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(54) Title: PROTEIN L FOR ACTIVATION AND EXPANSION OF CHIMERIC ANTIGEN RECEPTOR-MODIFIED IMMUNE CELLS

(57) Abstract: Provided herein are methods for the activation and expansion of T cells. Further provided are methods for the use of the T cells for therapy.

DESCRIPTION

PROTEIN L FOR ACTIVATION AND EXPANSION OF CHIMERIC ANTIGEN RECEPTOR-MODIFIED IMMUNE CELLS

[0001] This application claims the benefit of United States Provisional Patent Application No. 62/733,291, filed September 19, 2018, the entirety of which is incorporated herein by reference.

BACKGROUND

1. Field

[0002] The present invention relates generally to the fields of immunology and medicine. More particularly, it concerns methods and compositions of immune cells engineered to express a chimeric antigen receptor.

2. Description of Related Art

[0003] Adoptive cancer immunotherapy comprises the transfer of immune cells modified with chimeric antigen receptor (CAR) specific to tumor antigens which can be generated by *in vitro* expansion of CAR-modified T cells (CAR-T). Currently, CAR-T cells are expanded *in vitro* using traditional methods with CD3-activating antibodies (Ab). In this method, all T cells regardless of CAR expression are induced to proliferate and not in a CAR-specific manner. This method may lead to the "silencing" of CAR expression and function in the CD3-expanded CAR-T population.

[0004] Although CAR-T cells could be selectively expanded in cultures with CAR-specific tumor antigens either in isolated form or expressed by feeder cells, antigen culture is only applicable for specific CAR types. This approach is not universal or very practical, especially for therapeutic cell manufacturing. Therefore, similar to pan-T cell expansion cultures based on CD3 activation, the CAR-T cell therapeutic field would benefit from universal pan-CAR-specific *in vitro* cultures where CAR-T cells could be activated and expanded in a CAR-specific manner.

SUMMARY

[0005] In a first embodiment, the present disclosure provides an *in vitro* method for activation and/or expansion of CAR-modified immune cells comprising obtaining a starting

population of CAR-modified immune cells; and culturing said population of CAR-modified immune cells in the presence of Protein L for a sufficient period of time to produce a population of activated and/or expanded CAR-modified immune cells.

[0006] In certain aspects, the Protein L is present at a concentration of 0.1 to 5 $\mu g/cm^2$, such as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0. 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, or 2.5 $\mu g/cm^2$. In particular, the Protein L may be present at a concentration of 1 to 5 μg/mL, such as 1.5, 2, 2.5, 3, 3.5, 4, or 4.5 μg/mL. In some aspects, the Protein L is coated on a culture surface. For example, the culture surface can be a culture plate, culture flask, microcarrier, microparticle, hydrogel particle, or culture bag.

[0007] In some aspects, the culture does not comprise anti-CD3 antibody and/or antigen-specific target cells. In certain aspects, the CAR-modified immune cells are T cells, NK cells, dendritic cells, and/or macrophages. In particular aspects, the T cells are $CD8^+$ T cells, $CD4^+$ T cells, αβ T cells or γδ T cells. In certain aspects, the method further comprising selecting for $CD8^+$ T cells.

[0008] In certain aspects, the CAR-modified immune cells are allogeneic. In other aspects, the CAR-modified immune cells are autologous. In specific aspects, the CAR-modified immune cells are derived from pluripotent stem cells (PSCs). In particular aspects, the PSCs are induced pluripotent stem cells (iPSCs). In some aspects, the iPSCs are reprogrammed from blood cells or T cells. In specific aspects, the iPSCs are episomally reprogrammed. In some aspects, the CAR-modified immune cells are derived from primary peripheral blood mononuclear cells (PBMCs) or primary hematopoietic stem cells. In certain aspects, the iPSCs are differentiated to $CD34^+$ progenitors through cytokine-directed differentiation. In particular aspects, the iPSCs are differentiated to $CD34^+$ progenitors through forward programming. For example, the CAR can comprise an antigen-binding domain selected from the group consisting of F(ab')2, Fab', Fab, Fv, and scFv. In some aspects, the CAR comprises CD28 co-stimulatory and CD3ζ signaling domains.

[0009] In some aspects, the culture surface is further coated with retronectin, fibronectin, or VCAM1. In particular aspects, the retronectin is added to the culture at a concentration of 0.1-1 $\mu g/cm^2$, such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 $\mu g/cm^2$ or higher. In additional aspects, the culture plate is further coated with Notch ligand DLL4. In specific

aspects, the DLL4 is added to the culture at a concentration of 0.1-1 μg/cm^2, such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 μg/cm^2 or higher.

[0010] In additional aspects, the culture further comprises IL-2 and/or IL-15. In some aspects, the IL-12 and/or IL-15 are present at a concentration of 5-15 ng/mL, such as 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 ng/mL or higher.

[0011] In certain aspects, the culturing is under hypoxic conditions. In some aspects, the hypoxic conditions comprise 5% oxygen.

[0012] In some aspects, the sufficient period of time is 8-12 days, such as 8, 9 or 10 days. In some aspects, the culturing is in medium comprising SCF, TPO, FLT3L, and/or IL-7. In certain aspects, the SCF, TPO, FLT3L, and/or IL-7 are at a concentration of 50 ng/mL. In some aspects, the medium further comprises nicotinamide.

[0013] In certain aspects, the method results in the selective expansion of CAR-modified immune cells as compared to non-CAR-modified immune cells. In some aspects, at least 40% or 50% of the expanded population of CAR-modified immune cells are CAR-modified immune cells. In some aspects, the expanded population of CAR-modified T cells comprises at least 25% $CD3^+CD8^+$ CAR-modified T cells. In particular aspects, the expanded population of CAR-modified T cells comprises 2-3 fold higher cytotoxic activity as compared to anti-CD3 expanded CAR-modified T cells. In some aspects, the expanded population of CAR-modified T cells comprises increased IFNγ and/or TNFα levels compared to anti-CD3 expanded CAR-modified T cells.

[0014] In another embodiment, there is provided a population of activated and/or expanded CAR-modified immune cells of the embodiments provided herein and aspects thereof. Further provided herein is a pharmaceutical composition comprising the population of CAR-modified immune cells of the embodiments and a pharmaceutically acceptable carrier.

[0015] The present disclosure further provides a method of treating a cancer in a subject comprising administering a therapeutically effective amount expanded CAR-modified immune cells of the embodiments to the subject. In some aspects, the CAR-modified immune cells are allogenic. In other aspects, the CAR-modified immune cells are autologous.

[0016] In additional aspects, the method further comprises administering at least a second therapeutic agent. In some aspects, the at least a second therapeutic agent is a therapeutically effective amount of an immunomodulatory or an immunosuppressive agents. In some aspects, the at least second therapeutic agent is selected from the group consisting of chemotherapy, radiotherapy, and immunotherapy. In certain aspects, the CAR-modified immune cells and/or the at least a second therapeutic agent are administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion.

[0017] Use of a composition of activated and/or expanded CAR-modified immune cells of the embodiments provided herein for the treatment of cancer in subject in need thereof. In some aspects, the CAR-modified immune cells are allogenic. In other aspects, the CAR-modified immune cells are autologous.

[0018] Further provided herein is a composition comprising CAR-modified immune cells and Protein L. In certain aspects, the Protein L is present at a concentration of 0.1 to 5 $\mu g/cm^2$, such as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0. 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, or 2.5 $\mu g/cm^2$. In particular, the Protein L may be present at a concentration of 1 to 5 μg/mL, such as 1.5, 2, 2.5, 3, 3.5, 4, or 4.5 μg/mL. In some aspects, the Protein L is coated on a culture surface. For example, the culture surface can be a culture plate, culture flask, microcarrier, microparticle, hydrogel particle, or culture bag.

[0019] In some aspects, the culture surface is further coated with retronectin, fibronectin, or VCAM1. In particular aspects, the retronectin is added to the culture at a concentration of 0.1-1 $\mu g/cm^2$, such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 $\mu g/cm^2$ or higher. In additional aspects, the culture plate is further coated with Notch ligand DLL4. In specific aspects, the DLL4 is added to the culture at a concentration of 0.1-1 $\mu g/cm^2$, such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 $\mu g/cm^2$ or higher.

[0020] In some aspects, the culture does not comprise anti-CD3 antibody and/or antigen-specific target cells. In certain aspects, the CAR-modified immune cells are T cells, NK cells, dendritic cells, and/or macrophages. In particular aspects, the T cells are $CD8^+$ T cells, $CD4^+$ T cells, αβ T cells or γδ T cells. In certain aspects, the method further comprising selecting for $CD8^+$ T cells.

[0021] In certain aspects, the CAR-modified immune cells are allogeneic. In other aspects, the CAR-modified immune cells are autologous. In specific aspects, the CAR-modified immune cells are derived from pluripotent stem cells (PSCs). In particular aspects, the PSCs are induced pluripotent stem cells (iPSCs). In some aspects, the iPSCs are reprogrammed from blood cells or T cells. In specific aspects, the iPSCs are episomally reprogrammed. In some aspects, the CAR-modified immune cells are derived from primary peripheral blood mononuclear cells (PBMCs) or primary hematopoietic stem cells. In certain aspects, the iPSCs are differentiated to $CD34^+$ progenitors through cytokine-directed differentiation. In particular aspects, the iPSCs are differentiated to $CD34^+$ progenitors through forward programming. For example, the CAR can comprise an antigen-binding domain selected from the group consisting of F(ab')2, Fab', Fab, Fv, and scFv. In some aspects, the CAR comprises CD28 co-stimulatory and CD3ζ signaling domains.

[0022] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0024] FIG. 1: Protein L induces expression of CAR-T cells in dose-dependent manner. Fold expansion of T cells is shown in presence of Protein L alone or in combination with retronectin and/or DLL4.

[0025] FIG. 2: Protein L and retronectin combination resulted in efficient CAR-T expansion as compared to Protein L alone. Flow cytometry analysis of CD8 and CD3 expression is shown for T cell expanded in the present of Protein L alone or in combination with retronectin and/or DLL4.

[0026] FIG. 3: Comparison of T cell expansion with anti-CD3 monoclonal antibody versus Protein L.

[0027] FIGS. 4A-4B: (FIG. 4A) Cultures with $CD19^+$ P15 cells revealed inducible production of IFNγ, TNFα, Granzyme B, sFasL, CCL3, CCL2, GM-CSF, IL2 and IL13. Comparison of cytokine production in T cell expansion with anti-CD3 monoclonal antibody versus Protein L. (FIG. 4B) CAR-dependent *in vitro* cytokine production induced by Protein L.

[0028] FIG. 5: Both anti-CD3 mAb and Protein L expanded CAR-T cells display strong cytotoxic activity against $CD19^+$ P815, Daudi and Raji cells.

[0029] FIG. 6: Tumor growth in mice treated with CAR-T cells up to 6 weeks after CAR-T injection.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0030] Protein L is a bacterial protein with unique specificity to bind light chains of immunoglobulins, including a minimal antigen-binding moiety-single-chain variable domain fragments (scFv), which constitute an antigen-binding site of CARs. It was shown that protein L can bind a cell surface CAR (Zheng *et al.,* 2012). In addition, Zheng *et al.* demonstrated the potential of Protein L as a reagent to detect the expression of CARs by flow cytometry.

[0031] Protein L is a bacterial protein that binds immunoglobulins (Ig) through specific high-affinity interaction with variable light chains without interfering with antigen-binding site. Protein L can also bind the light chain component within single chain variable fragments (scFv), which constitute the antigen-binding domain of CARs. Another unique feature of Protein L is its multi- domain structure with five identical Ig-binding sites in one molecule. Multivalent binding, and therefore potential cross-linking of target molecules expressed on cell surface, indicate that Protein L may function as potent cell activator.

[0032] Protein L, when bound to the scFv portion of the CAR, while not directly binding and blocking the antigen-binding site, can mimic antigen binding and induce CAR activation. In this way, it can be used as a universal CAR-activation reagent in cell culture

applications with CAR-modified cells, including functional analysis of CAR-induced cell responses, and expansion of CAR-expressing cells for therapeutic applications.

[0033] Accordingly, in certain embodiments, the present disclosure provides methods and compositions comprising the use of Protein L for the activation and/or expansion of CAR-modified effector immune cells, such as CAR-T cells, CAR-NK cells, and CAR-macrophage cells. Thus, the present methods provide a universal cell culture system for activation and expansion of CAR-modified cells that is independent of its antigen-specificity.

[0034] Using anti-human CD19 CAR-modified PSC-derived T cells, the present studies provide experimental evidence that immobilized protein L induces a specific proliferative response in CAR-modified T cells, but not in non-modified T cells. It was found that retronectin (recombinant fibronectin fragment) supported the CAR-inductive function of Protein L, while Notch ligand DLL4 could further improve the proliferative response and expansion of $CD19^+$ CAR-T cells. When CAR-T cells expanded in the anti-CD3 monoclonal antibody and Protein L cultures were compared, superior CAR-mediated cytokine production, *in vitro* and *in vivo* anti-tumor activity were found in the Protein L-expanded CAR-T cells.

[0035] Thus, Protein L was identified as a cell culture reagent for specific activation, expansion and analysis of CAR-modified cells *in vitro*. Protein L may be used for GMP-compliant cultures suitable for therapeutic applications as it is produced in animal-free conditions. The present methods may be used for the development of PSC-derived CAR-T therapeutic products. Specific applications include the selection of an optimal CAR configuration for PSC-derived T cells, quality control assays for CAR-T cells and expansion of CAR-T cells for preclinical animal studies.

I. Definitions

[0036] As used herein, "essentially free," in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%, preferably below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0037] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

[0038] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0039] The term "about" refers to the stated value plus or minus 5%.

[0040] As used herein, a composition that is "substantially free" of a specified substance or material contains 30%, 20%, 15%, more preferably 10%, even more preferably 5%, or most preferably 1% of the substance or material.

[0041] By "expression construct" or "expression cassette" is meant a nucleic acid molecule that is capable of directing transcription. An expression construct includes, at a minimum, one or more transcriptional control elements (such as promoters, enhancers or a structure functionally equivalent thereof) that direct gene expression in one or more desired cell types, tissues or organs. Additional elements, such as a transcription termination signal, may also be included.

[0042] A "vector" or "construct" (sometimes referred to as a gene delivery system or gene transfer "vehicle") refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either *in vitro* or *in vivo*.

[0043] A "plasmid," a common type of a vector, is an extra-chromosomal DNA molecule separate from the chromosomal DNA that is capable of replicating independently of the chromosomal DNA. In certain cases, it is circular and double-stranded.

[0044] As used herein, the term "patient" or "subject" refers to a living mammalian organism, such as a human, monkey, cow, sheep, goat, dog, cat, mouse, rat, guinea pig, or transgenic species thereof. In certain embodiments, the patient or subject is a primate. Non-limiting examples of human patients are adults, juveniles, infants and fetuses.

[0045] The terms "tumor-associated antigen," "tumor antigen" and "cancer cell antigen" are used interchangeably herein. In each case, the terms refer to proteins, glycoproteins or carbohydrates that are specifically or preferentially expressed by cancer cells.

[0046] An "epitope" is the site on an antigen recognized by an antibody as determined by the specificity of the amino acid sequence. Two antibodies are said to bind to the same epitope if each competitively inhibits (blocks) binding of the other to the antigen as measured in a competitive binding assay. Alternatively, two antibodies have the same epitope if most amino acid mutations in the antigen that reduce or eliminate binding of one antibody reduce or eliminate binding of the other. Two antibodies are said to have overlapping epitopes if each partially inhibits binding of the other to the antigen, and/or if some amino acid mutations that reduce or eliminate binding of one antibody reduce or eliminate binding of the other.

[0047] "Treating" or treatment of a disease or condition refers to executing a protocol, which may include administering one or more drugs to a patient, in an effort to alleviate signs or symptoms of the disease. Desirable effects of treatment include decreasing the rate of disease progression, ameliorating or palliating the disease state, and remission or improved prognosis. Alleviation can occur prior to signs or symptoms of the disease or condition appearing, as well as after their appearance. Thus, "treating" or "treatment" may include "preventing" or "prevention" of disease or undesirable condition. In addition, "treating" or "treatment" does not require complete alleviation of signs or symptoms, does not require a cure, and specifically includes protocols that have only a marginal effect on the patient.

[0048] The term "effective," as that term is used in the specification and/or claims, means adequate to accomplish a desired, expected, or intended result. "Effective amount," "Therapeutically effective amount" or "pharmaceutically effective amount" when used in the context of treating a patient or subject with a compound means that amount of the compound which, when administered to a subject or patient for treating or preventing a disease, is an amount sufficient to effect such treatment or prevention of the disease.

[0049] "Treatment" or "treating" includes (1) inhibiting a disease in a subject or patient experiencing or displaying the pathology or symptomatology of the disease (*e.g.*, arresting further development of the pathology and/or symptomatology), (2) ameliorating a disease in a subject or patient that is experiencing or displaying the pathology or symptomatology of the disease (*e.g.*, reversing the pathology and/or symptomatology), and/or (3) effecting any

measurable decrease in a disease or symptom thereof in a subject or patient that is experiencing or displaying the pathology or symptomatology of the disease.

[0050] "Prevention" or "preventing" includes: (1) inhibiting the onset of a disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease, and/or (2) slowing the onset of the pathology or symptomatology of a disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease.

[0051] The term "forward programming" refers to the programming of a multipotent or pluripotent cell, as opposed to a differentiated somatic cell that has no pluripotency, by the provision of one or more specific lineage-determining genes or gene products to the multipotent or pluripotent cell.

[0052] As generally used herein "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues, organs, and/or bodily fluids of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

[0053] "Pharmaceutically acceptable salts" means salts of compounds disclosed herein which are pharmaceutically acceptable, as defined above, and which possess the desired pharmacological activity. Such salts include acid addition salts formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or with organic acids such as 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, 2-naphthalenesulfonic acid, 3-phenylpropionic acid, 4,4′-methylenebis(3-hydroxy-2-ene-1-carboxylic acid), 4-methylbicyclo[2.2.2]oct-2-ene-1-carboxylic acid, acetic acid, aliphatic mono- and dicarboxylic acids, aliphatic sulfuric acids, aromatic sulfuric acids, benzenesulfonic acid, benzoic acid, camphorsulfonic acid, carbonic acid, cinnamic acid, citric acid, cyclopentanepropionic acid, ethanesulfonic acid, fumaric acid, glucoheptonic acid, gluconic acid, glutamic acid, glycolic acid, heptanoic acid, hexanoic acid, hydroxynaphthoic acid, lactic acid, laurylsulfuric acid, maleic acid, malic acid, malonic acid, mandelic acid, methanesulfonic acid, muconic acid, *o*-(4-hydroxybenzoyl)benzoic acid, oxalic acid, *p*-chlorobenzenesulfonic acid, phenyl-substituted alkanoic acids, propionic acid, *p*-toluenesulfonic acid, pyruvic acid,

salicylic acid, stearic acid, succinic acid, tartaric acid, tertiarybutylacetic acid, trimethylacetic acid, and the like. Pharmaceutically acceptable salts also include base addition salts which may be formed when acidic protons present are capable of reacting with inorganic or organic bases. Acceptable inorganic bases include sodium hydroxide, sodium carbonate, potassium hydroxide, aluminum hydroxide and calcium hydroxide. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, *N*-methylglucamine and the like. It should be recognized that the particular anion or cation forming a part of any salt of this invention is not critical, so long as the salt, as a whole, is pharmacologically acceptable. Additional examples of pharmaceutically acceptable salts and their methods of preparation and use are presented in *Handbook of Pharmaceutical Salts: Properties, and Use* (P. H. Stahl & C. G. Wermuth eds., Verlag Helvetica Chimica Acta, 2002).

[0054] A "pharmaceutically acceptable carrier," "drug carrier," or simply "carrier" is a pharmaceutically acceptable substance formulated along with the active ingredient medication that is involved in carrying, delivering and/or transporting a chemical agent. Drug carriers may be used to improve the delivery and the effectiveness of drugs, including for example, controlled-release technology to modulate drug bioavailability, decrease drug metabolism, and/or reduce drug toxicity. Some drug carriers may increase the effectiveness of drug delivery to the specific target sites. Examples of carriers include: liposomes, microspheres (*e.g.*, made of poly(lactic-co-glycolic) acid), albumin microspheres, synthetic polymers, nanofibers, protein-DNA complexes, protein conjugates, erythrocytes, virosomes, and dendrimers.

[0055] The term "chimeric antigen receptors (CARs)," as used herein, may refer to artificial T cell receptors, chimeric T cell receptors, or chimeric immunoreceptors, for example, and encompass engineered receptors that graft an artificial specificity onto a particular immune effector cell. CARs may be employed to impart the specificity of a monoclonal antibody onto a T cell, thereby allowing a large number of specific T cells to be generated, for example, for use in adoptive cell therapy. In specific embodiments, CARs direct specificity of the cell to a tumor associated antigen, for example. In some embodiments, CARs comprise an intracellular activation domain, a transmembrane domain, and an extracellular domain comprising a tumor associated antigen binding region. In particular aspects, CARs comprise fusions of single-chain variable fragments (scFv) derived from monoclonal antibodies, fused to CD3-zeta a transmembrane domain and endodomain. The specificity of other CAR designs may be derived from ligands of receptors (*e.g.*, peptides) or from pattern-recognition receptors, such as Dectins.

In certain cases, the spacing of the antigen-recognition domain can be modified to reduce activation-induced cell death. In certain cases, CARs comprise domains for additional co-stimulatory signaling, such as CD3ζ, FcR, CD27, CD28, CD137, DAP10, and/or OX40. In some cases, molecules can be co-expressed with the CAR, including co-stimulatory molecules, reporter genes for imaging (*e.g.*, for positron emission tomography), gene products that conditionally ablate the T cells upon addition of a pro-drug, homing receptors, chemokines, chemokine receptors, cytokines, and cytokine receptors.

[0056] The term "culturing" refers to the *in vitro* maintenance, differentiation, and/or propagation of cells in suitable media. By "enriched" is meant a composition comprising cells present in a greater percentage of total cells than is found in the tissues where they are present in an organism.

[0057] An "anti-cancer" agent is capable of negatively affecting a cancer cell/tumor in a subject, for example, by promoting killing of cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer.

II. CAR-Modified Immune Cells

[0058] Certain embodiments of the present disclosure concern immune cells which express a CAR. The immune cells may be T cells (*e.g.*, regulatory T cells, $CD4^+$ T cells, $CD8^+$ T cells, alpha-beta T cell or gamma-delta T cells), NK cells, invariant NK cells, NKT cells, or stem cells (*e.g.*, mesenchymal stem cells (MSCs) or induced pluripotent stem (iPSC) cells). In some embodiments, the cells are monocytes or granulocytes, *e.g.*, myeloid cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and/or basophils. Also provided herein are methods of producing and engineering the immune cells as well as methods of using and administering the cells for adoptive cell therapy, in which case the cells may be autologous or allogeneic. Thus, the immune cells may be used as immunotherapy, such as to target cancer cells.

[0059] Methods are provided herein the for activation and/or expansion of the immune cells to selectively activate and/or expand CAR-modified immune cells, such as CAR-T cells,

in the presence of Protein L. The Protein L may be present in the culture alone or in combination with retronectin and/or DLL4.

[0060] The immune cells may be isolated from subjects, particularly human subjects. The immune cells can be obtained from a subject of interest, such as a subject suspected of having a particular disease or condition, a subject suspected of having a predisposition to a particular disease or condition, or a subject who is undergoing therapy for a particular disease or condition. Immune cells can be collected from any location in which they reside in the subject including, but not limited to, blood, cord blood, spleen, thymus, lymph nodes, and bone marrow. The isolated immune cells may be used directly, or they can be stored for a period of time, such as by freezing.

[0061] The immune cells may be enriched/purified from any tissue where they reside including, but not limited to, blood (including blood collected by blood banks or cord blood banks), spleen, bone marrow, tissues removed and/or exposed during surgical procedures, and tissues obtained via biopsy procedures. Tissues/organs from which the immune cells are enriched, isolated, and/or purified may be isolated from both living and non-living subjects, wherein the non-living subjects are organ donors.

A. T Cells

[0062] In some embodiments, the immune cells are T cells. Several basic approaches for the derivation, activation and expansion of functional anti-tumor effector cells have been described in the last two decades. These include: autologous cells, such as tumor-infiltrating lymphocytes (TILs); T cells activated *ex-vivo* using autologous DCs, lymphocytes, artificial antigen-presenting cells (APCs) or beads coated with T cell ligands and activating antibodies, or cells isolated by virtue of capturing target cell membrane; allogeneic cells naturally expressing anti-host tumor T cell receptor (TCR); and non-tumor-specific autologous or allogeneic cells genetically reprogrammed or "redirected" to express tumor-reactive TCR or chimeric TCR molecules displaying antibody-like tumor recognition capacity known as "T-bodies". These approaches have given rise to numerous protocols for T cell preparation and immunization which can be used in the methods described herein.

[0063] In some embodiments, the T cells are derived from the blood, bone marrow, lymph, umbilical cord, or lymphoid organs. In some aspects, the cells are human cells. The cells typically are primary cells, such as those isolated directly from a subject and/or isolated

from a subject and frozen. In some embodiments, the cells include one or more subsets of T cells or other cell types, such as whole T cell populations, $CD4^+$ cells, $CD8^+$ cells, and subpopulations thereof, such as those defined by function, activation state, maturity, potential for differentiation, expansion, recirculation, localization, and/or persistence capacities, antigen- specificity, type of antigen receptor, presence in a particular organ or compartment, marker or cytokine secretion profile, and/or degree of differentiation. With reference to the subject to be treated, the cells may be allogeneic and/or autologous. In some embodiments, the methods include isolating cells from the subject, preparing, processing, culturing, and/or engineering them, as described herein, and re-introducing them into the same patient, with or without cryopreservation.

[0064] Among the sub-types and subpopulations of T cells (*e.g.*, $CD4^+$ and/or $CD8^+$ T cells) are naive T (T_N) cells, effector T cells (T_{EFF}), memory T cells and sub-types thereof, such as stem cell memory T (TSC_M), central memory T (TC_M), effector memory T (T_{EM}), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells, mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, alpha/beta T cells, and delta/gamma T cells.

[0065] In some embodiments, one or more of the T cell populations is enriched for or depleted of cells that are positive for a specific marker, such as surface markers, or that are negative for a specific marker. In some cases, such markers are those that are absent or expressed at relatively low levels on certain populations of T cells (*e.g.*, non-memory cells) but are present or expressed at relatively higher levels on certain other populations of T cells (*e.g.*, memory cells).

[0066] In some embodiments, T cells are separated from a PBMC sample by negative selection of markers expressed on non-T cells, such as B cells, monocytes, or other white blood cells, such as CD14. In some aspects, a $CD4^+$ or $CD8^+$ selection step is used to separate $CD4^+$ helper and $CD8^+$ cytotoxic T cells. Such $CD4^+$ and $CD8^+$ populations can be further sorted into sub-populations by positive or negative selection for markers expressed or expressed to a relatively higher degree on one or more naive, memory, and/or effector T cell subpopulations.

[0067] In some embodiments, $CD8^+$ T cells are further enriched for or depleted of naive, central memory, effector memory, and/or central memory stem cells, such as by positive or negative selection based on surface antigens associated with the respective subpopulation. In some embodiments, enrichment for central memory T (T_{CM}) cells is carried out to increase efficacy, such as to improve long-term survival, expansion, and/or engraftment following administration, which in some aspects is particularly robust in such sub-populations.

[0068] In some embodiments, the T cells are autologous T cells. In this method, tumor samples are obtained from patients and a single cell suspension is obtained. The single cell suspension can be obtained in any suitable manner, *e.g.*, mechanically (disaggregating the tumor using, *e.g.*, a gentleMACS™ Dissociator, Miltenyi Biotec, Auburn, Calif.) or enzymatically (*e.g.*, collagenase or DNase). Single-cell suspensions of tumor enzymatic digests are cultured in interleukin-2 (IL-2).

[0069] The cultured T cells can be pooled and rapidly expanded. Rapid expansion provides an increase in the number of antigen-specific T-cells of at least about 50-fold (*e.g.*, 50-, 60-, 70-, 80-, 90-, or 100-fold, or greater) over a period of about 10 to about 14 days. More preferably, rapid expansion provides an increase of at least about 200-fold (*e.g.*, 200-, 300-, 400-, 500-, 600-, 700-, 800-, 900-, or greater) over a period of about 10 to about 14 days.

[0070] Expansion can be accomplished by any of a number of methods as are known in the art. For example, T cells can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of feeder lymphocytes and either interleukin-2 (IL-2) or interleukin-15 (IL-15), with IL-2 being preferred. The non-specific T-cell receptor stimulus can include around 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody (available from Ortho-McNeil®, Raritan, N.J.). Alternatively, T cells can be rapidly expanded by stimulation of peripheral blood mononuclear cells (PBMC) *in vitro* with one or more antigens (including antigenic portions thereof, such as epitope(s), or a cell) of the cancer, which can be optionally expressed from a vector, such as an human leukocyte antigen A2 (HLA-A2) binding peptide, in the presence of a T-cell growth factor, such as 300 IU/ml IL-2 or IL-15, with IL-2 being preferred. The *in vitro*-induced T-cells are rapidly expanded by re-stimulation with the same antigen(s) of the cancer pulsed onto HLA-A2-expressing antigen-presenting cells. Alternatively, the T-cells can be re-stimulated with irradiated, autologous lymphocytes or with irradiated $HLA\text{-}A2^+$ allogeneic lymphocytes and IL-2, for example.

[0071] The autologous T cells can be modified to express a T cell growth factor that promotes the growth and activation of the autologous T cells. Suitable T cell growth factors include, for example, interleukin (IL)-2, IL-7, IL-15, and IL-12. Suitable methods of modification are known in the art. See, for instance, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 2001; and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, NY, 1994. In particular aspects, modified autologous T cells express the T cell growth factor at high levels. T cell growth factor coding sequences, such as that of IL-12, are readily available in the art, as are promoters, the operable linkage of which to a T cell growth factor coding sequence promote high-level expression.

[0072] In some embodiments, the T cells are activated and/or expanded in the presence of protein L. The protein L may be added to the culture at a concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 1.5, 1.6, 1.7, 1.8, 1.9, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or 3.0 $\mu g/cm^2$. The protein L may be added to the culture immobilized on a surface or soluble in the media. The culture surface is a culture plate, culture flask, microcarrier, microparticle, hydrogel particle, or culture bag. The protein L can be added in combination with an extracellular matrix protein, such as retronectin or fibronectin.

B. Stem Cells

[0073] In some embodiments, the CAR-modified immune cells of the present disclosure may be, or are derived from, stem cells, such as induced pluripotent stem cells (PSCs), mesenchymal stem cells (MSCs), or hematopoietic stem cells (HSCs).

[0074] With the exception of germ cells, red blood cells, and platelets, any cell can be used as a starting point for iPSCs. For example, cell types could be keratinocytes, fibroblasts, hematopoietic cells, mesenchymal cells, liver cells, or stomach cells. There is no limitation on the degree of cell differentiation or the age of an animal from which cells are collected; even undifferentiated progenitor cells (including somatic stem cells) and finally differentiated mature cells can be used as sources of somatic cells in the methods disclosed herein.

[0075] Somatic cells can be reprogrammed to produce iPS cells using methods known to one of skill in the art. Generally, nuclear reprogramming factors are used to produce pluripotent stem cells from a somatic cell. In some embodiments, at least three, or at least four,

of Klf4, c-Myc, Oct3/4, Sox2, Nanog, and Lin28 are utilized. In other embodiments, Oct3/4, Sox2, c-Myc and Klf4 are utilized or Oct3/4, Sox2, Nanog, and Lin28.

[0076] Once derived, iPSCs can be cultured in a medium sufficient to maintain pluripotency. In certain embodiments, undefined conditions may be used; for example, pluripotent cells may be cultured on fibroblast feeder cells or a medium that has been exposed to fibroblast feeder cells in order to maintain the stem cells in an undifferentiated state. In some embodiments, the cell is cultured in the co-presence of mouse embryonic fibroblasts treated with radiation or an antibiotic to terminate the cell division, as feeder cells. Alternately, pluripotent cells may be cultured and maintained in an essentially undifferentiated state using a defined, feeder-independent culture system, such as a TESR™ medium or E8™/Essential 8™ medium.

C. Cytokine-Directed Differentiation

[0077] Certain embodiments of the present disclosure concern the differentiation of PSCs to HPCs. The PSCs can be differentiated into HPCs by methods known in the art such as described in U.S. Patent No. 8,372,642, which is incorporated by reference herein. In one method, combinations of BMP4, VEGF, Flt3 ligand, IL-3, and GM-CSF may be used to promote hematopoietic differentiation. In certain embodiments, the sequential exposure of cell cultures to a first media to prepare PSCs for differentiation, a second media that includes BMP4, VEGF, and FGF, followed by culture in a third media that includes Flt3 ligand, SCF, TPO, IL-3, and IL-6 can differentiate pluripotent cells into HPCs and hematopoietic cells. The second defined media can also comprise heparin. Further, inclusion of FGF-2 (50 ng/ml) in the media containing BMP4 and VEGF can enhance the efficiency of the generation of hematopoietic precursor cells from pluripotent cells. In addition, inclusion of a Glycogen synthase kinase 3 (GSK3) inhibitor (*e.g.*, CHIR99021, BIO, and SB-216763) in the first defined media can further enhance the production of HPCs.

[0078] Generally, differentiation of pluripotent cells into hematopoietic precursor cells may be performed using defined or undefined conditions. It will be appreciated that defined conditions are generally preferable in embodiments where the resulting cells are intended to be administered to a human subject. Hematopoietic stem cells may be derived from pluripotent stem cells under defined conditions (*e.g.*, using a TeSR media), and hematopoietic cells may be generated from embryoid bodies derived from pluripotent cells. In other embodiments,

pluripotent cells may be co-cultured on OP9 cells or mouse embryonic fibroblast cells and subsequently differentiated.

[0079] Pluripotent cells may be allowed to form embryoid bodies or aggregates as a part of the differentiation process. The formation of "embryoid bodies" (EBs), or clusters of growing cells, in order to induce differentiation generally involves *in vitro* aggregation of human pluripotent stem cells into EBs and allows for the spontaneous and random differentiation of human pluripotent stem cells into multiple tissue types that represent endoderm, ectoderm, and mesoderm origins. Three-dimensional EBs can thus be used to produce some fraction of hematopoietic cells and endothelial cells.

[0080] EBs may be formed using the following protocol. Undifferentiated iPSCs adapted to feeder free growth on MATRIGEL™ coated plates may be harvested at confluency using 0.5M EDTA treatment for about 8-10 minutes at room temperature. The EDTA is aspirated after the incubation and the EBs may be formed by collecting the cells in SFD media containing rock inhibitor or blebbistatin. The media may be changed the next day to EB1 differentiation media containing different cytokine formulations. The cells are plated at a density of 0.25-0.5 million cells per ml to promote aggregate formation.

[0081] To promote aggregate formation, the cells may be transferred to low-attachment plates for an overnight incubation in serum–free differentiation (SFD) medium, consisting of 75% IMDM (Gibco), 25% Ham's Modified F12 (Cellgro) supplemented with 0.05% N2 and B-27 without RA supplements, 200 mM 1-glutamine, 0.05 mg/ml Ascorbic Acid-2-phosphate Magnesium Salt (Asc 2-P) (WAKO), and 4.5 x 10^{-4} MTG. The next day the cells may be collected from each well and centrifuged. The cells may then be resuspended in "EB differentiation media," which consists of SFD basal media supplemented with about 50 ng/ml bone morphogenetic factor (BMP4), about 50 ng/ml vascular endothelial growth factor (VEGF), and 50 ng/ml zb FGF for the first four days of differentiation. The cells are half fed ever 48 hrs. On the fifth day of differentiation the media is replaced with a second media comprised of SFD media supplemented with 50 ng/ml stem cell factor (SCF), about 50 ng/ml Flt-3 ligand (Flt-3L), 50 ng/ml interleukin-6 (IL-6), 50 ng/ml interleukin-3 (IL-3), 50 ng/ml thrombopoieitin (TPO). The cells are half fed every 48 hrs with fresh differentiation media. The media changes are performed by spinning down the differentiation cultures at 300 g for 5 minutes and aspirating half the volume from the differentiating cultures and replenishing it with fresh media. In certain embodiments, the EB differentiation media may include about

BMP4 (*e.g.*, about 50 ng/ml), VEGF (*e.g.*, about 50 ng/ml), and optionally FGF-2 (*e.g.*, about 25-75 ng/ml or about 50 ng/ml). The supernatant may be aspirated and replaced with fresh differentiation medium. Alternately the cells may be half fed every two days with fresh media. The cells may be harvested at different time points during the differentiation process.

[0082] HPCs may be cultured from pluripotent stem cells using a defined medium. Methods for the differentiation of pluripotent cells into hematopoietic $CD34^+$ stem cells using a defined media are described, *e.g.*, in U.S. Application 12/715,136 which is incorporated by reference in its entirety. It is anticipated that these methods may be used with the present disclosure.

[0083] For example, a defined medium may be used to induce hematopoietic $CD34^+$ differentiation. The defined medium may contain the growth factors BMP4, VEGF, Flt3 ligand, IL-3 and/or GMCSF. Pluripotent cells may be cultured in a first defined media comprising BMP4, VEGF, and optionally FGF-2, followed by culture in a second media comprising either (Flt3 ligand, IL-3, and GMCSF) or (Flt3 ligand, IL-3, IL-6, and TPO). The first and second media may also comprise one or more of SCF, IL-6, G-CSF, EPO, FGF-2, and/or TPO. Substantially hypoxic conditions (*e.g.*, less than 20% O2) may further promote hematopoietic or endothelial differentiation.

[0084] Cells may be substantially individualized via mechanical or enzymatic means (*e.g.*, using a trypsin or TrypLE™). A ROCK inhibitor (*e.g.*, H1152 or Y-27632) may also be included in the media. It is anticipated that these approaches may be automated using, *e.g.*, robotic automation.

[0085] In certain embodiments, substantially hypoxic conditions may be used to promote differentiation of pluripotent cells into hematopoietic progenitor cells. As would be recognized by one of skill in the art, an atmospheric oxygen content of less than about 20.8% would be considered hypoxic. Human cells in culture can grow in atmospheric conditions having reduced oxygen content as compared to ambient air. This relative hypoxia may be achieved by decreasing the atmospheric oxygen exposed to the culture media. Embryonic cells typically develop *in vivo* under reduced oxygen conditions, generally between about 1% and about 6% atmospheric oxygen, with carbon dioxide at ambient levels. Without wishing to be bound by theory, it is anticipated that hypoxic conditions may mimic an aspect of certain embryonic developmental conditions. As shown in the below examples, hypoxic conditions

can be used in certain embodiments to promote additional differentiation of pluripotent cells, such as iPSC or hESC, into a more differentiated cell type, such as HPCs.

[0086] The following hypoxic conditions may be used to promote differentiation of pluripotent cells into hematopoietic progenitor cells. In certain embodiments, an atmospheric oxygen content of less than about 20%, less than about 19%, less than about 18%, less than about 17%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less than about 12%, less than about 11%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, about 5%, about 4%, about 3%, about 2%, or about 1% may be used to promote differentiation into hematopoietic precursor cells. In certain embodiments, the hypoxic atmosphere comprises about 5% oxygen gas.

[0087] In certain embodiments, a dissolved oxygen content of less than about 95%, less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, about 25%, about 20%, about 15%, about 10%, or about 5% may be used to promote differentiation into hematopoietic precursor cells. In certain embodiments, the dissolved oxygen content levels are about 25% oxygen.

[0088] Regardless of the specific medium being used in any given hematopoietic progenitor cell expansion, the medium used is preferably supplemented with at least one cytokine at a concentration from about 0.1 ng/mL to about 500 ng mL, more usually 10 ng/mL to 100 ng/mL. Suitable cytokines, include but are not limited to, c-kit ligand (KL) (also called steel factor (StI), mast cell growth factor (MGF), and stem cell factor (SCF)), IL-6, G-CSF, IL-3, GM-CSF, IL-1α, IL-11 MIP-1α, LIF, c-mpl ligand/TPO, and flk2/flk3 ligand (Flt2L or Flt3L). Particularly, the culture will include at least one of SCF, Flt3L and TPO. More particularly, the culture will include SCF, Flt3L and TPO.

[0089] In some embodiments, the HPCs exhibit disrupted Methyl-CpG Binding Protein 2 (MeCP2) and are cultured under conditions to promote myeloid differentiation or lymphoid differentiation. In some aspects, the HPCs express a non-functional MeCP2 that has essentially no binding to methylated DNA. In certain aspects, the HPCs do not express MeCP2 at levels that are sufficient to effect MeCP2 DNA binding activity. In particular aspects, the MeCP2 is

non-functional by virtue of a truncation or mutation in the MeCP2 gene. In some aspects, obtaining HPCs that exhibit disrupted MeCP2 comprises contacting the HPCs with siRNA, shRNA or a small molecule inhibitor of MeCP2.

D. Forward Programming

[0090] Certain embodiments of the present disclosure provide HPCs by forward programming of the CAR-PSCs via expression of a combination of programming genes important for hematopoietic cell differentiation/function. In one method, the PSCs are modified to express at least three hematopoietic precursor programming genes such as an ETS gene (*e.g.*, ETC2 or ERG), a hematopoietic development gene (*e.g.*, GATA2), and a homoebox gene (*e.g.*, HOXA9), such as described in PCT/US2016/057893, incorporated herein by reference in its entirety. In particular aspects, the ETV2/ERG, GATA2, and HOXA9 genes are co-expressed by one vector, such as an inducible *PiggyBac* vector, using a bi-directional Tight promoter which is transfected into the CAR-PSCs.

[0091] Further, the EGH-CAR-PSCs may be further modified to express additional genes for long-term engraftment potential. Exemplary genes include HMGA2, MYCN, NR4A2, SOX17, TFEC, MEIS1, HOXA4, ZNF414, KLF4, ZNF131, BCL2, ETV6, ZNF350, and/or RBAK. For example, the PSCs may be transfected with one or more vectors to express HMGA2, MYCN, NR4A2, SOX17, TFEC, MEIS1, and HOXA4.

[0092] Preferably, the ETV2/GAT2/HOXA9 genes are expressed for only a period of time sufficient to forward program the PSCs into hematopoietic precursor cells. Accordingly, the hematopoietic precursor programming genes can be under the control of an inducible promoter. Thus, the expression of the hematopoietic precursor programming genes can be induced in the PSCs for a period of time sufficient to forward program to the multi-lineage hematopoietic precursor cells. The period of time can be about 1 day to about 20 days, such as about 3, 4, 5, 6, 7, 8, 9, or 10 days. Alternatively, the hematopoietic precursor programming genes can be introduced to the PSCs by an episomal vector. Thus, the hematopoietic precursor programming genes could be transiently expressed in the PSCs.

E. Antigens

[0093] The CARs provided herein may have any antigenic specificity useful in the treatment of a disease or disorder. Among the antigens targeted by the genetically engineered antigen receptors are those expressed in the context of a disease, condition, or cell type to be

targeted via the adoptive cell therapy. Among the diseases and conditions are proliferative, neoplastic, and malignant diseases and disorders, including cancers and tumors, including hematologic cancers, cancers of the immune system, such as lymphomas, leukemias, and/or myelomas, such as B, T, and myeloid leukemias, lymphomas, and multiple myelomas. In some embodiments, the antigen is selectively expressed or overexpressed on cells of the disease or condition, *e.g.*, the tumor or pathogenic cells, as compared to normal or non-targeted cells or tissues, antigens associated with autoimmune or alloimmune disorders, or pathogen-specific antigens. In other embodiments, the antigen is expressed on normal cells and/or is expressed on the engineered cells.

[0094] Any suitable antigen may find use in the present method. Exemplary antigens include, but are not limited to, antigenic molecules from infectious agents, auto-/self-antigens, tumor-/cancer-associated antigens, and tumor neoantigens. In particular aspects, the antigens include NY-ESO, EGFRvIII, Muc-1, Her2, CA-125, WT-1, Mage-A3, Mage-A4, Mage-A10, TRAIL/DR4, and CEA.

[0095] Tumor-associated antigens may be derived from prostate, breast, colorectal, lung, pancreatic, renal, mesothelioma, ovarian, or melanoma cancers. Exemplary tumor-associated antigens or tumor cell-derived antigens include MAGE 1, 3, and MAGE 4; PRAME; BAGE; RAGE, Lage (also known as NY ESO 1); SAGE; and HAGE or GAGE. These non-limiting examples of tumor antigens are expressed in a wide range of tumor types such as melanoma, lung carcinoma, sarcoma, and bladder carcinoma. Prostate cancer tumor-associated antigens include, for example, prostate specific membrane antigen (PSMA), prostate-specific antigen (PSA), prostatic acid phosphates, NKX3.1, and six-transmembrane epithelial antigen of the prostate (STEAP).

[0096] Other tumor associated antigens include Plu-1, HASH-1, HasH-2, Cripto and Criptin. Additionally, a tumor antigen may be a self peptide hormone, such as whole length gonadotrophin hormone releasing hormone, a short 10 amino acid long peptide, useful in the treatment of many cancers.

[0097] Tumor antigens include tumor antigens derived from cancers that are characterized by tumor-associated antigen expression, such as HER-2/neu expression. Tumor-associated antigens of interest include lineage-specific tumor antigens such as the melanocyte-melanoma lineage antigens MART-1/Melan-A, gp100, gp75, mda-7, tyrosinase and

tyrosinase-related protein. Illustrative tumor-associated antigens include, but are not limited to, tumor antigens derived from or comprising any one or more of, p53, Ras, c-Myc, cytoplasmic serine/threonine kinases (*e.g.,* A-Raf, B-Raf, and C-Raf, cyclin-dependent kinases), MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MART-1, BAGE, DAM-6, -10, GAGE-1, -2, -8, GAGE-3, -4, -5, -6, -7B, NA88-A, MART-1, MC1R, Gp100, PSA, PSM, Tyrosinase, TRP-1, TRP-2, ART-4, CAMEL, CEA, Cyp-B, hTERT, hTRT, iCE, MUC1, MUC2, Phosphoinositide 3-kinases (PI3Ks), TRK receptors, PRAME, P15, RU1, RU2, SART-1, SART-3, Wilms' tumor antigen (WT1), AFP, -catenin/m, Caspase-8/m, CEA, CDK-4/m, ELF2M, GnT-V, G250, HSP70-2M, HST-2, KIAA0205, MUM-1, MUM-2, MUM-3, Myosin/m, RAGE, SART-2, TRP-2/INT2, 707-AP, Annexin II, CDC27/m, TPI/mbcr-abl, BCR-ABL, interferon regulatory factor 4 (IRF4), ETV6/AML, LDLR/FUT, Pml/RAR, Tumor-associated calcium signal transducer 1 (TACSTD1) TACSTD2, receptor tyrosine kinases (*e.g.,* Epidermal Growth Factor receptor (EGFR) (in particular, EGFRvIII), platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), cytoplasmic tyrosine kinases (*e.g.,* src-family, syk-ZAP70 family), integrin-linked kinase (ILK), signal transducers and activators of transcription STAT3, STAT5, and STAT6, hypoxia inducible factors (*e.g.,* HIF-1 and HIF-2), Nuclear Factor-Kappa B (NF-B), Notch receptors (*e.g.,* Notch1-4), c-Met, mammalian targets of rapamycin (mTOR), WNT, extracellular signal-regulated kinases (ERKs), and their regulatory subunits, PMSA, PR-3, MDM2, Mesothelin, renal cell carcinoma-5T4, SM22-alpha, carbonic anhydrases I (CAI) and IX (CAIX) (also known as G250), STEAD, TEL/AML1, GD2, proteinase3, hTERT, sarcoma translocation breakpoints, EphA2, ML-IAP, EpCAM, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, ALK, androgen receptor, cyclin B1, polysialic acid, MYCN, RhoC, GD3, fucosyl GM1, mesothelian, PSCA, sLe, PLAC1, GM3, BORIS, Tn, GLoboH, NY-BR-1, RGsS, SART3, STn, PAX5, OY-TES1, sperm protein 17, LCK, HMWMAA, AKAP-4, SSX2, XAGE 1, B7H3, legumain, TIE2, Page4, MAD-CT-1, FAP, MAD-CT-2, fos related antigen 1, CBX2, CLDN6, SPANX, TPTE, ACTL8, ANKRD30A, CDKN2A, MAD2L1, CTAG1B, SUNC1, LRRN1 and idiotype.

[0098] Antigens may include epitopic regions or epitopic peptides derived from genes mutated in tumor cells or from genes transcribed at different levels in tumor cells compared to normal cells, such as telomerase enzyme, survivin, mesothelin, mutated ras, bcr/abl rearrangement, Her2/neu, mutated or wild-type p53, cytochrome P450 1B1, and abnormally expressed intron sequences such as N-acetylglucosaminyltransferase-V; clonal rearrangements

of immunoglobulin genes generating unique idiotypes in myeloma and B-cell lymphomas; tumor antigens that include epitopic regions or epitopic peptides derived from oncoviral processes, such as human papilloma virus proteins E6 and E7; Epstein bar virus protein LMP2; nonmutated oncofetal proteins with a tumor-selective expression, such as carcinoembryonic antigen and alpha-fetoprotein.

[0099] In other embodiments, an antigen is obtained or derived from a pathogenic microorganism or from an opportunistic pathogenic microorganism (also called herein an infectious disease microorganism), such as a virus, fungus, parasite, and bacterium. In certain embodiments, antigens derived from such a microorganism include full-length proteins.

[00100] Illustrative pathogenic organisms whose antigens are contemplated for use in the method described herein include human immunodeficiency virus (HIV), herpes simplex virus (HSV), respiratory syncytial virus (RSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), Influenza A, B, and C, vesicular stomatitis virus (VSV), vesicular stomatitis virus (VSV), polyomavirus (*e.g.,* BK virus and JC virus), adenovirus, *Staphylococcus* species including Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Streptococcus* species including *Streptococcus pneumoniae*. As would be understood by the skilled person, proteins derived from these and other pathogenic microorganisms for use as antigen as described herein and nucleotide sequences encoding the proteins may be identified in publications and in public databases such as GENBANK®, Swiss-Prot®, and TrEMBL®.

[00101] Antigens derived from human immunodeficiency virus (HIV) include any of the HIV virion structural proteins (*e.g.,* gp120, gp41, p17, p24), protease, reverse transcriptase, or HIV proteins encoded by tat, rev, nef, vif, vpr and vpu.

[00102] Antigens derived from herpes simplex virus (*e.g.,* HSV 1 and HSV2) include, but are not limited to, proteins expressed from HSV late genes. The late group of genes predominantly encodes proteins that form the virion particle. Such proteins include the five proteins from (UL) which form the viral capsid: UL6, UL18, UL35, UL38 and the major capsid protein UL19, UL45, and UL27, each of which may be used as an antigen as described herein. Other illustrative HSV proteins contemplated for use as antigens herein include the ICP27 (H1, H2), glycoprotein B (gB) and glycoprotein D (gD) proteins. The HSV genome comprises at least 74 genes, each encoding a protein that could potentially be used as an antigen.

[00103] Antigens derived from cytomegalovirus (CMV) include CMV structural proteins, viral antigens expressed during the immediate early and early phases of virus replication, glycoproteins I and III, capsid protein, coat protein, lower matrix protein pp65 (ppUL83), p52 (ppUL44), IE1 and 1E2 (UL123 and UL122), protein products from the cluster of genes from UL128-UL150, envelope glycoprotein B (gB), gH, gN, and pp150. As would be understood by the skilled person, CMV proteins for use as antigens described herein may be identified in public databases such as GenBank®, Swiss-Prot®, and TrEMBL®.

[00104] Antigens derived from Epstein-Barr virus (EBV) that are contemplated for use in certain embodiments include EBV lytic proteins gp350 and gp110, EBV proteins produced during latent cycle infection including Epstein-Ban nuclear antigen (EBNA)-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-leader protein (EBNA-LP) and latent membrane proteins (LMP)-1, LMP-2A and LMP-2B.

[00105] Antigens derived from respiratory syncytial virus (RSV) that are contemplated for use herein include any of the eleven proteins encoded by the RSV genome, or antigenic fragments thereof: NS 1, NS2, N (nucleocapsid protein), M (Matrix protein) SH, G and F (viral coat proteins), M2 (second matrix protein), M2-1 (elongation factor), M2-2 (transcription regulation), RNA polymerase, and phosphoprotein P.

[00106] Antigens derived from Vesicular stomatitis virus (VSV) that are contemplated for use include any one of the five major proteins encoded by the VSV genome, and antigenic fragments thereof: large protein (L), glycoprotein (G), nucleoprotein (N), phosphoprotein (P), and matrix protein (M).

[00107] Antigens derived from an influenza virus that are contemplated for use in certain embodiments include hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix proteins M1 and M2, NS1, NS2 (NEP), PA, PB1, PB1-F2, and PB2.

[00108] Exemplary viral antigens also include, but are not limited to, adenovirus polypeptides, alphavirus polypeptides, calicivirus polypeptides (*e.g.*, a calicivirus capsid antigen), coronavirus polypeptides, distemper virus polypeptides, Ebola virus polypeptides, enterovirus polypeptides, flavivirus polypeptides, hepatitis virus (AE) polypeptides (a hepatitis B core or surface antigen, a hepatitis C virus E1 or E2 glycoproteins, core, or non-structural proteins), herpesvirus polypeptides (including a herpes simplex virus or varicella zoster virus glycoprotein), infectious peritonitis virus polypeptides, leukemia virus polypeptides, Marburg

virus polypeptides, orthomyxovirus polypeptides, papilloma virus polypeptides, parainfluenza virus polypeptides (*e.g.*, the hemagglutinin and neuraminidase polypeptides), paramyxovirus polypeptides, parvovirus polypeptides, pestivirus polypeptides, picorna virus polypeptides (*e.g.*, a poliovirus capsid polypeptide), pox virus polypeptides (*e.g.*, a vaccinia virus polypeptide), rabies virus polypeptides (*e.g.*, a rabies virus glycoprotein G), reovirus polypeptides, retrovirus polypeptides, and rotavirus polypeptides.

[00109] In certain embodiments, the antigen may be bacterial antigens. In certain embodiments, a bacterial antigen of interest may be a secreted polypeptide. In other certain embodiments, bacterial antigens include antigens that have a portion or portions of the polypeptide exposed on the outer cell surface of the bacteria.

[00110] Antigens derived from *Staphylococcus* species including Methicillin-resistant *Staphylococcus aureus* (MRSA) that are contemplated for use include virulence regulators, such as the Agr system, Sar and Sae, the Arl system, Sar homologues (Rot, MgrA, SarS, SarR, SarT, SarU, SarV, SarX, SarZ and TcaR), the Srr system and TRAP. Other *Staphylococcus* proteins that may serve as antigens include Clp proteins, HtrA, MsrR, aconitase, CcpA, SvrA, Msa, CfvA and CfvB (see, *e.g.*, *Staphylococcus*: Molecular Genetics, 2008 Caister Academic Press, Ed. Jodi Lindsay). The genomes for two species of *Staphylococcus aureus* (N315 and Mu50) have been sequenced and are publicly available, for example at PATRIC (PATRIC: The VBI PathoSystems Resource Integration Center, Snyder *et al.*, 2007). As would be understood by the skilled person, *Staphylococcus* proteins for use as antigens may also be identified in other public databases such as GenBank®, Swiss-Prot®, and TrEMBL®.

[00111] Antigens derived from *Streptococcus pneumoniae* that are contemplated for use in certain embodiments described herein include pneumolysin, PspA, choline-binding protein A (CbpA), NanA, NanB, SpnHL, PavA, LytA, Pht, and pilin proteins (RrgA; RrgB; RrgC). Antigenic proteins of *Streptococcus pneumoniae* are also known in the art and may be used as an antigen in some embodiments. The complete genome sequence of a virulent strain of *Streptococcus pneumoniae* has been sequenced and, as would be understood by the skilled person, *S. pneumoniae* proteins for use herein may also be identified in other public databases such as GenBank®, Swiss-Prot®, and TrEMBL®. Proteins of particular interest for antigens according to the present disclosure include virulence factors and proteins predicted to be exposed at the surface of the pneumococci.

[00112] Examples of bacterial antigens that may be used as antigens include, but are not limited to, *Actinomyces* polypeptides, *Bacillus* polypeptides, *Bacteroides* polypeptides, *Bordetella* polypeptides, *Bartonella* polypeptides, *Borrelia* polypeptides (*e.g., B. burgdorferi* OspA), *Brucella* polypeptides, *Campylobacter* polypeptides, Capnocytophaga polypeptides, *Chlamydia* polypeptides, *Corynebacterium* polypeptides, *Coxiella* polypeptides, *Dermatophilus* polypeptides, *Enterococcus* polypeptides, *Ehrlichia* polypeptides, *Escherichia* polypeptides, *Francisella* polypeptides, *Fusobacterium* polypeptides, *Haemobartonella* polypeptides, *Haemophilus* polypeptides (*e.g., H. influenzae* type b outer membrane protein), *Helicobacter* polypeptides, *Klebsiella* polypeptides, L-form bacteria polypeptides, *Leptospira* polypeptides, *Listeria* polypeptides, *Mycobacteria* polypeptides, *Mycoplasma* polypeptides, *Neisseria* polypeptides, *Neorickettsia* polypeptides, *Nocardia* polypeptides, *Pasteurella* polypeptides, *Peptococcus* polypeptides, *Peptostreptococcus* polypeptides, *Pneumococcus* polypeptides (i.e., *S. pneumoniae* polypeptides) (see description herein), *Proteus* polypeptides, *Pseudomonas* polypeptides, *Rickettsia* polypeptides, *Rochalimaea* polypeptides, *Salmonella* polypeptides, *Shigella* polypeptides, *Staphylococcus* polypeptides, group A *streptococcus* polypeptides (*e.g., S. pyogenes* M proteins), group B *streptococcus* (*S. agalactiae*) polypeptides, *Treponema* polypeptides, and *Yersinia* polypeptides (*e.g., Y pestis* F1 and V antigens).

[00113] Examples of fungal antigens include, but are not limited to, *Absidia* polypeptides, *Acremonium* polypeptides, *Alternaria* polypeptides, *Aspergillus* polypeptides, *Basidiobolus* polypeptides, *Bipolaris* polypeptides, *Blastomyces* polypeptides, *Candida* polypeptides, *Coccidioides* polypeptides, *Conidiobolus* polypeptides, *Cryptococcus* polypeptides, *Curvalaria* polypeptides, *Epidermophyton* polypeptides, *Exophiala* polypeptides, *Geotrichum* polypeptides, *Histoplasma* polypeptides, *Madurella* polypeptides, *Malassezia* polypeptides, *Microsporum* polypeptides, *Moniliella* polypeptides, *Mortierella* polypeptides, *Mucor* polypeptides, *Paecilomyces* polypeptides, *Penicillium* polypeptides, *Phialemonium* polypeptides, *Phialophora* polypeptides, *Prototheca* polypeptides, *Pseudallescheria* polypeptides, *Pseudomicrodochium* polypeptides, *Pythium* polypeptides, *Rhinosporidium* polypeptides, *Rhizopus* polypeptides, *Scolecobasidium* polypeptides, *Sporothrix* polypeptides, *Stemphylium* polypeptides, *Trichophyton* polypeptides, *Trichosporon* polypeptides, and *Xylohypha* polypeptides.

[00114] Examples of protozoan parasite antigens include, but are not limited to, *Babesia* polypeptides, *Balantidium* polypeptides, *Besnoitia* polypeptides, *Cryptosporidium* polypeptides, *Eimeria* polypeptides, *Encephalitozoon* polypeptides, *Entamoeba* polypeptides, *Giardia* polypeptides, *Hammondia* polypeptides, *Hepatozoon* polypeptides, *Isospora* polypeptides, *Leishmania* polypeptides, *Microsporidia* polypeptides, *Neospora* polypeptides, *Nosema* polypeptides, *Pentatrichomonas* polypeptides, *Plasmodium* polypeptides. Examples of helminth parasite antigens include, but are not limited to, *Acanthocheilonema* polypeptides, *Aelurostrongylus* polypeptides, *Ancylostoma* polypeptides, *Angiostrongylus* polypeptides, *Ascaris* polypeptides, *Brugia* polypeptides, *Bunostomum* polypeptides, *Capillaria* polypeptides, *Chabertia* polypeptides, *Cooperia* polypeptides, *Crenosoma* polypeptides, *Dictyocaulus* polypeptides, *Dioctophyme* polypeptides, *Dipetalonema* polypeptides, *Diphyllobothrium* polypeptides, *Diplydium* polypeptides, *Dirofilaria* polypeptides, *Dracunculus* polypeptides, *Enterobius* polypeptides, *Filaroides* polypeptides, *Haemonchus* polypeptides, *Lagochilascaris* polypeptides, *Loa* polypeptides, *Mansonella* polypeptides, *Muellerius* polypeptides, *Nanophyetus* polypeptides, *Necator* polypeptides, *Nematodirus* polypeptides, *Oesophagostomum* polypeptides, *Onchocerca* polypeptides, *Opisthorchis* polypeptides, *Ostertagia* polypeptides, *Parafilaria* polypeptides, *Paragonimus* polypeptides, *Parascaris* polypeptides, *Physaloptera* polypeptides, *Protostrongylus* polypeptides, *Setaria* polypeptides, *Spirocerca* polypeptides *Spirometra* polypeptides, *Stephanofilaria* polypeptides, *Strongyloides* polypeptides, *Strongylus* polypeptides, *Thelazia* polypeptides, *Toxascaris* polypeptides, *Toxocara* polypeptides, *Trichinella* polypeptides, *Trichostrongylus* polypeptides, *Trichuris* polypeptides, *Uncinaria* polypeptides, and *Wuchereria* polypeptides. (*e.g., P. falciparum* circumsporozoite (PfCSP)), sporozoite surface protein 2 (PfSSP2), carboxyl terminus of liver state antigen 1 (PfLSA1 c-term), and exported protein 1 (PfExp-1), *Pneumocystis* polypeptides, *Sarcocystis* polypeptides, *Schistosoma* polypeptides, *Theileria* polypeptides, *Toxoplasma* polypeptides, and *Trypanosoma* polypeptides.

[00115] Examples of ectoparasite antigens include, but are not limited to, polypeptides (including antigens as well as allergens) from fleas; ticks, including hard ticks and soft ticks; flies, such as midges, mosquitoes, sand flies, black flies, horse flies, horn flies, deer flies, tsetse flies, stable flies, myiasis-causing flies and biting gnats; ants; spiders, lice; mites; and true bugs, such as bed bugs and kissing bugs.

F. Chimeric Antigen Receptors

[00116] In some embodiments, the CAR contains an extracellular antigen-recognition domain that specifically binds to an antigen. In some embodiments, the antigen is a protein expressed on the surface of cells. In some embodiments, the CAR is a TCR-like CAR and the antigen is a processed peptide antigen, such as a peptide antigen of an intracellular protein, which, like a TCR, is recognized on the cell surface in the context of a major histocompatibility complex (MHC) molecule.

[00117] In some embodiments, the chimeric antigen receptor comprises: a) an intracellular signaling domain, b) a hinge and transmembrane domain, and c) an extracellular domain comprising an antigen binding region.

[00118] In some embodiments, the engineered antigen receptors include chimeric antigen receptors (CARs), including activating or stimulatory CARs, costimulatory CARs (see WO2014/055668), and/or inhibitory CARs (iCARs, see Fedorov *et al.*, 2013). The CARs generally include an extracellular antigen (or ligand) binding domain linked to one or more intracellular signaling components, in some aspects via linkers and/or transmembrane domain(s). Such molecules typically mimic or approximate a signal through a natural antigen receptor, a signal through such a receptor in combination with a costimulatory receptor, and/or a signal through a costimulatory receptor alone.

[00119] Certain embodiments of the present disclosure concern the use of nucleic acids, including nucleic acids encoding an antigen-specific CAR polypeptide, including a CAR that has been humanized to reduce immunogenicity (hCAR), comprising an intracellular signaling domain, a transmembrane domain, and an extracellular domain comprising one or more signaling motifs. In certain embodiments, the CAR may recognize an epitope comprising the shared space between one or more antigens. In certain embodiments, the binding region can comprise complementary determining regions of a monoclonal antibody, variable regions of a monoclonal antibody, and/or antigen binding fragments thereof. In another embodiment, that specificity is derived from a peptide (*e.g.*, cytokine) that binds to a receptor.

[00120] It is contemplated that the human CAR nucleic acids may be human genes used to enhance cellular immunotherapy for human patients. In a specific embodiment, the invention includes a full-length CAR cDNA or coding region. The antigen binding regions or domain can comprise a fragment of the V_H and V_L chains of a single-chain variable fragment

(scFv) derived from a particular human monoclonal antibody, such as those described in U.S. Patent 7,109,304, incorporated herein by reference. The fragment can also be any number of different antigen binding domains of a human antigen-specific antibody. In a more specific embodiment, the fragment is an antigen-specific scFv encoded by a sequence that is optimized for human codon usage for expression in human cells.

[00121] The arrangement could be multimeric, such as a diabody or multimers. The multimers are most likely formed by cross pairing of the variable portion of the light and heavy chains into a diabody. The hinge portion of the construct can have multiple alternatives from being totally deleted, to having the first cysteine maintained, to a proline rather than a serine substitution, to being truncated up to the first cysteine. The Fc portion can be deleted. Any protein that is stable and/or dimerizes can serve this purpose. One could use just one of the Fc domains, *e.g.*, either the CH2 or CH3 domain from human immunoglobulin. One could also use the hinge, CH2 and CH3 region of a human immunoglobulin that has been modified to improve dimerization. One could also use just the hinge portion of an immunoglobulin. One could also use portions of CD8alpha.

[00122] In some embodiments, the CAR nucleic acid comprises a sequence encoding other costimulatory receptors, such as a transmembrane domain and a modified CD28 intracellular signaling domain. Other costimulatory receptors include, but are not limited to one or more of CD28, CD27, OX-40 (CD134), DAP10, DAP12, and 4-1BB (CD137).

[00123] In some embodiments, CAR is constructed with a specificity for a particular antigen (or marker or ligand), such as an antigen expressed in a particular cell type to be targeted by adoptive therapy, *e.g.*, a cancer marker, and/or an antigen intended to induce a dampening response, such as an antigen expressed on a normal or non-diseased cell type. Thus, the CAR typically includes in its extracellular portion one or more antigen binding molecules, such as one or more antigen-binding fragment, domain, or portion, or one or more antibody variable domains, and/or antibody molecules. In some embodiments, the CAR includes an antigen-binding portion or portions of an antibody molecule, such as a single-chain antibody fragment (scFv) derived from the variable heavy (V_H) and variable light (V_L) chains of a monoclonal antibody (mAb).

[00124] In certain embodiments of the chimeric antigen receptor, the antigen-specific portion of the receptor (which may be referred to as an extracellular domain comprising

an antigen binding region) comprises a tumor associated antigen or a pathogen-specific antigen binding domain. Antigens include carbohydrate antigens recognized by pattern-recognition receptors, such as Dectin-1. A tumor associated antigen may be of any kind so long as it is expressed on the cell surface of tumor cells. Exemplary embodiments of tumor associated antigens include CD19, CD319 (CS1), CD20, carcinoembryonic antigen, alphafetoprotein, CA-125, MUC-1, CD56, EGFR, c-Met, AKT, Her2, Her3, epithelial tumor antigen, melanoma-associated antigen, mutated p53, mutated ras, and so forth. In certain embodiments, the CAR may be co-expressed with a cytokine to improve persistence when there is a low amount of tumor-associated antigen. For example, CAR may be co-expressed with IL-15.

[00125] The sequence of the open reading frame encoding the chimeric receptor can be obtained from a genomic DNA source, a cDNA source, or can be synthesized (*e.g.*, *via* PCR), or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, it may be desirable to use cDNA or a combination thereof as it is found that introns stabilize the mRNA. Also, it may be further advantageous to use endogenous or exogenous non-coding regions to stabilize the mRNA.

[00126] It is contemplated that the chimeric construct can be introduced into immune cells as naked DNA or in a suitable vector. Methods of stably transfecting cells by electroporation using naked DNA are known in the art. Naked DNA generally refers to the DNA encoding a chimeric receptor contained in a plasmid expression vector in proper orientation for expression.

[00127] Alternatively, a viral vector (*e.g.*, a retroviral vector, adenoviral vector, adeno-associated viral vector, or lentiviral vector) can be used to introduce the chimeric construct into immune cells. Suitable vectors for use in accordance with the method of the present disclosure are non-replicating in the immune cells. A large number of vectors are known that are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain the viability of the cell, such as, for example, vectors based on HIV, SV40, EBV, HSV, or BPV.

[00128] In some aspects, the antigen-specific binding, or recognition component is linked to one or more transmembrane and intracellular signaling domains. In some embodiments, the CAR includes a transmembrane domain fused to the extracellular domain of the CAR. In one embodiment, the transmembrane domain that naturally is associated with one

of the domains in the CAR is used. In some instances, the transmembrane domain is 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.

[00129] The transmembrane domain in some embodiments is derived either from a natural or from a synthetic source. Where the source is natural, the domain in some aspects is derived from any membrane-bound or transmembrane protein. Transmembrane regions include those derived from (*i.e.* comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T cell receptor, CD28, CD3 zeta, CD3 epsilon, CD3 gamma, CD3 delta, CD45, CD4, CD5, CD8, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD154, ICOS/CD278, and GITR/CD357 molecules. Alternatively the transmembrane domain in some embodiments is synthetic. In some aspects, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. In some aspects, a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.

III. Methods of Use

[00130] In some embodiments, the present disclosure provides methods for immunotherapy comprising administering an effective amount of the immune cells expressing CARs of the present disclosure. In one embodiment, a medical disease or disorder is treated by transfer of an immune cell population that elicits an immune response. In certain embodiments of the present disclosure, cancer or infection is treated by transfer of an immune cell population that elicits an immune response. Provided herein are methods for treating or delaying progression of cancer in an individual comprising administering to the individual an effective amount an antigen-specific cell therapy. The present methods may be applied for the treatment of immune disorders, solid cancers, hematologic cancers, and viral infections.

[00131] In particular embodiments, methods are provided for treating cancer patients by administering immune cells, such as NK cells and/or T cells, expressing CARs provided herein with an antigen-binding domain specific for the antigen expressed by said cancer.

[00132] Tumors for which the present treatment methods are useful include any malignant cell type, such as those found in a solid tumor or a hematological tumor. Exemplary

solid tumors can include, but are not limited to, a tumor of an organ selected from the group consisting of pancreas, colon, cecum, stomach, brain, head, neck, ovary, kidney, larynx, sarcoma, lung, bladder, melanoma, prostate, and breast. Exemplary hematological tumors include tumors of the bone marrow, T or B cell malignancies, leukemias, lymphomas, blastomas, myelomas, and the like. Further examples of cancers that may be treated using the methods provided herein include, but are not limited to, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, gastric or stomach cancer (including gastrointestinal cancer and gastrointestinal stromal cancer), pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, various types of head and neck cancer, and melanoma.

[00133] The cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant;

paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; lentigo malignant melanoma; acral lentiginous melanomas; nodular melanomas; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; hodgkin's disease; hodgkin's; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; B-cell lymphoma; low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; Waldenstrom's macroglobulinemia; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; hairy cell leukemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); acute myeloid leukemia (AML); and chronic myeloblastic leukemia.

[00134] Particular embodiments concern methods of treatment of leukemia. Leukemia is a cancer of the blood or bone marrow and is characterized by an abnormal proliferation (production by multiplication) of blood cells, usually white blood cells (leukocytes). It is part of the broad group of diseases called hematological neoplasms. Leukemia is a broad term covering a spectrum of diseases. Leukemia is clinically and pathologically split into its acute and chronic forms.

[00135] In certain embodiments of the present disclosure, immune cells are delivered to an individual in need thereof, such as an individual that has cancer or an infection. The cells then enhance the individual's immune system to attack the respective cancer or pathogenic cells. In some cases, the individual is provided with one or more doses of the immune cells. In cases where the individual is provided with two or more doses of the immune cells, the duration between the administrations should be sufficient to allow time for propagation in the individual, and in specific embodiments the duration between doses is 1, 2, 3, 4, 5, 6, 7, or more days.

[00136] Certain embodiments of the present disclosure provide methods for treating or preventing an immune-mediated disorder. In one embodiment, the subject has an autoimmune disease. Non-limiting examples of autoimmune diseases include: alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac spate-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Graves' disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erthematosus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, nephrotic syndrome (such as minimal change disease, focal glomerulosclerosis, or mebranous nephropathy), pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis,

Raynaud's phenomenon, Reiter's syndrome, Rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, ulcerative colitis, uveitis, vasculitides (such as polyarteritis nodosa, takayasu arteritis, temporal arteritis/giant cell arteritis, or dermatitis herpetiformis vasculitis), vitiligo, and Wegener's granulomatosis. Thus, some examples of an autoimmune disease that can be treated using the methods disclosed herein include, but are not limited to, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosis, type I diabetes mellitus, Crohn's disease; ulcerative colitis, myasthenia gravis, glomerulonephritis, ankylosing spondylitis, vasculitis, or psoriasis. The subject can also have an allergic disorder such as Asthma.

[00137] Therapeutically effective amounts of immune cells can be administered by a number of routes, including parenteral administration, for example, intravenous, intraperitoneal, intramuscular, intrasternal, or intraarticular injection, or infusion.

[00138] The therapeutically effective amount of immune cells for use in adoptive cell therapy is that amount that achieves a desired effect in a subject being treated. For instance, this can be the amount of immune cells necessary to inhibit advancement, or to cause regression of an autoimmune or alloimmune disease, or which is capable of relieving symptoms caused by an autoimmune disease, such as pain and inflammation. It can be the amount necessary to relieve symptoms associated with inflammation, such as pain, edema and elevated temperature. It can also be the amount necessary to diminish or prevent rejection of a transplanted organ.

[00139] The immune cell population can be administered in treatment regimens consistent with the disease, for example a single or a few doses over one to several days to ameliorate a disease state or periodic doses over an extended time to inhibit disease progression and prevent disease recurrence. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. The therapeutically effective amount of immune cells will be dependent on the subject being treated, the severity and type of the affliction, and the manner of administration. In some embodiments, doses that could be used in the treatment of human subjects range from at least 3.8×10^4, at least 3.8×10^5, at least 3.8×10^6, at least 3.8×10^7, at least 3.8×10^8, at least 3.8×10^9, or at least 3.8×10^{10} immune cells/m^2. In a certain embodiment, the dose used in the treatment of human subjects ranges from about 3.8×10^9 to about 3.8×10^{10} immune cells/m^2. In additional embodiments, a therapeutically effective amount of immune cells can vary from about 5×10^6

cells per kg body weight to about 7.5×10^8 cells per kg body weight, such as about 2×10^7 cells to about 5×10^8 cells per kg body weight, or about 5×10^7 cells to about 2×10^8 cells per kg body weight. The exact amount of immune cells is readily determined by one of skill in the art based on the age, weight, sex, and physiological condition of the subject. Effective doses can be extrapolated from dose-response curves derived from in vitro or animal model test systems.

[00140] The immune cells may be administered in combination with one or more other therapeutic agents for the treatment of the immune-mediated disorder. Combination therapies can include, but are not limited to, one or more anti-microbial agents (for example, antibiotics, anti-viral agents and anti-fungal agents), anti-tumor agents (for example, fluorouracil, methotrexate, paclitaxel, fludarabine, etoposide, doxorubicin, or vincristine), immune-depleting agents (for example, fludarabine, etoposide, doxorubicin, or vincristine), immunosuppressive agents (for example, azathioprine, or glucocorticoids, such as dexamethasone or prednisone), anti-inflammatory agents (for example, glucocorticoids such as hydrocortisone, dexamethasone or prednisone, or non-steroidal anti-inflammatory agents such as acetylsalicylic acid, ibuprofen or naproxen sodium), cytokines (for example, interleukin-10 or transforming growth factor-beta), hormones (for example, estrogen), or a vaccine. In addition, immunosuppressive or tolerogenic agents including but not limited to calcineurin inhibitors (*e.g.*, cyclosporin and tacrolimus); mTOR inhibitors (*e.g.*, Rapamycin); mycophenolate mofetil, antibodies (*e.g.*, recognizing CD3, CD4, CD40, CD154, CD45, IVIG, or B cells); chemotherapeutic agents (*e.g.*, Methotrexate, Treosulfan, Busulfan); irradiation; or chemokines, interleukins or their inhibitors (*e.g.*, BAFF, IL-2, anti-IL-2R, IL-4, JAK kinase inhibitors) can be administered. Such additional pharmaceutical agents can be administered before, during, or after administration of the immune cells, depending on the desired effect. This administration of the cells and the agent can be by the same route or by different routes, and either at the same site or at a different site.

B. Pharmaceutical Compositions

[00141] Also provided herein are pharmaceutical compositions and formulations comprising immune cells (*e.g.*, T cells or NK cells) and a pharmaceutically acceptable carrier.

[00142] Pharmaceutical compositions and formulations as described herein can be prepared by mixing the active ingredients (such as an antibody or a polypeptide) having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences 22nd edition, 2012), in the form of lyophilized

formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn- protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG).

C. Combination Therapies

[00143] In certain embodiments, the compositions and methods of the present embodiments involve an immune cell population in combination with at least one additional therapy. The additional therapy may be radiation therapy, surgery (*e.g.*, lumpectomy and a mastectomy), chemotherapy, gene therapy, DNA therapy, viral therapy, RNA therapy, immunotherapy, bone marrow transplantation, nanotherapy, monoclonal antibody therapy, or a combination of the foregoing. The additional therapy may be in the form of adjuvant or neoadjuvant therapy.

[00144] In some embodiments, the additional therapy is the administration of small molecule enzymatic inhibitor or anti-metastatic agent. In some embodiments, the additional therapy is the administration of side- effect limiting agents (*e.g.*, agents intended to lessen the occurrence and/or severity of side effects of treatment, such as anti-nausea agents, *etc.*). In some embodiments, the additional therapy is radiation therapy. In some embodiments, the additional therapy is surgery. In some embodiments, the additional therapy is a combination of radiation therapy and surgery. In some embodiments, the additional therapy is gamma irradiation. In some embodiments, the additional therapy is therapy targeting PBK/AKT/mTOR pathway, HSP90 inhibitor, tubulin inhibitor, apoptosis inhibitor, and/or

chemopreventative agent. The additional therapy may be one or more of the chemotherapeutic agents known in the art.

[00145] An immune cell therapy may be administered before, during, after, or in various combinations relative to an additional cancer therapy, such as immune checkpoint therapy. The administrations may be in intervals ranging from concurrently to minutes to days to weeks. In embodiments where the immune cell therapy is provided to a patient separately from an additional therapeutic agent, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the two compounds would still be able to exert an advantageously combined effect on the patient. In such instances, it is contemplated that one may provide a patient with the antibody therapy and the anti-cancer therapy within about 12 to 24 or 72 h of each other and, more particularly, within about 6-12 h of each other. In some situations it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6, or 7) to several weeks (1, 2, 3, 4, 5, 6, 7, or 8) lapse between respective administrations.

[00146] Various combinations may be employed. For the example below a CAR immune cell therapy is "A" and an anti-cancer therapy is "B":

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B
B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A
B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[00147] Administration of any compound or therapy of the present embodiments to a patient will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the agents. Therefore, in some embodiments there is a step of monitoring toxicity that is attributable to combination therapy.

1. Chemotherapy

[00148] A wide variety of chemotherapeutic agents may be used in accordance with the present embodiments. The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to

intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

[00149] Examples of chemotherapeutic agents include alkylating agents, such as thiotepa and cyclosphosphamide; alkyl sulfonates, such as busulfan, improsulfan, and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines, including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide, and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards, such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitrosureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics, such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegaI1); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, pteropterin, and trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs, such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens, such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals, such as mitotane and trilostane; folic acid replenisher, such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic

acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids, such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKpolysaccharide complex; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; taxoids, *e.g.*, paclitaxel and docetaxel gemcitabine; 6-thioguanine; mercaptopurine; platinum coordination complexes, such as cisplatin, oxaliplatin, and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (*e.g.*, CPT-11); topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids, such as retinoic acid; capecitabine; carboplatin, procarbazine,plicomycin, gemcitabien, navelbine, farnesyl-protein tansferase inhibitors, transplatinum, and pharmaceutically acceptable salts, acids, or derivatives of any of the above.

2. Radiotherapy

[00150] Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated, such as microwaves, proton beam irradiation, and UV-irradiation. It is most likely that all of these factors affect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

3. Immunotherapy

[00151] The skilled artisan will understand that additional immunotherapies may be used in combination or in conjunction with methods of the embodiments. In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and

molecules to target and destroy cancer cells. Rituximab (RITUXAN®) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells

[00152] Antibody–drug conjugates (ADCs) comprise monoclonal antibodies (MAbs) that are covalently linked to cell-killing drugs and may be used in combination therapies. This approach combines the high specificity of MAbs against their antigen targets with highly potent cytotoxic drugs, resulting in "armed" MAbs that deliver the payload (drug) to tumor cells with enriched levels of the antigen. Targeted delivery of the drug also minimizes its exposure in normal tissues, resulting in decreased toxicity and improved therapeutic index. Exemplary ADC drugs inlcude ADCETRIS® (brentuximab vedotin) and KADCYLA® (trastuzumab emtansine or T-DM1).

[00153] In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present embodiments. Common tumor markers include CD20, carcinoembryonic antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, laminin receptor, erb B, and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines, such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines, such as MIP-1, MCP-1, IL-8, and growth factors, such as FLT3 ligand.

[00154] Examples of immunotherapies include immune adjuvants, *e.g.*, Mycobacterium bovis, Plasmodium falciparum, dinitrochlorobenzene, and aromatic compounds); cytokine therapy, *e.g.*, interferons α, β, and γ, IL-1, GM-CSF, and TNF; gene therapy, *e.g.*, TNF, IL-1, IL-2, and p53; and monoclonal antibodies, *e.g.*, anti-CD20, anti-ganglioside GM2, and anti-p185. It is contemplated that one or more anti-cancer therapies may be employed with the antibody therapies described herein.

[00155] In some embodiments, the immunotherapy may be an immune checkpoint inhibitor. Immune checkpoints either turn up a signal (*e.g.*, co-stimulatory molecules) or turn down a signal. Inhibitory immune checkpoints that may be targeted by immune checkpoint blockade include adenosine A2A receptor (A2AR), B7-H3 (also known as CD276), B and T lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), indoleamine 2,3-dioxygenase (IDO), killer-cell immunoglobulin (KIR), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA). In particular, the immune checkpoint inhibitors target the PD-1 axis and/or CTLA-4.

[00156] The immune checkpoint inhibitors may be drugs such as small molecules, recombinant forms of ligand or receptors, or, in particular, are antibodies, such as human antibodies. Known inhibitors of the immune checkpoint proteins or analogs thereof may be used, in particular chimerized, humanized or human forms of antibodies may be used. As the skilled person will know, alternative and/or equivalent names may be in use for certain antibodies mentioned in the present disclosure. Such alternative and/or equivalent names are interchangeable in the context of the present disclosure. For example it is known that lambrolizumab is also known under the alternative and equivalent names MK-3475 and pembrolizumab.

[00157] In some embodiments, the PD-1 binding antagonist is a molecule that inhibits the binding of PD-1 to its ligand binding partners. In a specific aspect, the PD-1 ligand binding partners are PDL1 and/or PDL2. In another embodiment, a PDL1 binding antagonist is a molecule that inhibits the binding of PDL1 to its binding partners. In a specific aspect, PDL1 binding partners are PD-1 and/or B7-1. In another embodiment, the PDL2 binding antagonist is a molecule that inhibits the binding of PDL2 to its binding partners. In a specific aspect, a PDL2 binding partner is PD-1. The antagonist may be an antibody, an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide.

[00158] In some embodiments, the PD-1 binding antagonist is an anti-PD-1 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody). In some embodiments, the anti-PD-1 antibody is selected from the group consisting of nivolumab, pembrolizumab, and CT-011. In some embodiments, the PD-1 binding antagonist is an immunoadhesin (*e.g.*, an immunoadhesin comprising an extracellular or PD-1 binding portion

of PDL1 or PDL2 fused to a constant region (*e.g.*, an Fc region of an immunoglobulin sequence). In some embodiments, the PD-1 binding antagonist is AMP- 224. Nivolumab, also known as MDX-1106-04, MDX-1106, ONO-4538, BMS-936558, and OPDIVO®, is an anti-PD-1 antibody that may be used. Pembrolizumab, also known as MK-3475, Merck 3475, lambrolizumab, KEYTRUDA®, and SCH-900475, is an exemplary anti-PD-1 antibody. CT-011, also known as hBAT or hBAT-1, is also an anti-PD-1 antibody. AMP-224, also known as B7-DCIg, is a PDL2-Fc fusion soluble receptor.

[00159] Another immune checkpoint that can be targeted in the methods provided herein is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also known as CD152. The complete cDNA sequence of human CTLA-4 has the Genbank accession number L15006. CTLA-4 is found on the surface of T cells and acts as an "off" switch when bound to CD80 or CD86 on the surface of antigen-presenting cells. CTLA4 is a member of the immunoglobulin superfamily that is expressed on the surface of Helper T cells and transmits an inhibitory signal to T cells. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Intracellular CTLA4 is also found in regulatory T cells and may be important to their function. T cell activation through the T cell receptor and CD28 leads to increased expression of CTLA-4, an inhibitory receptor for B7 molecules.

[00160] In some embodiments, the immune checkpoint inhibitor is an anti-CTLA-4 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody), an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide.

[00161] Anti-human-CTLA-4 antibodies (or VH and/or VL domains derived therefrom) suitable for use in the present methods can be generated using methods well known in the art. Alternatively, art recognized anti-CTLA-4 antibodies can be used. An exemplary anti-CTLA-4 antibody is ipilimumab (also known as 10D1, MDX- 010, MDX- 101, and Yervoy®) or antigen binding fragments and variants thereof. In other embodiments, the antibody comprises the heavy and light chain CDRs or VRs of ipilimumab. Accordingly, in one embodiment, the antibody comprises the CDR1, CDR2, and CDR3 domains of the VH region of ipilimumab, and the CDR1, CDR2 and CDR3 domains of the VL region of ipilimumab. In another embodiment, the antibody competes for binding with and/or binds to the same epitope on CTLA-4 as the above- mentioned antibodies. In another embodiment, the

antibody has at least about 90% variable region amino acid sequence identity with the above-mentioned antibodies (*e.g.*, at least about 90%, 95%, or 99% variable region identity with ipilimumab).

4. Surgery

[00162] Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed and may be used in conjunction with other therapies, such as the treatment of the present embodiments, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy, and/or alternative therapies. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically-controlled surgery (Mohs' surgery).

[00163] Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection, or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

5. Other Agents

[00164] It is contemplated that other agents may be used in combination with certain aspects of the present embodiments to improve the therapeutic efficacy of treatment. These additional agents include agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Increases in intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with certain aspects of the present embodiments to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present embodiments. Examples of cell adhesion inhibitors are

focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with certain aspects of the present embodiments to improve the treatment efficacy.

IV. Articles of Manufacture or Kits

[00165] An article of manufacture or a kit is provided comprising immune cells is also provided herein. The article of manufacture or kit can further comprise a package insert comprising instructions for using the immune cells to treat or delay progression of cancer in an individual or to enhance immune function of an individual having cancer. Any of the antigen-specific immune cells described herein may be included in the article of manufacture or kits. Suitable containers include, for example, bottles, vials, bags and syringes. The container may be formed from a variety of materials such as glass, plastic (such as polyvinyl chloride or polyolefin), or metal alloy (such as stainless steel or hastelloy). In some embodiments, the container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture or kit may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use. In some embodiments, the article of manufacture further includes one or more of another agent (*e.g.*, a chemotherapeutic agent, and anti-neoplastic agent). Suitable containers for the one or more agent include, for example, bottles, vials, bags and syringes.

V. Examples

[00166] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Protein L Activation and Expansion of CAR-T Cells

[00167] *Protein L induces CAR-T cell expansion*: E11 pluripotent stem cells reprogrammed from peripheral blood T cells (TiPSCs) using episomal vectors were modified for constitutive expression of 2nd generation anti-human CD19 chimeric antigen receptor (CAR) consisting of FMC63 monoclonal antibody-derived human CD19-binding scFv domain, CD28 co-stimulatory and CD3ζ signaling domains.

[00168] Non-modified and CAR-modified E11 TiPSCs were differentiated to T/NK $CD34^+$ progenitors through cytokine-directed differentiation. Isolated CD34+ progenitors were further differentiated to CD3+ T cells in 2 week hypoxic culture on DLL4/retronectin-coated plates in T cell differentiation medium (TCDM) consisting of StemSpan SFEM (Stem Cell Technologies) supplemented with ascorbic acid magnesium phosphate (0.25 mM), nicotinamide (2 mM), GlutaMax (Gibco) and cytokines (SCF, TPO, FLT3L, IL7; each at 50 ng/ml).

[00169] CAR-T cells were cultured on plates coated with different concentrations of Protein L (Pierce; 0, 0.1, 0.5, 2.5 μg/cm^2) with or without retronectin (Takara; 0.5 μg/cm^2) and human DLL4-Fc (Acro Biosystems; 0.5 μg/cm^2) at density of 10,000 $CD3^+$ T cells/cm^2 in TCDM supplemented with IL2 and IL15 (both at 10 ng/mL). Cultures were maintained in hypoxic (5% O_2) conditions and harvested after 10 days. Total harvested cells were counted, percent of $CD3^+$ cells determined, and fold T cell expansion calculated (output/input T cell numbers).

[00170] Protein L induced expansion of CAR-T cells in dose-dependent manner; however, retronectin enhanced the proliferative response (FIG. 1). DLL4 is a complementary T cell growth factor that also significantly improved Protein L-dependent expansion of CAR-T cells. A hypoxic environment was used to achieve efficient CAR-T cell expansion, as parallel normoxic cultures showed inferior expansion yields in the tested variants.

[00171] *DLL4 promotes CD8+ CAR-T expansion:* CD8α expression was determined in CAR-T cells harvested from day 10 expansion cultures on Protein L in combination with retronectin and DLL4-Fc (all used at 0.5 μg/cm^2) (FIG. 2).

[00172] The Protein L and retronectin combination resulted in efficient CAR-T expansion as compared to Protein L alone, however, despite the much higher expansion rate,

CD8 expression remained low. In contrast, when DLL4 was added, along with a further ~25% improvement in CAR-T expansion rate, a significant proportion of $CD3^+$ T cells acquired CD8α expression (FIG. 2).

[00173] *CAR-specific T cell expansion induced by Protein L:* The specificity of CAR-induced T cell expansion in Protein L cultures was verified with CAR-modified versus non-modified T cells plated for expansion in both CD3-induced pan-T cell (control) and Protein L-induced expansion cultures. CAR-modified and non-modified E11-derived T cells were cultured on plates coated with anti-CD3 monoclonal antibody (OKT3 clone; 0.5 $\mu g/cm^2$) or Protein L (0.5 $\mu g/cm^2$) along with retronectin and DLL4-Fc in both culture variants (each at 0.5 $\mu g/cm^2$). Cells were plated at 10,000 $CD3^+$ T cells/cm^2 density in TCDM supplemented with IL2 and IL15 (both at 10 ng/ml). After 8 days, cells were collected, counted, percent of $CD3^+$ determined and fold T cell expansion calculated (output/input T cell numbers) (FIG. 3).

[00174] In contrast to CD3-activated culture where both T and CAR-T cells proliferated with similar efficiency, exclusive expansion of CAR-T cells was observed in Protein L cultures. Efficiency of CAR-T cell expansion in anti-CD3 and Protein L cultures was comparable. When CAR expression in expanded CAR-T cells was determined by Protein L staining, a significantly higher proportion of CAR^+ T cells was found in Protein L-expanded CAR-T population, indicating positive selection of CAR^+ T cells in Protein L cultures.

[00175] *Cytokine production in Protein L-expanded CAR-T cells:* CAR-T cells expanded 8 days in the anti-CD3 mAb and Protein L cultures were incubated with non-transfected P815 cells (control) and P815 cells transfected with human CD19 CAR antigen. CAR-T effector (E) and P815 target (T) cells were added in co-cultures at 10^5 and $2x10^5$/ml (1:2 E/T ratio). Supernatants were collected after 24 hours for cytokine analysis by LegendPlex multiplex flow cytometry assay (BioLegend).

[00176] While CAR-T co-cultures with non-transfected P815 cells showed no significant target cell-inducible cytokine production, cultures with $CD19^+$ P815 cells revealed inducible production of IFNγ, TNFα, Granzyme B, sFasL, CCL3, CCL2, GM-CSF, IL2 and IL13; thus, defining a set of cytokines produced in response to CAR activation. Cytokine secretion profile was similar in CAR-T cells expanded either in anti-CD3 mAb or Protein- L cultures, though higher IFNγ and TNFα levels were detected in the Protein L expanded CAR-T cells (FIG. 4A).

[00177] *CAR-dependent in vitro cytokine production induced by Protein L:* Cytokine production induced by CAR activation was evaluated by incubating CAR-T cells with plastic-adsorbed Protein L. E11 PSC-derived CAR-T cells were added to the wells coated with Protein L and retronectin (both at 0.5 $\mu g/cm^2$), or retronectin alone (control). Cells were plated at 20000/well density in 0.5 mL TCDM. After 24-hour incubation, supernatants were collected for cytokine analysis by LegendPlex multiplex flow cytometry assay (BioLegend).

[00178] CAR-T cells produced granzyme B (GzmB), CCL3 and low levels of GM-CSF and IL13 during 24-hour incubation in the wells coated with retronectin alone. When CAR-T cells were incubated in the wells coated with Protein L, induction of TNFα, IP10, IFNγ, sFasL and IL6 was detected, thus revealing CAR-dependent cytokine production response induced by Protein L (FIG. 4B). The treatment of CAR-modified immune cells with Protein L can be used to evaluate CAR-dependent functional responses *in vitro*, including cytokine production.

[00179] *Cytotoxic activity in Protein L-expanded CAR-T cells:* Cytotoxic function of CAR-T cells expanded 8 days in the anti-CD3 monoclonal antibody and Protein L cultures was evaluated using *in vitro* cytotoxicity assay against luciferase-expressing non-transfected P815 cells (control), P815 cells transfected with human CD19 CAR antigen, CD19+ B cell lymphomas Daudi and Raji (FIG 5). CAR-T effector (E) cells were incubated 24 hours with tumor target (T) cells at 1:2 E/T ratio, and luciferase activity was quantitated in culture lysates by Steady-Glo luciferase assay reagent (Promega). The absolute number of target cells in 24-hour cytotoxicity cultures was determined by calibration against serial dilutions of respective target cell standards. Cytotoxicity was expressed as absolute number of target cells lysed by single effector cell and calculated by formula: (T control – T experiment) / E, where "experiment" and "control" – target cell cultures with and without effector cells, respectively; T and E – absolute numbers of target and effector cells, respectively. In addition, the cytotoxic function of CAR-T cells expanded in Protein L cultures was verified against GFP-expressing Raji cells by real-time GFP+ cell counting using Incucyte S3 live cell analysis system (Essen Biosciences).

[00180] While minimal cytotoxicity was detected in cultures with CD19 CAR antigen-negative P815 cells, both anti-CD3 monoclonal antibody and Protein L expanded CAR-T cells displayed strong cytotoxic activity against $CD19^+$ P815, Daudi and Raji cells. Noteworthy, Protein L-expanded CAR-T cells revealed ~2-3x higher cytotoxic activity than

CAR-T cells expanded in anti-CD3 monoclonal antibody cultures. Cytolytic activity of Protein L-expanded CAR-T cells was also confirmed in cultures with GFP^+ Raji cells, where significant decrease in GFP^+ live cell counts was detected only in cultures with CAR-T cells, not with non-modified T cells.

[00181] *Anti-tumor potential of Protein L-expanded CAR-T cells:* 8-week old NSG mice were injected intra-peritoneally (ip) with luciferase-expressing Daudi cells. After 4 days, when tumor cell inoculation was confirmed by control imaging, mice were divided into control (tumor only) and 2 experimental groups treated with CAR-T cells expanded either in the anti-CD3 monoclonal antibody or Protein L cultures. CAR-T cells were injected twice with a 2-day interval. During CAR-T injections and one additional week, all mice were injected (ip) with IL2 and IL15 cytokines. Tumor progression was monitored every week by bioluminescent *in vivo* imaging. Anesthetized mice injected (ip) with In Vivo-Glo luciferin (Promega) were analyzed within 15 min after luciferin injection using a Pearl Trilogy *in vivo* imager (LiCor). Images of mice in the indicated groups are shown (left panel). Tumor growth *in vivo* was estimated by quantitation of bioluminescence signal intensity (BLI) using Image Studio software (LiCor). Graph shows mean ± STD BLI values in each group over time.

[00182] It was observed that tumor growth was significantly suppressed in mice treated with CAR-T cells up to 6 weeks after CAR-T injections (FIG. 6). In accordance with cytotoxic potential *in vitro*, anti-tumor effect *in vivo* was higher in CAR-T cells expanded in Protein L cultures.

* * *

[00183] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, NY, 1994

International Patent Application No. PCT/US2016/057893

Remington's Pharmaceutical Sciences 22nd edition, 2012.

Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* 3rd ed., 2001.

U.S. Patent Application No. 12/715,136

U.S. Patent No. 8,372,642

Zheng *et al., J Transl Med,* 10:29, 2012.

WHAT IS CLAIMED IS:

1. An *in vitro* method for activation and/or expansion of CAR-modified immune cells comprising:

   (a) obtaining a starting population of CAR-modified immune cells; and

   (b) culturing said population of CAR-modified immune cells in the presence of Protein L for a sufficient period of time to produce a population of activated and/or expanded CAR-modified immune cells.

2. The method of claim 1, wherein the Protein L is coated on a culture surface.

3. The method of claim 2, wherein the culture surface is a culture plate, culture flask, microcarrier, microparticle, hydrogel particle, or culture bag.

4. The method of claim 2, wherein the culturing is performed in the absence of anti-CD3 antibody and/or antigen-specific target cells.

5. The method of claim 1, wherein the CAR-modified immune cells are T cells, NK cells, dendritic cells, and/or macrophages.

6. The method of claim 5, wherein the T cells are $CD8^+$ T cells, $CD4^+$ T cells, αβ T cells or γδ T cells.

7. The method of claim 6, wherein the method further comprises selecting for $CD8^+$ T cells.

8. The method of claim 1, wherein the CAR-modified immune cells are allogeneic.

9. The method of claim 1, wherein the CAR-modified immune cells are autologous.

10. The method of claim 1, wherein the CAR-modified immune cells are derived from pluripotent stem cells (PSCs).

11. The method of claim 10, wherein the PSCs are induced pluripotent stem cells (iPSCs).

12. The method of claim 11, wherein the iPSCs are reprogrammed from blood cells.

13. The method of claim 11, wherein the iPSCs are reprogrammed from T cells.

14. The method of claim 11, wherein the iPSCs are episomally reprogrammed.

15. The method of claim 1, wherein the CAR-modified immune cells are derived from primary peripheral blood mononuclear cells (PBMCs) or primary hematopoietic stem cells.

16. The method of any one of claims 10-14, wherein the iPSCs are differentiated to $CD34^+$ progenitors through cytokine-directed differentiation.

17. The method of any one of claims 10-14, wherein the iPSCs are differentiated to $CD34^+$ progenitors through forward programming.

18. The method of claim 1, wherein the CAR comprises an antigen-binding domain selected from the group consisting of F(ab')2, Fab', Fab, Fv, and scFv.

19. The method of claim 1, wherein the CAR comprises CD28 co-stimulatory and CD3ζ signaling domains.

20. The method of claim 2, wherein the culture surface is further coated with retronectin, fibronectin, or VCAM1.

21. The method of claim 20, wherein the retronectin is at a concentration of 0.1-1 $\mu g/cm^2$.

22. The method of claim 20, wherein the retronectin is at a concentration of 0.5 $\mu g/cm^2$.

23. The method of claim 2 or 20, wherein the culture surface is further coated with Notch ligand DLL4.

24. The method of claim 23, wherein the DLL4 is at a concentration of 0.1-1 $\mu g/cm^2$.

25. The method of claim 23, wherein the DLL4 is at a concentration of 0.5 $\mu g/cm^2$.

26. The method of claim 1, wherein the culturing is performed in the presence of IL-2 and/or IL-15.

27. The method of claim 26, wherein the IL-12 and/or IL-15 are present at a concentration of 5-15 ng/mL.

28. The method of claim 26, wherein the IL-12 and/or IL-15 are present at a concentration of 10 ng/mL.

29. The method of claim 1, wherein the culturing is under hypoxic conditions.

30. The method of claim 29, wherein the hypoxic conditions comprise 5% oxygen.

31. The method of claim 1, wherein the sufficient period of time is 8-12 days.

32. The method of claim 31, wherein the sufficient period of time is 8, 9, or 10 days.

33. The method of claim 1, wherein the culturing is in medium comprising SCF, TPO, FLT3L, and/or IL-7.

34. The method of claim 33, wherein the SCF, TPO, FLT3L, and/or IL-7are at a concentration of 50 ng/mL.

35. The method of claim 33, wherein the medium further comprises nicotinamide.

36. The method of claim 1, wherein the method results in the selective expansion of CAR-modified immune cells as compared to non-CAR-modified immune cells.

37. The method of claim 36, wherein at least 40% or 50% of the expanded population of CAR-modified immune cells are CAR-modified immune cells.

38. The method of claim 5, wherein the expanded population of CAR-modified T cells comprises at least 25% $CD3^+CD8^+$ CAR-modified T cells.

39. The method of claim 5, wherein the expanded population of CAR-modified T cells comprises 2-3 fold higher cytotoxic activity as compared to anti-CD3 expanded CAR-modified T cells.

40. The method of claim 5, wherein the expanded population of CAR-modified T cells comprises increased IFNγ and/or TNFα levels compared to anti-CD3 expanded CAR-modified T cells.

41. A population of activated and/or expanded CAR-modified immune cells produced according to the methods of any one of claims 1-40.

42. A pharmaceutical composition comprising the population of activated and/or expanded CAR-modified immune cells of claim 41 and a pharmaceutically acceptable carrier.

43. A method of treating a cancer in a subject comprising administering a therapeutically effective amount of the activated and/or expanded CAR-modified immune cells of claim 41 to the subject.

44. The method of claim 43, wherein the activated and/or expanded CAR-modified immune cells are allogenic.

45. The method of claim 43, wherein the activated and/or expanded CAR-modified immune cells are autologous.

46. The method of claim 43, further comprising administering at least a second therapeutic agent.

47. The method of claim 46, wherein the at least a second therapeutic agent is a therapeutically effective amount of an immunomodulatory or an immunosuppressive agent.

48. The method of claim 47, wherein the at least second therapeutic agent is selected from the group consisting of chemotherapy, radiotherapy, and immunotherapy.

49. The method of claim 46, wherein the activated and/or expanded CAR-modified immune cells, and/or the at least a second therapeutic agent are administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, by direct injection, or by perfusion.

50. Use of a composition of claim 41 or 42 for the treatment of cancer in subject in need thereof.

51. A composition comprising CAR-modified immune cells and Protein L.

52. The composition of claim 51, wherein the Protein L is coated on a culture surface.

53. The composition of claim 51, further comprising retronectin.

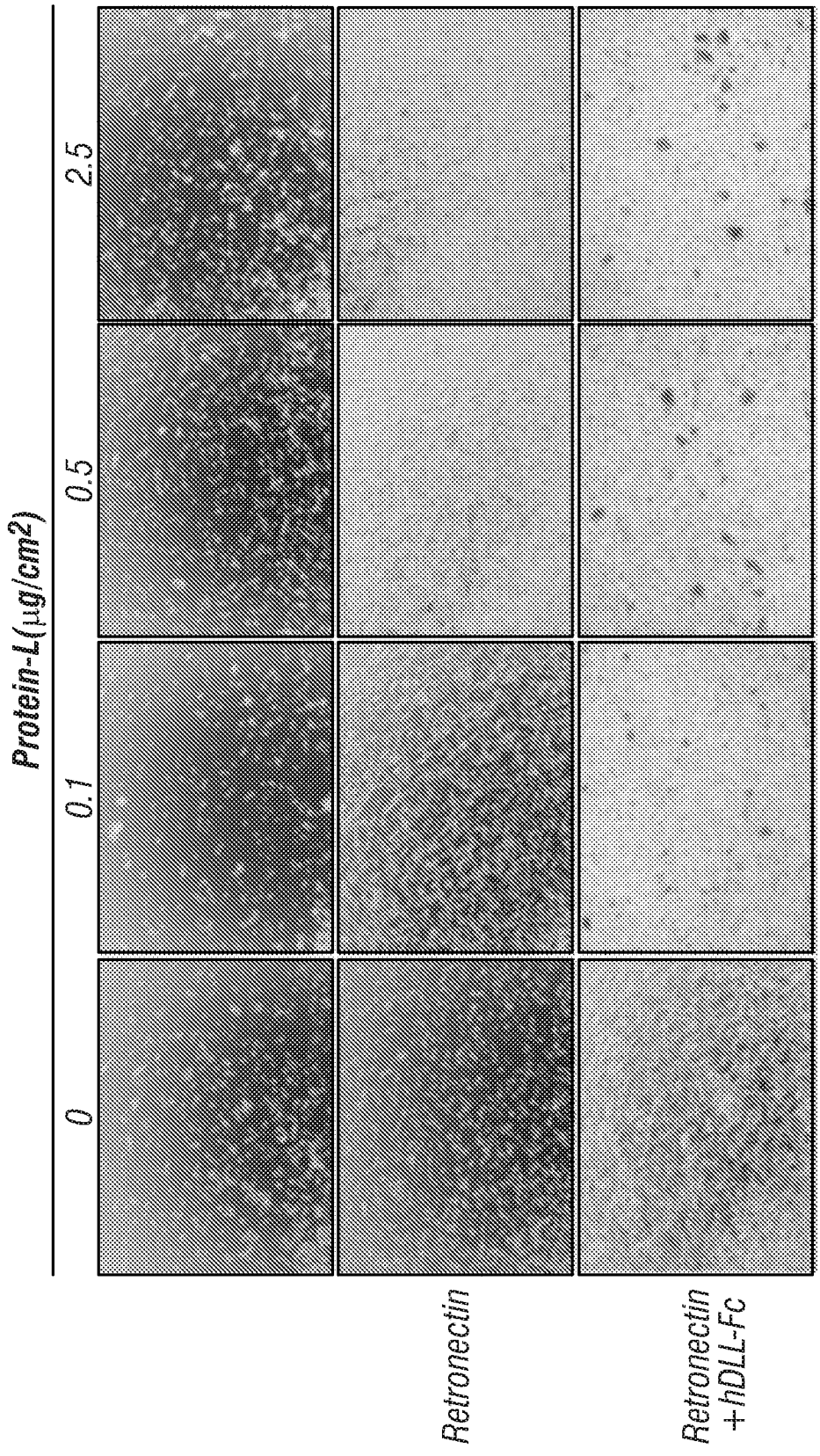

FIG. 1

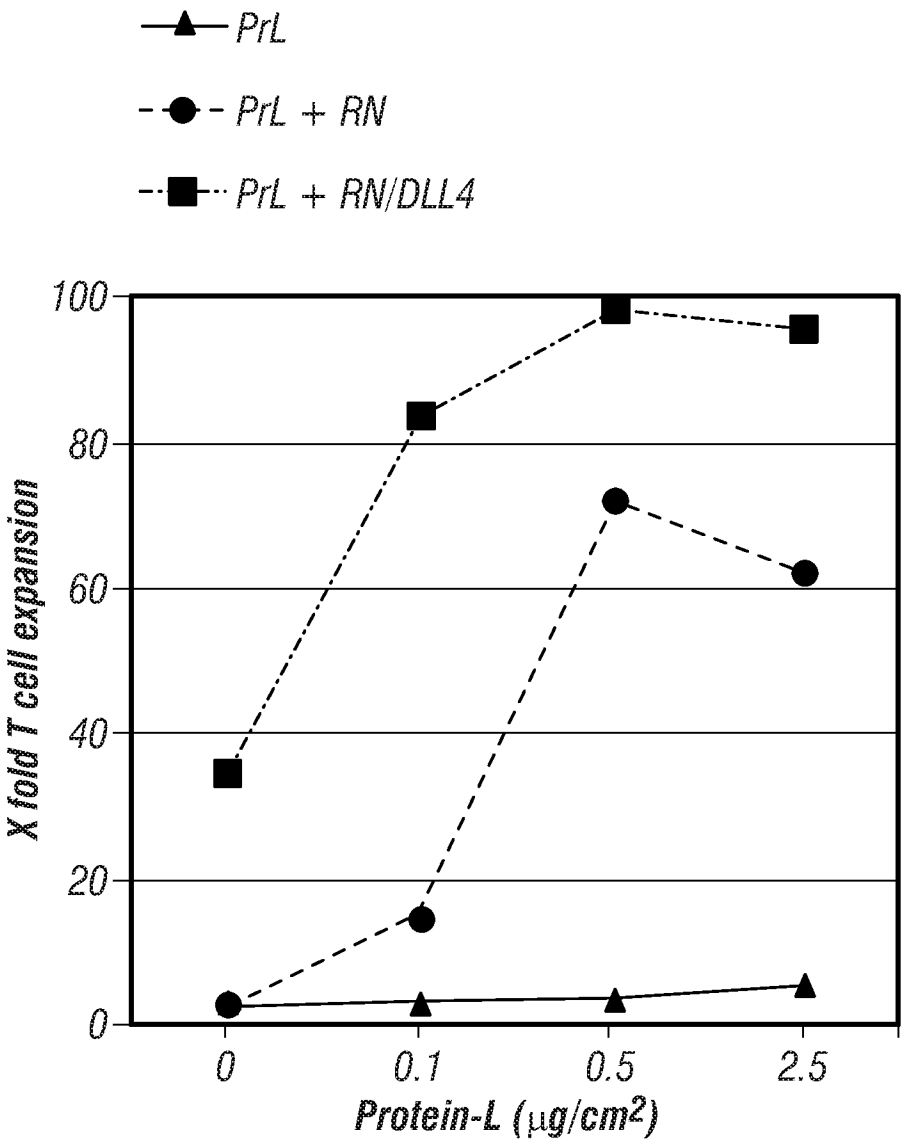
PrL
PrL + RN
PrL + RN/DLL4
100
80
60
40
20
0
X fold T cell expansion
0
0.1
0.5
2.5
Protein-L (μg/cm2)

FIG. 1
(Cont'd)

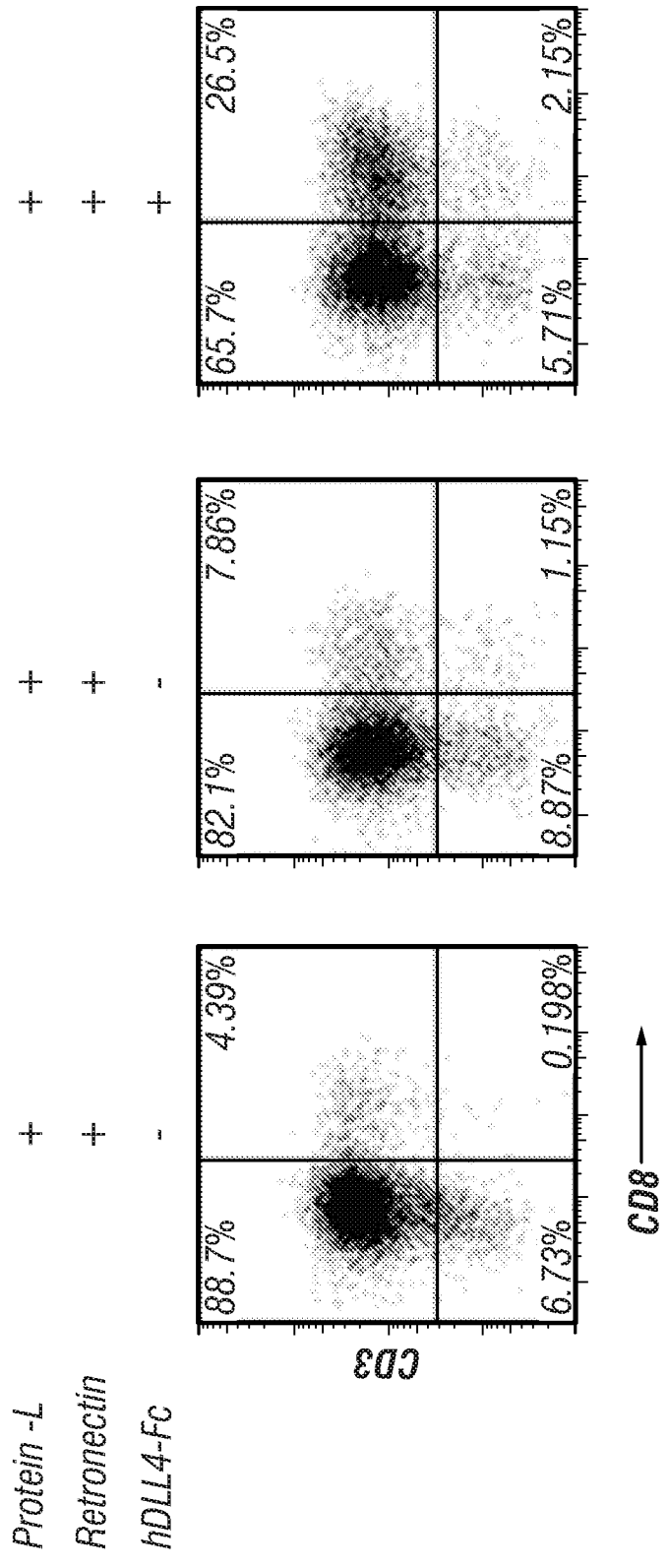
Protein -L
Retronectin
hDLL4-Fc
+ + -
+ + -
+ + +
88.7%
4.39%
6.73%
0.198%
82.1%
7.86%
8.87%
1.15%
65.7%
26.5%
5.71%
2.15%
CD3
CD8

FIG. 2

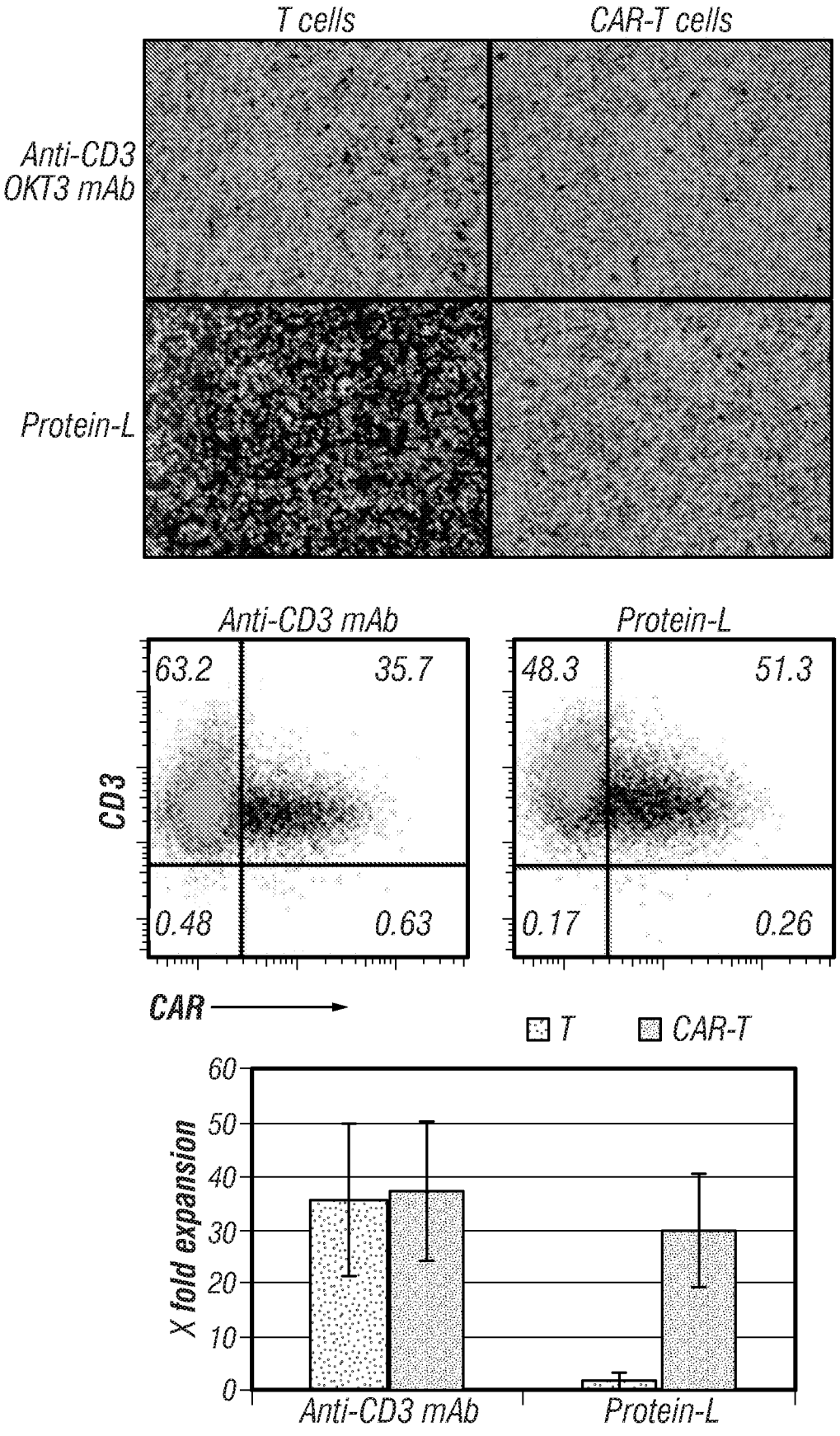
T cells
CAR-T cells
Anti-CD3 OKT3 mAb
Protein-L
Anti-CD3 mAb
Protein-L
63.2
35.7
48.3
51.3
CD3
0.48
0.63
0.17
0.26
CAR
T
CAR-T
60
50
40
30
20
10
0
X fold expansion
Anti-CD3 mAb
Protein-L

FIG. 3

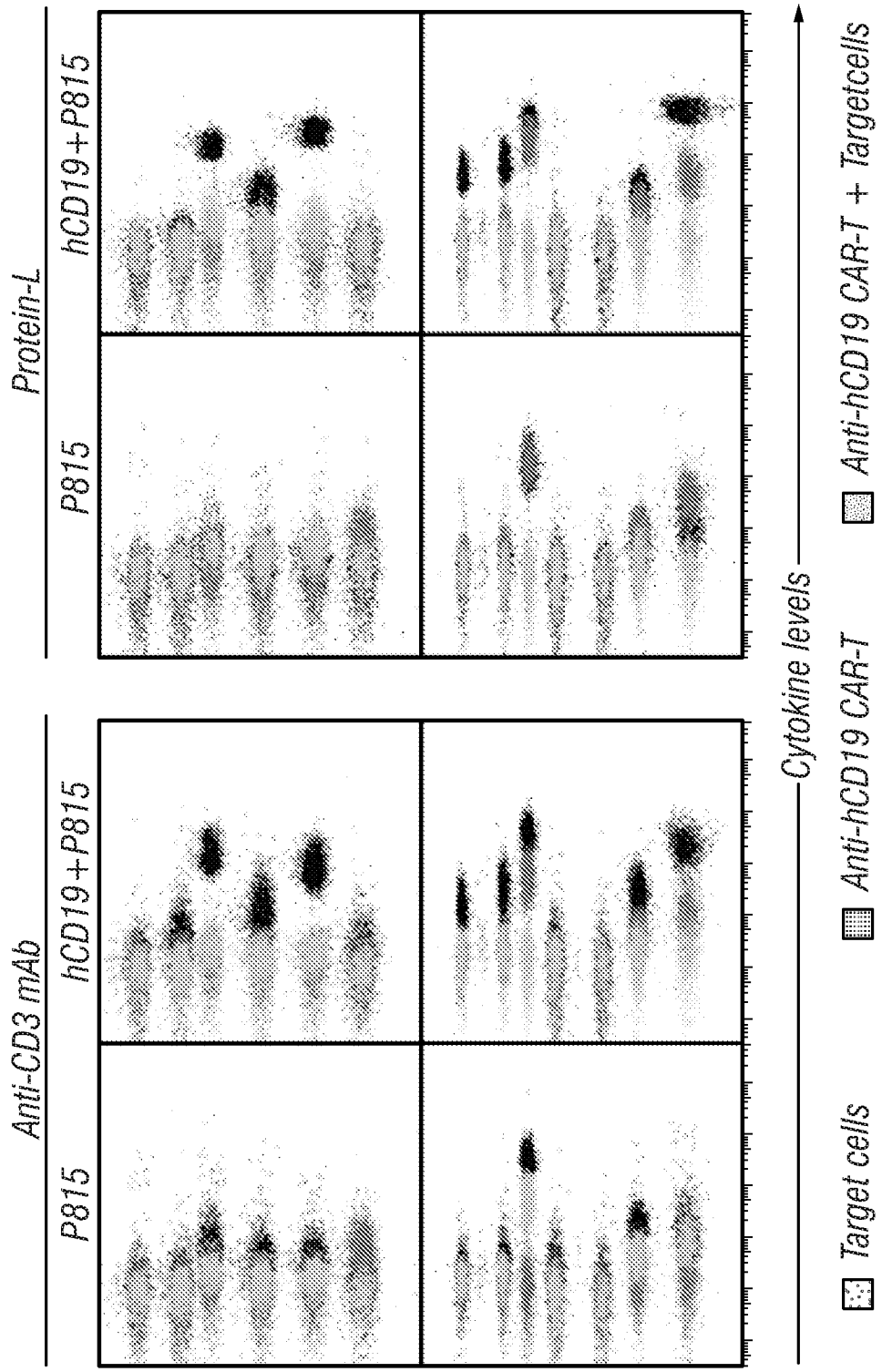
Anti-CD3 mAb
Protein-L
P815
hCD19+P815
P815
hCD19+P815
IP10
CCL2
GM-CSF
IL2
IL 13
IL8
IFNγ
TNFα
Granzyme B
IL6
IL 17A
sFasL
CCL3
Cytokine levels
Target cells
Anti-hCD19 CAR-T
Anti-hCD19 CAR-T + Targetcells

FIG. 4A

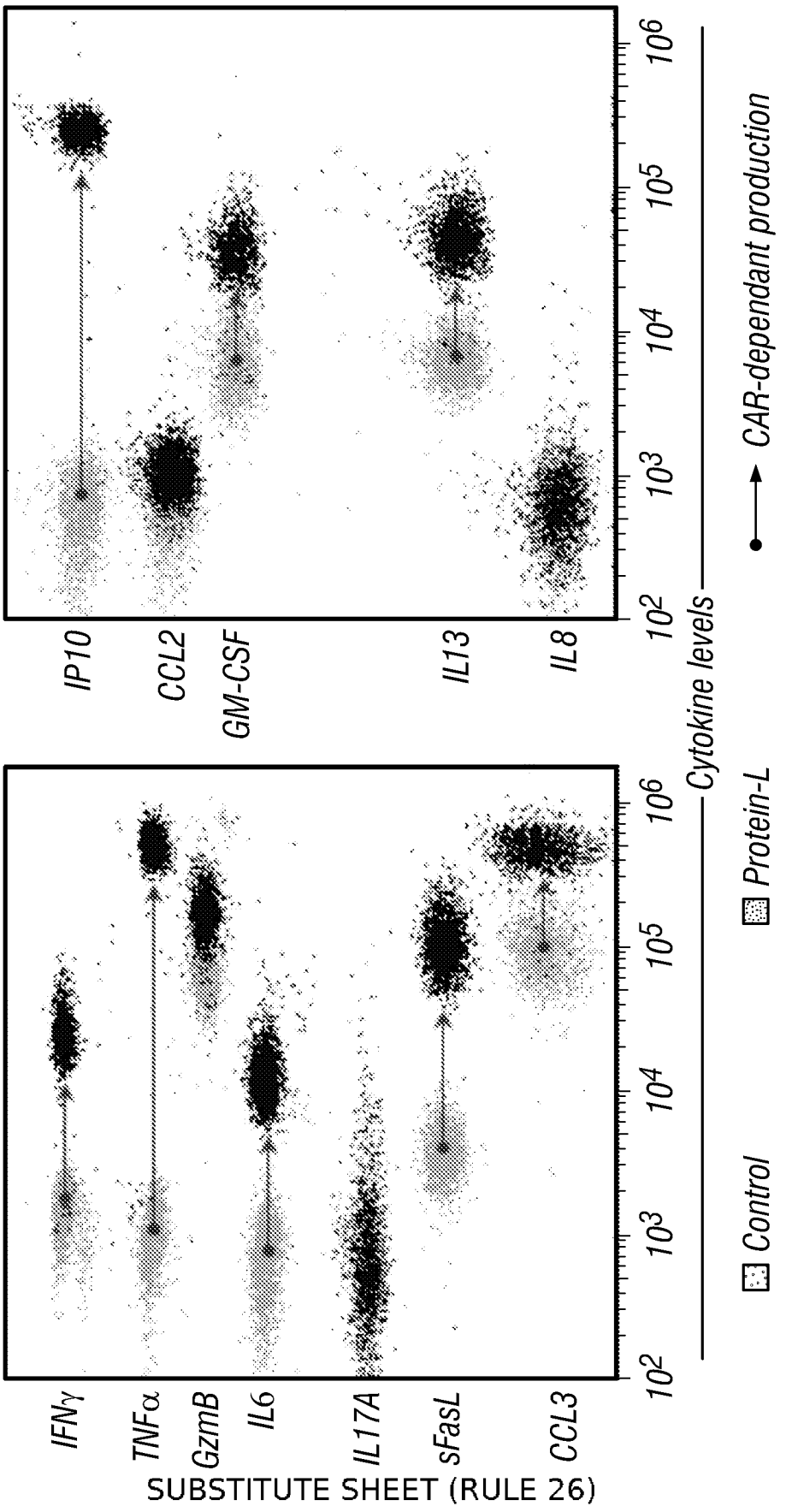
IFNγ
TNFα
GzmB
IL6
IL17A
sFasL
CCL3
IP10
CCL2
GM-CSF
IL13
IL8
10^2
10^3
10^4
10^5
10^6
Cytokine levels
Control
Protein-L
CAR-dependant production

FIG. 4B

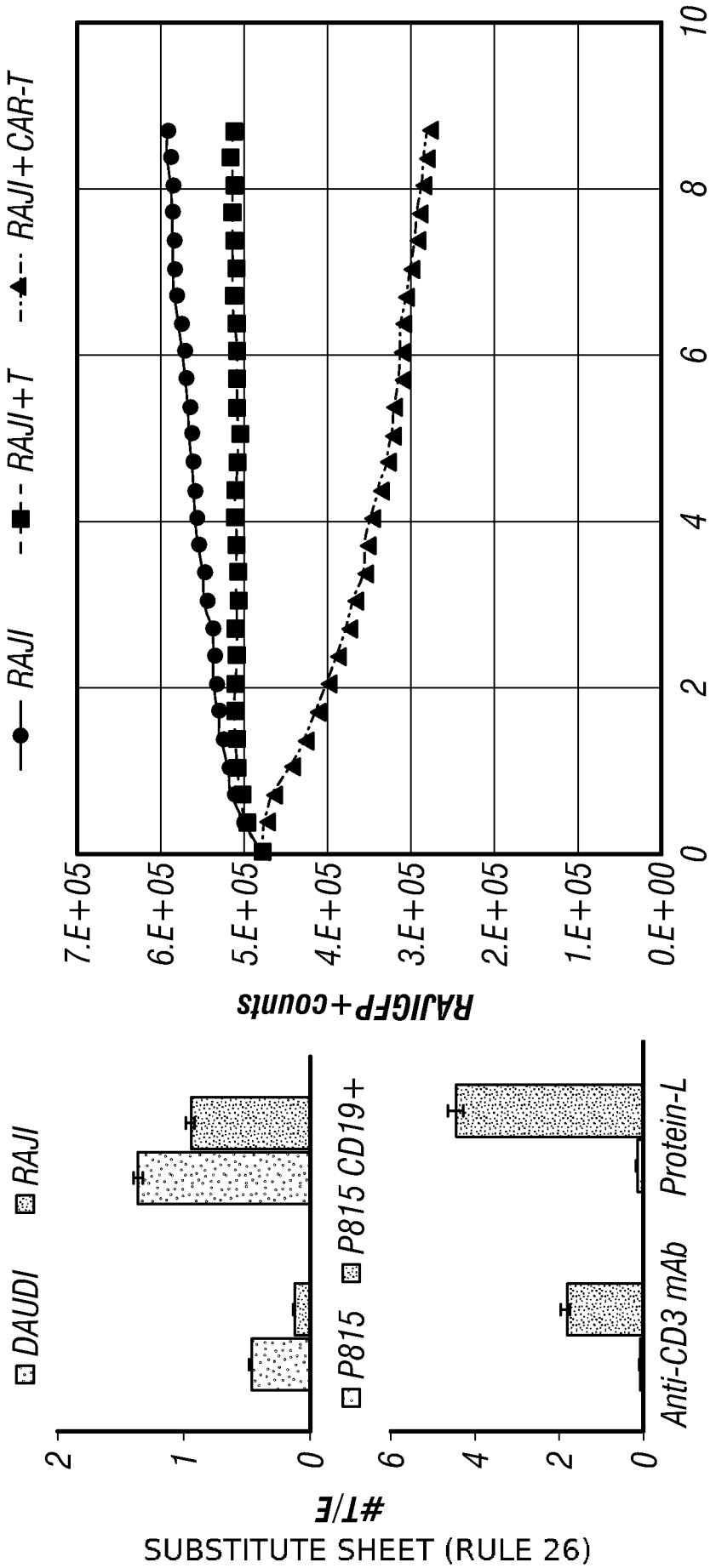
RAJI
RAJI+T
RAJI+CAR-T
RAJIGFP+counts
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
0
2
4
6
8
10
Hours
DAUDI
RAJI
P815
P815 CD19+
#T/E
2
1
0
6
4
2
0
Anti-CD3 mAb
Protein-L

FIG. 5

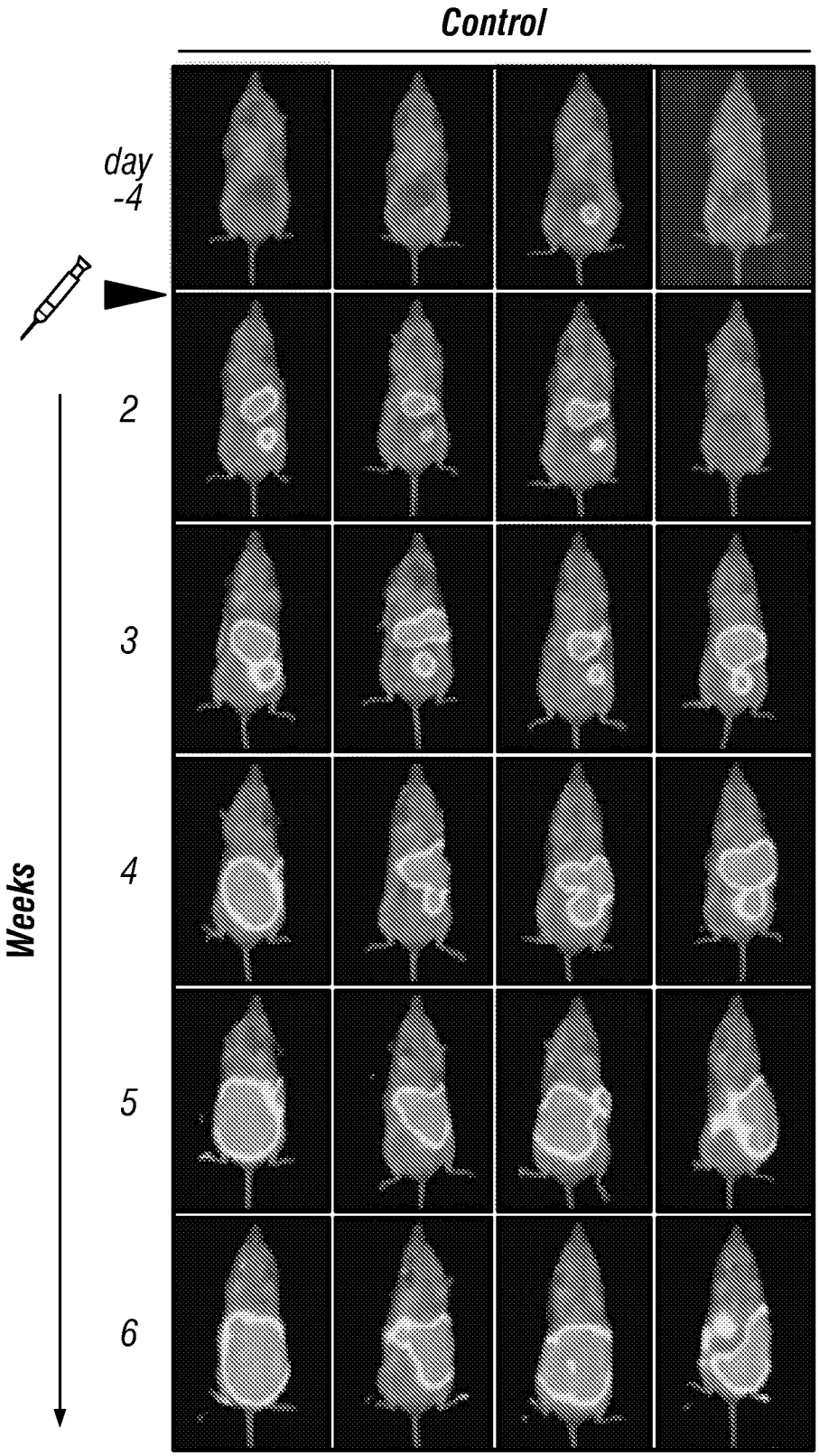

FIG. 6

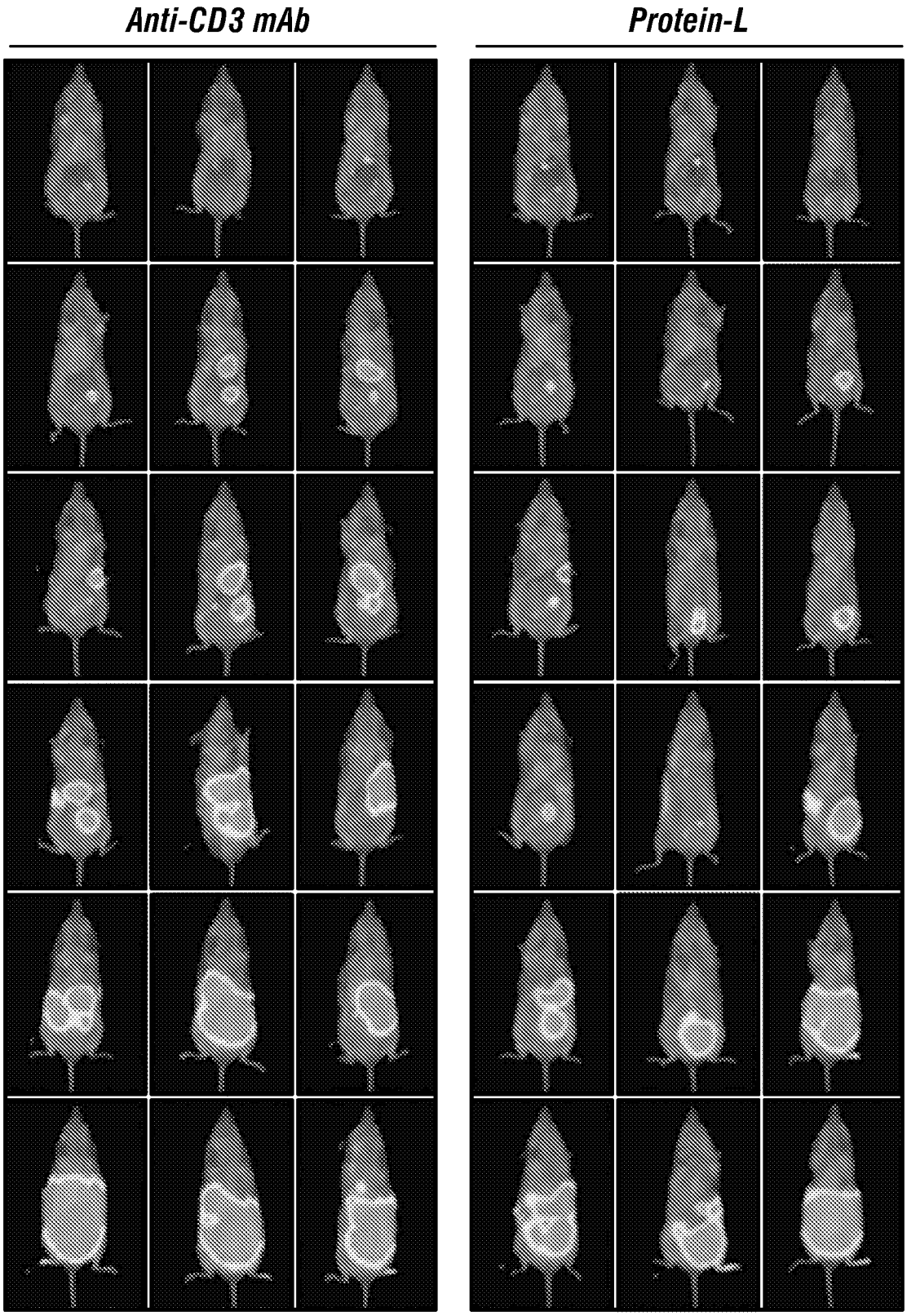

FIG. 6
(Cont'd)

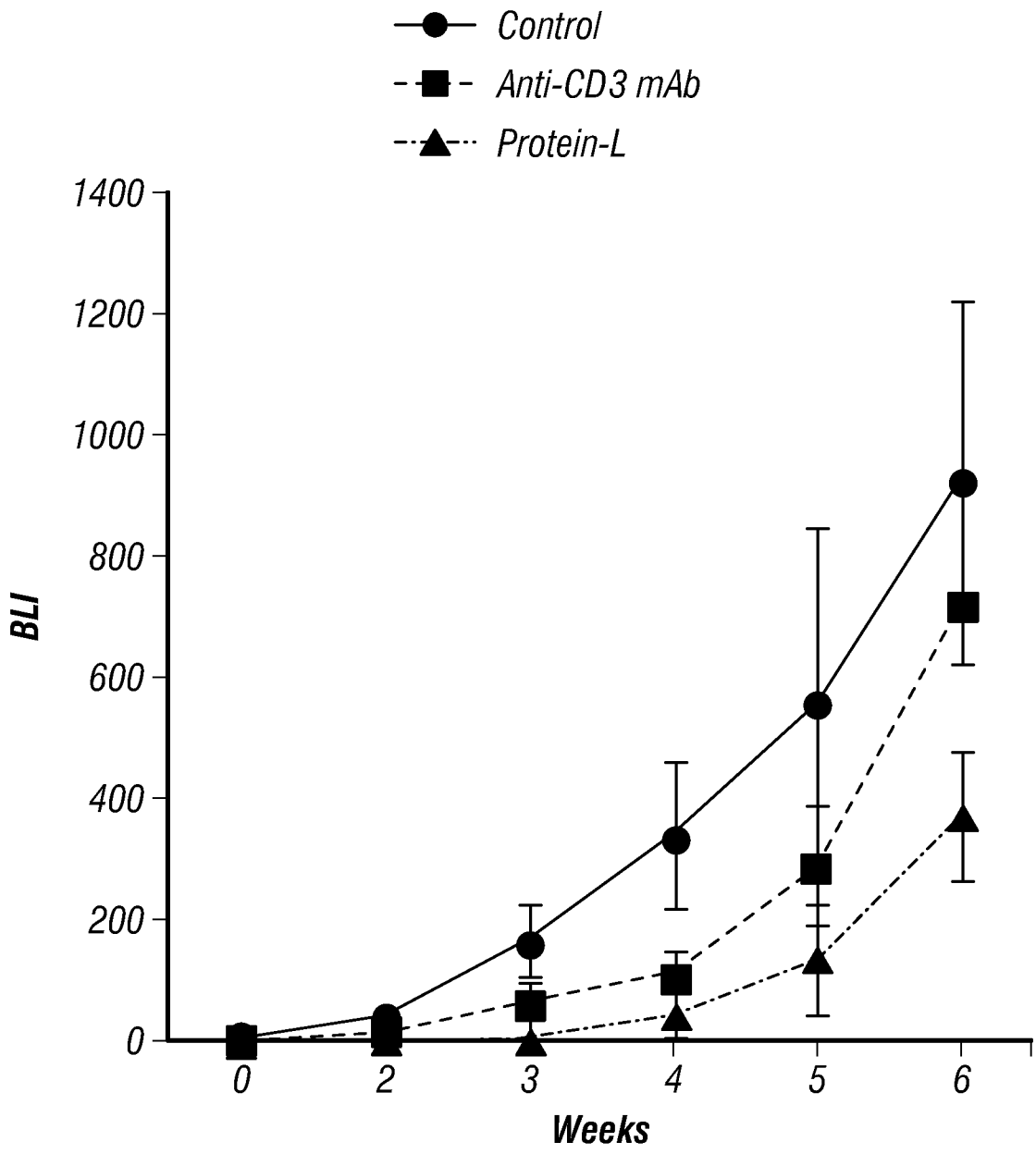
Control
Anti-CD3 mAb
Protein-L
1400
1200
1000
800
600
400
200
0
BLI
0
2
3
4
5
6
Weeks

FIG. 6
(Cont'd)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/051832

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0783 A61K35/17
ADD.

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)
C12N A61K

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)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/102795 A2 (UNIV SOUTHERN CALIFORNIA [US]) 7 June 2018 (2018-06-07) abstract page 226, paragraph 357 page 334, paragraph 609 ----- -/--	1-53

[X] Further documents are listed in the continuation of Box C.

[X] 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
26 November 2019	04/12/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Grötzinger, Thilo

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALEC J. WALKER ET AL: "Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase", MOLECULAR THERAPY, vol. 25, no. 9, 1 September 2017 (2017-09-01), pages 2189-2201, XP055568995, ISSN: 1525-0016, DOI: 10.1016/j.ymthe.2017.06.008 abstract page 2193, right-hand column, last paragraph - page 2195, left-hand column, paragraph 1; figure 5 page 2198, right-hand column, last paragraph - page 2199, left-hand column, paragraph 1	1-53
Y	WO 2017/161092 A1 (NEXIMMUNE INC [US]; UNIV JOHNS HOPKINS [US]) 21 September 2017 (2017-09-21) abstract page 1, line 24 - line 29 page 5, line 26 - line 30 page 6, line 15 - line 17 page 9, line 19 - line 30 page 10, line 3 - line 23 page 19, line 5 - line 15 page 20, line 1 - line 3	1-53
Y	DANIELA LORENZO ET AL: "A B-Cell Superantigen Induces the Apoptosis of Murine and Human Malignant B Cells", PLOS ONE, vol. 11, no. 9, 7 September 2016 (2016-09-07), page e0162456, XP055450455, DOI: 10.1371/journal.pone.0162456 abstract introduction; pages 1-2	1-53

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/051832

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 3044682 A1	07-06-2018
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		CO 2019007092 A2	18-09-2019
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		KR 20190101979 A	02-09-2019
		PH 12019501227 A1	21-10-2019
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		EP 3445399 A1	27-02-2019
		JP 2019513412 A	30-05-2019
		KR 20190026645 A	13-03-2019
		SG 11201807940X A	30-10-2018
		WO 2017161092 A1	21-09-2017