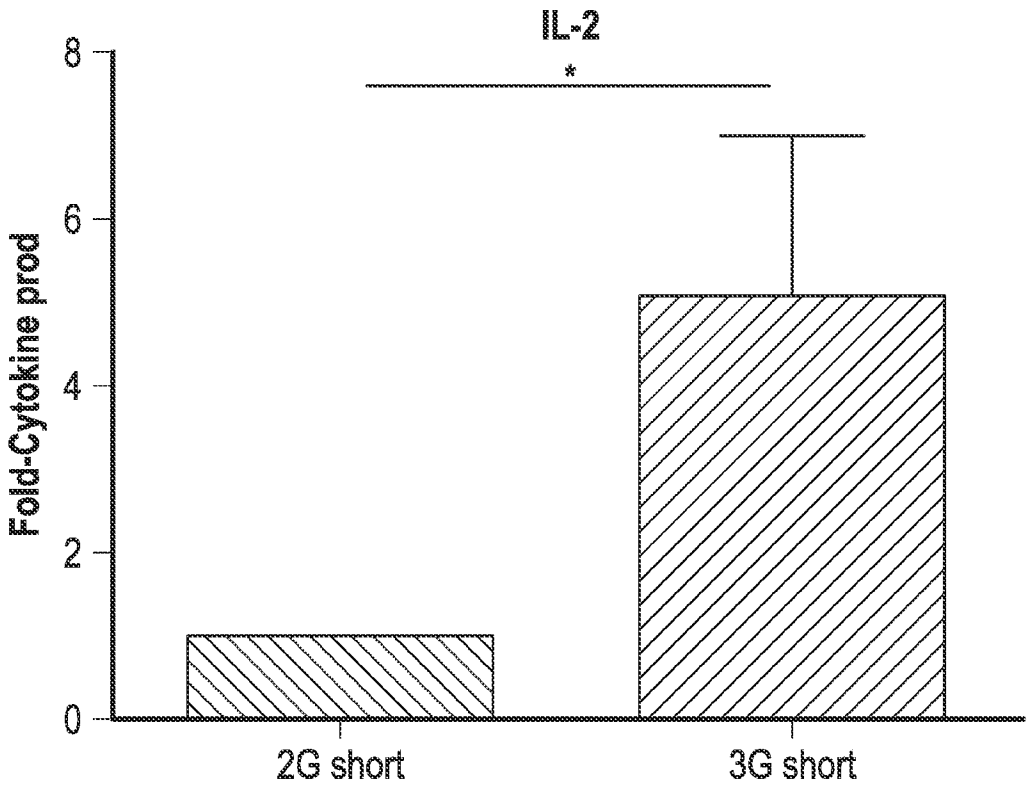
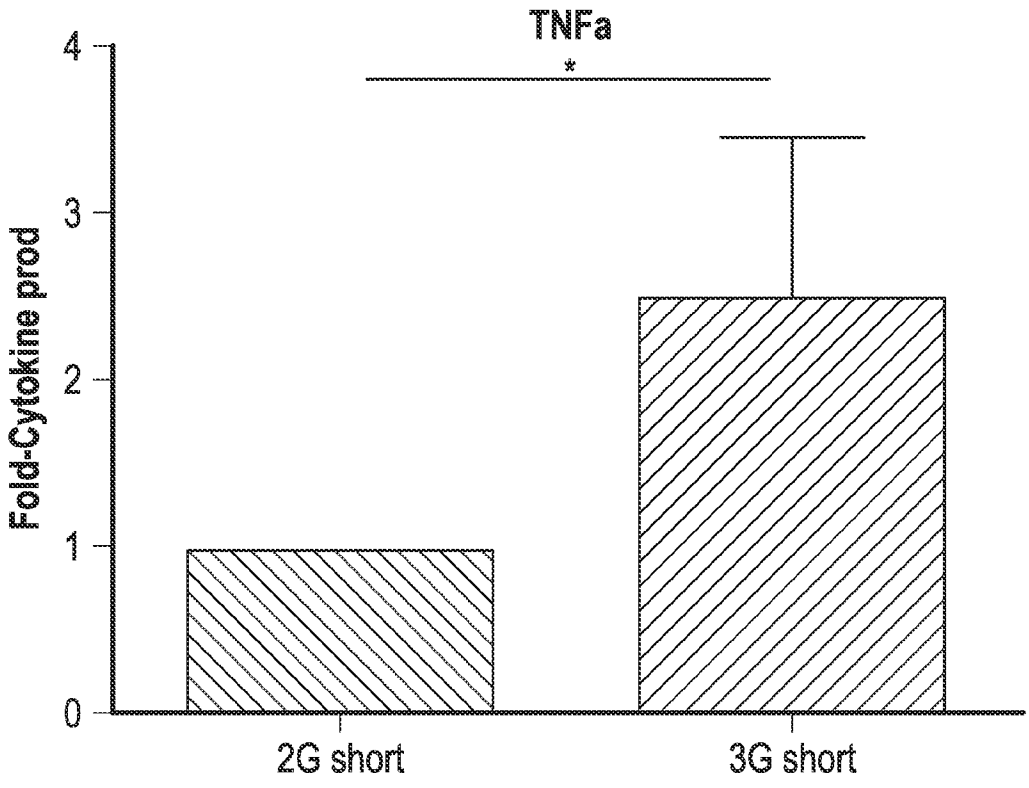


FIG. 16F (Continued)





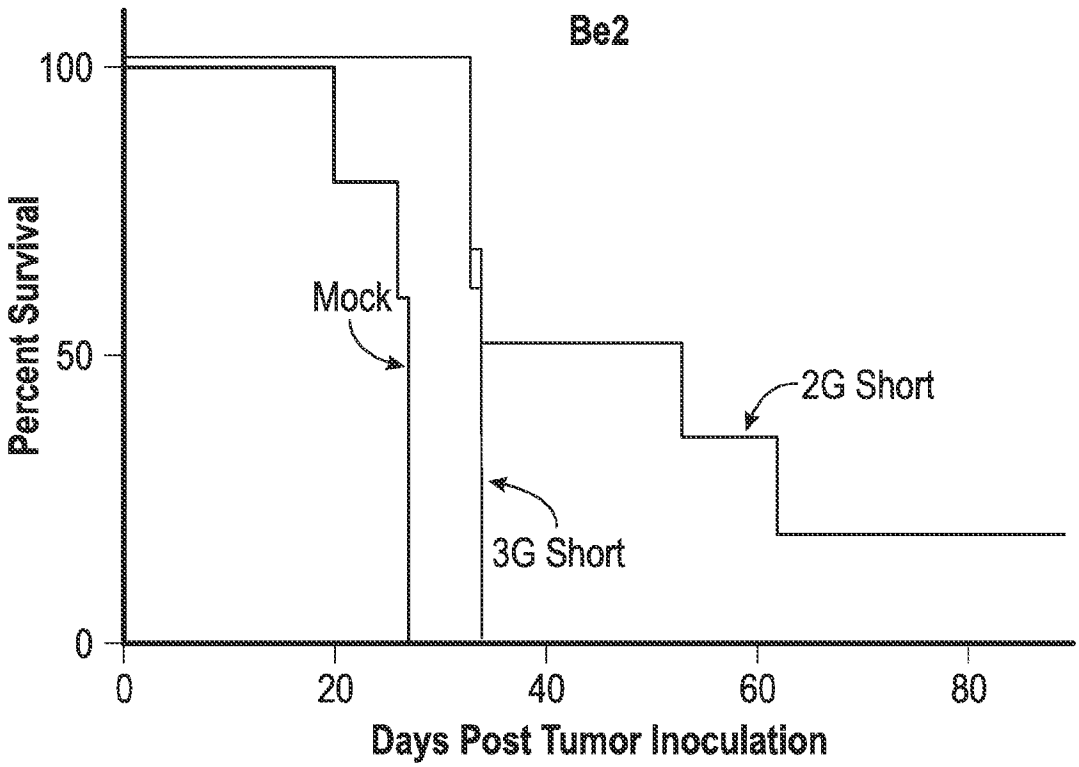


FIG. 17A

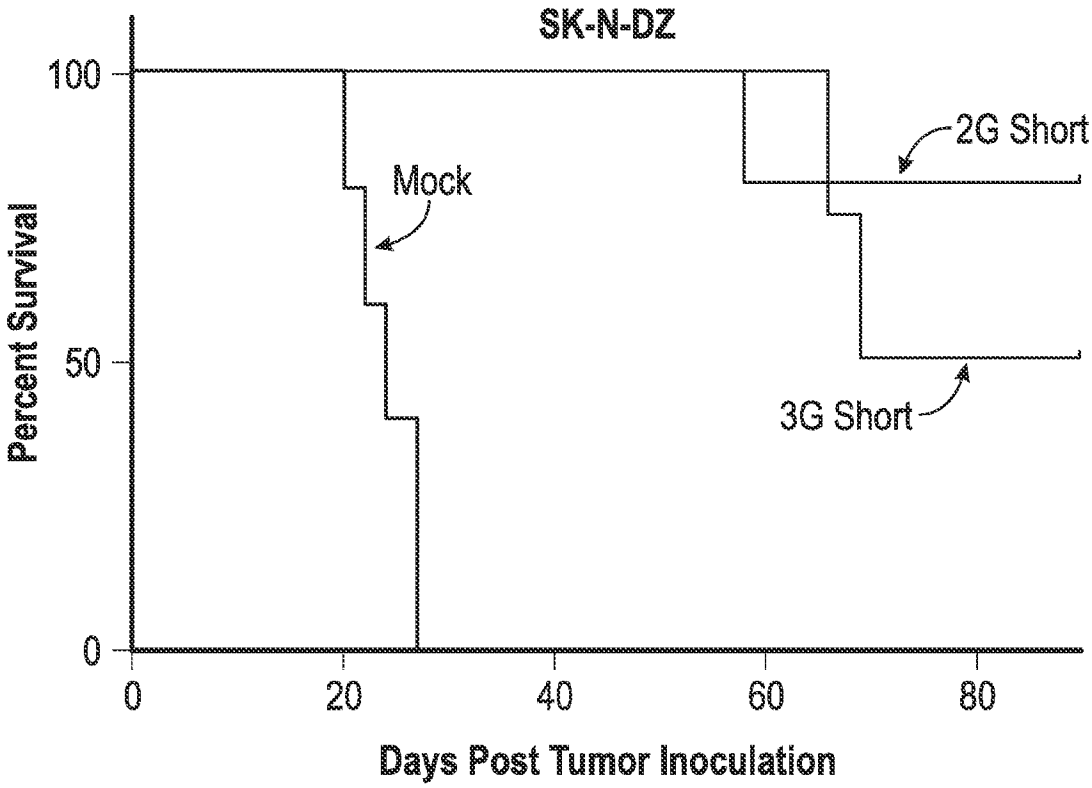
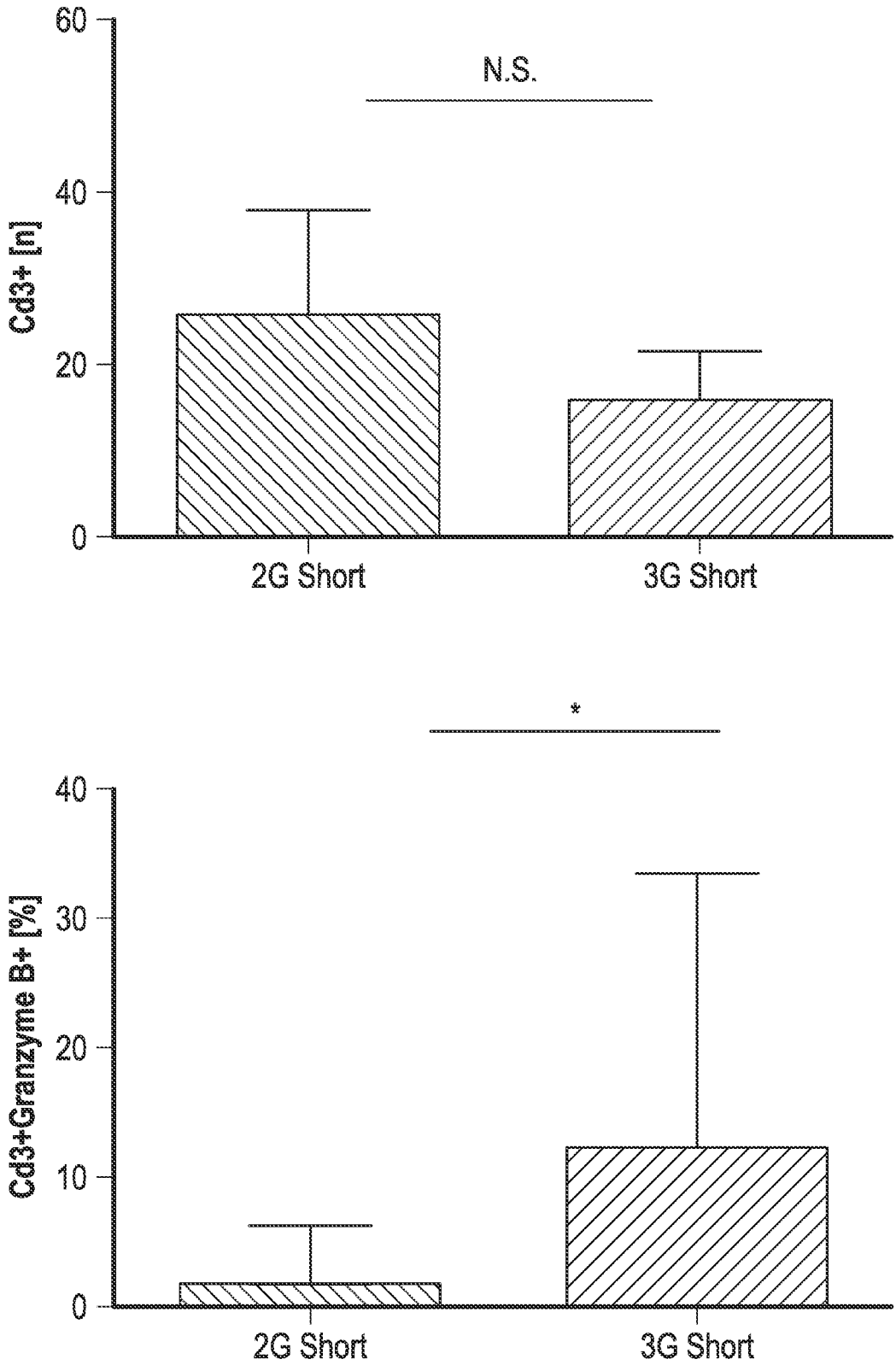


FIG. 17B

FIG. 17C


60
40
20
0
Cd3+ [n]
N.S.
2G Short
3G Short
*
40
30
20
10
0
Cd3+Granzyme B+ [%]
2G Short
3G Short

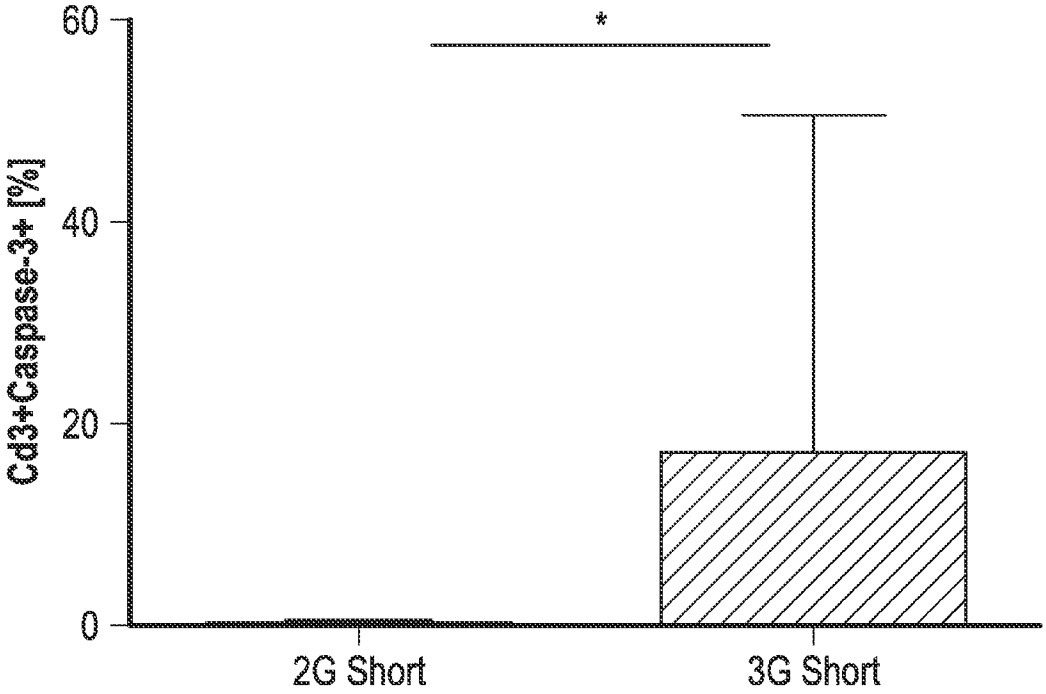


FIG. 17C (Continued)

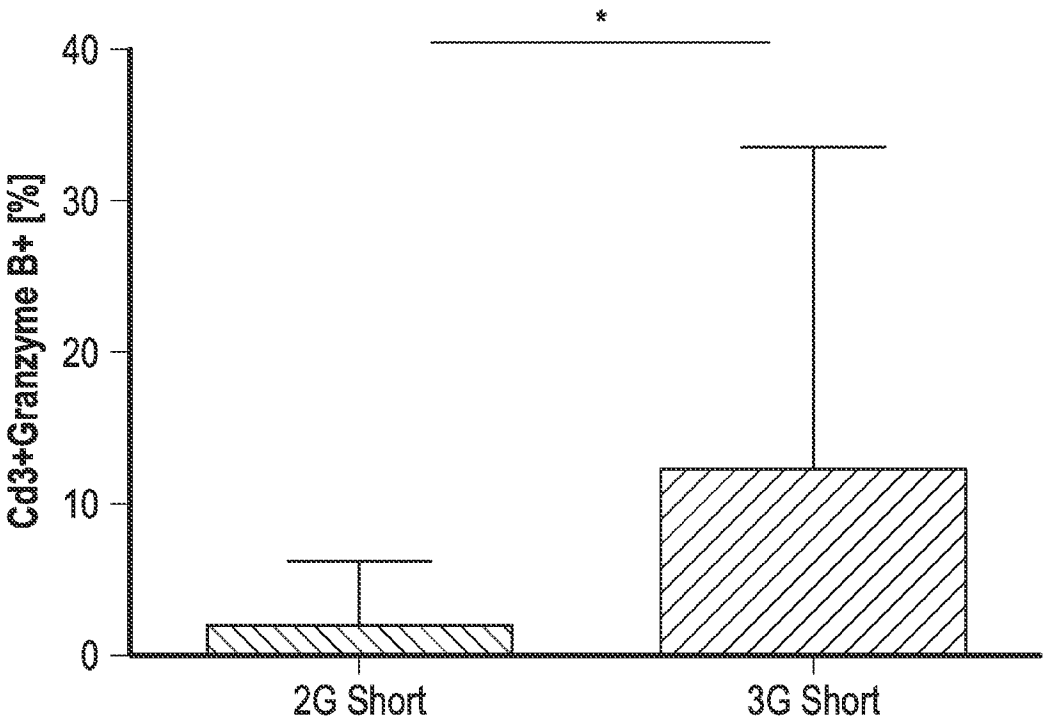


FIG. 17D

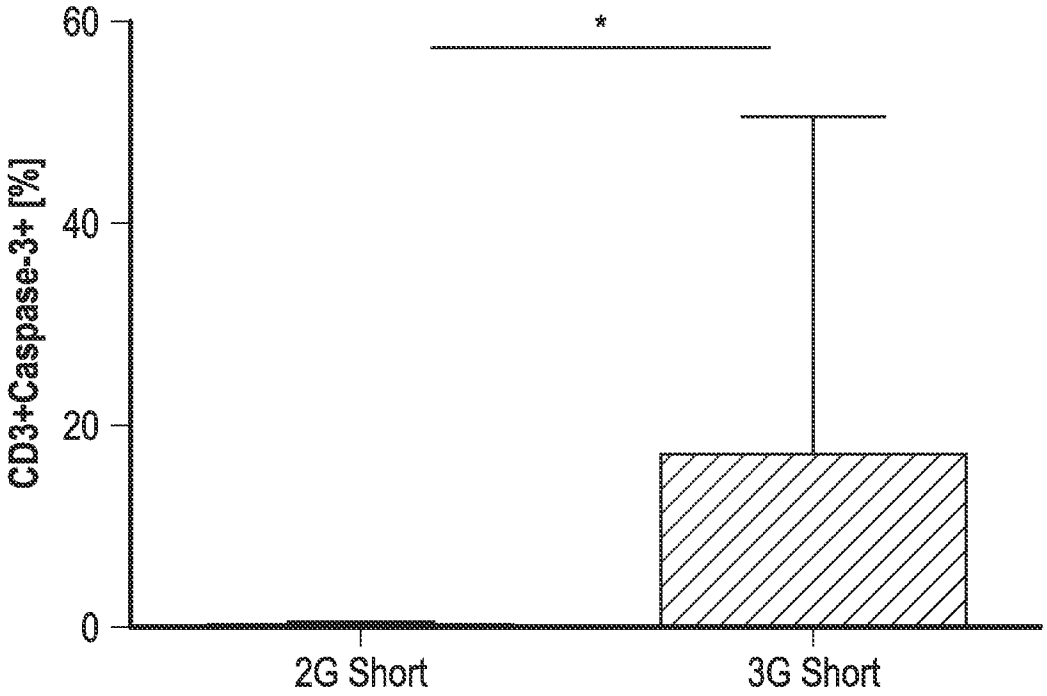


FIG. 17D (Continued)

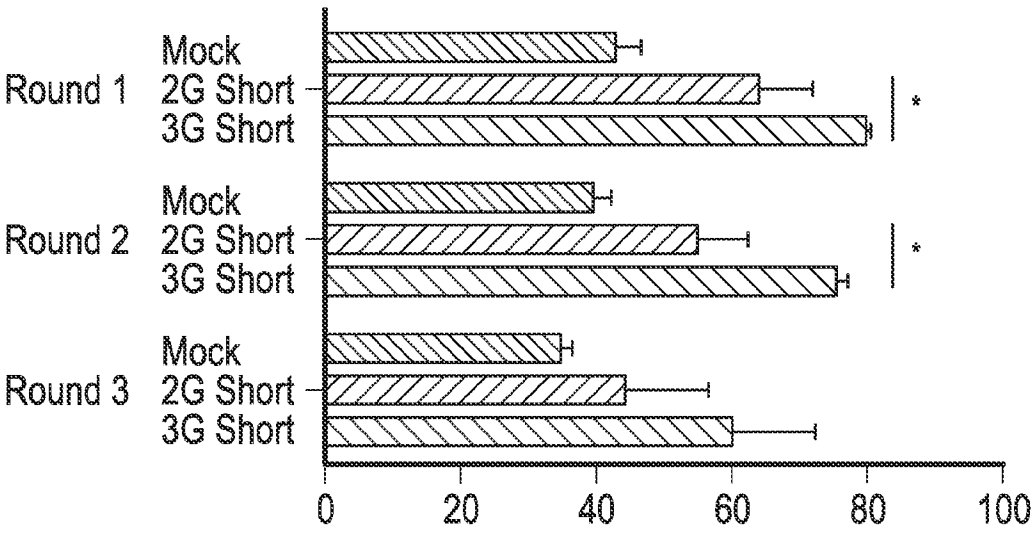


FIG. 18A

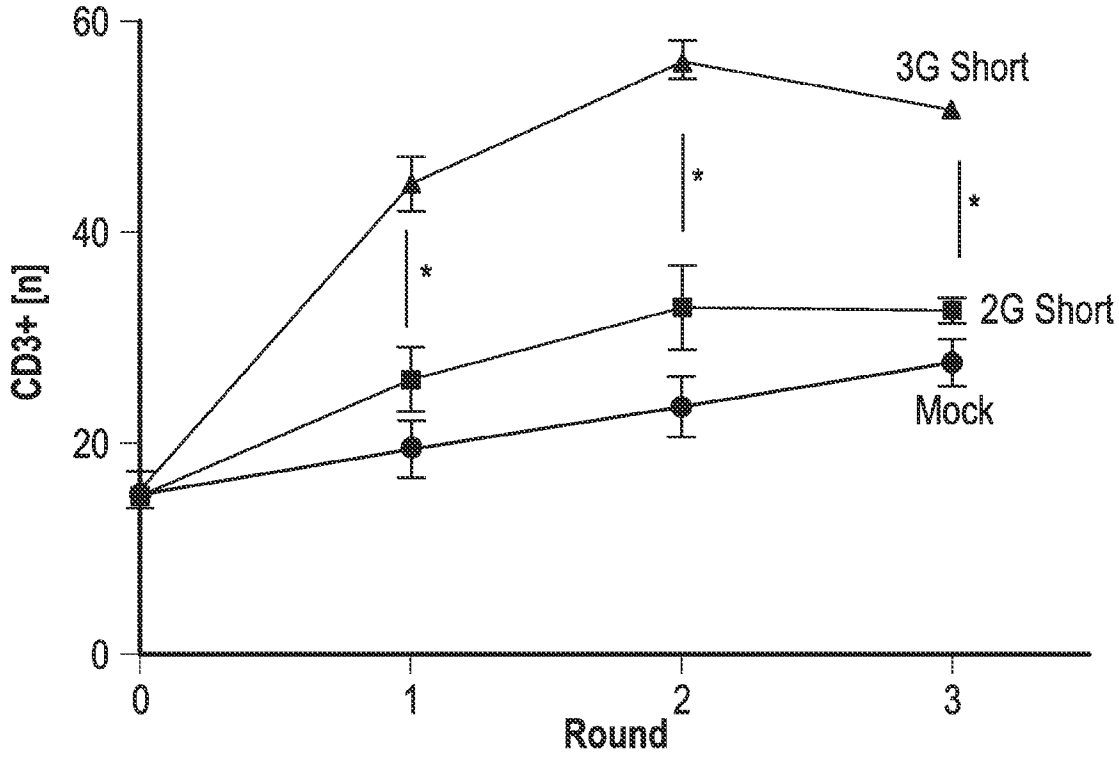


FIG. 18B

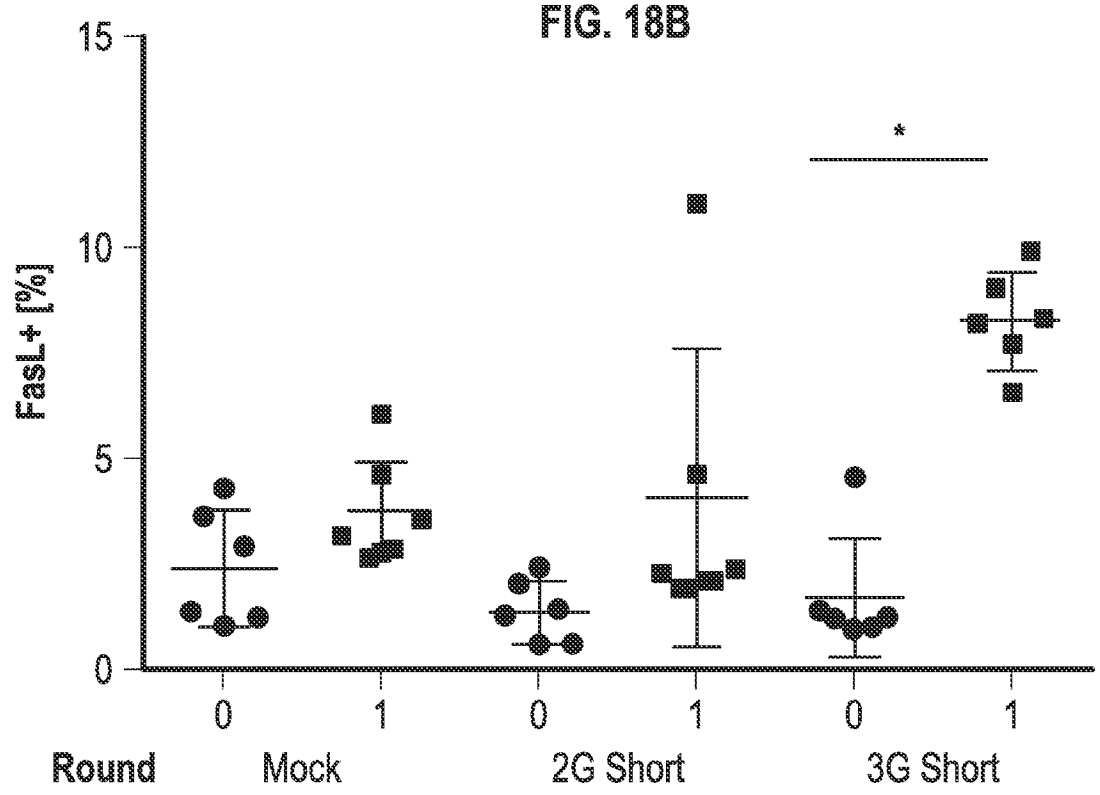


FIG. 18C

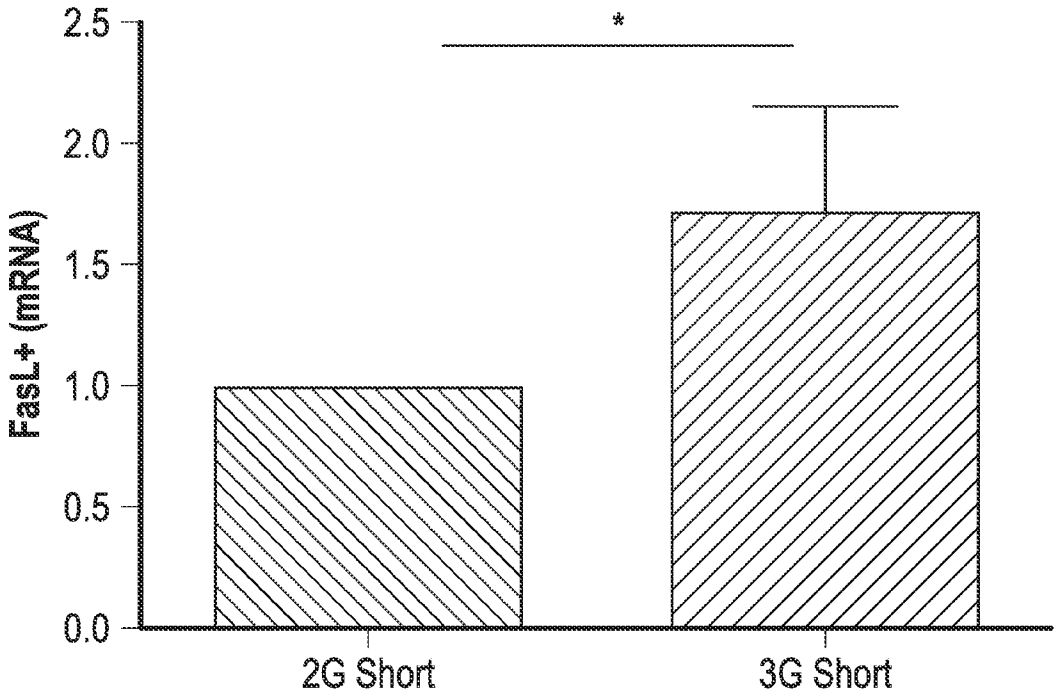


FIG. 18D

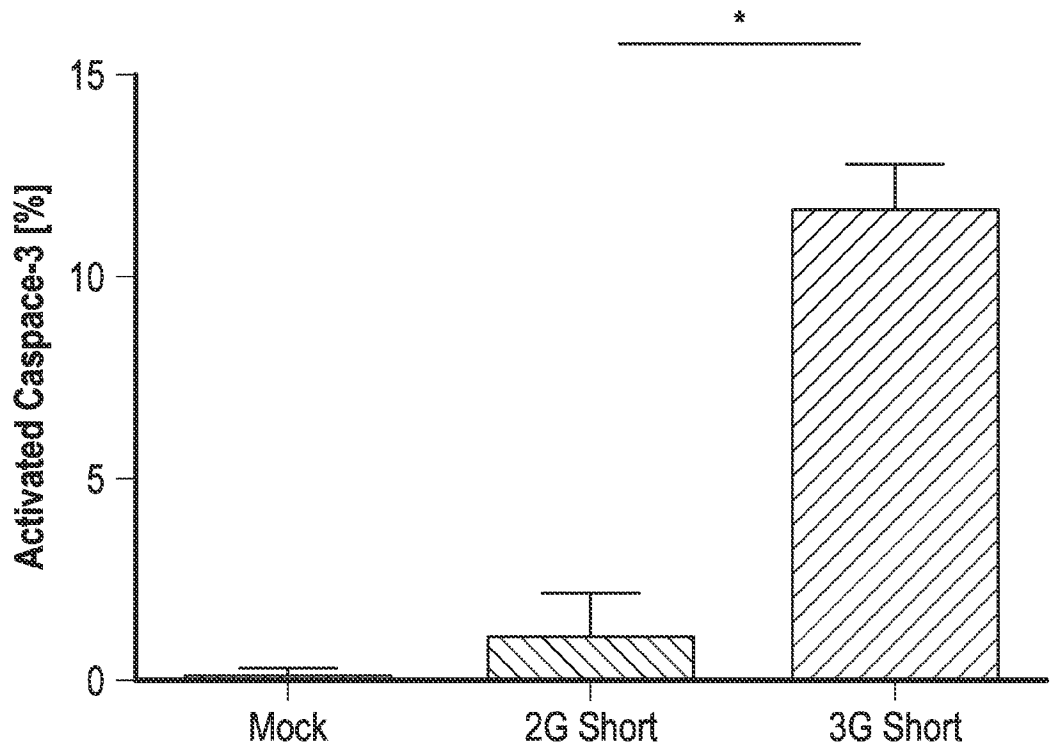


FIG. 18E

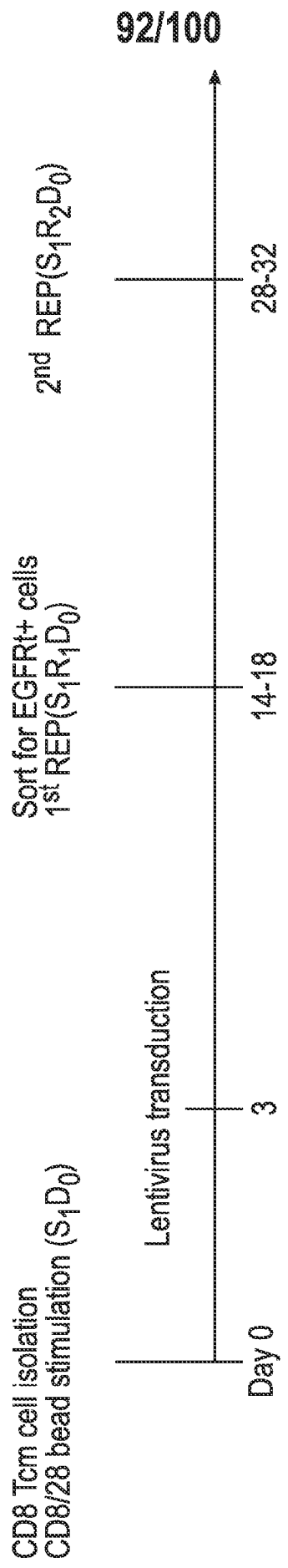

CD8 Tcm cell isolation
CD8/28 bead stimulation (S_1D_0)
Day 0
Lentivirus transduction
3
Sort for EGFRt+ cells
1st REP($S_1R_1D_0$)
14-18
2nd REP($S_1R_2D_0$)
28-32

FIG. 19A

FIG. 19B

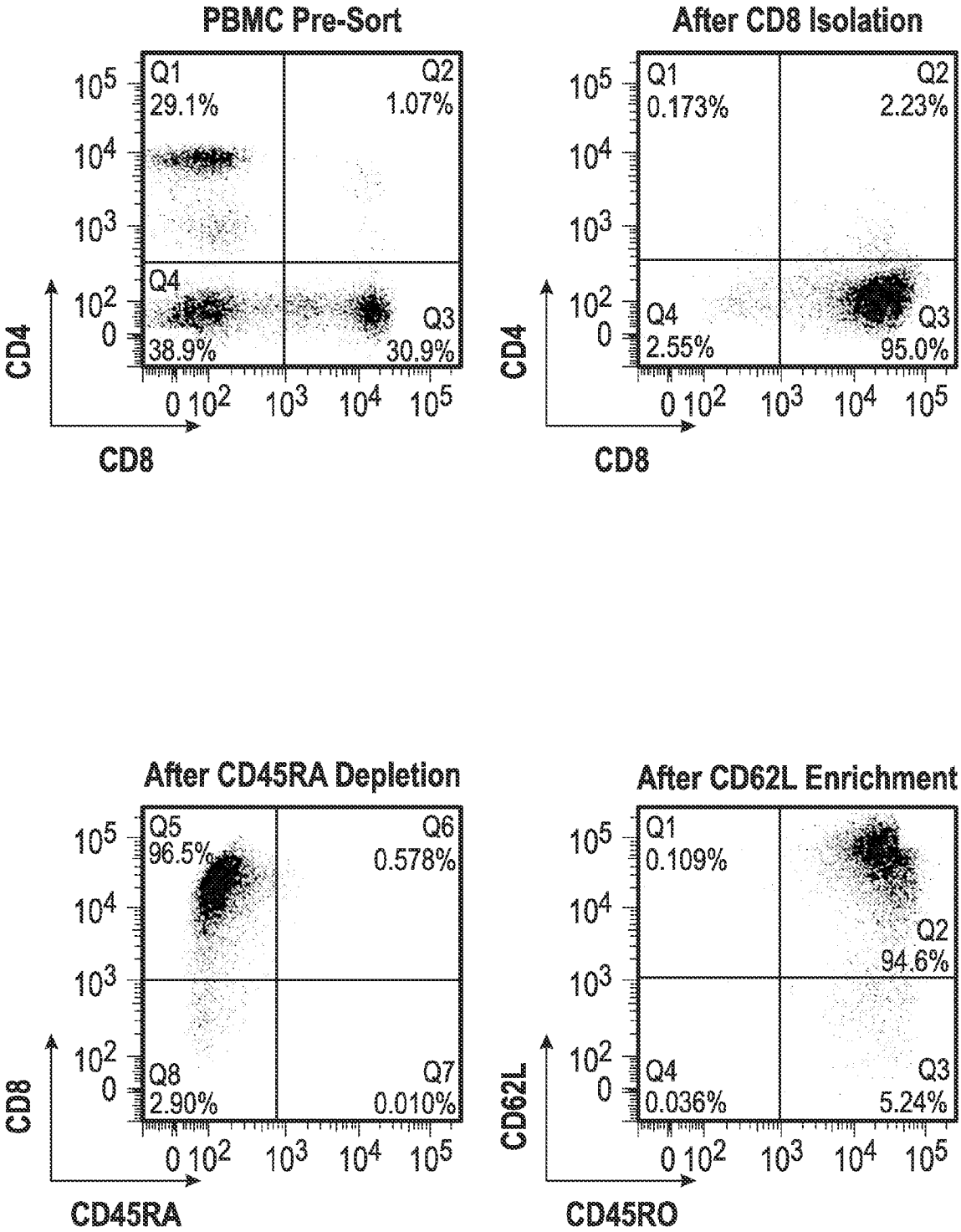

PBMC Pre-Sort
Q1
29.1%
Q2
1.07%
Q4
38.9%
Q3
30.9%
CD4
CD8
After CD8 Isolation
Q1
0.173%
Q2
2.23%
Q4
2.55%
Q3
95.0%
CD4
CD8
After CD45RA Depletion
Q5
96.5%
Q6
0.578%
Q8
2.90%
Q7
0.010%
CD8
CD45RA
After CD62L Enrichment
Q1
0.109%
Q2
94.6%
Q4
0.036%
Q3
5.24%
CD62L
CD45RO

FIG. 19C

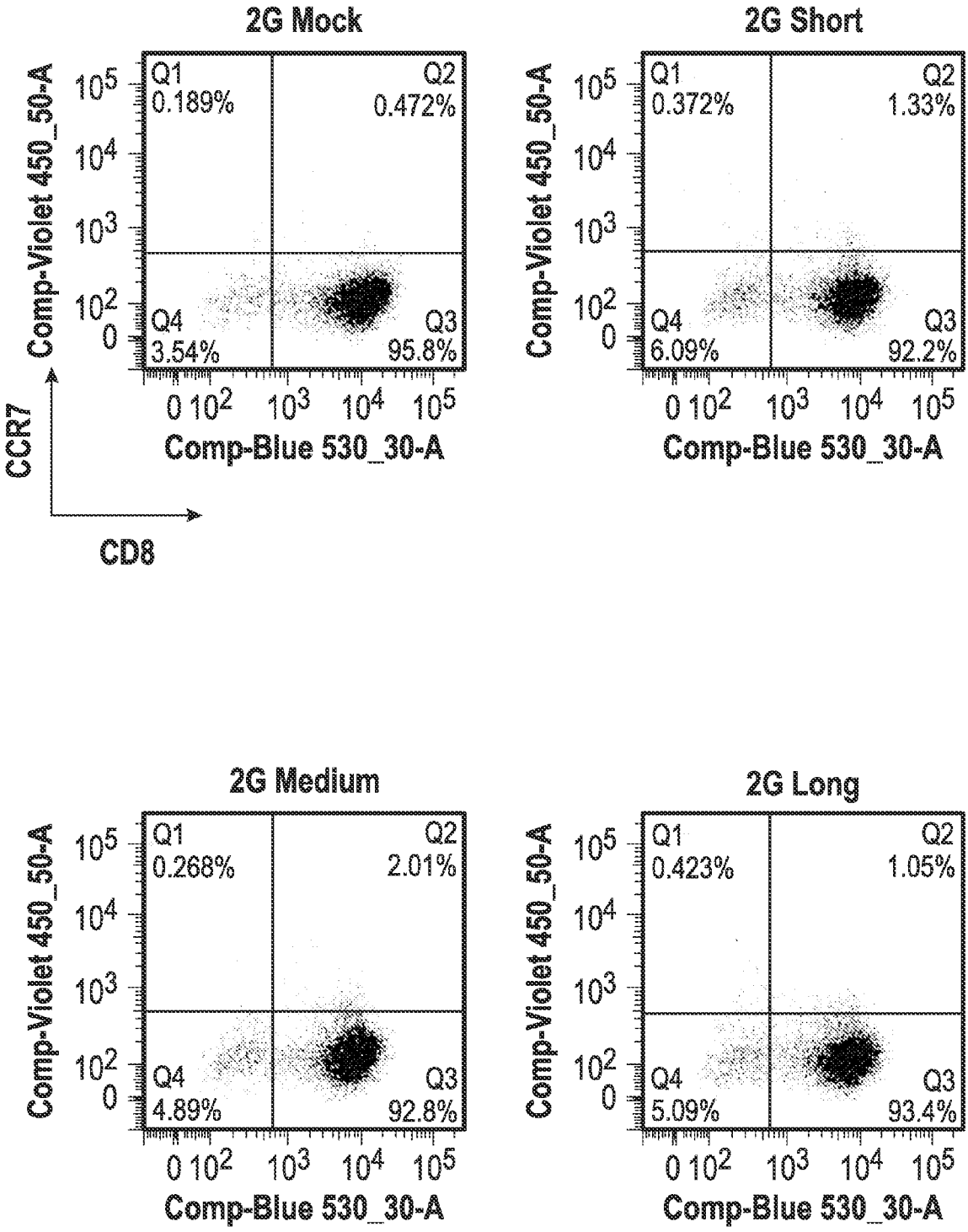

2G Mock
Q1
0.189%
Q2
0.472%
Q4
3.54%
Q3
95.8%
Comp-Violet 450_50-A
Comp-Blue 530_30-A
CCR7
CD8
2G Short
Q1
0.372%
Q2
1.33%
Q4
6.09%
Q3
92.2%
2G Medium
Q1
0.268%
Q2
2.01%
Q4
4.89%
Q3
92.8%
2G Long
Q1
0.423%
Q2
1.05%
Q4
5.09%
Q3
93.4%

FIG. 19C (Continued)

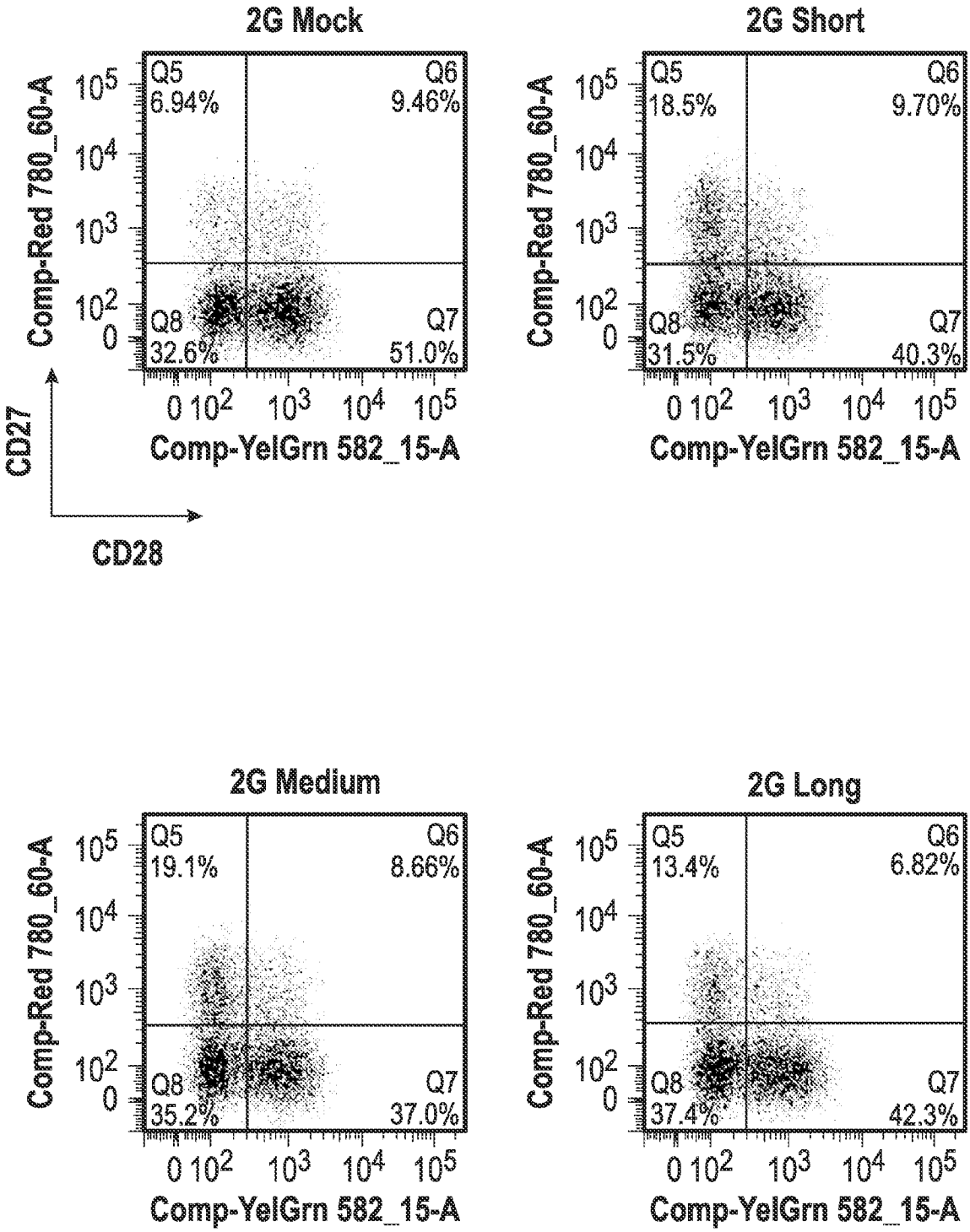
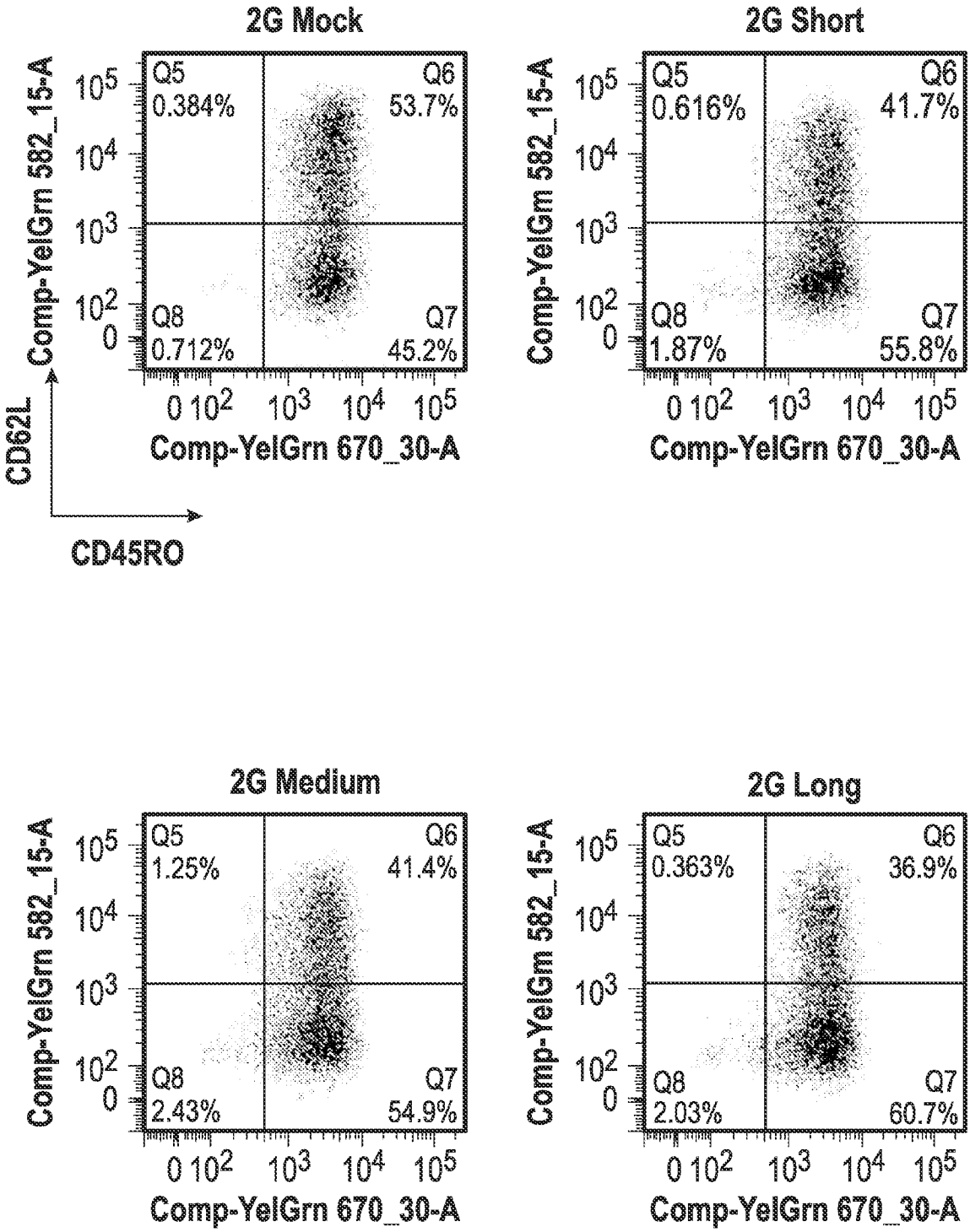


FIG. 19C (Continued)



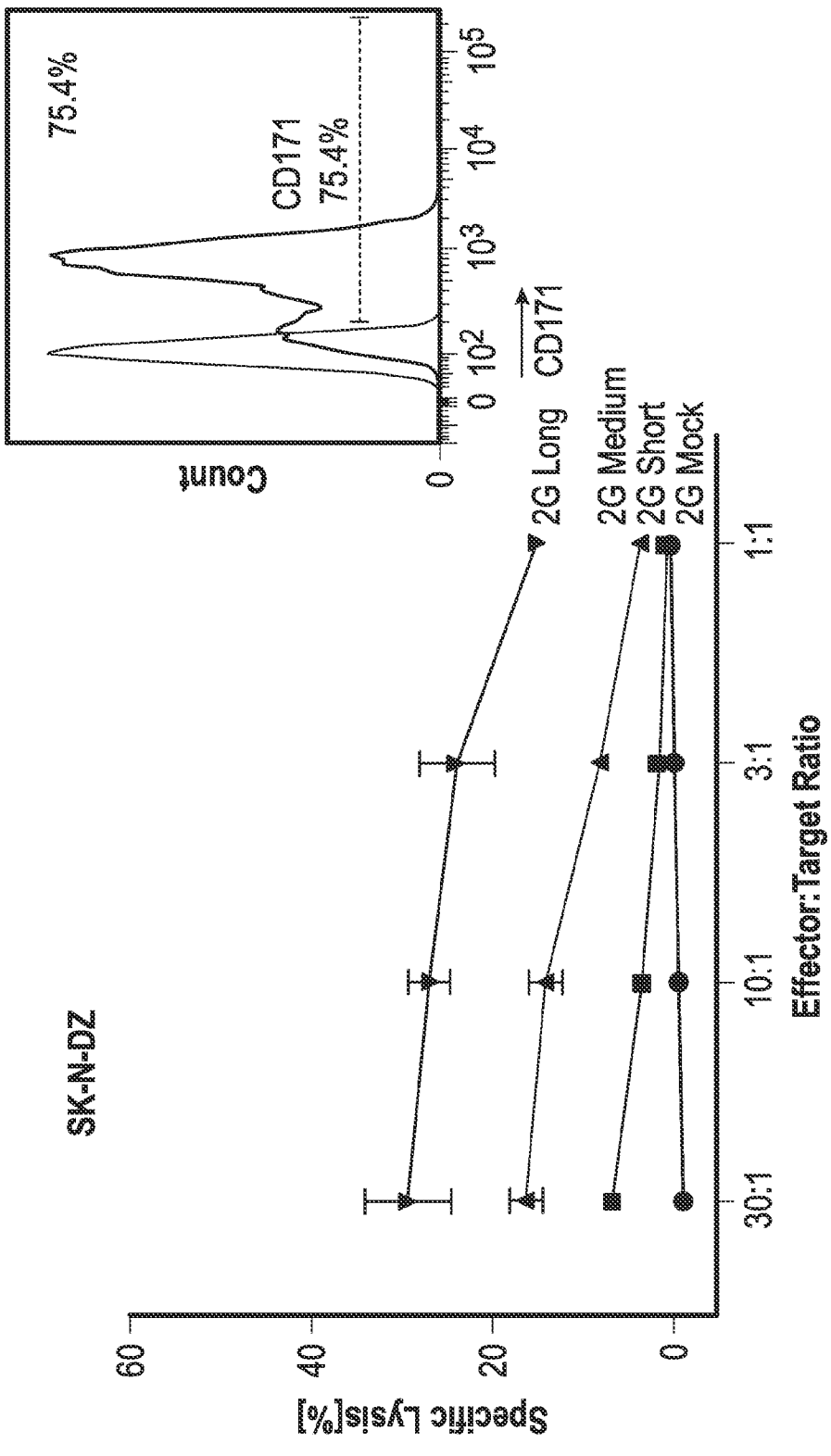

Specific Lysis[%]
60
40
20
0
SK-N-DZ
30:1
10:1
3:1
1:1
Effector:Target Ratio
2G Mock
2G Short
2G Medium
2G Long
CD171
Count
0
0 10^2
10^3
10^4
10^5
CD171
75.4%
75.4%

FIG. 20A

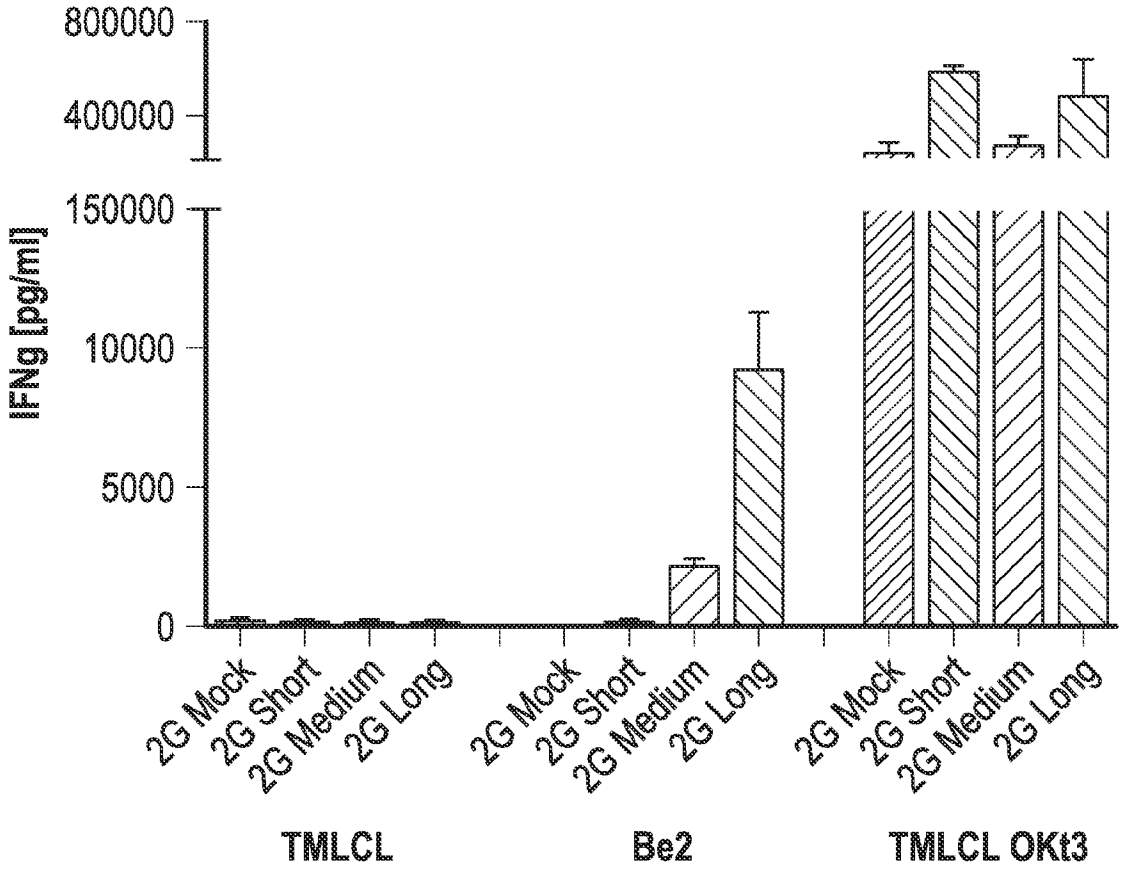

800000
400000
150000
10000
5000
0
IFNg [pg/ml]
2G Mock
2G Short
2G Medium
2G Long
2G Mock
2G Short
2G Medium
2G Long
2G Mock
2G Short
2G Medium
2G Long
TMLCL
Be2
TMLCL OKt3

FIG. 20B

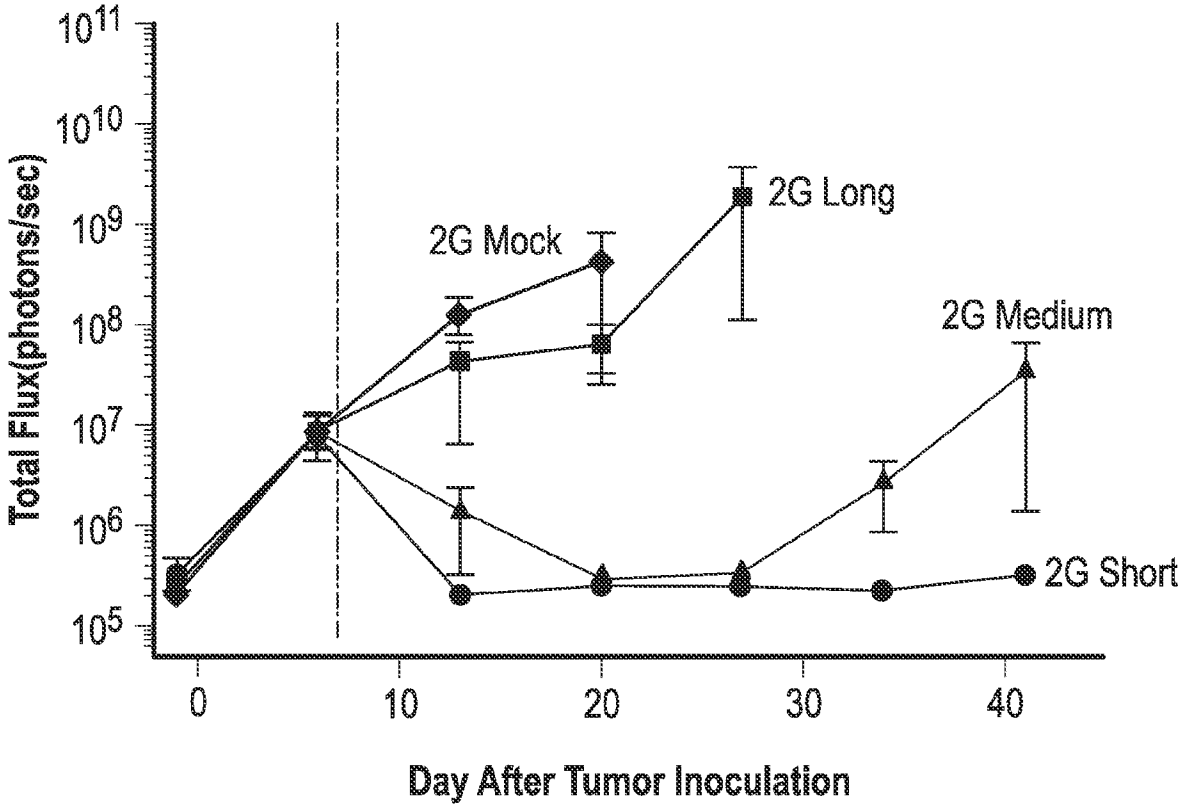


FIG. 20C

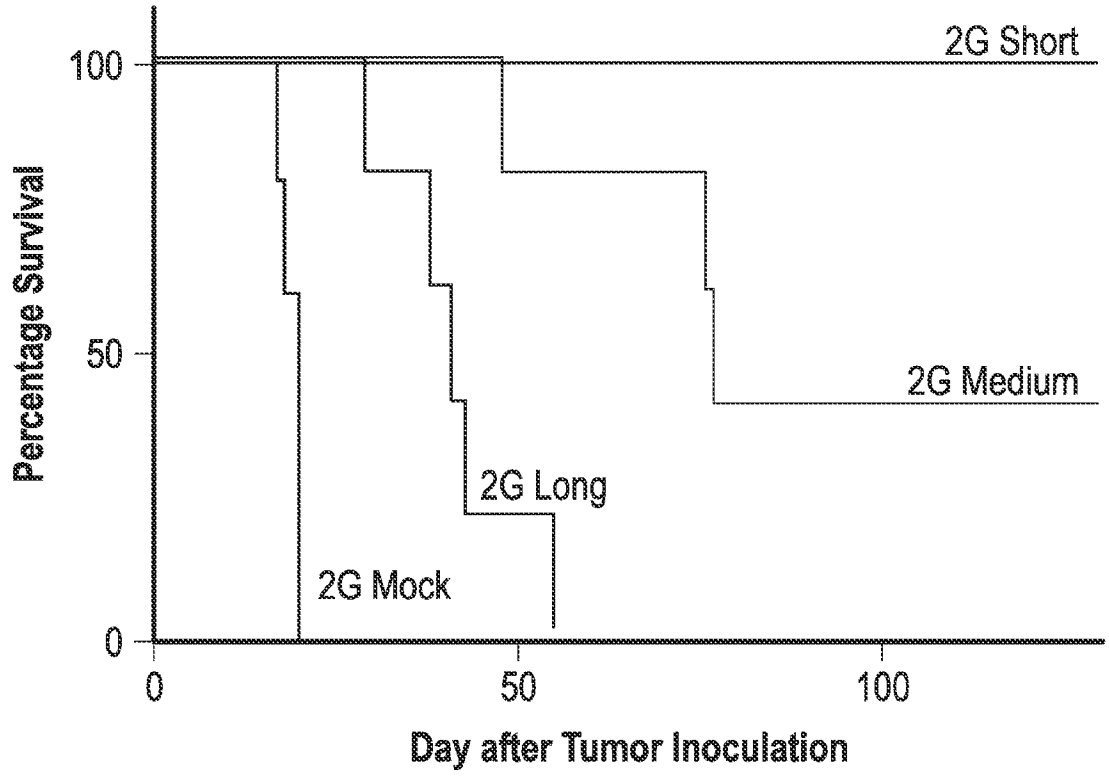
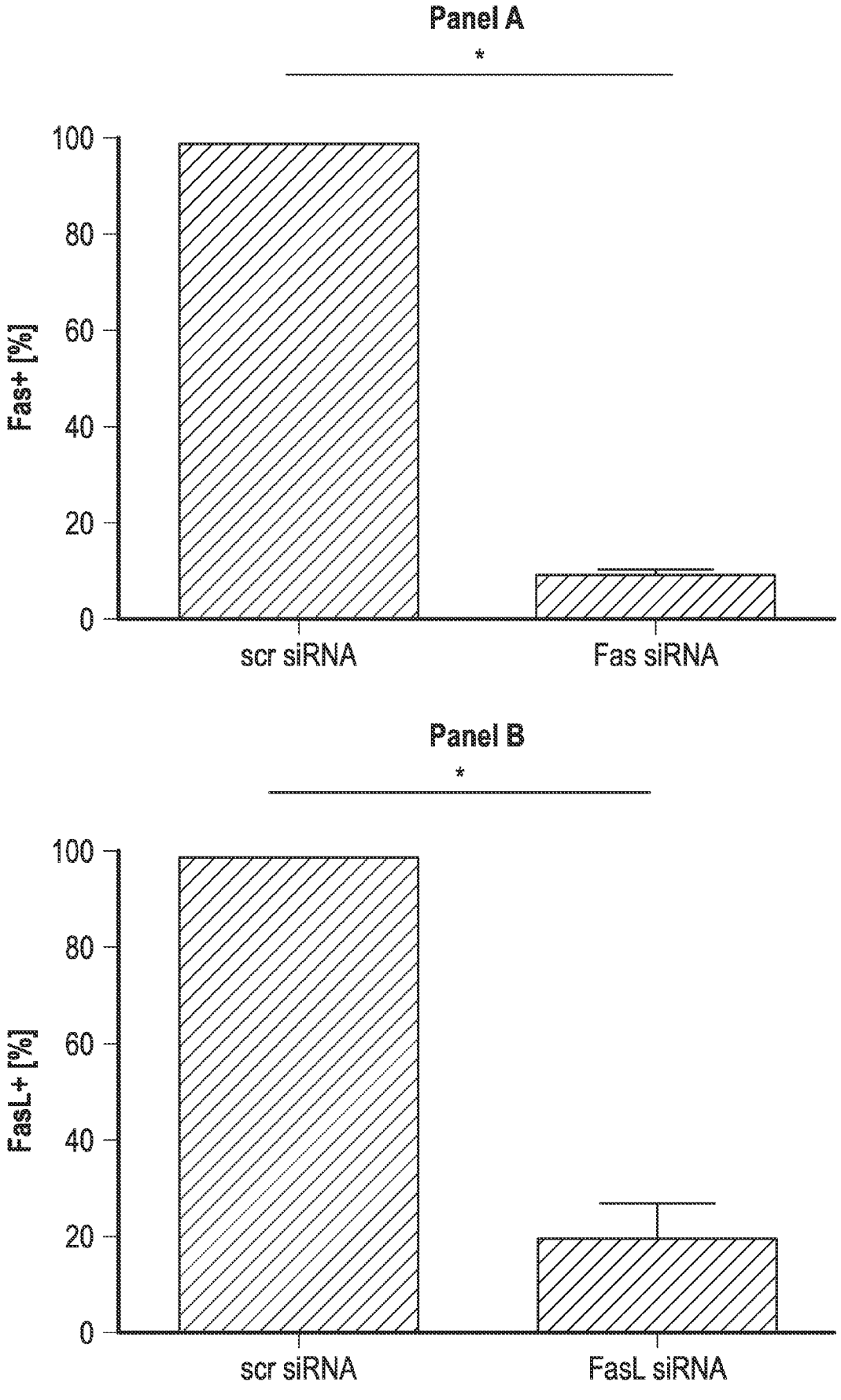


FIG. 20D

FIG. 21


Panel A
*
100
80
60
40
20
0
Fas+ [%]
scr siRNA
Fas siRNA
Panel B
*
100
80
60
40
20
0
FasL+ [%]
scr siRNA
FasL siRNA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/24882

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/0789, 15/62; C07K 16/28 (2015.01)
CPC - C07K 16/30; A61K 38/177; C12N 15/625
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): C12N 5/10, 5/0783, 5/0789, 15/62, 15/63, 15/85; C07K 16/28, 16/30; C12P 19/34 (2015.01)
CPC: C07K 16/28, 16/30, 2319/32, 2319/03, 2319/30; A61K 38/177; C12N 15/10, 15/625, 15/62, 15/63, 15/85

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); EBSCO; PubMed; Google; Google Scholar; Google Patents; 'fusion protein,' 'fusion receptor,' 'chimeric antigen receptor,' 'cd171,' 'L1-CAM,' 'L1CAM,' 'L1 protein,' polypeptide, 'amino acid,' domain, region, spacer, hinge, optimize, customize, 'specific length,' transmembrane, intracellular

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/031687 A1 (JENSEN, M) February 27, 2014; page 2, lines 9-31	1-4, 29
Y	PARK, JR et al. Adoptive Transfer Of Chimeric Antigen Receptor Re-Directed Cytolytic T Lymphocyte Clones In Patients With Neuroblastoma. Mol Ther. April 2007, Vol. 15, No. 4; pages 825-833; page 825, first column, first paragraph to second column, second paragraph; DOI:10.1038/sj.mt.6300104.	1-4, 29
A	WO 2013/123061 A1 (SEATTLE CHILDREN'S HOSPITAL D/B/A SEATTLE CHILDREN'S RESEARCH INSTITUTE) August 22, 2013; abstract	1-4, 29
A	WO 2012/031744 A1 (CHEMOTHERAPEUTISCHES FORSCHUNGSINSTITUT) March 15, 2012; abstract	1-4, 29
A	WO 2009/013359 A2 (FRAUNHOFER GESELLSCHAFT ZUR FORDERUNG DER ANGEWANDTEN FORSCHUNG E.V., et al.) January 29, 2009; abstract	1-4, 29
A	MAHER, J. Immunotherapy Of Malignant Disease Using Chimeric Antigen Receptor Engrafted T Cells. ISRN Oncology. November 14, 2012, Vol. 2012, Article ID 278093; pages 1-23. DOI: 10.5402/2012/278093.	1-4, 29
P, X	KUNKELE, A et al. Functional Tuning Of CARs Reveals Signaling Threshold Above Which CD8 + CTL Antitumor Potency Is Attenuated Due To Cell Fas-FasL-Dependent AICD. Cancer Immunol Res. 09 January 2015, Vol. 3, No. 4; pages 368-379; DOI: 10.1158/2326-6066.CIR-14-0200.	1-4, 29
E, X	WO 2015/066551 A2 (FRED HUTCHINSON CANCER RESEARCH CENTER, et al.) May 07, 2015; abstract	1-4, 29

☐ 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	Date of mailing of the international search report
23 June 2015 (23.06.2015)	10 JUL 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/24882

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.: 5-28, 30, 31
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.