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(54) Title: IMMUNOSUPPRESSIVE TGF- $\beta$  SIGNAL CONVERTER

(57) Abstract: Embodiments of the disclosure concern cell therapy methods and compositions utilizing cells expressing at least a chimeric TGF $\beta$  receptor including the exodomain of a TGF $\beta$ II receptor and an endodomain that is not from TGF $\beta$  receptor, thereby converting the negative signal of TGF $\beta$  for T cell proliferation into a T cell activation signal. In at least certain aspects, cells harbor- ing the chimeric TGF $\beta$  receptor also harbor one or more chimeric antigen receptors.



## IMMUNOSUPPRESSIVE TGF- $\beta$ SIGNAL CONVERTER

**[0001]** This application claims priority to U.S. Provisional Patent Application Serial No. 61/812,917, filed April 17, 2013, which is incorporated by reference herein in its entirety.

### STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

**[0002]** This invention was made with government support under W81XWH-11-1-0625 awarded by the Department of Defense. The government has certain rights in the invention.

### TECHNICAL FIELD

**[0003]** Embodiments of the disclosure encompass at least the fields of immunology, cell biology, molecular biology, and medicine, including cancer medicine.

### BACKGROUND

**[0004]** Transforming growth factor beta (TGF $\beta$ ) has been demonstrated to play an important role in the regulation of the immune response, primarily through its suppressive function towards cells of the immune system. TGF $\beta$  is a suppressor of antigen-specific T cell proliferation at least through reduction of the cell-cycle rate, as opposed to induction of apoptosis. In particular, TGF $\beta$  acts on cytotoxic T lymphocytes (CTLs) to specifically inhibit the expression of at least five cytolytic gene products: perforin, granzyme A, granzyme B, Fas ligand, and interferon gamma that are important for CTL-mediated tumor cytotoxicity (Thomas and Massagué, 2005).

### BRIEF SUMMARY

**[0005]** Embodiments of the disclosure include methods and compositions for cancer therapy. In particular embodiments, there are methods and compositions related to cell therapy for cancer. Although particular embodiments may be useful for any cancer, in specific embodiments the methods and compositions are particularly effective against cancers that secrete TGF $\beta$  or that reside in a tumor microenvironment (including tumor stroma) that releases TGF $\beta$ . Exemplary cancers that are suitable for treatment with the compositions and methods disclosed herein include but are not limited to cancers of the prostate, breast, melanoma, pancreatic, lung,

brain, colon, esophageal, liver, kidney, testicular, ovarian, cervical, gall bladder, thyroid, anal, endometrial, bladder, pituitary gland, leukemia, lymphoma, stomach, and spleen.

**[0006]** Aspects of the disclosure utilize cells that are modified to express one or more non-native molecules that render the cells effective against cancers that secrete TGF $\beta$ . The cells may be utilized for adoptive cell therapy, in specific embodiments. In particular aspects, the cells are T cells, NK cells, NKT cells, cytotoxic T lymphocytes, antigen-specific T cells, including tumor- or pathogen (such as viral or bacterial)-specific T cells, T cells having  $\alpha\beta$  T cell receptors (TCR), T cells that comprise at least one chimeric antigen receptor, and so forth. Cells may be genetically engineered to be effective in the presence of TGF $\beta$ , and in addition may or may not have other non-naturally occurring genetic modifications. In addition to the chimeric TGF $\beta$  receptor, the cells may express other non-native molecules. In particular embodiments, the non-native molecules are cell surface receptors. The molecules may be a chimeric antigen receptor (CAR) specific for a tumor cell surface molecule or  $\alpha\beta$  TCRs, for example. In certain aspects, a cell may employ one or more chimeric antigen receptors and a chimeric TGF $\beta$  receptor.

**[0007]** In embodiments of the disclosure, the cells of the disclosure convert the normally inhibitory signal from TGF $\beta$  into an activation stimulus for T cells by utilizing a chimeric TGF $\beta$  receptor that employs an extracellular receptor for TGF $\beta$  on the cell surface linked to an endodomain from another entity that is capable of an activation signal. In particular embodiments, T cells modified with such chimeric receptors have increased potency compared to cells that lack such chimeric receptors. Specific embodiments of the disclosure employ a chimeric TGF $\beta$  receptor expressing the exodomain of TGF $\beta$ RII linked to an endodomain from another molecule. In specific embodiments the exodomain and the endodomain are linked through a transmembrane domain, and in certain cases the transmembrane domain is the naturally occurring transmembrane domain for the selected endodomain. Although the receptor for TGF $\beta$  can be part or all of any one of TGF $\beta$  receptor 1, TGF $\beta$  receptor 2, or TGF $\beta$  receptor 3, in specific embodiments it comprises the TGF $\beta$  receptor 2 isoform of the receptor. In specific embodiments, the endodomain comprises the endodomain of the exemplary toll-like receptor (TLR) 4, although others such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD28, 4-1BB, OX40, CD3zeta, CD40, CD27, or a combination thereof, may be

employed, for example. In specific embodiments, the chimeric cytokine receptor is labeled, such as with GFP, mOrange, blue fluorescent protein, and so forth.

**[0008]** In certain aspects of the disclosure, the cell that comprises a chimeric TGF $\beta$  receptor also comprises a chimeric antigen receptor. The chimeric antigen receptor (CAR) may be of any kind, but in specific embodiments the CAR targets the cancer that also secretes the TGF $\beta$  molecule. Exemplary CARs include at least CARs specific for any one or more of the following: EphA2, HER2, GD2, Glypican-3, 5T4, 8H9,  $\alpha_v\beta_6$  integrin, B cell maturation antigen (BCMA) B7-H3, B7-H6, CAIX, CA9, CD19, CD20, CD22, kappa light chain, CD30, CD33, CD38, CD44, CD44v6, CD44v7/8, CD70, CD123, CD138, CD171, CEA, CSPG4, EGFR, EGFRvIII, EGP2, EGP40, EPCAM, ERBB3, ERBB4, ErbB3/4, FAP, FAR, FBP, fetal AchR, Folate Receptor  $\alpha$ , GD2, GD3, HLA-AI MAGE A1, HLA-A2, IL11Ra, IL13Ra2, KDR, Lambda, Lewis-Y, MCSP, Mesothelin, Muc1, Muc16, NCAM, NKG2D ligands, NY-ESO-1, PRAME, PSCA, PSC1, PSMA, ROR1, Sp17, SURVIVIN, TAG72, TEM1, TEM8, VEGFR2, carcinoembryonic antigen, HMW-MAA, VEGF receptors, and/or other exemplary antigens that are present within the extracellular matrix of tumors, such as oncofetal variants of fibronectin, tenascin, or necrotic regions of tumors and other tumor-associated antigens or actionable mutations that are identified through genomic analysis and/or differential expression studies of tumors, for example, or a combination thereof. The cells of the disclosure may have more than one CAR, and a CAR may have more than one scFv specific for different antigens.

**[0009]** In certain aspects of the disclosure, the cell that comprises (such as expresses) a chimeric TGF $\beta$  receptor also comprises (such as expresses) an antigen-specific receptor. The antigen may be a tumor antigen or a pathogen antigen, including viral or bacterial, for example.

**[0010]** Exemplary tumor antigens include at least the following: carcinoembryonic antigen (CEA) for bowel cancers; CA-125 for ovarian cancer; MUC-1 or epithelial tumor antigen (ETA) or CA15-3 for breast cancer; tyrosinase or melanoma-associated antigen (MAGE) for malignant melanoma; and abnormal products of ras, p53 for a variety of types of tumors; alphafetoprotein for hepatoma, ovarian, or testicular cancer; beta subunit of hCG for men with testicular cancer; prostate specific antigen for prostate cancer; beta 2 microglobulin for multiple myeloma and in some lymphomas; CA19-9 for colorectal, bile duct, and pancreatic cancer;

chromogranin A for lung and prostate cancer; TA90 for melanoma, soft tissue sarcomas, and breast, colon, and lung cancer. Examples of tumor antigens are known in the art, for example in Cheever *et al.*, 2009, which is incorporated by reference herein in its entirety. Specific examples of tumor antigens include at least CEA, gp100, mesothelin, TRP1, CD40, EGFP, Her2, TCR alpha, trp2, MUC1, cdr2, ras, GITR, WT1, MUC1, LMP2, HPV E6 E7, EGFRvIII, HER-2/neu, MAGE A3, p53 nonmutant, NY-ESO-1, PSMA, GD2, Melan A/MART1, Ras mutant, gp 100, p53 mutant, Proteinase3 (PR1), bcr-abl, Tyrosinase, Survivin, PSA, hTERT, EphA2, PAP, ML-IAP, AFP, EpCAM, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, ALK, Androgen receptor, Cyclin B1, Polysialic acid, MYCN, RhoC, TRP-2, GD3, Fucosyl GM1, Mesothelin, PSCA, MAGE A1, sLe(a), CYP1B1, PLAC1, GM3, BORIS, Tn, GloboH, ETV6-AML, NY-BR-1, RGS5, SART3, STn, Carbonic anhydrase IX, PAX5, OY-TES1, Sperm protein 17, LCK, HMWMAA, AKAP-4, SSX2, XAGE 1, B7H3, Legumain, Tie 2, Page4, VEGFR2, MAD-CT-1, FAP, PDGFR- $\beta$ , MAD-CT-2, and Fos-related antigen 1, for example.

**[0011]** Specific viruses for which an antigen receptor T cell may be directed includes one selected from the group consisting of EBV, CMV, Adenovirus, BK virus, HHV6, RSV, Influenza, Parainfluenza, Bocavirus, Coronavirus, LCMV, Mumps, Measles, Metapneumovirus, Parvovirus B, Rotavirus, West Nile Virus, JC, HHV7, and a combination thereof. In some cases, the virus is EBV and the antigen is selected from the group consisting of EBNA1, LMP2, and BZLF1; the virus may be CMV and the antigen may be selected from the group consisting of IE1 and pp65; the virus may be Adv and the antigen may be selected from the group consisting of Hexon and penton; the virus may be BK virus and the antigen may be selected from the group consisting of LT and VP-1; the virus may be HHV6 and the antigen may be selected from the group consisting of U14, U11, U71, U54, and U90; the virus may be RSV and the antigen may be selected from the group consisting of N and F; the virus may be Influenza and the antigen may be selected from the group consisting of MP1 and NP1.

**[0012]** In embodiments of the disclosure, there is transgenic expression of a novel immunosuppressive signal converter on T cells. The signal converter changes the normally inhibitory action of TGF $\beta$  on an immune effector cell into a stimulatory signal for the cell through the use of the chimeric TGF $\beta$  receptor that has the TGF $\beta$  exodomain linked to an endodomain that is not from TGF $\beta$  receptor and provides an activation signal.

**[0013]** In embodiments of the disclosure, there is a polynucleotide comprising a nucleotide sequence encoding a chimeric TGF $\beta$  receptor, wherein the receptor comprises an exodomain of TGF $\beta$  receptor and a non-TGF $\beta$  receptor endodomain. In specific embodiments, the endodomain comprises an endodomain of an immune stimulant protein, or functional fragment thereof, wherein the endodomain or fragment thereof is from toll-like receptor (TLR)1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD28, 41BB, OX40, CD40, CD27, or a combination thereof. In specific cases, the endodomain is from toll-like receptor (TLR) 4. In particular embodiments, the polynucleotide further comprises a polynucleotide sequence that encodes a chimeric antigen receptor (CAR) or an  $\alpha\beta$  T cell receptor (TCR). In some embodiments, the expression of the chimeric TGF $\beta$  receptor and the expression of the CAR and/or  $\alpha\beta$  TCR are under control of the same or different regulatory element or elements.

**[0014]** In embodiments wherein one or more polynucleotides are utilized, the polynucleotide may be further defined as an expression construct or vector, and the vector may be a viral vector (such as a retroviral vector, lentiviral vector, adenoviral vector, or adeno-associated viral vector) or a non-viral vector, such as plasmid or RNA, including mRNA.

**[0015]** In certain embodiments, provided herein are one or more cells that comprise a polynucleotide as provided herein. Such cells may be, for example, T cells, natural killer (NK) cells, natural killer T (NKT) cells, antigen-specific T cells, or T cells comprising an  $\alpha\beta$ TCR, or CTLs. In a specific embodiment, the antigen-specific T cells are further defined as tumor-specific T cells or pathogen-specific T cells. In specific cases, the TGF $\beta$  receptor comprises part or all of TGF $\beta$  receptor II. In some cases, the antigen-specificity of the antigen-specific T cells is natural, whereas in certain cases, the antigen-specificity of the antigen-specific T cells is recombinantly generated. In particular cases, the cell is an antigen-specific T cell, and/or comprises an  $\alpha\beta$  TCR, which may be native to the T cell or is an engineered molecule. In particular aspects, the antigen-specific T cell is further defined as a tumor-specific T cell or pathogen-specific T cell. In some cases, the antigen-specificity of the antigen-specific T cell is natural, whereas in certain cases, the antigen-specificity of the antigen-specific T cell is recombinantly generated. In some cases, the  $\alpha\beta$  TCR on the cell is natural, whereas in other cases the  $\alpha\beta$  TCR on the cell is recombinantly generated; the  $\alpha\beta$  TCR may be selected for, such as from a library.

**[0016]** In embodiments of the disclosure, there are one or more cells, comprising a first polynucleotide that encodes a chimeric TGF $\beta$  receptor, wherein the chimeric TGF $\beta$  receptor comprises an exodomain of TGF $\beta$  receptor and a non- TGF $\beta$  receptor endodomain; and a second polynucleotide that encodes a CAR and/or a  $\alpha\beta$  TCR. In some cases, the first and second expression construct are the same or different molecule. The antigen-specific T cell may be a tumor-specific T cell receptor or a pathogen-specific T cell receptor. The CAR may be specific for an antigen such as one of the following: PSCA, HER2, CD19, CD20, CD22, Kappa or light chain, CD30, CD33, CD123, CD38, ROR1, ErbB3/4, EGFR, EGFRvIII, EphA2, FAP, carcinoembryonic antigen, EGP2, EGP40, mesothelin, TAG72, PSMA, NKG2D ligands, B7-H6, IL-13 receptor  $\alpha$ 2, IL-11 receptor R  $\alpha$ , MUC1, MUC16, CA9, GD2, GD3, HMW-MAA, CD171, Lewis Y, G250/CAIX, HLA-AI MAGE A1, HLA-A2 NY-ESO-1, PSC1, folate receptor-  $\alpha$ , CD44v7/8, 8H9, NCAM, VEGF receptors, 5T4, Fetal AchR, NKG2D ligands, CD44v6, TEM1, TEM8 or a combination thereof.

**[0017]** In some embodiments, provided herein are one or more cells, defined as: 1) being an antigen-specific T cell, and/or a cell comprising a CAR, and/or a cell comprising an  $\alpha\beta$  T cell receptor; and 2) comprising a chimeric TGF $\beta$  receptor, wherein the receptor comprises an exodomain of TGF $\beta$  receptor and a non- TGF $\beta$  receptor endodomain. In specific embodiments, the cell is autologous with respect to a recipient of said cell. In some cases, the cell is allogenic with respect to a recipient of said cell.

**[0018]** In embodiments of the disclosure, provided herein is a method of treating and/or preventing cancer in an individual, comprising the step of delivering a therapeutically effective amount of a plurality of cells as provided herein to the individual, wherein the individual has a cancer that has cells that secrete TGF $\beta$  and/or the cancer has a microenvironment that produces TGF $\beta$ . Although in some cases the cancer may be of any kind, in specific cases, the cancer is prostate, breast, melanoma, pancreatic, lung, brain, colon, esophageal, liver, kidney, testicular, ovarian, cervical, gall bladder, thyroid, anal, endometrial, bladder, pituitary gland, leukemia, lymphoma, stomach, spleen, or myeloma. In specific embodiments, the cells are delivered intravenously, intraperitoneally, intratumorally, intrathecally, and/or transrectally. In some embodiments, exposure of TGF $\beta$  to the cell protects or enhances the anti-tumor activity of the cell. In particular aspects, the individual is provided with another cancer therapy, such as surgery, chemotherapy, immunotherapy, hormone therapy,

radiation, or a combination thereof. In certain embodiments, the cells are allogenic or autologous to said individual. In some aspects, the methods of the disclosure further comprise the step of obtaining peripheral blood mononuclear cells (PBMCs) from the individual or from another individual. In specific embodiments, the method comprises the step of obtaining T cells from the PBMCs and modifying the T cells to comprise a polynucleotide comprising a nucleotide sequence encoding a chimeric TGF $\beta$  receptor, wherein the receptor comprises an exodomain of TGF $\beta$  receptor and a non-TGF $\beta$  receptor endodomain. In embodiments of the disclosure, the cells are provided to the individual more than once. In specific cases, when the cells are provided more than once and the cells comprise a CAR specific for a particular tumor antigen, upon one or more subsequent deliveries the cells comprise a CAR specific for a different tumor antigen.

**[0019]** In an embodiment of the disclosure, there is a method of converting a T cell-inhibitory cytokine signal into a T cell-stimulatory signal, comprising the step of exposing the inhibitory cytokine to a receptor on a T cell, said receptor having an exodomain and an endodomain that is not naturally linked to the exodomain, wherein the exodomain is capable of binding the inhibitory cytokine and the endodomain provides a stimulatory signal to the T cell, wherein the exodomain and the endodomain pair as homodimers. In specific embodiment, the cytokine is TGF $\beta$  or VEGF.

**[0020]** In an embodiment of the disclosure, there is a method of converting a T cell-inhibitory cytokine signal into a T cell-stimulatory signal, comprising the step of exposing the inhibitory cytokine to a receptor on a T cell, said receptor having an exodomain and an endodomain that is not naturally linked to the exodomain, wherein the exodomain is capable of binding the inhibitory cytokine and the endodomain provides a stimulatory signal to the T cell, wherein the inhibitory cytokine is not IL4 or IL7 or both. In specific embodiments, the cytokine is TGF $\beta$  or VEGF.

**[0021]** Embodiments of the disclosure provide improvements in activity of cells for adoptive transfer therapy *in vivo* in the presence of cancer cells that secrete the inhibitor TGF $\beta$ .

**[0022]** In an embodiment of the disclosure, there is a kit comprising a polynucleotides or polypeptides as described herein and/or a cell or cells as described herein.



**[0023]** The foregoing has outlined the features and technical advantages of the present disclosure in order that the detailed description of the disclosure that follows may be better understood. Additional features and advantages of the disclosure will be described hereinafter which form the subject of the claims of the disclosure. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present disclosure. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the disclosure as set forth in the appended claims. The novel features which are believed to be characteristic of the disclosure, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present disclosure.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0024]** For a more complete understanding of the present disclosure, reference is now made to the following descriptions taken in conjunction with the accompanying drawing, in which:

**[0025]** FIG. 1 illustrates an exemplary embodiment of arming T cells to withstand the inhibitory tumor microenvironment.

**[0026]** FIG. 2 provides exemplary retroviral vector maps of the control DNR11, RIID2 and RIID4.

**[0027]** FIG. 3 shows generation of DNR11, RIID2 and RIID4 transgenic T cells ;

**[0028]** FIGS. 4A-4B demonstrate determination of co-expression of DNR11, RIID2 and RIID4 with CAR-PSCA.2G.

**[0029]** FIG. 5 demonstrates whether RIID2 and RIID4 protect CAR-modified T cells exposed to TGF $\beta$ .

**[0030]** FIGS. 6A-6C show whether in suppressive conditions RIID2- and RIID4-modified T cells are protected.

**[0031]** FIG. 7 shows administration of TGF $\beta$ 1 selects RIID2 and RIID4-transduced T cells.

**[0032]** FIG. 8 demonstrates that CARPSCA.2G/RIID2 and /RIID4 T cells require antigen stimulation for their expansion.

**[0033]** FIG. 9 provides that RIID2 and RIID4 enhance anti-tumor effect of CARPSCA.2G against DU145 cells.

**[0034]** FIG. 10 shows the impact of TGF $\beta$  exposure on exemplary CAR T cells. A) signaling, B) cytolytic function, C) expansion, D) viability, and E) PD1 expression.

**[0035]** FIG. 11. illustrates A) wild type TGF $\beta$ R, and B) RIID4 signaling.

**[0036]** FIG. 12 illustrates A) the retroviral vector for an example of a RIID4 construct, B) expression of RIID4 on transduced cells, C) microscopic photographs of T cell cultures in presence or absence of TGF $\beta$ , and D) expression of PD1 in T cells as measured by flow.

**[0037]** FIG. 13 illustrates exemplary chimeric cytokine retroviral receptors.

**[0038]** FIG. 14 demonstrates that TGF $\beta$ 1 does not induce pSmad 2/3 in RIID4-modified T cells.

**[0039]** FIG. 15 shows protection by RIID4 of T cells from TGF $\beta$ 1-induced apoptosis.

**[0040]** FIG. 16 demonstrates that RIID4 prevents PD1 upregulation.

**[0041]** FIG. 17 demonstrates that RIID4 prevents TGF $\beta$ 1-induced inhibition of cytolytic function.

**[0042]** FIG. 18 shows that RIID4+ cells are selected by TGF $\beta$ 1 exposure.

[0043] FIG. 19 demonstrates that withdrawal of antigen and TGF $\beta$ 1 leads to culture failure.

[0044] FIG. 20 illustrates an example of a co-culture experimental set-up.

[0045] FIG. 21 provides evidence that 2G.CAR-PSCA/RIID4 cells eliminated tumors in the presence of TGF $\beta$ 1.

[0046] FIG. 22 shows that 2G.CAR-PSCA T cells did not proliferate in the presence of TGF $\beta$ 1.

[0047] FIG. 23 demonstrates that 2G.CAR-PSCA/RIID4 cells were able to expand and that the cells depend on antigen existence.

[0048] FIG. 24 illustrates that TGF $\beta$ 1 administration promotes proliferation of 2G.CAR-PSCA/RIID4 T cells.

#### DETAILED DESCRIPTION

[0049] The words “a” and “an” when used in the present specification in concert with the word comprising, including the claims, denote “one or more.” Some embodiments of the disclosure may consist of or consist essentially of one or more elements, method steps, and/or methods of the disclosure. It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

[0050] Embodiments of the present disclosure encompass cells for adoptive cell transfer that have been modified to switch an inhibitory signal that suppresses immune cell proliferation to a stimulatory signal that enhances immune cell proliferation and, at least in some cases, anti-tumor activity. In particular embodiments, immune cells of the disclosure are equipped with receptors that are designed to bind to inhibitory molecules but transmit a positive signal rather than a suppressive one. That is, the cells transform a “brake” signal (such as TGF $\beta$ ) into an “accelerator” signal to improve the anti-tumor effects of the respective immune cells.

**[0051]** The cells, in certain embodiments, comprise a chimeric cytokine receptor that encompasses a chimera of a TGF $\beta$  exodomain (for example) with an endodomain that is from another molecule such that binding of TGF $\beta$  to the exodomain causes the endodomain to stimulate activity of the T cell. The cells may or may not have other traits, natural and/or genetically engineered by man, including receptors for other molecules, including at least  $\alpha\beta$ TCR, antigen-specific receptors (including tumor-specific receptors), and/or chimeric antigen receptors, for example.

#### **I. [0052] Chimeric Cytokine Receptors**

**[0053]** In embodiments of the disclosure, there are compositions that comprise chimeric cytokine receptors that bind to a Th2 or immunosuppressive cytokine (TGF $\beta$  is an example) but induce immune stimulatory signaling instead of immunosuppression, resulting in the maintenance of a Th1 (effector) phenotype, proliferation and cytotoxic profile. The receptors as polypeptides and nucleotides that encode them are encompassed in the disclosure.

**[0054]** Embodiments of the disclosure include polypeptides (and the polynucleotides that encode them) that comprise an exodomain of a receptor for an immunosuppressive cytokine fused with an endodomain of an immunostimulatory molecule. In specific embodiments, the exodomain of TGF $\beta$ R is fused with one or more stimulatory endodomains including for example TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD28, 4-1BB, OX40, or combinations of any of the foregoing. Cells harboring such chimeric cytokine receptors protect tumor-reactive immune cells from immunosuppressive cytokines such as TGF $\beta$ .

**[0055]** In certain embodiments, provided herein are chimeric TGF $\beta$  receptors that include only the exodomain of TGF $\beta$  receptor or a TGF $\beta$ -binding fragment thereof.

**[0056]** The chimeric TGF $\beta$  receptor, in certain embodiments, comprises the exodomain of the TGF $\beta$  receptor. Any isoform of the natural TGF $\beta$  receptor may be used for its exodomain, including TGF $\beta$  receptor 1, TGF $\beta$  receptor 2, and TGF $\beta$  receptor 3. In particular embodiments, the exodomain for TGF $\beta$  receptor 2 is employed in compositions (including expression constructs and cells, for example) of the disclosure. Exemplary nucleotide sequences encoding TGF $\beta$  receptor 2 include GenBank® Accession Nos. NM\_001024847.2 (SEQ ID

NO:3) (polypeptide is NP\_001020018; SEQ ID NO:4) or NM\_003242.5 (polypeptide is NM\_003242). An example of an endodomain is TLR4, and an exemplary sequence for TLR4 is at GenBank® Accession No. U88880 (SEQ ID NO:5) (polypeptide is AAC34135; SEQ ID NO:6).

**[0057]** Polynucleotides for the receptor may include a nucleotide sequence encoding the signal peptide of TGFβ receptor, in specific embodiments. In a certain embodiment, the signal peptide is as follows (amino acid position 1-22 from TGFβ<sub>2</sub> receptor 2): MGRGLLRGLWPLHIVLWTRIAS (SEQ ID NO:1). In a specific embodiment, the exodomain (which may be referred to as the extracellular domain) includes amino acid position 23-166 from TGFβ receptor 2 as shown in SEQ ID NO:2:  
 TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQE  
 VCVAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCSSD  
 ECNDNIIFSEEYNTSNPDLLLVIQ (SEQ ID NO:2). In other specific embodiments, the exodomain is from TGFβ receptor 1, TGFβ receptor 3, or from any other TGFβ receptor or variant thereof.

**[0058]** The disclosure encompasses polynucleotides and polypeptides for the chimeric TGFβ receptors and cells that harbor them. The disclosure includes use of the chimeric TGFβ receptors in methods of treating cancers that secrete TGFβ or that are in a tumor environment that provides TGFβ. The chimeric TGFβ receptors may be used in conjunction on cells that also include one or more CARs.

**[0059]** In particular embodiments, the chimeric TGFβ receptor of the disclosure comprises an endodomain that is not from the endogenous TGFβ receptor. The endodomain may be of any kind so long as it imparts an activation signal to the immune effector cell in which it resides upon binding of TGFβ to the chimeric TGFβ receptor exodomain, *e.g.*, causes T cells that express the chimeric TGFβ receptor to proliferate in the presence of TGFβ. Exemplary endodomains include, for example, an immune stimulant endodomain or fragment thereof from one or more of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD28, 4-1BB, OX40, CD3zeta, CD40, CD27, or a combination thereof.

**[0060]** In embodiments of the disclosure, the chimeric TGFβ receptor may also include a transmembrane domain between the exodomain and endodomain. The transmembrane

domain may come from any source, although in specific embodiments it is from the same molecule as the endodomain. In particular embodiments, the chimeric receptor comprises the exodomain of the TGF $\beta$  receptor, an endodomain of an immunostimulatory molecule and a transmembrane domain; in particular aspects the transmembrane domain is from the same molecule as the immunostimulatory molecule. In specific embodiments, the chimeric TGF $\beta$  receptor comprises a transmembrane domain and one or more additional amino acids (such as one, two, three, four, five, or more additional amino acids (aa)) from the same molecule as the transmembrane domain (TM). An exemplary pattern for the chimeric TGF $\beta$  receptor is as follows:

**[0061]** TGF $\beta$ R exodomain—1,2, 3, or more aa from TM—TM—non-TGF $\beta$ R endodomain

**[0062]** In certain embodiments, the exodomain may not include the entire exodomain but instead comprise an epitope and, in some cases, a spacer connected to the endodomain.

## **II. [0063] Host Cells Comprising Chimeric Cytokine Receptors**

**[0064]** In embodiments of the disclosure, cells are employed for therapy. The cells encompassed in the disclosure may be immune cells, such as immune effector cells that encompass a T cell, including a cytotoxic T cell (also known as TC, Cytotoxic T Lymphocyte, CTL, T-Killer cell, cytolytic T cell, CD8+ T-cells or killer T cell); NK cells; NKT cells; and other immune cells that can elicit an effector function. In specific embodiments, cells that incorporate the receptors of the disclosure exhibit a bystander effect at the tumor microenvironment at least by depleting inhibitory cytokines from the tumor microenvironment, for example.

**[0065]** As used herein, the terms “cell,” “cell line,” and “cell culture” may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, “host cell” refers to a eukaryotic cell that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be “transfected” or “transformed,” which refers to a process by which

exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous nucleic acid sequence, such as, for example, a vector, has been introduced. Therefore, recombinant cells are distinguishable from naturally occurring cells which do not contain an introduced recombinant nucleic acid.

**[0066]** In certain embodiments, it is contemplated that RNAs or proteinaceous sequences may be co-expressed with other selected RNAs or proteinaceous sequences in the same cell, such as the same CTL. Co-expression may be achieved by co transfecting or co-transducing the CTL with two or more distinct recombinant vectors. Alternatively, a single recombinant vector may be constructed to include multiple distinct coding regions for RNAs, which could then be expressed in CTLs transfected or transduced with the single vector.

**[0067]** Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

**[0068]** The cells can be autologous cells, syngeneic cells, allogenic cells and even in some cases, xenogeneic cells, in relation to the individual that receives them.

**[0069]** In many situations one may wish to be able to kill the modified cells, where one wishes to terminate the treatment, the cells become neoplastic, in research where the absence of the cells after their presence is of interest, or other event. For this purpose one can provide for the expression of certain gene products in which one can kill the modified cells under controlled conditions, such as inducible suicide genes.

#### **A. Additional Cellular Characteristics**

**[0070]** In addition to the cell comprising a chimeric cytokine receptor, the cell may have one or more other characteristics that are useful for cellular immunotherapy. Such additional characteristics may be inherent to the cell or may be a part of the cell following genetic manipulation by man. There may be more than one characteristic in addition to the

chimeric TGF $\beta$  receptor. In specific embodiments, in addition to the cell having a chimeric TGF $\beta$  receptor, the cell may have a chimeric antigen receptor (CAR), an  $\alpha\beta$  T cell receptor, and/or an antigen-specific receptor, such as a tumor-specific receptor.

**[0071]** In one embodiment, the host cell is a T-cell comprising one or more chimeric TGF $\beta$  receptors and comprising one or more of an engineered  $\alpha\beta$ TCR receptor, a native receptor specific for a tumor antigen, or a CAR; in each case, the additional modification may target a tumor antigen of choice.

**[0072]** Naturally occurring T-cell receptors comprise two subunits, an  $\alpha$ -subunit and a  $\beta$ -subunit, each of which is a unique protein produced by recombination event in each T-cell's genome, and libraries of TCRs may be screened for their selectivity to particular target antigens, including tumor antigens. An "engineered TCR" refers to a natural TCR, which has a high-avidity and reactivity toward target antigens that is selected, cloned, and/or subsequently introduced into a population of T-cells used for adoptive immunotherapy. In contrast to engineered TCRs, CARs are engineered to bind target antigens in an MHC- independent manner. In particular embodiments, a CAR comprises an extracellular binding domain including, but not limited to, an antibody or antigen binding fragment thereof; a transmembrane domain; one or more intracellular costimulatory signaling domains and a primary signaling domain.

**[0073]** In embodiments wherein T cell receptors are generated in cells that express or will express a chimeric cytokine receptor, in specific embodiments the methods use exposure of peripheral mononuclear blood cells with libraries of mixture of peptide from a tumor antigen (see PCT/US2013/025342, which is incorporated by reference herein in its entirety).

**[0074]** In certain embodiments of the disclosure, there are immune effector cells that include the chimeric TGF $\beta$  receptor and also that are modified to comprise at least one CAR that allows bypass of tumor immune escape mechanisms that are due to abnormalities in protein-antigen processing and presentation.

**[0075]** In particular cases, the cells include a CAR that is chimeric, non-natural and engineered at least in part by the hand of man. In particular cases, the engineered CAR has one, two, three, four, or more components, and in some embodiments the one or more components facilitate targeting or binding of the cell (such as a T lymphocyte) to the tumor antigen-



comprising cancer cell. In specific embodiments, the CAR comprises a part of an antibody for the tumor antigen, part or all of a cytoplasmic signaling domain, and/or part or all of one or more co-stimulatory molecules, for example endodomains of co-stimulatory molecules. In specific embodiments, the antibody is a single-chain variable fragment (scFv). In certain aspects the antibody is directed at target antigens on the cell surface of cancer cells that secrete TGF $\beta$ , for example. In certain embodiments, a cytoplasmic signaling domain, such as those derived from the T cell receptor zeta-chain, is employed as at least part of the chimeric receptor in order to produce stimulatory signals for T lymphocyte proliferation and effector function following engagement of the chimeric receptor with the target antigen. Examples include, but are not limited to, endodomains from co-stimulatory molecules such as CD27, CD28, 4-1BB, and OX40. In particular embodiments, co-stimulatory molecules are employed to enhance the activation, proliferation, and cytotoxicity of T cells produced by the CAR after antigen engagement. In specific embodiments, the co-stimulatory molecules are CD28, OX40, and 4-1BB.

**[0076]** The CAR may be first generation, second generation, or third generation (CAR in which signaling is provided by CD3 $\zeta$  together with co-stimulation provided by CD28 and a tumor necrosis factor receptor (TNFr), such as 4-1BB or OX40), for example. The CAR may be specific for PSCA, HER2, CD19, CD20, CD22, Kappa or light chain, CD30, CD33, CD123, CD38, ROR1, ErbB2, ErbB3/4, EGFR, EGFRvIII, EphA2, FAP, carcinoembryonic antigen, EGP2, EGP40, mesothelin, TAG72, PSMA, NKG2D ligands, B7-H6, IL-13 receptor  $\alpha$ 2, IL-11 receptor  $\alpha$ , MUC1, MUC16, CA9, GD2, GD3, HMW-MAA, CD171, Lewis Y, G250/CAIX, HLA-AI MAGE A1, HLA-A2 NY-ESO-1, PSC1, folate receptor-  $\alpha$ , CD44v7/8, 8H9, NCAM, VEGF receptors, 5T4, Fetal AchR, NKG2D ligands, CD44v6, TEM1, TEM8, viral-associated antigens expressed by the tumor, or other tumor-associated antigens that are identified through genomic analysis and or differential expression studies of tumors. A single cell may have multiple CARs, including CARs that target different tumor antigens.

**[0077]** In particular embodiments the CAR is encoded on an expression vector that may or may not also encode the chimeric TGF $\beta$  receptor. The vector may be bicistronic, in particular embodiments. When present on the same expression construct, the CAR coding sequence may be configured 5' or 3' to the chimeric TGF $\beta$  receptor coding sequence. The

expression of the CAR and the chimeric TGF $\beta$  receptor may be under the direction of the same or different regulatory sequences.

## **B. Introduction of Constructs into Host Cells**

**[0078]** Expression vectors that encode the chimeric cytokine receptor and optionally comprise at least one CAR,  $\alpha\beta$ TCR, and/or antigen-specific receptor can be introduced as one or more DNA molecules or constructs, where there may be at least one marker that will allow for selection of host cells that contain the construct(s). The constructs can be prepared in conventional ways, where the genes and regulatory regions may be isolated, as appropriate, ligated, cloned in an appropriate cloning host, analyzed by restriction or sequencing, or other convenient means. Particularly, using PCR, individual fragments including all or portions of a functional unit may be isolated, where one or more mutations may be introduced using "primer repair", ligation, *in vitro* mutagenesis, *etc.*, as appropriate. The construct(s) once completed and demonstrated to have the appropriate sequences may then be introduced into the CTL by any convenient means. The construct(s) may be integrated and packaged into non-replicating, defective viral genomes like Adenovirus, Adeno-associated virus (AAV), or Herpes simplex virus (HSV) or others, including retroviral vectors, for infection or transduction into cells. The constructs may include viral sequences for transfection, if desired. Alternatively, the construct may be introduced by fusion, electroporation, biolistics, transfection, lipofection, or the like. The host cells may be grown and expanded in culture before introduction of the construct(s), followed by the appropriate treatment for introduction of the construct(s) and integration of the construct(s). The cells are then expanded and screened by virtue of a marker present in the construct. Various markers that may be used successfully include hprt, neomycin resistance, thymidine kinase, hygromycin resistance, *etc.*

**[0079]** In some instances, one may have a target site for homologous recombination, where it is desired that a construct be integrated at a particular locus. For example,) can knock-out an endogenous gene and replace it (at the same locus or elsewhere) with the gene encoded for by the construct using materials and methods as are known in the art for homologous recombination. For homologous recombination, one may use either .OMEGA. or O-vectors. See, for example, Thomas and Capecchi, *Cell* (1987) 51, 503-512; Mansour, et al., *Nature* (1988) 336, 348-352; and Joyner, et al., *Nature* (1989) 338, 153-156.

[0080] The constructs may be introduced as a single DNA molecule encoding at least the chimeric TGF $\beta$  receptor and optional CAR(s), or different DNA molecules having one or more genes. The constructs may be introduced simultaneously or consecutively, each with the same or different markers.

[0081] Vectors containing useful elements such as bacterial or yeast origins of replication, selectable and/or amplifiable markers, promoter/enhancer elements for expression in prokaryotes or eukaryotes, etc. that may be used to prepare stocks of construct DNAs and for carrying out transfections are well known in the art, and many are commercially available.

### III. [0082] Polynucleotides Encoding Chimeric Cytokine Receptors

[0083] The present disclosure also encompasses a composition comprising a nucleic acid sequence encoding a chimeric cytokine receptor as defined above and cells harboring the nucleic acid sequence. The nucleic acid molecule is a recombinant nucleic acid molecule, in particular aspects, and it may be synthetic. It may comprise DNA, RNA as well as PNA (peptide nucleic acid) and it may be a hybrid thereof.

[0084] It is evident to the person skilled in the art that one or more regulatory sequences may be added to the nucleic acid molecule comprised in the composition of the disclosure. For example, promoters, transcriptional enhancers and/or sequences that allow for induced expression of the polynucleotide of the disclosure may be employed. A suitable inducible system is for example tetracycline-regulated gene expression as described, *e.g.*, by Gossen and Bujard (Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551) and Gossen et al. (Trends Biotech. 12 (1994), 58-62), or a dexamethasone-inducible gene expression system as described, *e.g.* by Crook (1989) EMBO J. 8, 513-519.

[0085] In certain embodiments, the chimeric TGF $\beta$  receptor is expressed constitutively by the cell or vector. In other embodiments, expression of the chimeric TGF $\beta$  receptor is under the control of an inducible promoter, *e.g.*, a promoter that is inducible by TGF $\beta$  or by another molecule present in the tumor microenvironment.

[0086] Furthermore, it is envisaged for further purposes that nucleic acid molecules may contain, for example, thioester bonds and/or nucleotide analogues. The modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in

the cell. The nucleic acid molecules may be transcribed by an appropriate vector comprising a chimeric gene that allows for the transcription of said nucleic acid molecule in the cell. In this respect, it is also to be understood that such polynucleotides can be used for "gene targeting" or "gene therapeutic" approaches. In another embodiment the nucleic acid molecules are labeled. Methods for the detection of nucleic acids are well known in the art, *e.g.*, Southern and Northern blotting, PCR or primer extension. This embodiment may be useful for screening methods for verifying successful introduction of the nucleic acid molecules described above during gene therapy approaches.

**[0087]** The nucleic acid molecule(s) may be a recombinantly produced chimeric nucleic acid molecule comprising any of the aforementioned nucleic acid molecules either alone or in combination. In specific aspects, the nucleic acid molecule is part of a vector.

**[0088]** The present disclosure therefore also relates to a composition comprising a vector comprising the nucleic acid molecule described in the present disclosure.

**[0089]** Many suitable vectors are known to those skilled in molecular biology, the choice of which would depend on the function desired and include plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering. Methods that are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook *et al.* (1989) and Ausubel, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Alternatively, the polynucleotides and vectors of the disclosure can be reconstituted into liposomes for delivery to target cells. A cloning vector may be used to isolate individual sequences of DNA. Relevant sequences can be transferred into expression vectors where expression of a particular polypeptide is required. Typical cloning vectors include pBluescript SK, pGEM, pUC9, pBR322 and pGBT9. Typical expression vectors include pTRE, pCAL-n-EK, pESP-1, pOP13CAT.

**[0090]** In specific embodiments, there is a vector that comprises a nucleic acid sequence that is a regulatory sequence operably linked to the nucleic acid sequence encoding a chimeric cytokine receptor construct defined herein. Such regulatory sequences (control elements) are known to the artisan and may include a promoter, a splice cassette, translation initiation codon, translation and insertion site for introducing an insert into the vector. In

specific embodiments, the nucleic acid molecule is operatively linked to said expression control sequences allowing expression in eukaryotic or prokaryotic cells.

**[0091]** It is envisaged that a vector is an expression vector comprising the nucleic acid molecule encoding a chimeric cytokine receptor construct defined herein. In specific aspects, the vector is a viral vector, such as a lentiviral vector. Lentiviral vectors are commercially available, including from Clontech (Mountain View, CA) or GeneCopoeia (Rockville, MD), for example.

**[0092]** The term "regulatory sequence" refers to DNA sequences that are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoters, ribosomal binding sites, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

**[0093]** The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is preferably used.

**[0094]** Thus, the recited vector is an expression vector, in certain embodiments. An "expression vector" is a construct that can be used to transform a selected host and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotes and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Possible regulatory elements permitting expression in prokaryotic host cells comprise,

*e.g.*, the P<sub>L</sub>, lac, trp or tac promoter in *E. coli*, and examples of regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells.

**[0095]** Beside elements that are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the recited nucleic acid sequence and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product; see *supra*. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pEF-Neo, pCDM8, pRc/CMV, pcDNA1, pcDNA3 (Invitrogen), pEF-DHFR and pEF-ADA, (Raum et al. Cancer Immunol Immunother (2001) 50(3), 141-150) or pSPORT1 (GIBCO BRL).

**[0096]** In some embodiments, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and as desired, the collection and purification of the polypeptide of the disclosure may follow.

**[0097]** Additional regulatory elements may include transcriptional as well as translational enhancers. Advantageously, the above-described vectors of the disclosure comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed cells are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life-Sci. Adv.) 13 (1994), 143-149); npt, which confers

resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histidinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from *Aspergillus terreus* which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

**[0098]** Useful scorable markers are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or  $\beta$ -glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a recited vector.

**[0099]** As described above, the recited nucleic acid molecule can be used in a cell, alone, or as part of a vector to express the encoded polypeptide in cells. The nucleic acid molecules or vectors containing the DNA sequence(s) encoding any one of the above described chimeric cytokine receptor constructs is introduced into the cells that in turn produce the polypeptide of interest. The recited nucleic acid molecules and vectors may be designed for direct introduction or for introduction *via* liposomes, or viral vectors (*e.g.*, adenoviral, retroviral) into a cell. In certain embodiments, the cells are T-cells, CAR T-cells, NK cells, NKT-cells, MSCs, neuronal stem cells, or hematopoietic stem cells, for example.

**[0100]** In accordance with the above, the present disclosure relates to methods to derive vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a nucleic acid molecule encoding the polypeptide sequence of a chimeric cytokine receptor construct defined herein. In particular embodiments, the vector is an expression vector and/or a gene transfer or targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine

papilloma virus, may be used for delivery of the recited polynucleotides or vector into targeted cell populations. Methods which are well known to those skilled in the art can be used to construct recombinant vectors; see, for example, the techniques described in Sambrook *et al.* (loc cit.), Ausubel (1989, loc cit.) or other standard text books. Alternatively, the recited nucleic acid molecules and vectors can be reconstituted into liposomes for delivery to target cells. The vectors containing the nucleic acid molecules of the disclosure can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts; see Sambrook, *supra*.

#### **IV. [0101] Pharmaceutical Compositions**

**[0102]** In accordance with this disclosure, the term "pharmaceutical composition" relates to a composition for administration to an individual and encompasses compositions of cells for immunotherapy. In specific embodiments, the cells for immunotherapy are engineered to express at least a chimeric cytokine receptor. In certain embodiments, the cells comprise one or more additional modifications, such as one or more receptors, including receptors for tumor antigens.

**[0103]** In a particular embodiment, the pharmaceutical composition comprises a composition for parenteral, transdermal, intraluminal, intra-arterial, intrathecal or intravenous administration or for direct injection into a cancer. It is in particular envisaged that said pharmaceutical composition is administered to the individual *via* infusion or injection. Administration of the suitable compositions may be effected by different ways, *e.g.*, by intravenous, subcutaneous, intraperitoneal, intramuscular, topical or intradermal administration.

**[0104]** The pharmaceutical composition of the present disclosure may further comprise a pharmaceutically acceptable carrier. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions, *etc.* Compositions comprising such carriers can be formulated by well-known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose.



[0105] The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A particular dosage for administration might be in the range of between  $5 \times 10^6$  per m<sup>2</sup> and  $3 \times 10^8$  per m<sup>2</sup>.

[0106] The compositions of the disclosure may be administered locally or systemically. The compositions provided herein, e.g., cells expressing the constructs provided herein, may, in certain embodiments, be administered parenterally, e.g., intravenous, intraarterial, intrathecal, subdermal or intramuscular administration. In certain other embodiments, DNA encoding the constructs provided herein may be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. In a preferred embodiment, the pharmaceutical composition is administered subcutaneously and in an even more preferred embodiment intravenously. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishes, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. In addition, the pharmaceutical composition of the present disclosure might comprise proteinaceous carriers, like, e.g., serum albumin or immunoglobulin, preferably of human origin. It is envisaged that the pharmaceutical composition of the disclosure might comprise, in addition to the proteinaceous chimeric cytokine receptor constructs or nucleic acid molecules or vectors encoding the same (as described in this disclosure), further biologically active agents, depending on the intended use of the pharmaceutical composition. In specific embodiments, the cells are infused intravenously in 10% DMSO, 40% saline and 50% human serum albumin.

**V. [0107] Therapeutic Uses of Chimeric Cytokine Receptors and Host T-cells  
Comprising Chimeric Cytokine Receptors**

**[0108]** In various embodiments chimeric cytokine receptor constructs, nucleic acid sequences, vectors, and/or host cells, as contemplated herein and/or pharmaceutical compositions comprising the same are used for the prevention, treatment or amelioration of a cancerous disease, such as a tumorous disease. In particular embodiments, the pharmaceutical composition of the present disclosure may be particularly useful in preventing, ameliorating and/or treating cancer, including solid tumors, for example. In certain cases, the cancer has a tumor antigen.

**[0109]** As used herein “treatment” or “treating,” includes any beneficial or desirable effect on the symptoms or pathology of a disease or pathological condition, and may include even minimal reductions in one or more measurable markers of the disease or condition being treated, *e.g.*, cancer. Treatment can involve optionally either the reduction or amelioration of symptoms of the disease or condition, or the delaying of the progression of the disease or condition. “Treatment” does not necessarily indicate complete eradication or cure of the disease or condition, or associated symptoms thereof.

**[0110]** As used herein, “prevent,” and similar words such as “prevented,” “preventing” *etc.*, indicate an approach for preventing, inhibiting, or reducing the likelihood of the occurrence or recurrence of, a disease or condition, *e.g.*, cancer. It also refers to delaying the onset or recurrence of a disease or condition or delaying the occurrence or recurrence of the symptoms of a disease or condition. As used herein, “prevention” and similar words also includes reducing the intensity, effect, symptoms and/or burden of a disease or condition prior to onset or recurrence of the disease or condition.

**[0111]** In particular embodiments, the present invention contemplates, in part, cells, chimeric cytokine receptor construct, nucleic acid molecules and vectors that can administered either alone or in any combination using standard vectors and/or gene delivery systems, and in at least some aspects, together with a pharmaceutically acceptable carrier or excipient. In certain embodiments, subsequent to administration, said nucleic acid molecules or vectors may be stably integrated into the genome of the subject.

**[0112]** In specific embodiments, viral vectors may be used that are specific for certain cells or tissues and persist in said cells. Suitable pharmaceutical carriers and excipients

are well known in the art. The compositions prepared according to the disclosure can be used for the prevention or treatment or delaying the above identified diseases.

**[0113]** Furthermore, the disclosure relates to a method for the prevention, treatment or amelioration of a tumorous disease comprising the step of administering to a subject in need thereof an effective amount of cells harboring the chimeric cytokine receptor molecule, a nucleic acid sequence, a vector, as contemplated herein and/or produced by a process as contemplated herein.

**[0114]** Possible indications for administration of the composition(s) of the exemplary chimeric cytokine receptor-comprising cells are cancerous diseases, including tumorous diseases, including breast, prostate, lung, and colon cancers or epithelial cancers/carcinomas such as breast cancer, colon cancer, prostate cancer, head and neck cancer, skin cancer, cancers of the genito-urinary tract, *e.g.* ovarian cancer, endometrial cancer, cervix cancer and kidney cancer, lung cancer, gastric cancer, cancer of the small intestine, liver cancer, pancreas cancer, gall bladder cancer, cancers of the bile duct, esophagus cancer, cancer of the salivary glands and cancer of the thyroid gland. The administration of the composition(s) of the disclosure is useful for all stages and types of cancer, including for minimal residual disease, early cancer, advanced cancer, and/or metastatic cancer and/or refractory cancer, for example.

**[0115]** The disclosure further encompasses co-administration protocols with other compounds, *e.g.* bispecific antibody constructs, targeted toxins or other compounds, which act *via* immune cells. The clinical regimen for co-administration of the inventive compound(s) may encompass co-administration at the same time, before or after the administration of the other component. Particular combination therapies include the use of a chemotherapeutic agent (*e.g.*, a chemotherapeutic agent listed in Section VII.A. below), radiation, surgery, hormone therapy, or other types of immunotherapy, in combination with the chimeric TGF $\beta$  receptor-expressing cells provided herein.

**[0116]** Embodiments relate to a kit comprising a chimeric cytokine receptor construct as defined above, a nucleic acid sequence as defined above, a vector as defined above and/or a host as defined above. It is also contemplated that the kit of this disclosure comprises a pharmaceutical composition as described herein above, either alone or in combination with

further medicaments to be administered to an individual in need of medical treatment or intervention.

[0117] By way of illustration, cancer patients or patients susceptible to cancer or suspected of having cancer may be treated as follows. Cells modified as described herein may be administered to the individual and retained for extended periods of time. The individual may receive one or more administrations of the cells, and the timing of separations of administrations may be on the order of days, weeks, months, or years. In specific embodiments, multiple administrations occur within weeks or months of each other, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more weeks or months. In some embodiments, the genetically modified cells are encapsulated to inhibit immune recognition and placed at the site of the tumor. In cases where cells are provided to the individual following tumor recurrence after initially treating with cells of the disclosure, the cells may be altered such that they recognize a different target tumor antigen. For example, when initial rounds include cells that harbor the chimeric cytokine receptor and another receptor specific for a particular antigen, upon subsequent rounds (including upon tumor recurrence, if it occurs) may utilize a receptor for a different particular antigen.

[0118] In particular cases the individual is provided with effective amounts of the therapeutic cells that encompass a chimeric TGF $\beta$  receptor and, optionally, 2) a CAR,  $\alpha\beta$  TCR, and/or antigen-specific receptor. The cells may be delivered at the same time or at different times from one or more other cancer therapies. The cells and the other cancer therapy may be delivered in the same or separate formulations. The cells and the other cancer therapy may be provided to the individual in separate delivery routes. The cells and/or the other cancer therapy may be delivered by injection at a tumor site or intravenously or orally, for example. Routine delivery routes for such compositions are known in the art.

[0119] The cells that have been modified with the construct(s) are then grown in culture under selective conditions and cells that are selected as having the construct may then be expanded and further analyzed, using, for example; the polymerase chain reaction for determining the presence of the construct in the host cells. Once the modified host cells have been identified, they may then be used as planned, *e.g.*, expanded in culture or introduced into a host organism.

**[0120]** Depending upon the nature of the cells, the cells may be introduced into a host organism, *e.g.*, a mammal, in a wide variety of ways. The cells may be introduced at the site of the tumor, in specific embodiments, although in alternative embodiments the cells hone to the cancer or are modified to hone to the cancer. The number of cells that are employed will depend upon a number of circumstances, the purpose for the introduction, the lifetime of the cells, the protocol to be used, for example, the number of administrations, the ability of the cells to multiply, the stability of the recombinant construct, and the like. The cells may be applied as a dispersion, generally being injected at or near the site of interest. The cells may be in a physiologically-acceptable medium.

**[0121]** The DNA introduction need not result in integration in every case. In some situations, transient maintenance of the DNA introduced may be sufficient. In this way, one could have a short term effect, where cells could be introduced into the host and then turned on after a predetermined time, for example, after the cells have been able to home to a particular site.

**[0122]** The cells may be administered as desired. In certain embodiments, the regimen parameters may be modulated using various protocols. In specific embodiments, the route or number or timing of administration, the life of the cells, and/or the number of cells present, may be varied. The number of administrations may depend upon the factors described above, for example, at least in part.

**[0123]** It should be appreciated that the system is subject to many variables, such as the cellular response to the ligand, the efficiency of expression and, as appropriate, the level of secretion, the activity of the expression product, the particular need of the individual, which may vary with time and circumstances, the rate of loss of the cellular activity as a result of loss of cells or expression activity of individual cells, and the like. Therefore, it is expected that for each individual patient, even if there were universal cells that could be administered to the population at large, each patient would be monitored for the proper dosage for the individual, and such practices of monitoring a patient are routine in the art.

**[0124]** In specific embodiments, there is a screening assay employed as part of, or not as part of, methods of the disclosure. For example, in certain embodiments, a biopsy is taken and the level of TGF $\beta$  production is assessed so as to determine whether the construct would

work in the individual from which the biopsy is taken. In specific embodiments, the assay method identified cancers that detectably express TGF $\beta$  or a certain level of TGF $\beta$ .

## **VI. [0125] Kits of the Invention**

[0126] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, chimeric cytokine receptor-expressing cells for use in cell therapy and/or the reagents to generate one or more cells for use in cell therapy that harbors recombinant expression vectors may be comprised in a kit. The kit components are provided in suitable container means.

[0127] Some components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there are more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present disclosure also will typically include a means for containing the components in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained.

[0128] When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly useful. In some cases, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit.

[0129] However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

**[0130]** In particular embodiments of the disclosure, cells that are to be used for cell therapy are provided in a kit, and in some cases the cells are essentially the sole component of the kit. The kit may comprise reagents and materials to make the desired cell. In specific embodiments, the reagents and materials include primers for amplifying desired sequences, nucleotides, suitable buffers or buffer reagents, salt, and so forth, and in some cases the reagents include vectors and/or DNA that encodes a chimeric cytokine receptor as described herein and/or regulatory elements therefor.

**[0131]** In particular embodiments, there are one or more apparatuses in the kit suitable for extracting one or more samples from an individual and/or for delivering cells to an individual. The apparatus may be a syringe, scalpel, and so forth.

**[0132]** In some cases of the disclosure, the kit, in addition to cell therapy embodiments, also includes a second cancer therapy, such as chemotherapy, hormone therapy, and/or another immunotherapy, for example. The kit(s) may be tailored to a particular cancer for an individual and comprise respective second cancer therapies for the individual.

## **VII. [0133] Combination Therapy**

**[0134]** In certain embodiments of the disclosure, methods of the present disclosure for clinical aspects are combined with other agents effective in the treatment of hyperproliferative disease, such as anti-cancer agents. An “anti-cancer” agent is capable of negatively affecting cancer in a subject, for example, by killing 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. More generally, these other compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cancer cells with the expression construct and the agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

**[0135]** Tumor cell resistance to chemotherapy and radiotherapy agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with other therapy. In the context of the present disclosure, it is contemplated that the cell therapy could be used similarly in conjunction with chemotherapeutic, radiotherapeutic, and/or immunotherapeutic intervention.

**[0136]** Alternatively, the present inventive cell therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and present disclosure are applied separately to the individual, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and inventive therapy would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several d (2, 3, 4, 5, 6 or 7) to several wk (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

**[0137]** Various combinations may be employed, present disclosure is “A” and the secondary agent, such as radio- or chemotherapy, is “B”:

**[0138]** 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

**[0139]** B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

**[0140]** B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

**[0141]** It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the inventive cell therapy.

#### **A. [0142] Chemotherapy**

**[0143]** Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination anti-cancer agents include, for example, acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; amsacrine; anastrozole; anthramycin; asparaginase; asperlin;



azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; celecoxib (COX-2 inhibitor); chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflomithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; fluorocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; iproplatin; irinotecan; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedopa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin; spirogermanium hydrochloride; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan sodium; taxotere; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; tricirbine phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vaporeotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; zorubicin hydrochloride; 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene;

adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzo chlorins; benzoylstauroporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; broprimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetorelix; chlorlins; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentantraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidenmin B; deslorelin; dexamethasone; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docetaxel; docosanol; dolasetron; doxifluridine; doxorubicin; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorubicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofofosine; ilomastat; imatinib (*e.g.*, GLEEVEC®), imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim;

lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon;  
 leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue;  
 lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin;  
 lombricine; lometrexol; lonidamine; losoxantrone; loxoribine; lurtotecan; lutetium texaphyrin;  
 lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprocol; maspin;  
 matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin;  
 methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim;  
 mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-  
 saporin; mitoxantrone; mofarotene; molgramostim; Erbitux, human chorionic gonadotrophin;  
 monophosphoryl lipid A+myobacterium cell wall sk; mopidamol; mustard anticancer agent;  
 mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted  
 benzamides; nafarelin; nagrestip; naloxone+pentazocine; napavin; naphterpin; nartograstim;  
 nedaplatin; nemorubicin; neridronic acid; nilutamide; nisamycin; nitric oxide modulators;  
 nitroxide antioxidant; nitrullyn; oblimersen (GENASENSE®); O.sup.6-benzylguanine;  
 octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral  
 cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel; paclitaxel  
 analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol;  
 panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium;  
 pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin;  
 phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin;  
 piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum  
 compounds; platinum-triamine complex; porfimer sodium; porfiromycin; prednisone; propyl bis-  
 acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein  
 kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase  
 inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine;  
 pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron;  
 ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine  
 demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rohitukine;  
 romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A;  
 sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides;  
 signal transduction inhibitors; sizofuran; sobuzoxane; sodium borocaptate; sodium  
 phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D;  
 spiromustine; splenopentin; spongistatin 1; squalamine; stipiamide; stromelysin inhibitors;

sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene bichloride; topsentin; toremifene; translation inhibitors; tretinoin; triacetylrudine; tricyribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer, or any analog or derivative variant of the foregoing and also combinations thereof.

[0144] In specific embodiments, chemotherapy for the individual is employed in conjunction with the disclosure, for example before, during and/or after administration of the disclosure.

#### **B. [0145] Radiotherapy**

[0146] Other factors that cause DNA damage and have been used extensively include what are commonly known as  $\gamma$ -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 and UV-irradiation. It is most likely that all of these factors effect 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.

[0147] The terms “contacted” and “exposed,” when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

**C. [0148] Immunotherapy**

[0149] Immunotherapeutics generally rely on the use of immune effector cells and molecules to target and destroy cancer cells, and immunotherapeutics other than the chimeric cytokine-expressing cells of the disclosure may be used, in certain embodiments. 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 effect 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 merely 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.

[0150] Immunotherapy other than the inventive therapy described herein could thus be used as part of a combined therapy, in conjunction with the present cell therapy. The general approach for combined therapy is discussed below. Generally, 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 disclosure. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155.

[0151] In certain other embodiments, the immunotherapy comprises use of an antibody against DLL4, Notch, or a Wnt pathway protein, for example.

**D. [0152] Genes**

[0153] In yet another embodiment, the secondary treatment is a gene therapy in which a therapeutic polynucleotide is administered before, after, or at the same time as the present disclosure clinical embodiments. A variety of expression products are encompassed within the disclosure, including inducers of cellular proliferation, inhibitors of cellular proliferation, or regulators of programmed cell death.

**E. [0154] Surgery**

**[0155]** Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present disclosure, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

**[0156]** Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. 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). It is further contemplated that the present disclosure may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

**[0157]** Upon excision of part of 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.

#### **F. [0158] Other agents**

**[0159]** It is contemplated that other agents may be used in combination with the present disclosure to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, or agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas / Fas ligand, DR4 or DR5 / TRAIL would potentiate the apoptotic inducing abilities of the present disclosure by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increases 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 the present disclosure to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present disclosure. 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 the present disclosure to improve the treatment efficacy.

## EXAMPLES

**[0160]** The following examples are included to demonstrate preferred embodiments of the disclosure. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the disclosure, 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 disclosure.

### EXAMPLE 1

#### EXEMPLARY EMBODIMENTS OF THE DISCLOSURE

**[0161]** FIG. 1 illustrates an exemplary method to arm T cells to overcome the inhibitory tumor microenvironment. The method permits conversion of an inhibitory signal from TGF $\beta$  into a positive signal for proliferation of tumor-specific CTLs (for example). The conversion location occurs at a receptor for TGF $\beta$  wherein the receptor employs the cytokine receptor exodomain with an endodomain that transmits the signal instead as a positive one. FIG. 2 provides exemplary vectors suitable for the disclosure by encoding chimeric TGF $\beta$  receptors, including one that employs a TLR2 endodomain (RIID2) or one that employs a TLR4 endodomain (referred to herein as RIID4). A control is shown that is a dominant negative receptor including the TGF $\beta$  receptor exodomain with no endodomain. Although any type of vector may include the expression construct, retroviral vectors were employed.

**[0162]** FIG. 3 demonstrates generation of the DNRII control and exemplary chimeric TGF $\beta$  receptors of RIID2 and RIID4. The flow cytometry images demonstrate the percentage of cells expressing the particular chimeric receptors (FIG. 3).

[0163] FIG. 4A demonstrates co-expression of the dominant negative control DNR<sup>II</sup> and the exemplary chimeric TGF $\beta$  receptors of RIID2 (labeled with mOrange) and RIID4 (labeled with GFP) with an exemplary second generation chimeric antigen receptor (CAR) specific for PSCA. FIG. 4B further demonstrates that RIID4 can be expressed on second generation CAR-PSCA T cells.

[0164] FIG. 5 shows that RIID2 and RIID4 protect CAR-modified T cells exposed to TGF $\beta$ . T cells that are in a healthy environment and proliferating are visualized under a microscope as clusters of cells (weekly antigen stimulation and administration of 5 ng/ml of TGF $\beta$ 1; no IL2 administration). FIG. 5 shows that although control DNR<sup>II</sup> and RIID2 show some clustering of the cells in the presence of TGF $\beta$ 1, the RIID4 cells are nearly indistinguishable when comparing the absence and presence of TGF $\beta$ 1.

[0165] FIGS. 6A through 6C show that in suppressive conditions RIID2- and RIID4-modified T cells are protected in the presence of TGF $\beta$ . FIG. 6A demonstrates that in control cells harboring only a CAR, T cell proliferation decreases, particularly fast in the presence of TGF $\beta$ 1. In the presence of the dominant negative receptor DNR, a decrease in proliferation occurs at a later time. In FIG. 6B, the presence of the chimeric TGF $\beta$  receptor RIID2 enhances proliferation, even in the presence of TGF $\beta$ . Even after almost 100 days, the cells proliferate in the presence of TGF $\beta$ . FIG. 6C also shows that in the presence of the chimeric TGF $\beta$  receptor RIID4 enhances proliferation, even in the presence of TGF $\beta$ .

[0166] FIG. 7 demonstrates that cells harboring the exemplary chimeric TGF $\beta$  receptors RIID2 and RIID4 are selected for in the presence of TGF $\beta$ 1, and FIG. 8 demonstrates that the cells harboring RIID2 and RIID4 require antigen stimulation for their expansion. Therefore, *in vivo* when the cells are successful in reducing tumor load, their proliferation will decrease, in specific embodiments.

[0167] FIG 9 shows that RIID2 and RIID4 enhance the anti-tumor effect of the exemplary PSCA CAR against the exemplary DU145 prostate cancer cells.



## EXAMPLE 2

IMMUNOSUPPRESSIVE TGF- $\beta$  SIGNAL CONVERTER

[0168] Chimeric antigen receptor (CAR)-transduced T cells are promising tools for the treatment of cancers. To extend this therapeutic modality to prostate cancer, the inventors generated a 2<sup>nd</sup> generation CAR targeting the tumor antigen PSCA (2G.CAR-PSCA), which provides cells with the ability to kill PSCA+ prostate tumor cells (51.7 $\pm$ 1.2% specific lysis of Du145 cells at 20:1 E:T). However, many tumors, including prostate cancer, secrete TGF $\beta$ , which inhibits *in vivo* T cell proliferation, activation and function. The present disclosure addresses this need in the art to overcome this *in vivo* limitation of T cells.

[0169] It has previously been demonstrated that adoptively-transferred T cells can be protected from the inhibitory effects of TGF $\beta$  through the transgenic expression of a truncated, dominant-negative receptor (DNRII), which blocks transmission of TGF $\beta$  signal. The inventors herein have extended this strategy by converting the inhibitory signal from TGF $\beta$  into an activation stimulus for T cells. The inventors generated a chimeric cytokine receptor expressing the exodomain of TGF $\beta$ RII linked to the endodomain of the exemplary toll-like receptor (TLR) 4 and GFP (RIID4).

[0170] The inventors generated a chimeric cytokine receptor expressing the exodomain of TGF $\beta$ RII linked to the endodomain of the exemplary toll-like receptor (TLR) 4 and GFP (RIID4). The inventors transduced primary T cells with RIID4 and obtained 69.3 $\pm$ 6.0% transduction that was stable for >60 days of culture.

[0171] To address whether transgenic expression of RIID4 protected against TGF $\beta$ , the inventors modified 2G.CAR-PSCA T cells to co-express either the dominant negative DNRII or RIID4 receptors. These T cells were then stimulated weekly with PSCA+ tumor cells (K562-PSCA) with or without exogenous TGF $\beta$ 1 (5ng/mL). In the absence of TGF $\beta$ 1, 2G.CAR-PSCA, 2G.CAR-PSCA(DNRII) or 2G.CAR-PSCA(RIID4) T cells proliferated at similar levels for 30 days (6.4 $\times$ 10<sup>2</sup>, 2.5 $\times$ 10<sup>3</sup>, 5.9 $\times$ 10<sup>3</sup> fold, respectively). But, in the presence of TGF $\beta$ 1, 2G.CAR-PSCA T cells did not expand, and cultures failed within 2 weeks. In contrast, transgenic expression of DNRII or RIID4 protected the cells from the inhibitory impact of this cytokine (7.4 and 21 fold at 2 weeks of culture, respectively). To determine whether there were long term differences between DNRII- and RIID4-modified cells, the inventors monitored cell expansion

and found that only RIID4-modified T cells were able to expand for >60 days in the presence of TGF $\beta$ 1 ( $3.0 \times 10^5$  fold) while DNR II cells began to contract after 30 days in culture (0.72 fold). Administration of TGF $\beta$ 1 also selected 2G.CAR-PSCA(RIID4) T cells, leading to an enrichment in this transgenic cell population over time (from 63.6% to 93.3%). This modification is safe, because the administration of TGF $\beta$ 1 alone was insufficient to drive transgenic T cell proliferation (0.04 fold), and the withdrawal of antigenic stimulation resulted in T cell contraction (0.02 fold). Finally, to address whether this modification could improve the anti-tumor activity of CAR-T cells, the inventors co-cultured  $1 \times 10^6$  firefly-luciferase-Du145 cells, which express PSCA and produce TGF $\beta$ 1, with  $1 \times 10^5$  2G.CAR-PSCA, 2G.CAR-PSCA(DNR II) or 2G.CAR-PSCA(RIID4) T cells. After 6 days there was superior control of tumor growth by RIID4-expressing T cells compared with DNR II or CAR alone conditions (Total Flux;  $9 \pm 0.1 \times 10^9$ ,  $10 \pm 1 \times 10^9$ ,  $20 \pm 1 \times 10^9$  p/s, respectively). Therefore, RIID4 not only protects cells from the inhibitory effects TGF $\beta$  but converts this cytokine signal into one that is stimulant.

### EXAMPLE 3

#### CHIMERIC CYTOKINE RECEPTORS IN THE TUMOR MICROENVIRONMENT

**[0172]** TGF $\beta$  has been detected at high levels in patients with cancer, including at least prostate cancer, where it acts by suppressing effector T cell function while promoting Treg development. Under wild type conditions, TGF $\beta$  engagement results in phosphorylation of Smad2/3, which triggers multiply inhibitory pathways (FIG. 10A). To evaluate the influence of TGF $\beta$  on tumor-specific T cells, CAR-PSCA T cells were cultured with TGF $\beta$  (5ng/ml) and administered twice weekly. As expected, this resulted in phosphorylation of Smad2/3 and decreased cytolytic function in CAR-PSCA T cells (FIG. 10A and 10B). In addition, TGF $\beta$  exposure resulted in a decrease in the expansion of CAR T cells, in part due to higher cell death (FIG. 10C and 10D). Interestingly, exposure of CAR-PSCA T cells to TGF $\beta$  also resulted in the upregulation of PD1, suggesting T cell exhaustion (FIG. 10E). Interestingly, TGF $\beta$ 1 does not induce pSmad2/3 in RIID4-modified T cells (FIG. 14).

**[0173]** Arming CAR-PSCA T cells against TGF $\beta$ : To determine whether Chimeric Cytokine Receptors could be utilized to protect tumor-specific T cells from the immunosuppressive effects of TGF $\beta$ , the TGF $\beta$  endodomain was substituted for that of an immunostimulatory molecule. One example of an endodomain derives from the Toll-like

Receptor (TLR) family, which constitutes an important component of the innate immune response. In particular, TLR4 signaling in T cells has been demonstrated to improve T cell activation while decreasing the need for co-stimulation. Therefore, with the purpose of transforming the inhibitory signal of TGF $\beta$  into an immunostimulatory one, the TGF $\beta$ R endodomain was substituted for the TLR4 endodomain (referred to herein as “RIID4”) (FIGS. 11B and 12A).

**[0174]** RIID4 expression protects CAR T cells from TGF $\beta$ . To evaluate whether a Chimeric Cytokine Receptor could protect CAR T cells from TGF $\beta$ , a retroviral vector was generated expressing the extracellular component of TGF $\beta$ R and the intracellular signaling domain of TLR4 (RIID4). This sequence was then linked with GFP using an IRES, allowing detection of the transgenic T cell population (FIG. 12A). Activated T cells were then transduced with the retroviral vector encoding for RIID4/GFP, as shown in FIG. 12B. RIID4 expression on T cells was stable as illustrated by a direct correlation of receptor expression with GFP. To evaluate the protective properties of the construct, there was co-expression of RIID4 on CAR-PSCA T cells and culturing of them in the presence of TGF $\beta$ . Importantly, only the CAR T cells expressing RIID4 were able to expand when co-cultured with TGF $\beta$ , as shown by the culture photographs in FIG. 12C. As expected, administering TGF $\beta$  to CAR T cells induced upregulation of PD1 expression. In contrast, T cells modified with the RIID4 construct did not express PD1, indicating the lack of exhaustion in these T cells (FIG. 12D).

**[0175]** Embodiments of the disclosure extend the target range of chimeric cytokine receptors. Specific embodiments include an array of different permutations of chimeric cytokine receptors expressing the exodomain of TGF $\beta$  receptor with the endodomain of different co-stimulatory molecules as shown by example in FIG. 13. Next, one can identify which of these constructs is the most efficient in enhancing T cell function in the presence of TGF $\beta$  and determine whether the incorporation of such a modification could provide a positive bystander effect by decreasing the function of suppressive TGF $\beta$ -producing Tregs.

**[0176]** Vector Generation and assessment: A variety of different TGF $\beta$ /immunostimulatory endodomain constructs (TGF $\beta$ R/Th1) may be generated by standard recombinant means in the art. An example of a retroviral vector expressing the exodomain of TGF $\beta$  receptor and the endodomain of TLR4, RIID4, is shown in FIG. 12A. Using this vector as an example of a template, one can subclone additional configurations by substituting the

transmembrane domain and endodomain of TLR4 with OX40, CD28, 41BB, and CD28/41BB, for example. In specific embodiments the vectors may also co-express a marker, such as GFP. These different vectors may then be compared for expression and function in T cells.

**[0177]** Construct Assessment: To characterize the different constructs for suitability to reverse the inhibitory signaling of TGF $\beta$ , one can generate viral supernatant and transduce T cells. Protein expression can be assessed by flow cytometric analysis by correlating TGF $\beta$  receptor with GFP expression as shown in FIG. 12B. Transgenic function can be assessed by comparing T cell (i) expansion, (ii) cytokine production, (iii) phenotypic profile including expression of effector, memory and exhaustion markers and (iv) cytolytic function in the presence or absence of TGF $\beta$ , for example.

**[0178]** In specific embodiments, the different TGF $\beta$  cytokine receptor constructs can achieve a transduction efficiency of >60%. In specific embodiments, the constructs are stably expressed on the surface of T cells as detected by flow cytometry analysis. In general embodiments, permutations of the TGF $\beta$ R/Th1 constructs exhibit superior T cell expansion, cytokine production, phenotypic profile and cytolytic function in the presence of TGF $\beta$ . However because of the difference in the co-stimulatory endodomains in certain embodiments the T cells modified with the CD28/41BB construct have a greater potential for expansion than cells expressing receptors containing a single endodomain. In certain aspects, this enhanced proliferative capacity leads to over-activation and cell exhaustion as measured by upregulation of PD1, TIM3 and LAG3, for example.

**[0179]** The in-vitro and in-vivo function of TGF $\beta$ R/Th1 in dual-specific CTLs may be evaluated. These studies can show whether TGF $\beta$ R/Th1 can be expressed by dual-specific CTLs, and whether transgenic CTLs maintain their proliferative capacity and anti-tumor activity even in the presence of TGF $\beta$ . One or more of the TGF $\beta$ R/Th1 constructs may be included in a bicistronic retroviral vector. In a specific example, the bicistronic retroviral vector encodes CAR-PSCA and TGF $\beta$ R/Th1.

**[0180]** One can confirm that CTLs maintain their function and anti-tumor activity in the presence of TGF $\beta$  by using IFN $\gamma$  ELISpot with PSMA and PSCA pepmixes as stimulators, and by measuring killing using TRPC tumor as a target in the presence of TGF $\beta$  in short (4hr

Cr<sup>51</sup> assay), long-term (4 day co-culture) cytotoxicity assays and in TRPC tumor-bearing mice, for example.

**[0181]** In particular embodiments, dual-specific CTLs expressing the TGFβR/Th1 retain the antigen specificity against their respective targets (such as PSCA and PSMA). However in specific embodiments, only TGFβR/Th1 expressing T cells are functional in the presence of TGFβ resulting in a more potent in-vitro and in-vivo anti-tumor effect (Table 1).

**[0182] Table 1: Expected Assessments of Dual-CTLs expressing Chimeric Cytokine Receptors**

	In-vitro assessment				In-vivo assessment	
	Cell Signaling		CTL expansion		Tumor Growth	
	+IL2	+TGFβ	+IL2	+TGFβ	+IL2	+TGFβ
Dual-CTL	Th1	Th2	+++	-	(-/+)	(++++)
Dual-CTL-TGFβR/Th1	Th1	Th1	+++	+++	(-/+)	(-)

**[0183]** Assessing the bystander effect of TGFβR/Th1 One can evaluate the ability of TGFβR/Th1 to provide a positive bystander effect on the recipient immune system by depleting inhibitory cytokines from the tumor microenvironment. To assess this one can recapitulate the immunosuppressive tumor environment in an animal model by performing an autologous transfer of TGFβ-producing Tregs, which will deliver TGFβ at the tumor site. Experimental groups may be subdivided into two: (i) dual-CTLs-TGFβR/Th1 or (ii) dual-CTLs as a control. Two weeks after T cell treatment a cohort of mice can be sacrificed to evaluate intra-tumor levels of TGFβ as well as the number and function of the Tregs.

**[0184]** In specific embodiments, there is only a marginal anti-tumor response from Dual-CTLs (alone) in presence of Tregs (Table 2). In particular embodiments, and in contrast, treatment with dual-CTLs expressing the TGFβR/Th1 depletes the levels of TGFβ from the

tumor microenvironment affecting the function and persistence of Tregs (Table 2), resulting in an overall enhanced anti-tumor response.

**[0185] Table 2: Example of Outcome with Dual-CTLs comprising a Chimeric Cytokine Receptor**

	Tumor levels of TGF $\beta$	Treg Function	Anti-tumor effect
Dual-CTLs	(++++)	(++++)	(+)
Dual-CTLs-TGF $\beta$ R/Th1	(-/ +)	(+)	(++++)

**[0186]** T cells harboring RIID4 are protected from TGF $\beta$ 1-induced apoptosis (FIG. 15); furthermore, RIID4 prevents PD1 upregulation (FIG. 16) and TGF $\beta$ 1-induced inhibition of cytolytic function (FIG. 17).

**[0187]** FIG. 18 shows that RIID4+ cells are selected by TGF $\beta$ 1 exposure, whereas withdrawal of antigen and TGF $\beta$ 1 leads to culture failure (FIG. 19).

**[0188]** FIG. 20 illustrates an example of a co-culture experimental set-up.

**[0189]** FIG. 21 demonstrates that 2G.CAR-PSCA/RIID4 cells eliminated tumors in the presence of TGF $\beta$ 1.

**[0190]** FIG. 22 shows that 2G.CAR-PSCA T cells did not proliferate in the presence of TGF $\beta$ 1.

**[0191]** FIG. 23 demonstrates that 2G.CAR-PSCA/RIID4 cells were able to expand dependent upon antigen existence. FIG. 24 illustrates TGF $\beta$ 1 administration promotes proliferation of 2G.CAR-PSCA/RIID4 T cells. Therefore, RIID4 modified T cells can use TGF $\beta$ 1 for their proliferation.

[0192] In specific embodiments, the TGF $\beta$ R/Th1 chimeric cytokine receptor may be used with another chimeric cytokine receptor. In specific embodiments, the TGF $\beta$ R/Th1 chimeric cytokine receptor is used in the same cell as a IL4R $\alpha$ /IL7R (4/7R) chimeric cytokine receptor. In specific embodiment, the TGF $\beta$ R/Th1 chimeric cytokine receptor and the additional chimeric cytokine receptor results in cooperative signaling. Therefore in specific embodiments, there are provided dual-CTLs expressing the (i) 4/7R (ii) the TGF $\beta$ R/Th1 or (iii) 4/7R and TGF $\beta$ R/Th1. In certain embodiments, the use of both 4/7R and TGF $\beta$ R/Th1 chimeric cytokine receptors results in a synergistic response.

[0193] Although the present disclosure and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the disclosure as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present disclosure, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present disclosure. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

## CLAIMS

What is claimed is:

1. A polynucleotide comprising a nucleotide sequence encoding a chimeric TGF $\beta$  receptor, wherein the receptor comprises an exodomain of TGF $\beta$  receptor and a non-TGF $\beta$  receptor endodomain.
2. The polynucleotide of claim 1, wherein the endodomain comprises an endodomain of an immune stimulant protein, or functional fragment thereof, wherein the endodomain or fragment thereof is from TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD28, 41BB, 41BB, OX40, CD3zeta, CD40, CD27, or a combination thereof.
3. The polynucleotide of claim 1, wherein the endodomain is from toll-like receptor (TLR) 4.
4. The polynucleotide of claim 1, further comprising a polynucleotide sequence that encodes a chimeric antigen receptor (CAR) or an  $\alpha\beta$  T cell receptor (TCR) that is native to the T cell or is an engineered TCR.
5. The polynucleotide of claim 4, wherein the polynucleotide that encodes a CAR.
6. The polynucleotide of claim 4, wherein the expression of the chimeric TGF $\beta$  receptor and the expression of the CAR and/or  $\alpha\beta$  TCR are under control of the same regulatory element or elements.
7. The polynucleotide of claim 1, wherein the expression of the chimeric TGF $\beta$  receptor and the expression of the CAR and/or  $\alpha\beta$  TCR are under control of a different regulatory element or elements.



8. The polynucleotide of any one of claims 4-7, wherein the comprises a targeting domain that binds to one or more of PSCA, HER2, CD19, CD20, CD22, Kappa or light chain, BCMA, CD30, CD33, CD123, CD38, ROR1, ErbB3/4, EGFR, EGFRvIII, EphA2, FAP, carcinoembryonic antigen (CEA), EGP2, EGP40, mesothelin, TAG72, PSMA, NKG2D ligands, B7-H6, IL-13 receptor  $\alpha$ 2, IL-11 receptor R  $\alpha$ , MUC1, MUC16, CA9, GD2, GD3, HMW-MAA, CD171, Lewis Y, G250/CAIX, HLA-AI MAGE A1, HLA-A2 NY-ESO-1, PSC1, folate receptor-  $\alpha$ , CD44v7/8, 8H9, NCAM, VEGF receptors, 5T4, Fetal AchR, NKG2D ligands, CD44v6, TEM1, TEM8 or a combination thereof.
9. The polynucleotide of any one of claims 1-8, wherein the polynucleotide is further defined as an expression construct or vector.
10. The polynucleotide of claim 9, wherein the vector is a viral vector or a non-viral vector.
11. The polynucleotide of claim 10, wherein the viral vector is a retroviral vector, lentiviral vector, adenoviral vector, or adeno-associated viral vector.
12. The polynucleotide of claim 11, wherein the non-viral vector is a plasmid or is mRNA.
13. The polynucleotide of any one of claims 1-12, wherein said polynucleotide is comprised in a cell.
14. The polynucleotide of claim 13, wherein the cell is a T cell, natural killer (NK) cell, natural killer T (NKT) cell, antigen-specific T cell, or T cell comprising an  $\alpha\beta$ TCR that is native to the T cell or is an engineered TCR.

15. The polynucleotide of claim 14, wherein the antigen-specific T cell is further defined as a tumor-specific T cell or pathogen-specific T cell.
16. The polynucleotide of any one of claims 1-15, wherein the TGF $\beta$  receptor comprises part or all of TGF $\beta$  receptor II.
17. A polypeptide encoded by the polynucleotide of any one of claims 1-16.
18. A composition comprising the polynucleotide of any one of claims 1-16.
19. The composition of claim 18, further comprising a polynucleotide comprising sequence that encodes a CAR or that encodes an  $\alpha\beta$ TCR that is native to the T cell or is an engineered TCR.
20. The composition of claim 19, wherein the polynucleotide comprising sequence encoding a chimeric TGF $\beta$  receptor further comprises the polynucleotide comprising sequence that encodes the CAR or the  $\alpha\beta$ TCR.
21. The composition of claim 19, wherein the polynucleotide comprising sequence encoding a chimeric TGF $\beta$  receptor does not further comprise the polynucleotide comprising sequence that encodes the CAR or the  $\alpha\beta$ TCR.
22. A composition comprising one or more cells that comprise the polynucleotide of any one of claims 1-16.
23. The composition of claim 22, wherein the cell is an immune cell.
24. The composition of claim 22, wherein the cell is a T cell, NK cell, or NKT cell.

25. The composition of claim 22, wherein the cell is an antigen-specific T cell, and/or comprises an  $\alpha\beta$  TCR that is native to the T cell or is an engineered TCR.
26. The composition of claim 25, wherein the antigen-specific T cell is further defined as a tumor-specific T cell or pathogen-specific T cell.
27. The composition of claim 25, wherein the antigen-specificity of the antigen-specific T cell is natural.
28. The composition of claim 25, wherein the antigen-specificity of the antigen-specific T cell is recombinantly generated.
29. The composition of claim 25, wherein the  $\alpha\beta$  TCR on the cell is natural.
30. The composition of claim 25, wherein the  $\alpha\beta$  TCR on the cell is recombinantly generated.
31. The composition of claim 22, wherein the cell comprises a chimeric antigen receptor.
32. A composition comprising cells, wherein one or more cells comprise:

a first polynucleotide that encodes a chimeric TGF $\beta$  receptor, wherein the chimeric TGF $\beta$  receptor comprises an exodomain of TGF $\beta$  receptor and a non-TGF $\beta$  receptor endodomain; and

a second polynucleotide that encodes a CAR and/or an  $\alpha\beta$  TCR.

33. The composition of claim 32, wherein the first and second expression construct are the same molecule.
34. The composition of claim 32, wherein the first and second expression constructs are different molecules.

35. The composition of claim 32, wherein the antigen-specific T cell is a tumor-specific T cell receptor or a pathogen-specific T cell receptor.
36. The composition of claim 32, wherein the CAR is specific for an antigen selected from the group consisting of PSCA, HER2, CD19, CD20, CD22, Kappa or light chain, CD30, CD33, CD123, CD38, ROR1, ErbB3/4, EGFR, EGFRvIII, EphA2, FAP, carcinoembryonic antigen, EGP2, EGP40, mesothelin, TAG72, PSMA, NKG2D ligands, B7-H6, IL-13 receptor  $\alpha$ 2, IL-11 receptor R  $\alpha$ , MUC1, MUC16, CA9, GD2, GD3, HMW-MAA, CD171, Lewis Y, G250/CAIX, HLA-AI MAGE A1, HLA-A2 NY-ESO-1, PSC1, folate receptor-  $\alpha$ , CD44v7/8, 8H9, NCAM, VEGF receptors, 5T4, Fetal AchR, NKG2D ligands, CD44v6, TEM1, TEM8 and a combination thereof.
37. A composition comprising one or more cells, wherein the cells are defined as:
  - 1) being an antigen-specific T cell, and/or a cell comprising a CAR, and/or a cell comprising an  $\alpha\beta$  T cell receptor; and
  - 2) comprising a chimeric TGF $\beta$  receptor, wherein the receptor comprises an exodomain of TGF $\beta$  receptor and a non-TGF $\beta$  receptor endodomain
38. The composition of any one of claims 22-37, wherein the cell is autologous with respect to a recipient of said cell.
39. The composition of any one of claims 22-37, wherein the cell is allogenic with respect to a recipient of said cell.
40. A method of treating and/or preventing cancer in an individual, comprising the step of delivering a therapeutically effective amount of a composition of any one of claims 15-29 to the individual, wherein the individual has a cancer that has cells that

secrete TGF $\beta$  and/or the cancer has a microenvironment that produces TGF $\beta$ .

41. The method of claim 40, wherein the cancer is prostate, breast, melanoma, pancreatic, lung, brain, colon, esophageal, liver, kidney, testicular, ovarian, cervical, gall bladder, thyroid, anal, endometrial, bladder, pituitary gland, leukemia, lymphoma, stomach, spleen, colon, gastric, or myeloma.
42. The method of claim 40, wherein the cells are delivered intravenously, intraperitoneally, intratumorally, intrathecally, and/or transrectally.
43. The method of claim 40, wherein exposure of TGF $\beta$  to the cell protects or enhances the anti-tumor activity of the cell.
44. The method of claim 40, wherein the individual is provided with another cancer therapy.
45. The method of claim 44, wherein the cancer therapy is surgery, chemotherapy, immunotherapy, hormone therapy, radiation, or a combination thereof.
46. The method of any one of claims 40-44, wherein the cells are allogeneic or autologous to said individual.
47. The method of any one of claims 40-44, further comprising the step of obtaining peripheral blood mononuclear cells (PBMCs) from the individual or from another individual.
48. The method of claim 47, comprising the step of obtaining immune cells from the PBMCs and modifying said immune cells to comprise a polynucleotide comprising a nucleotide sequence encoding a chimeric TGF $\beta$  receptor, wherein the receptor comprises an exodomain of TGF $\beta$  receptor and a non-TGF $\beta$  receptor endodomain.

49. The method of claim 48, wherein the immune cells are T cells, NKT cells, or NK cells.
50. The method of claim 40, wherein the composition is provided to the individual more than once.
51. The method of claim 50, wherein when the composition is provided more than once and the cells comprise a CAR specific for a particular tumor antigen, upon one or more subsequent deliveries the cells comprise a CAR specific for a different tumor antigen.
52. A method of converting a T cell-inhibitory cytokine signal into a T cell-stimulatory signal, comprising the step of exposing the inhibitory cytokine to a receptor on a T cell, said receptor having an exodomain and an endodomain that is not naturally linked to the exodomain, wherein the exodomain is capable of binding the inhibitory cytokine and the endodomain provides a stimulatory signal to the T cell, wherein the exodomain and the endodomain pair as homodimers.
53. The method of claim 52, wherein the cytokine is TGF $\beta$  or VEGF.
54. A method of converting a T cell-inhibitory cytokine signal into a T cell-stimulatory signal, comprising the step of exposing the inhibitory cytokine to a receptor on a T cell, said receptor having an exodomain and an endodomain that is not naturally linked to the exodomain, wherein the exodomain is capable of binding the inhibitory cytokine and the endodomain provides a stimulatory signal to the T cell, wherein the inhibitory cytokine is not IL4 or IL7 or both.
55. The method of claim 54, wherein the cytokine is TGF $\beta$ .
56. The method of claim 54, wherein the cytokine is VEGF and the exodomain is from the VEGF receptor.

57. The method of claim 56, wherein the endodomain is selected from the group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD28, 4-1BB, OX40, and a combination thereof.
58. A kit comprising the polynucleotide of any one of claims 1-16, the polypeptide of claim 17, a composition of any one of claims 18-39, or a combination thereof.

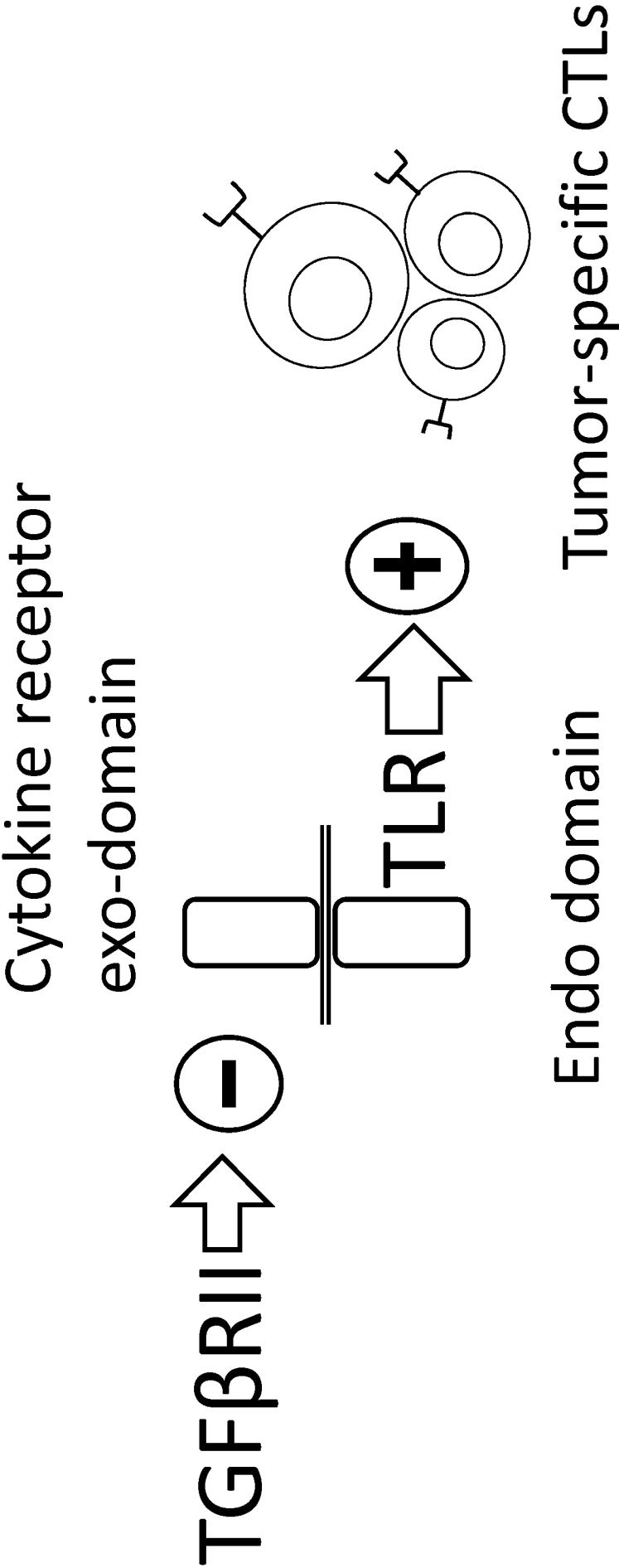


FIG. 1



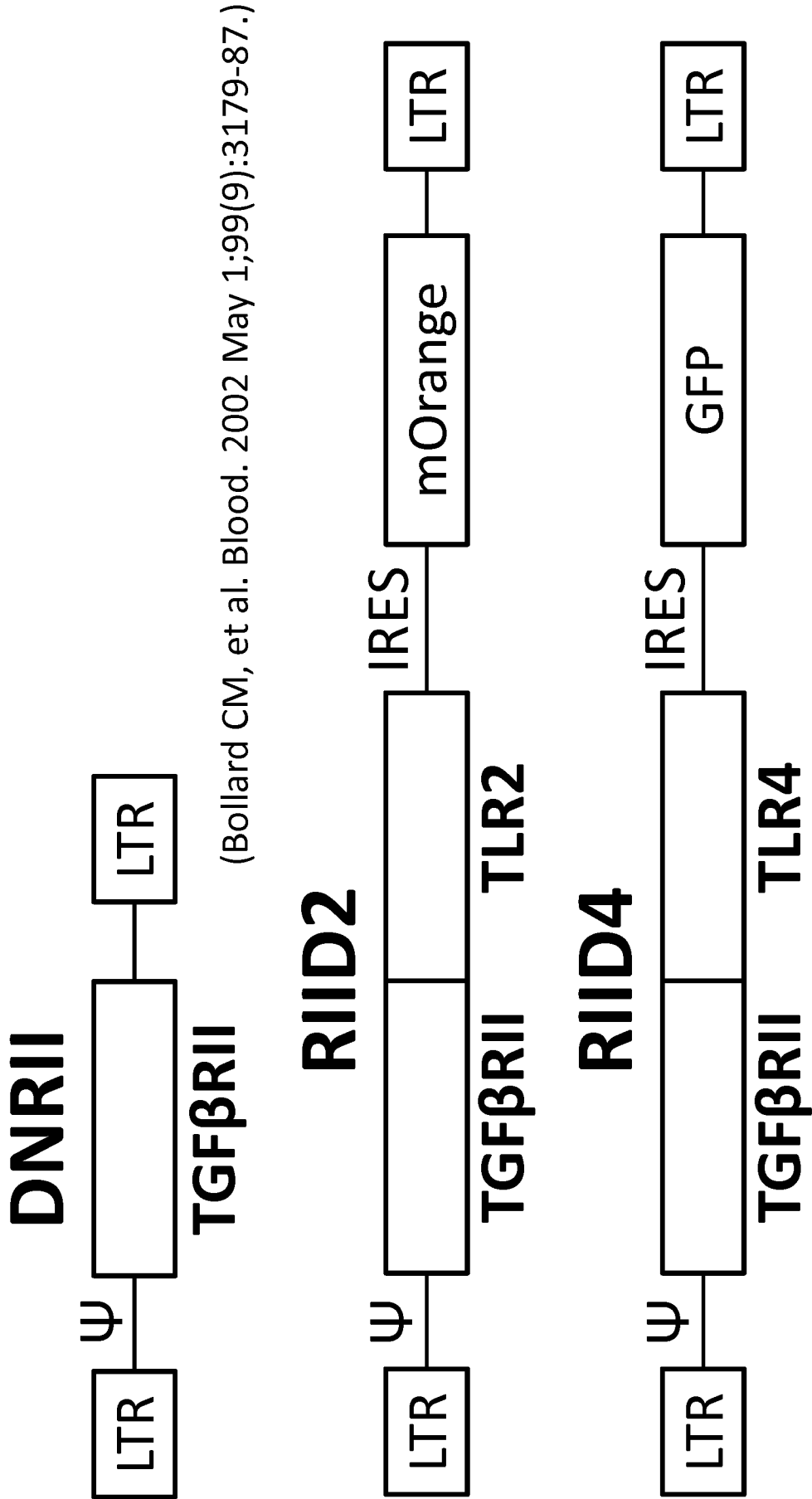


FIG. 2

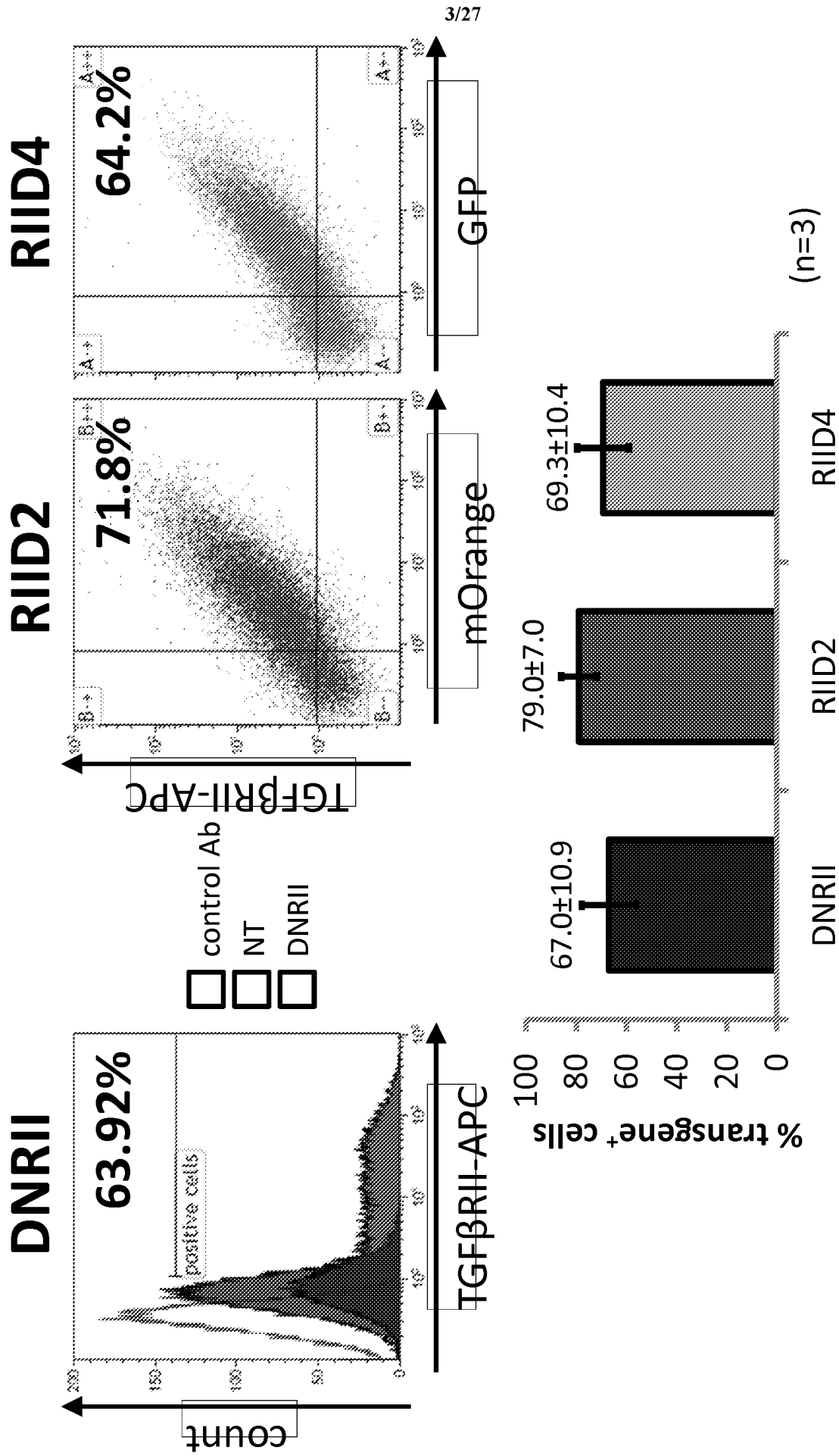


FIG. 3

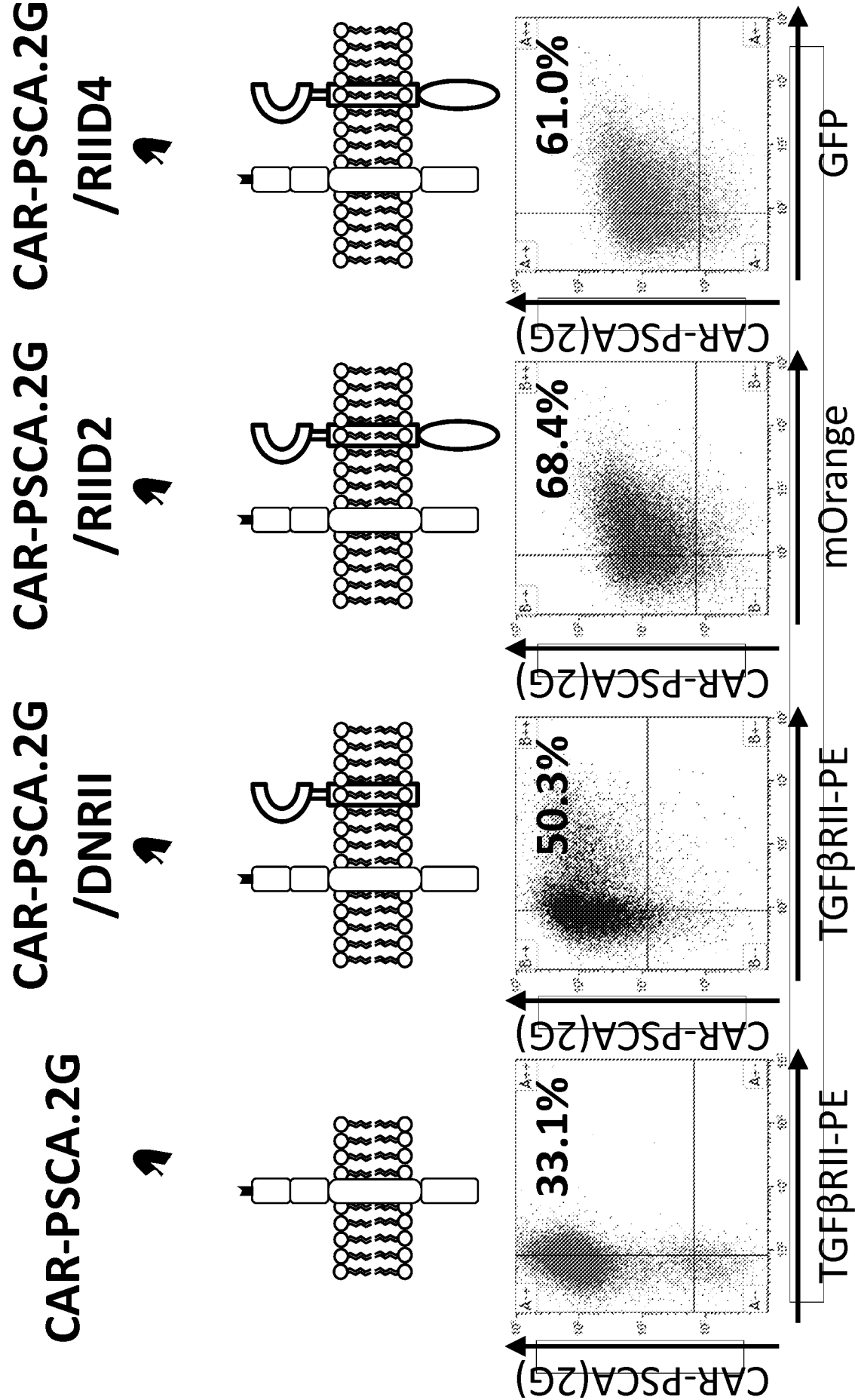


FIG. 4A

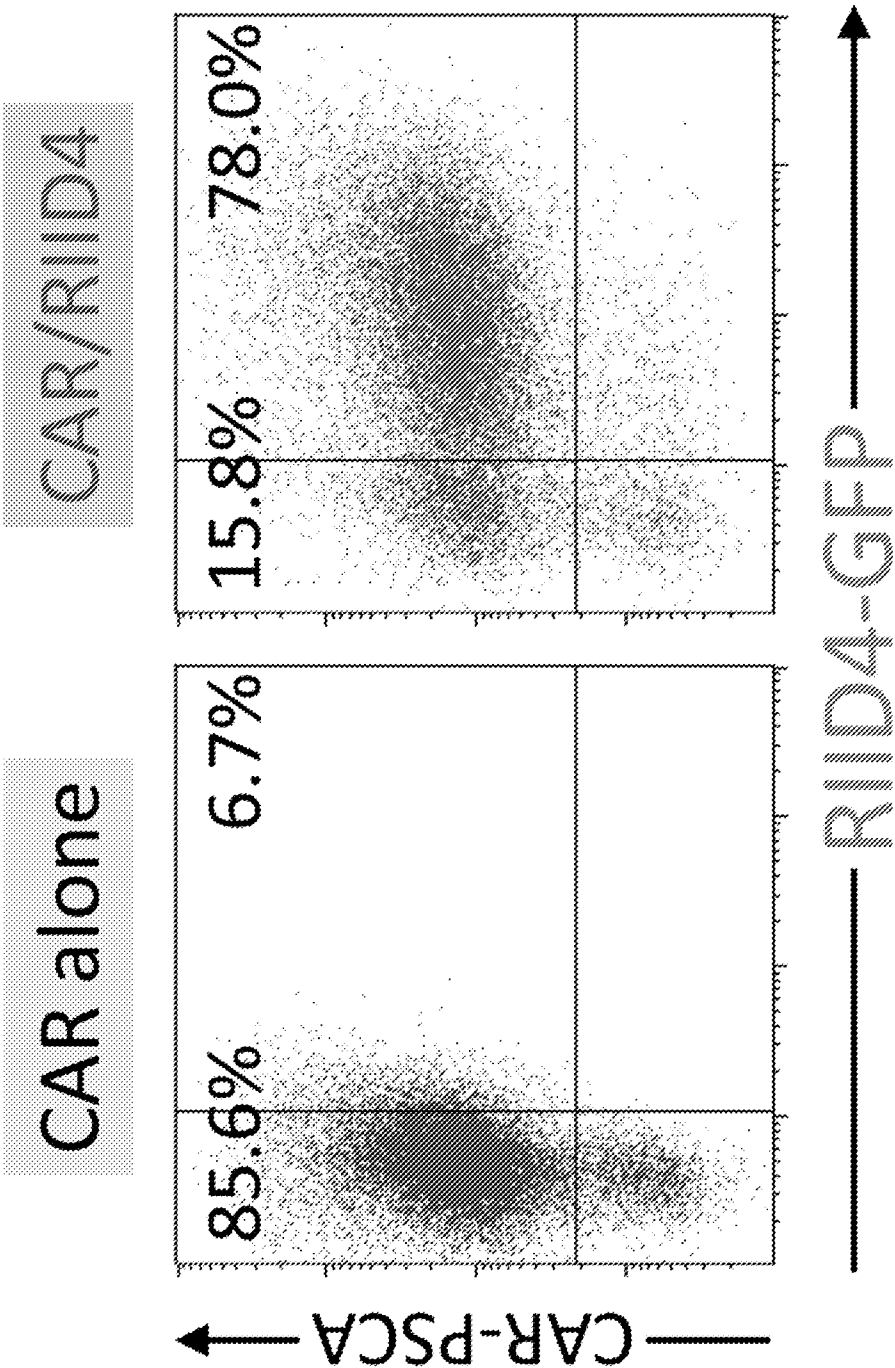


FIG. 4B

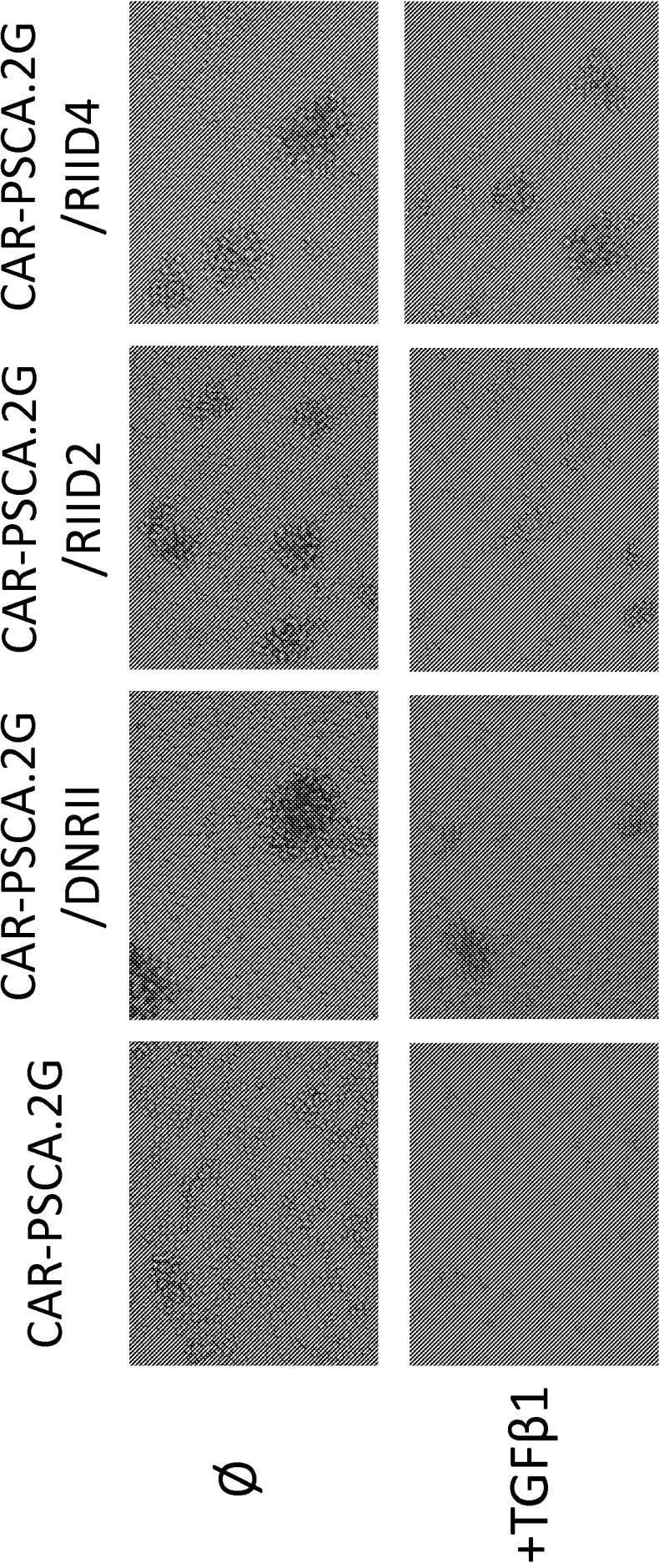
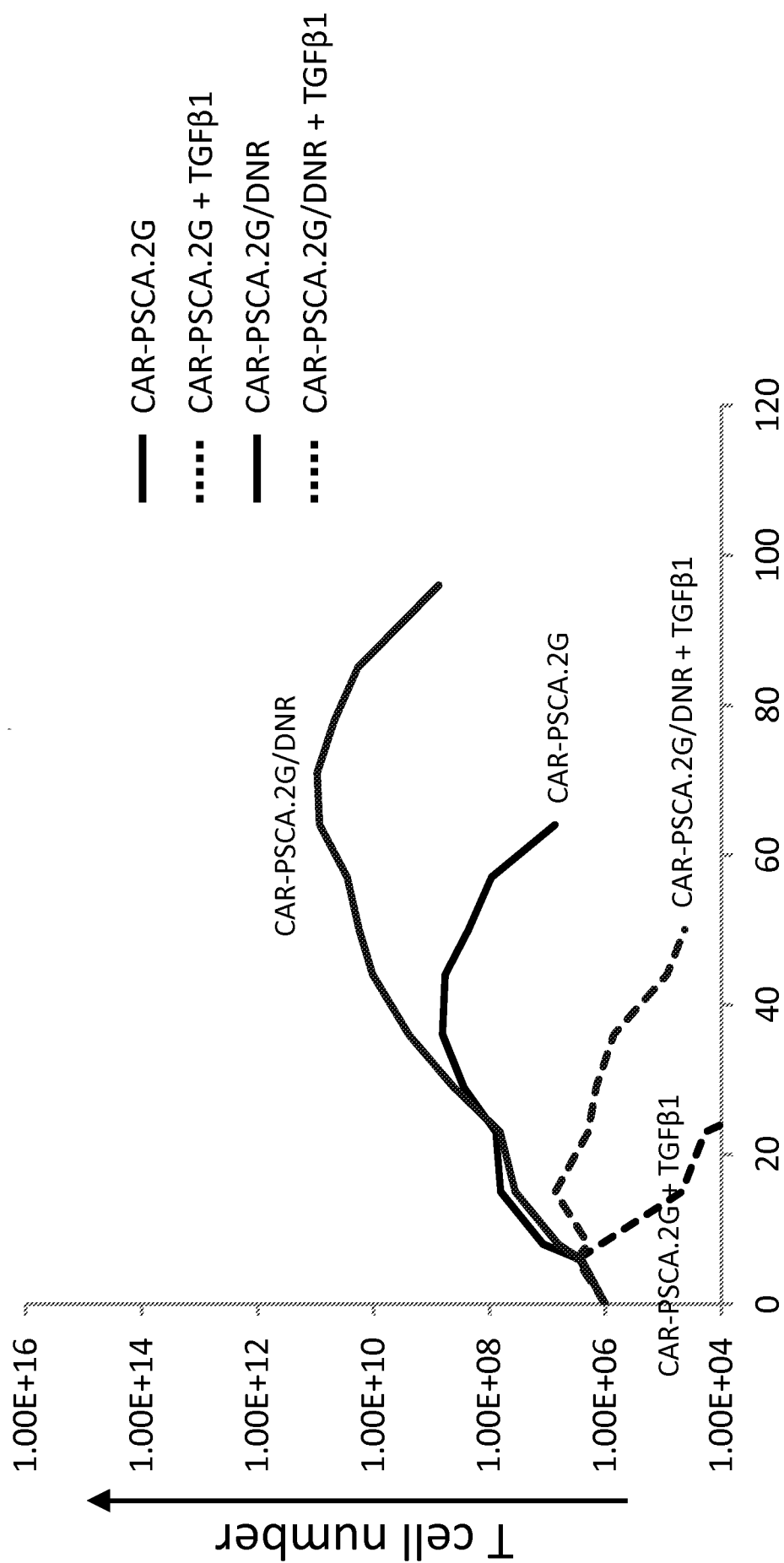
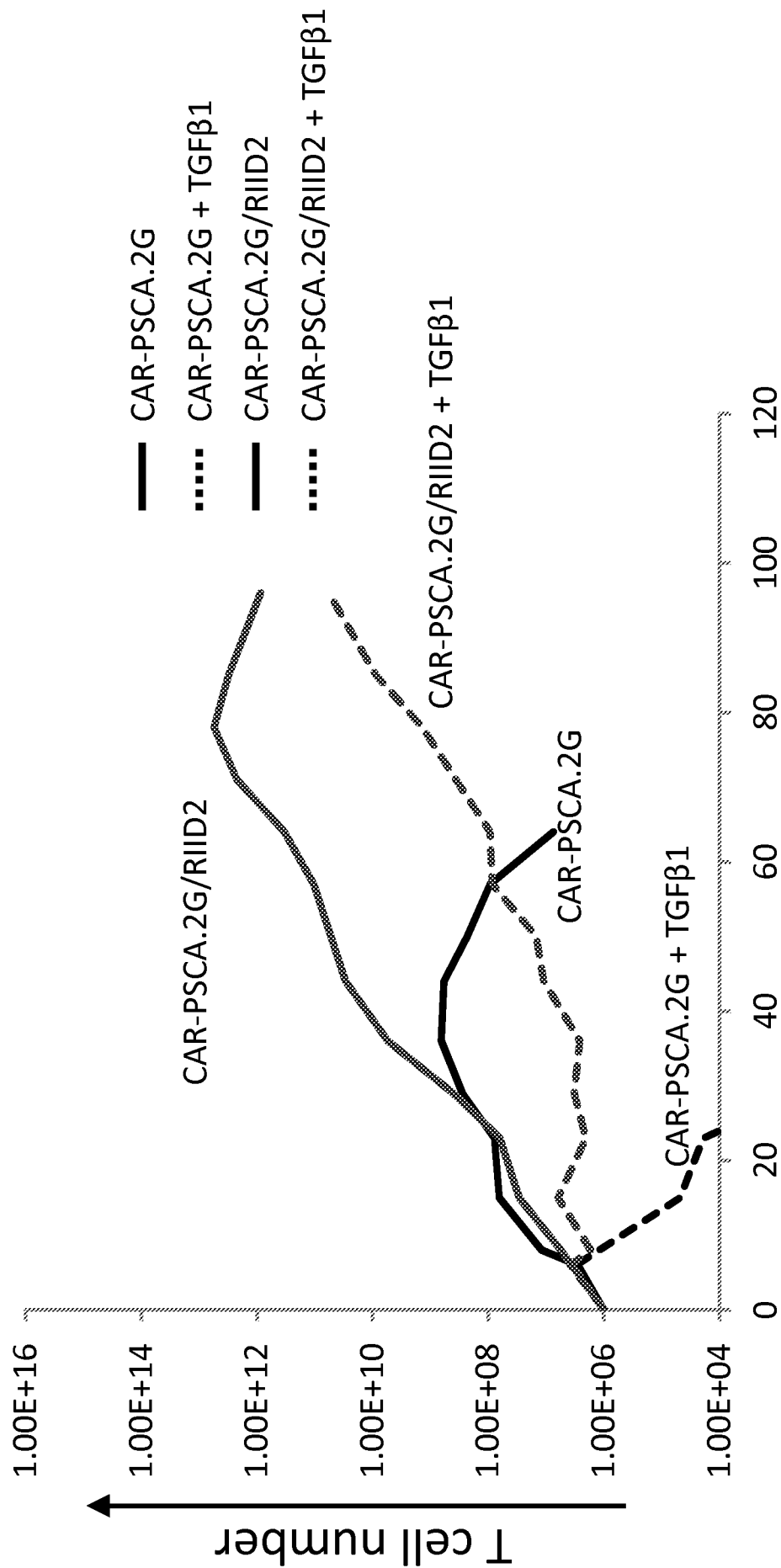


FIG. 5



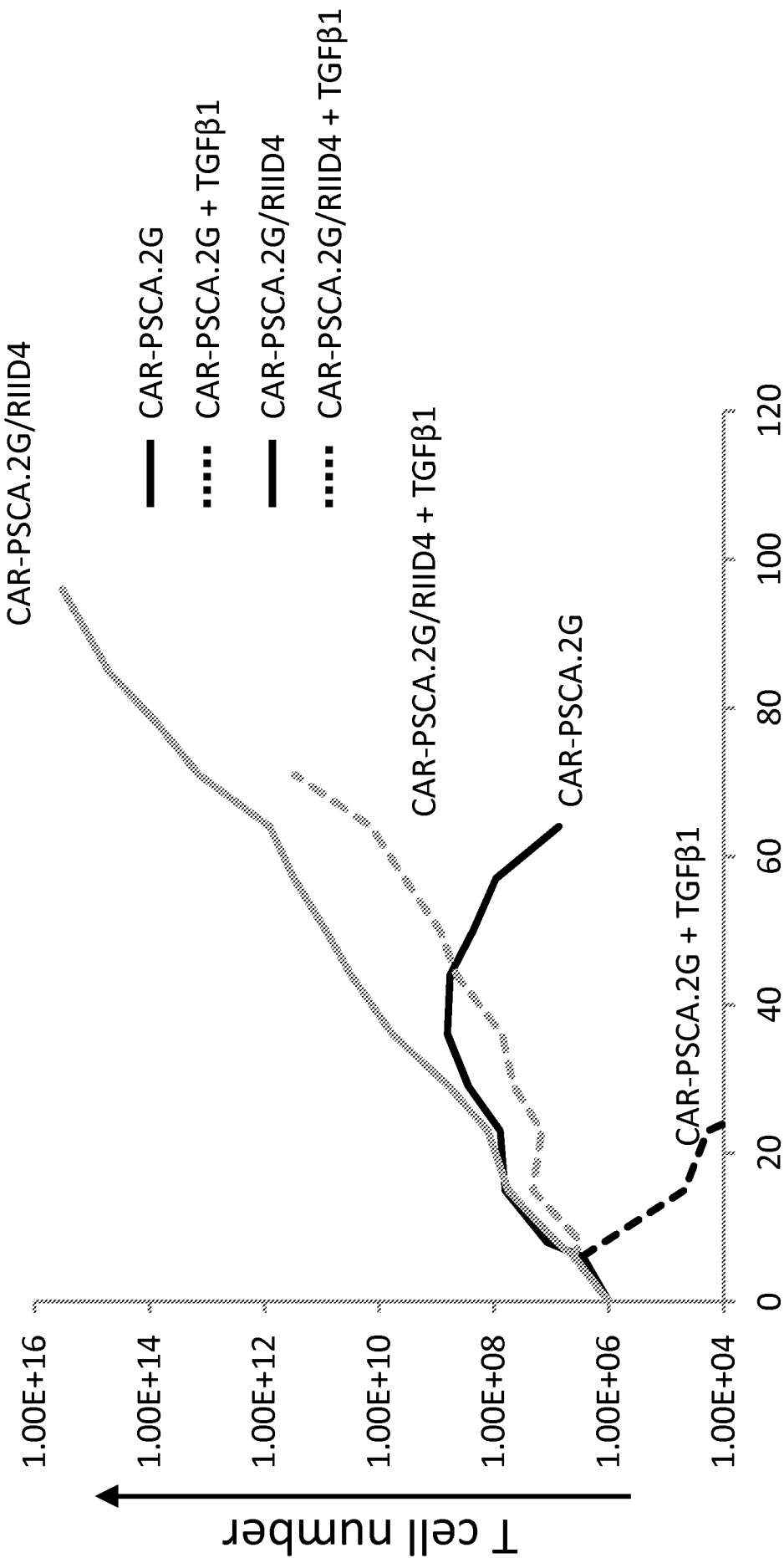
weekly antigen stimulation and administration of 5 ng/ml of TGFβ1, **No** IL2 administration

FIG. 6A



weekly antigen stimulation and administration of 5 ng/ml of TGFβ1, No IL2 administration

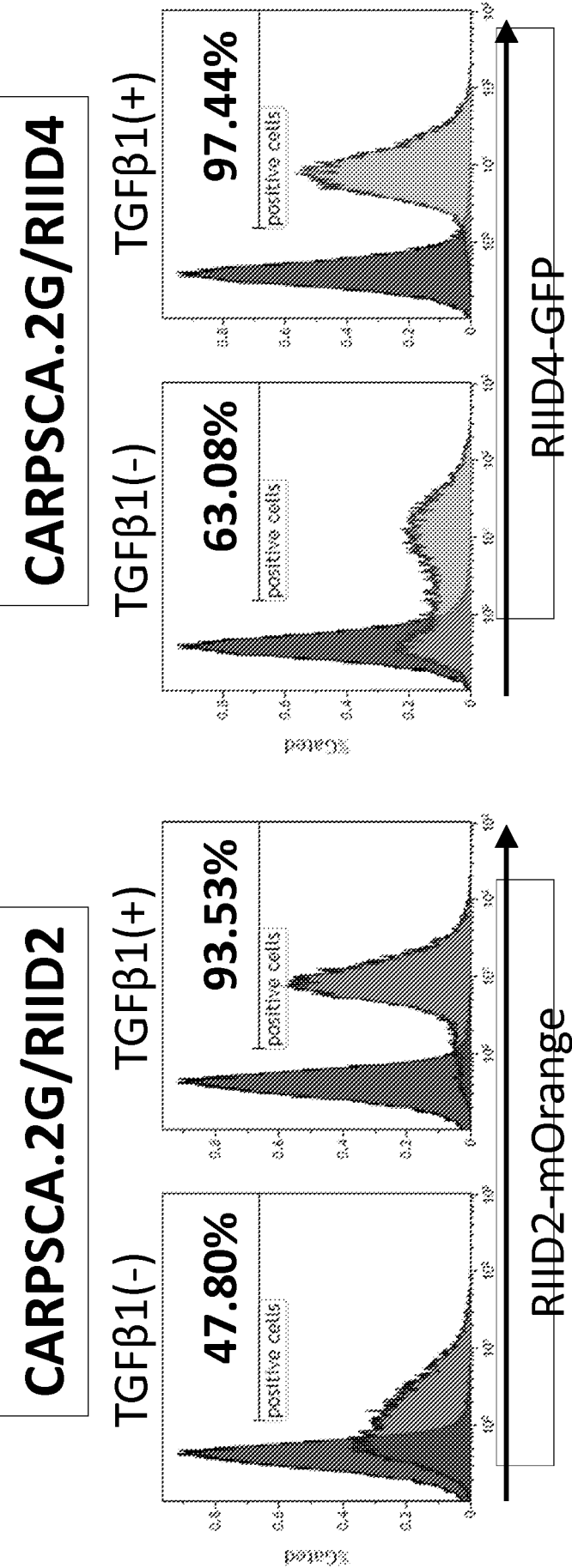
FIG. 6B



weekly antigen stimulation and administration of 5 ng/ml of TGFβ1, No IL2 administration

FIG. 6C



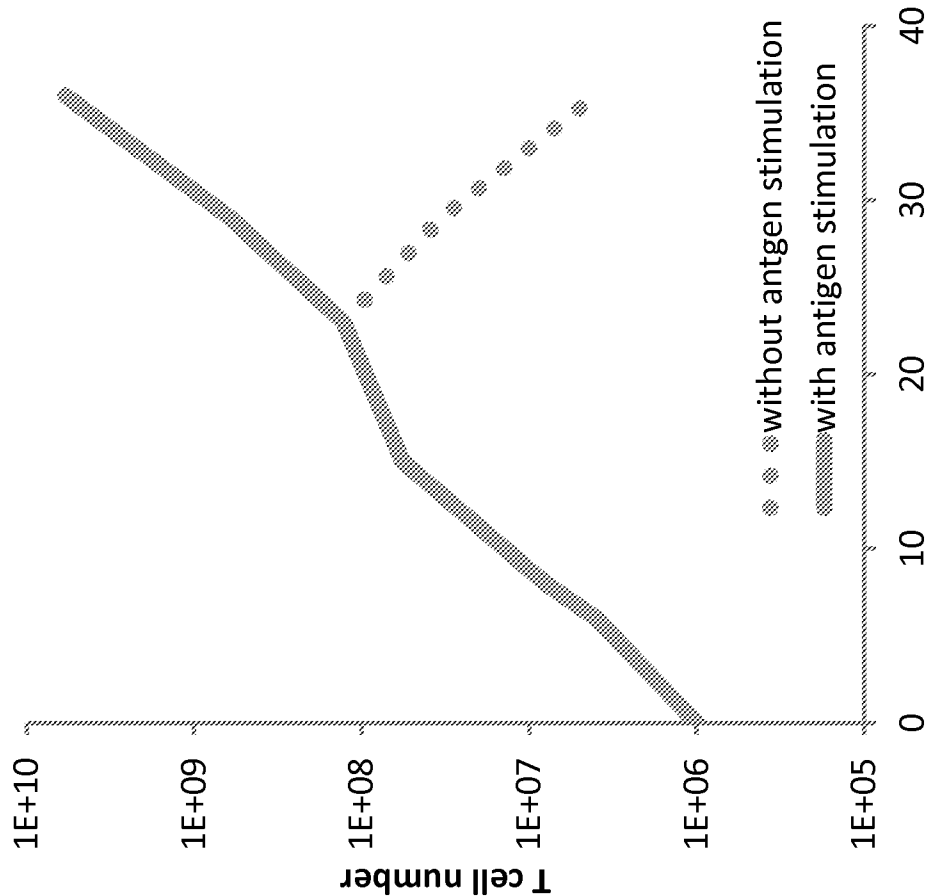


weekly antigen stimulation and administration of 5 ng/ml of TGFβ1, No IL2 administration

FIG. 7

11/27

# CARPSCA.2G/RIID4



# CARPSCA.2G/RIID2

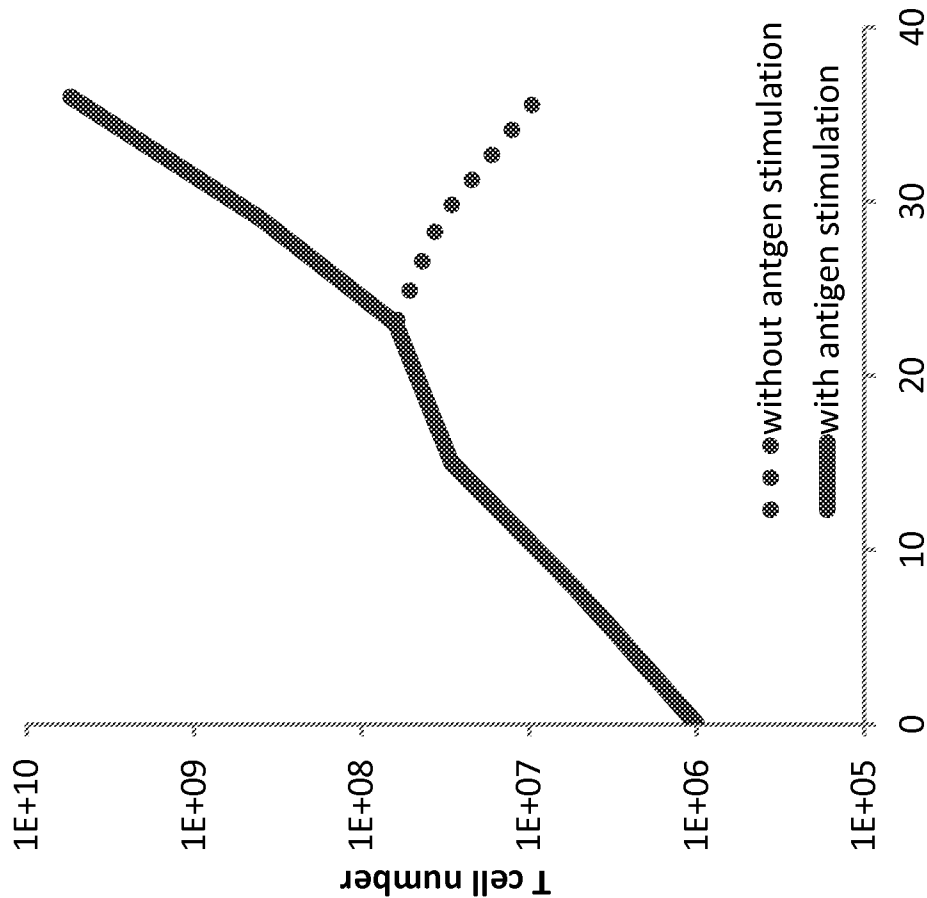


FIG. 8

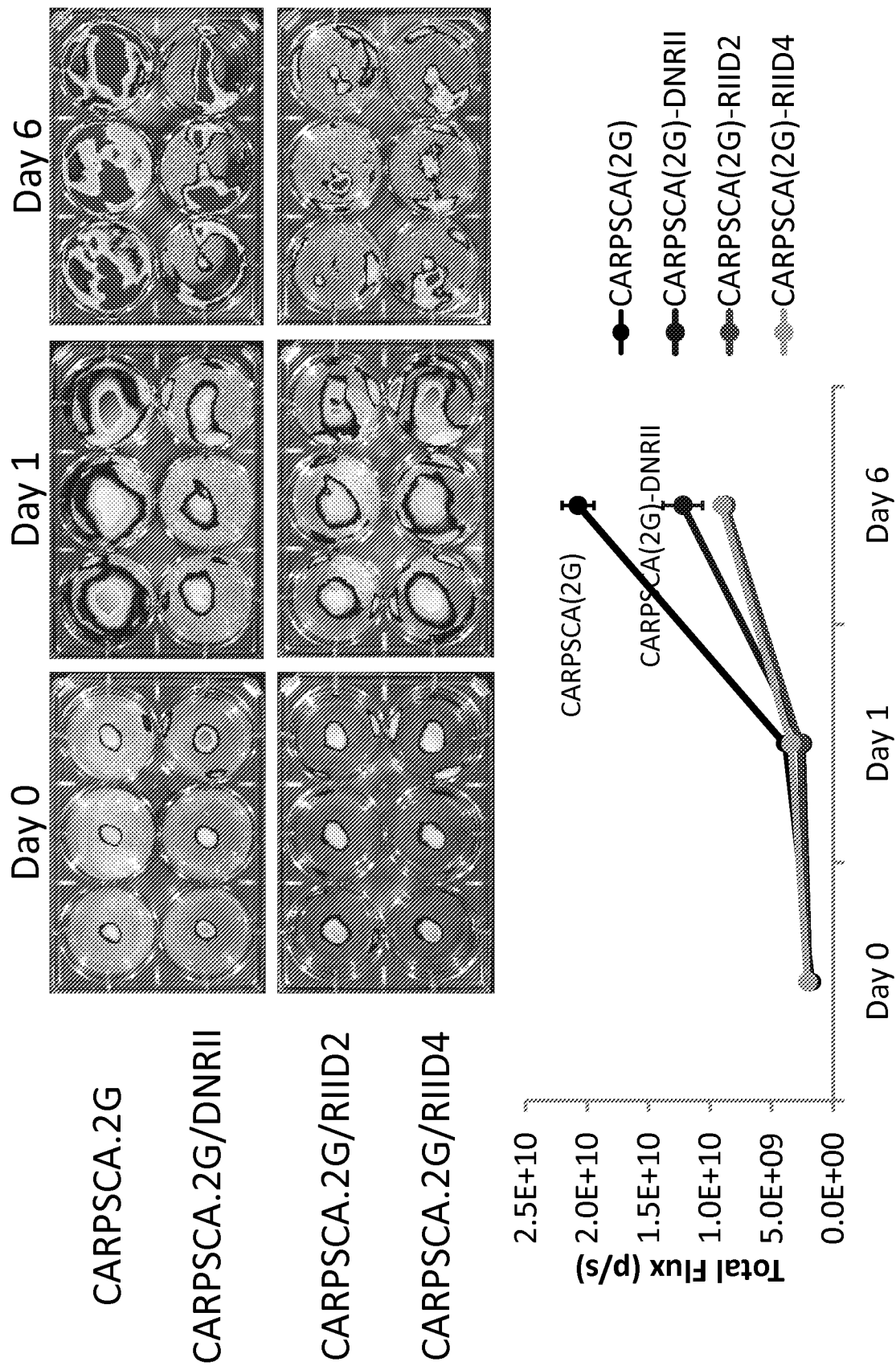
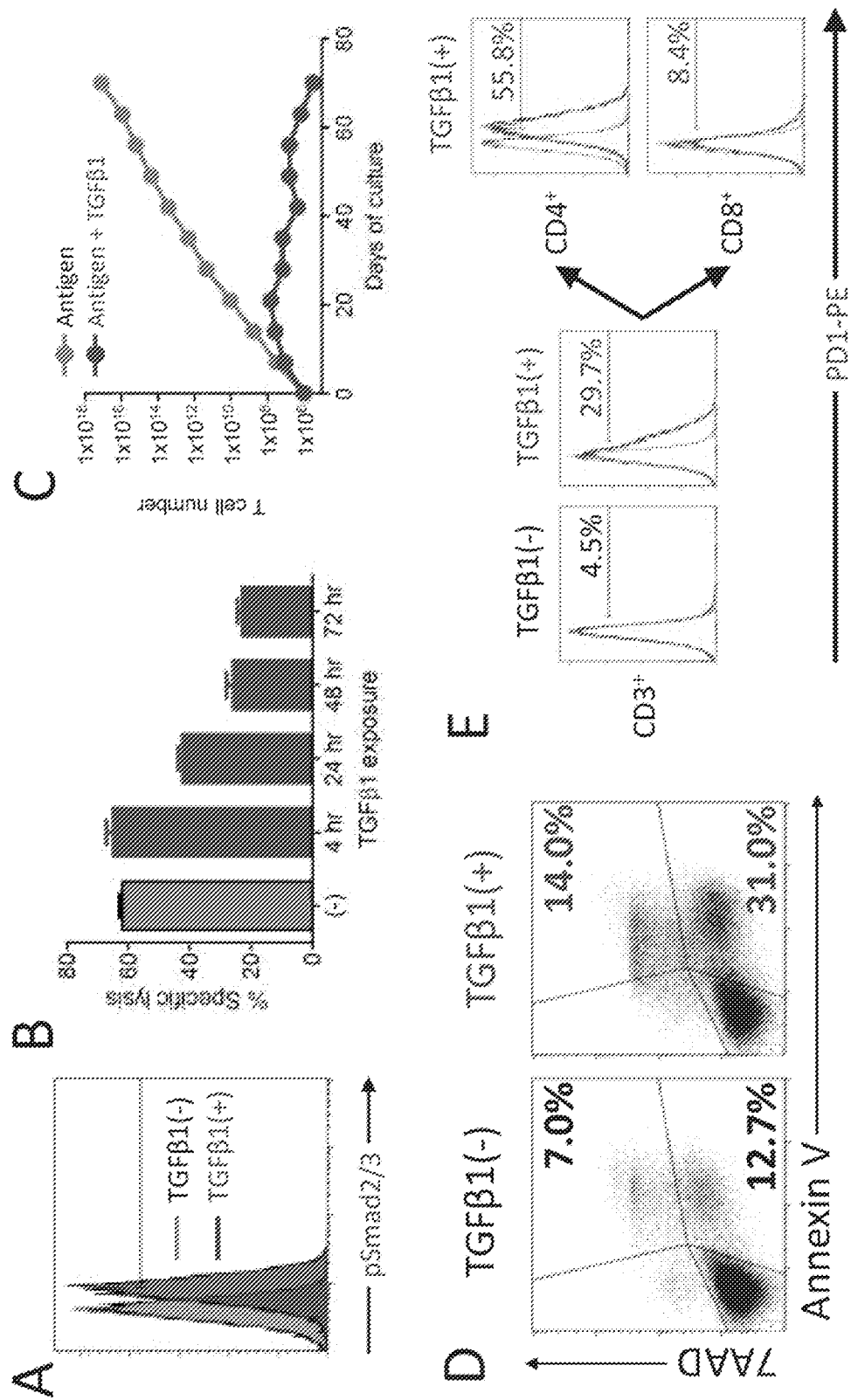


FIG. 9



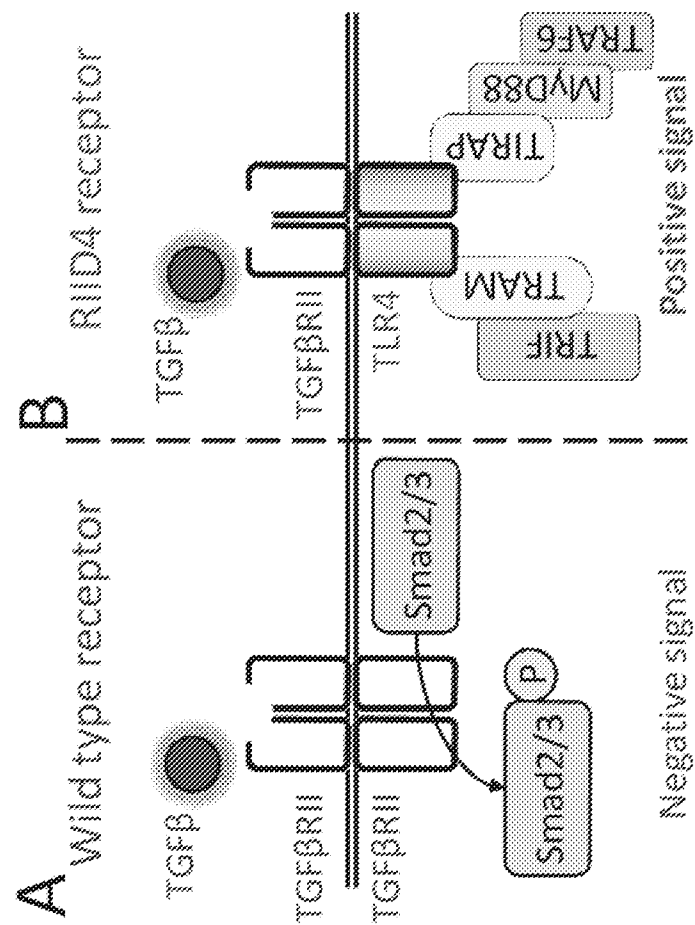


FIG. 11

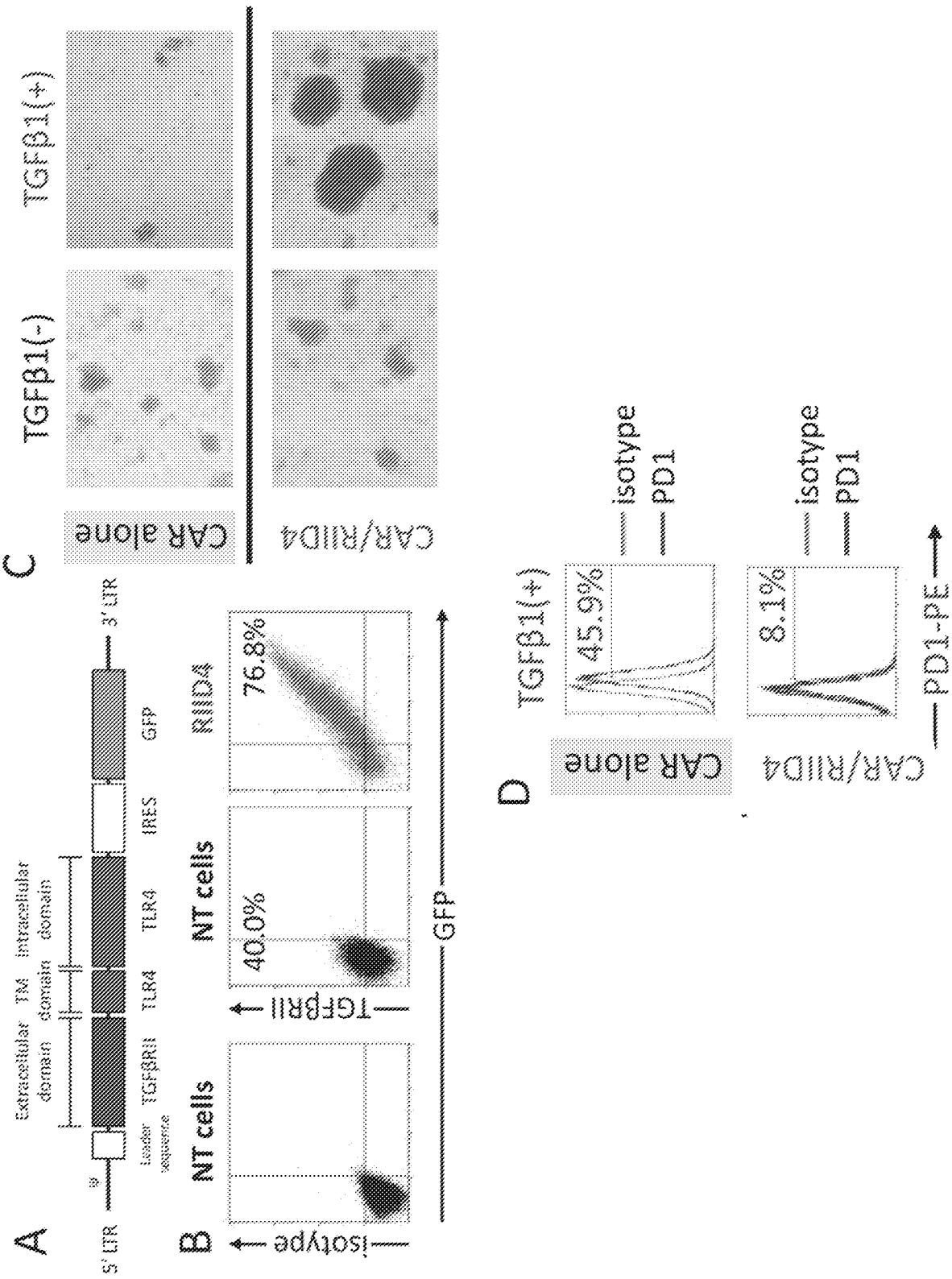


FIG. 12

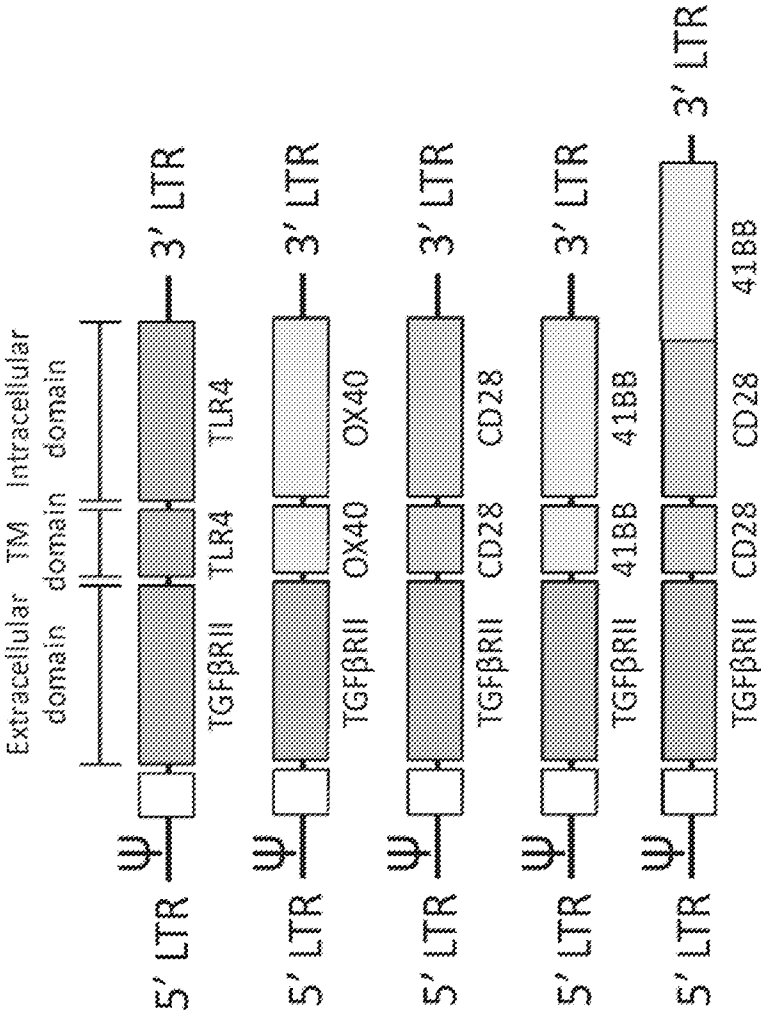


FIG. 13

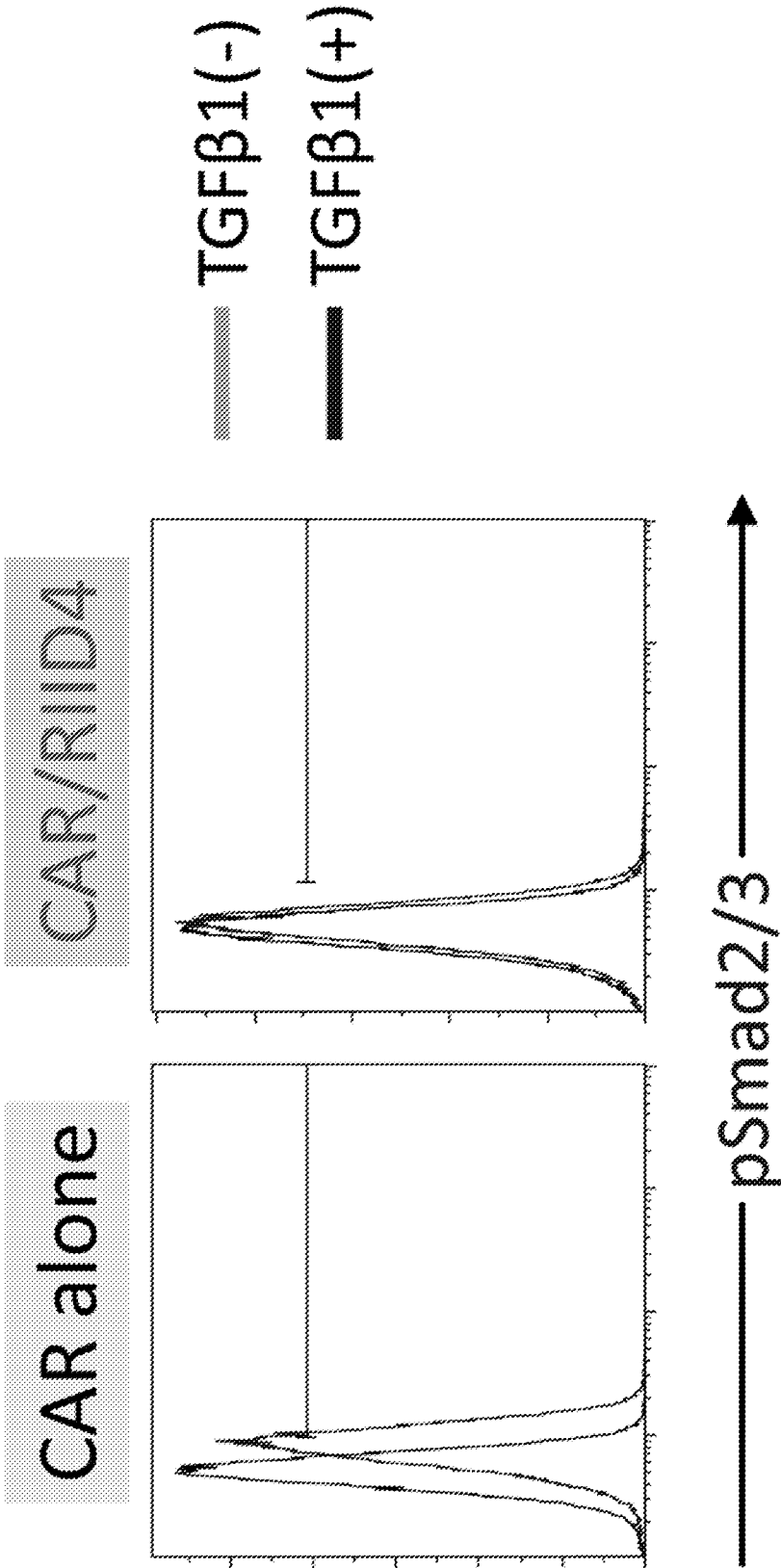


FIG. 14



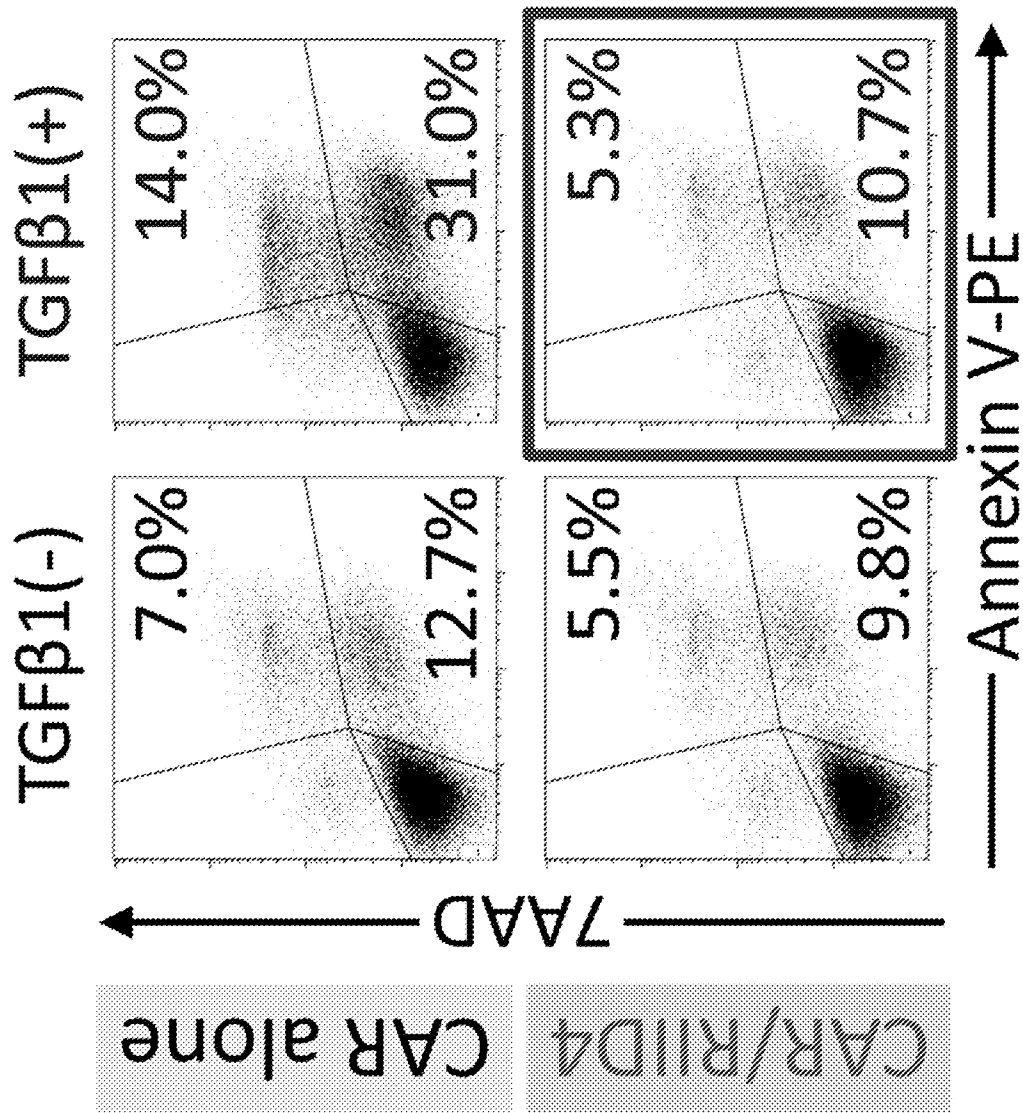


FIG. 15

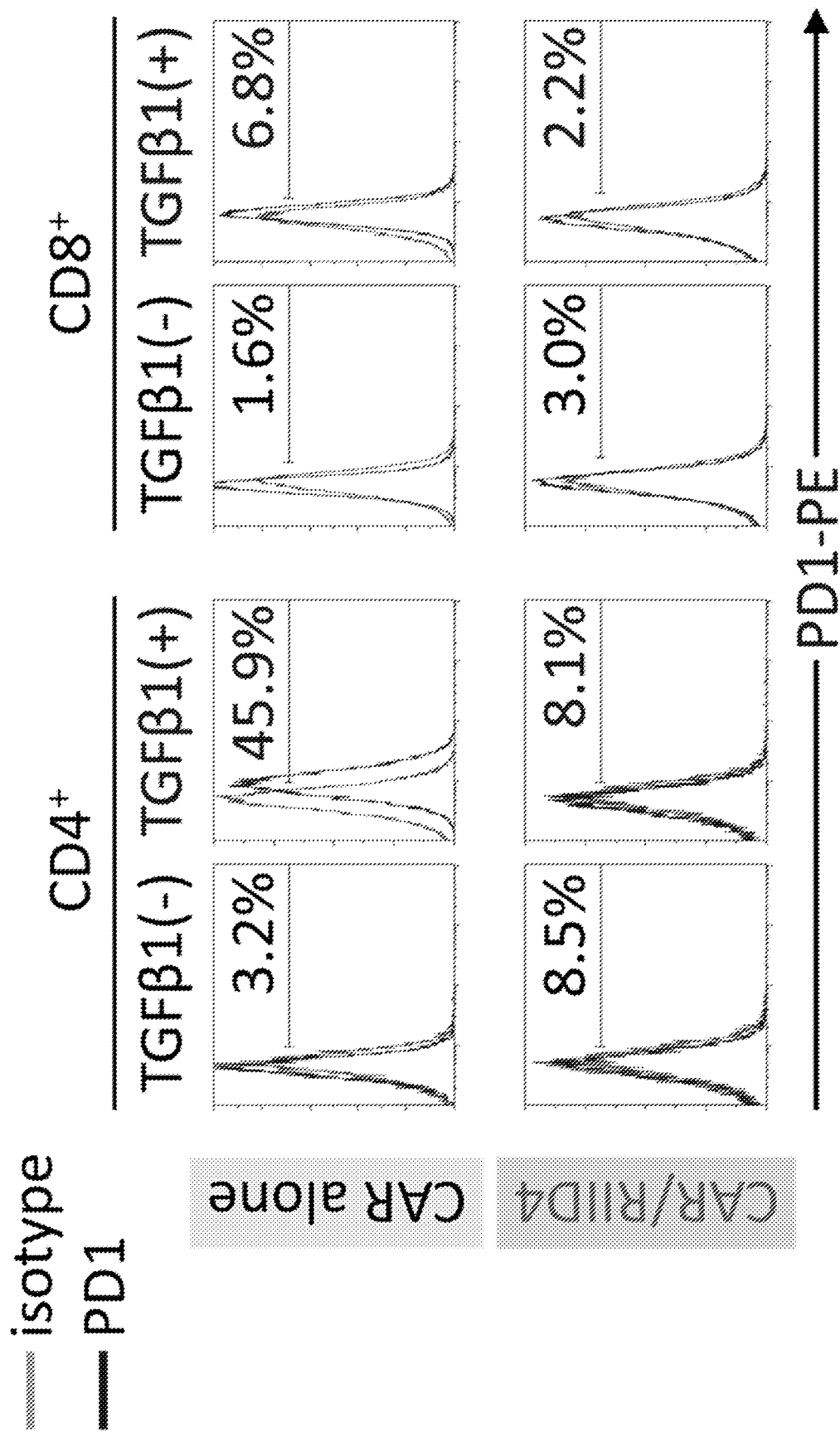


FIG. 16

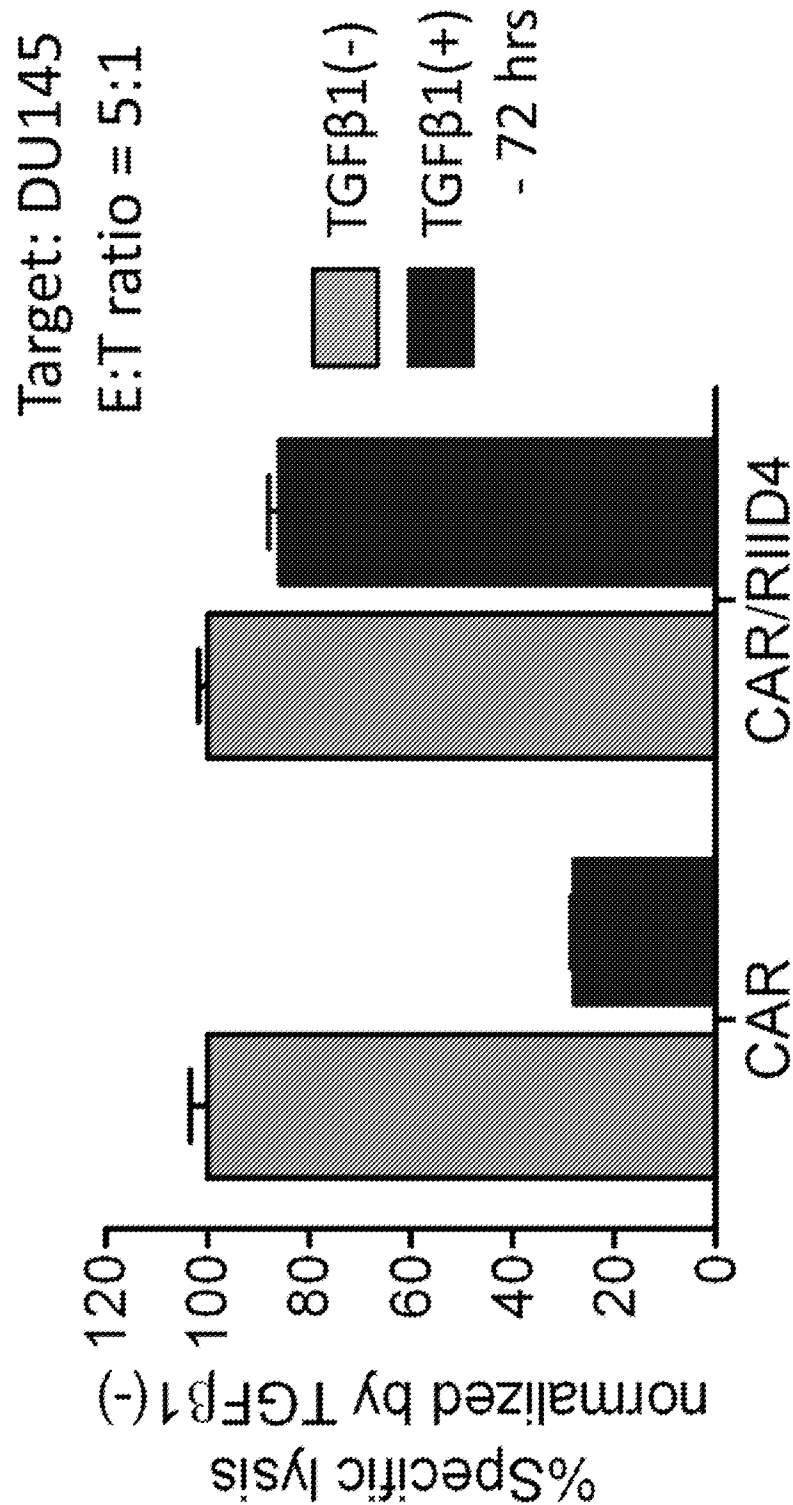


FIG. 17

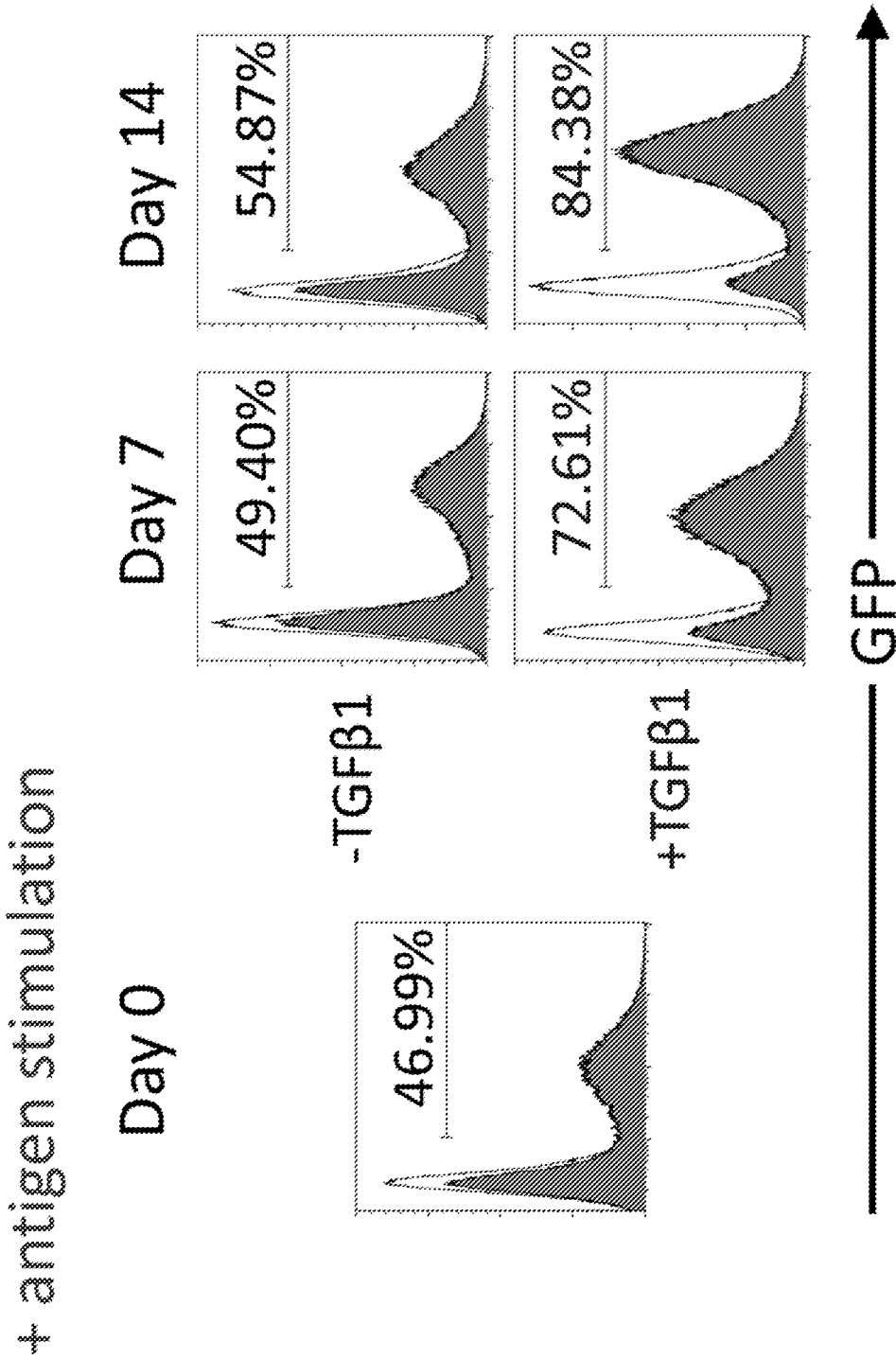


FIG. 18

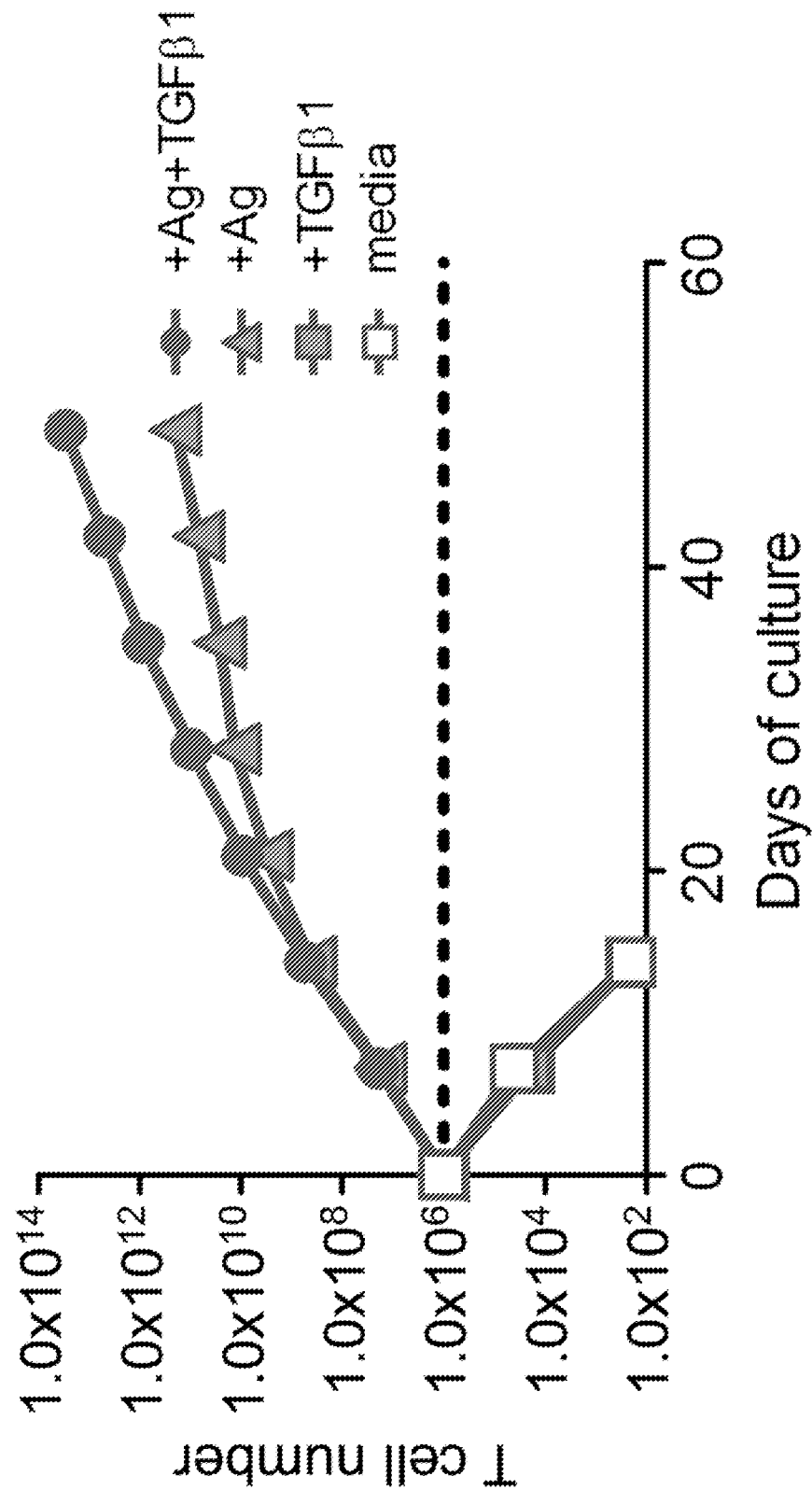


FIG. 19

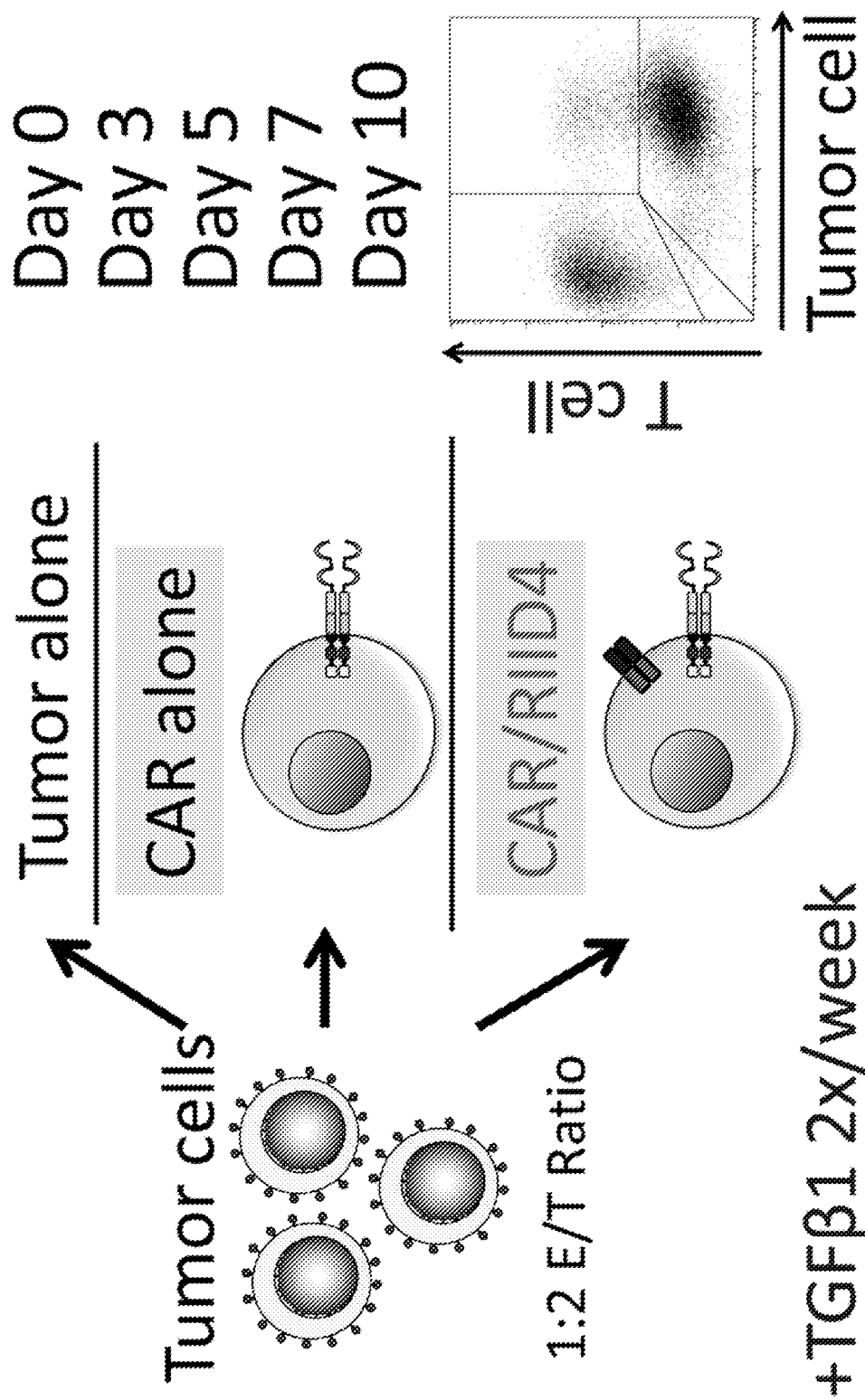


FIG. 20

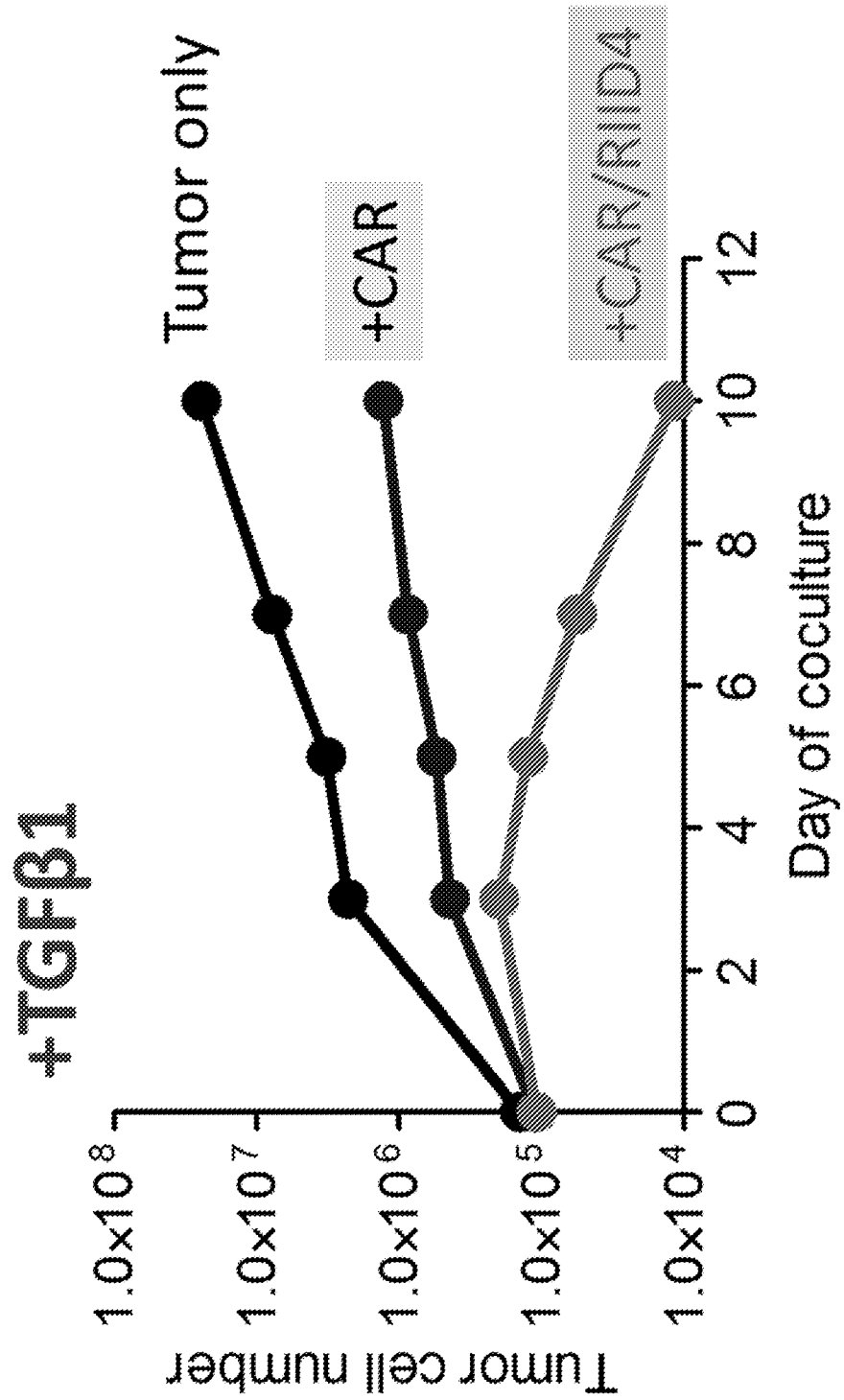


FIG. 21

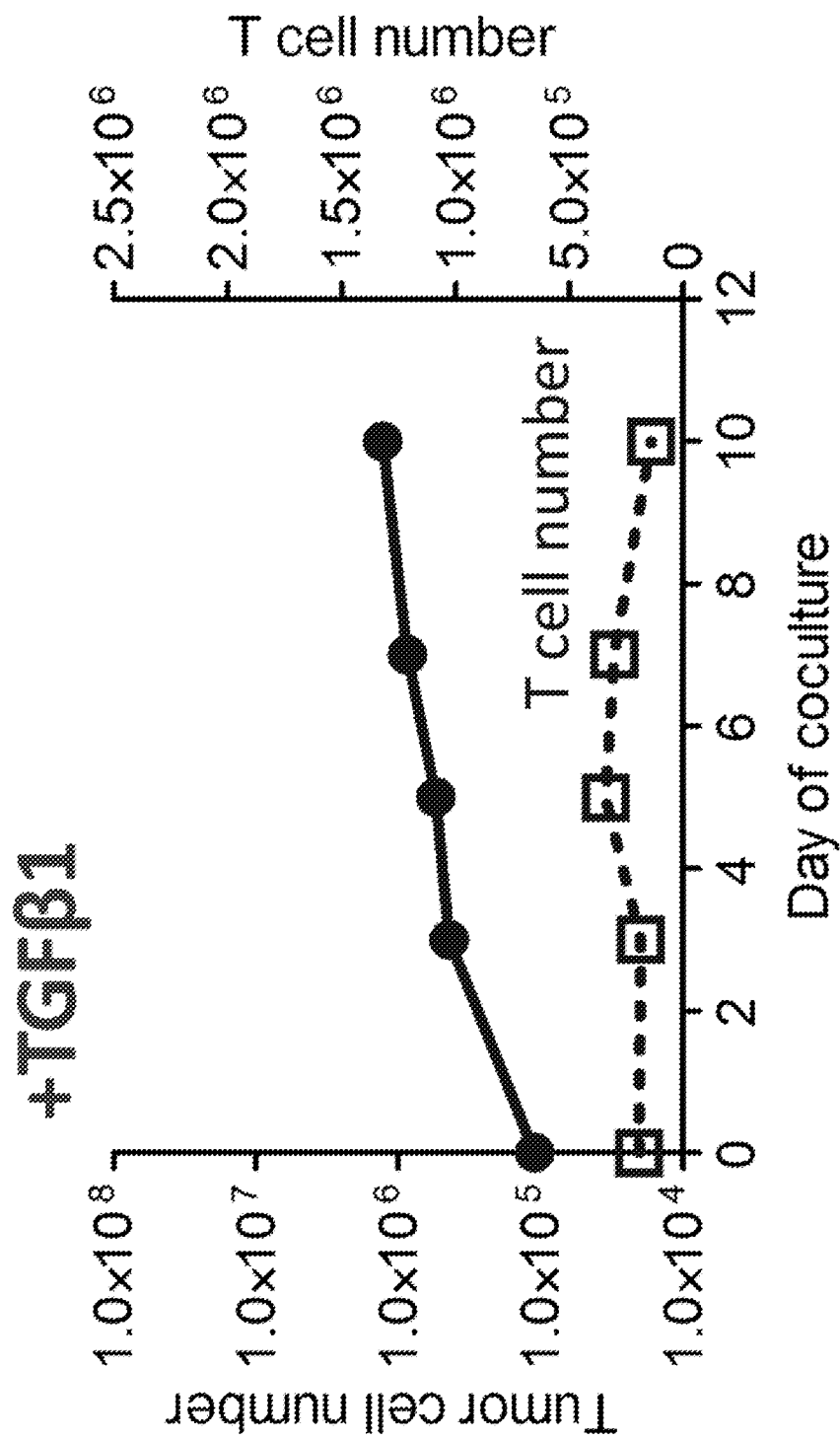


FIG. 22



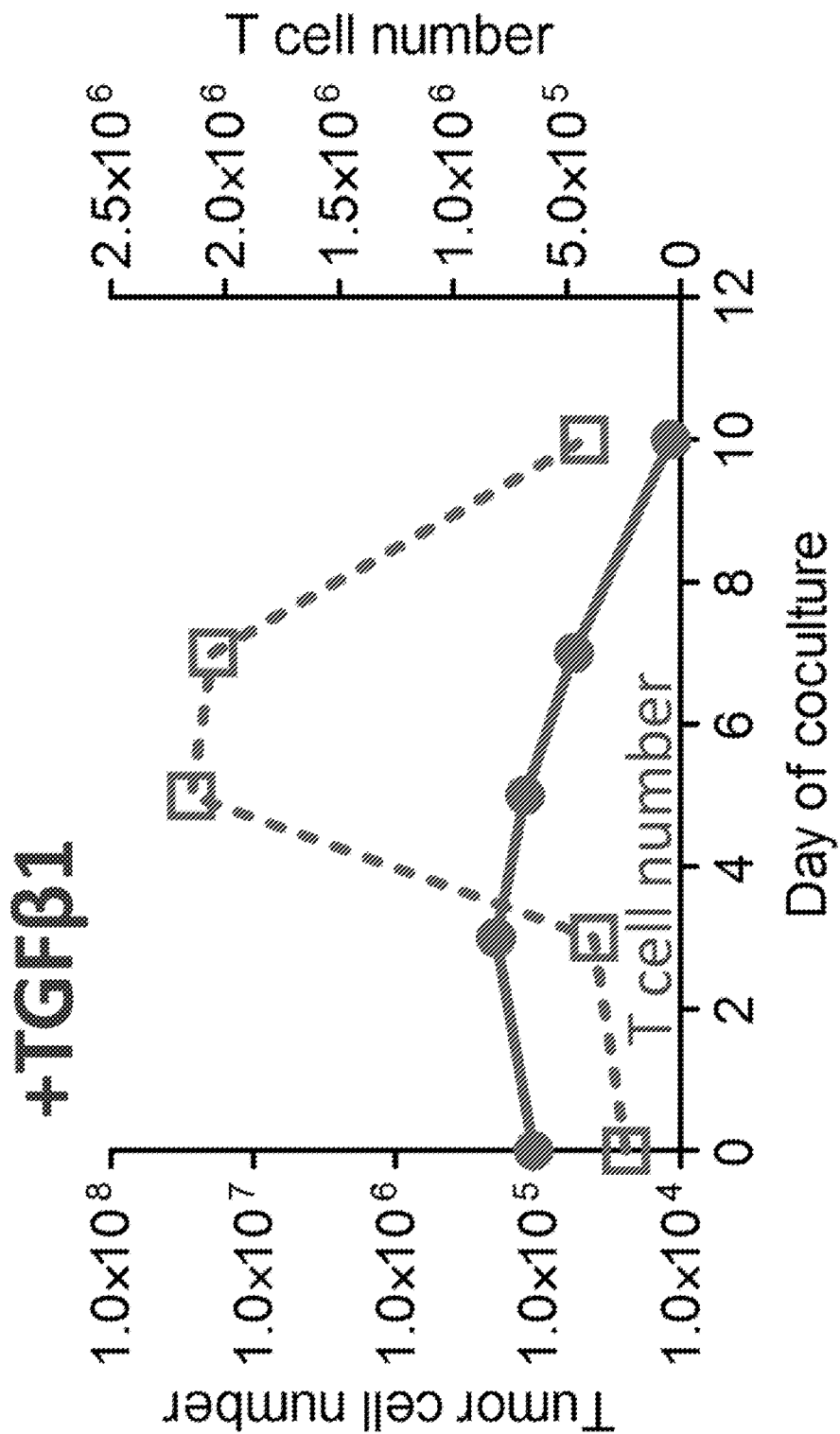


FIG. 23

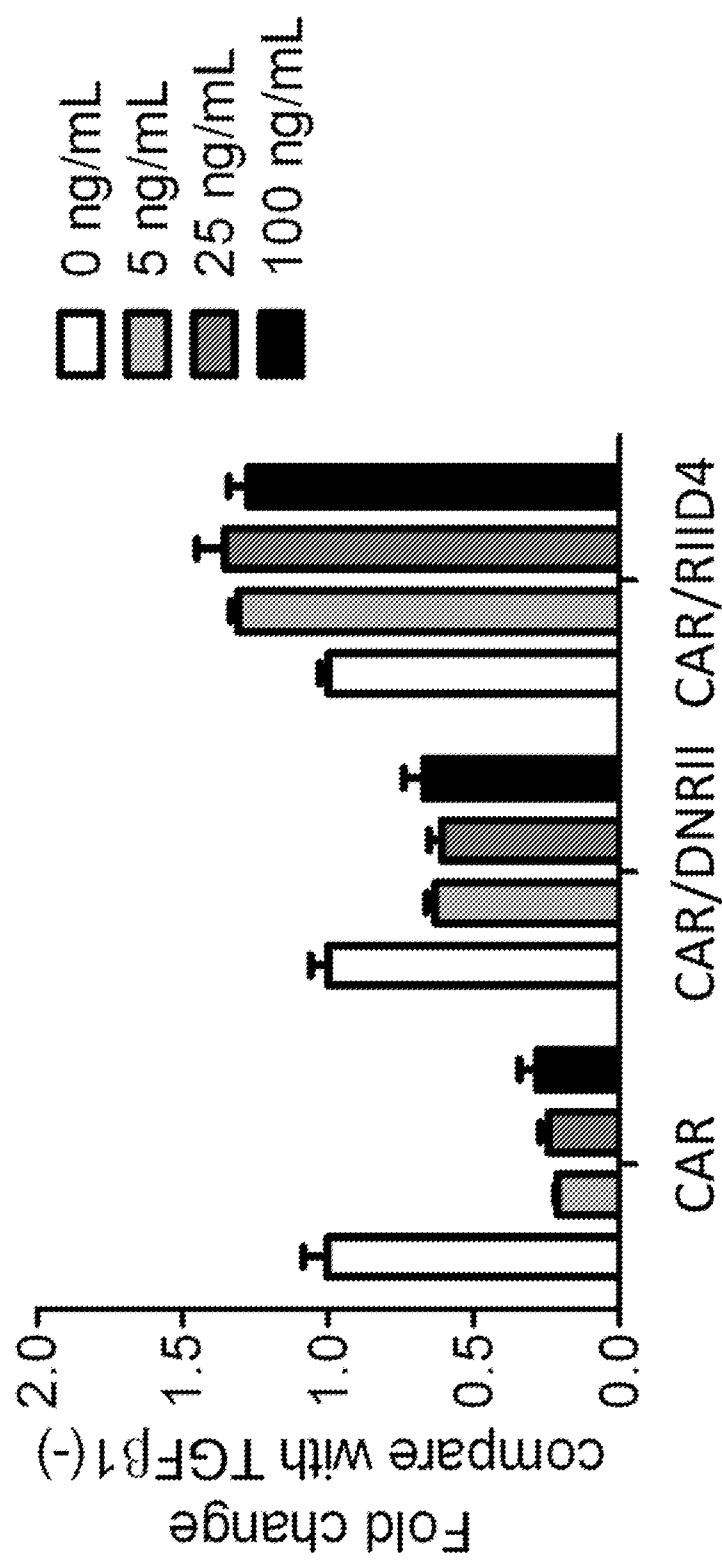


FIG. 24

## INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/034570

A. CLASSIFICATION OF SUBJECT MATTER  
 INV. C07K14/71  
 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)  
 C07K

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, BIOSIS, EMBASE, 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	<p>PERSSON U ET AL: "TRANSFORMING GROWTH FACTOR (TGF-BETA)-SPECIFIC SIGNALING BY CHIMERIC TGF-BETA TYPE II RECEPTOR WITH INTRACELLULAR DOMAIN OF ACTIVIN TYPE IIB RECEPTOR", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 272, no. 34, 22 August 1997 (1997-08-22), pages 21187-21194, XP002934435, ISSN: 0021-9258, DOI: 10.1074/JBC.272.34.21187 the whole document figure 1</p> <p style="text-align: center;">----- -/-</p>	<p>1,9-11, 13, 16-18, 22,58</p>



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

1 August 2014

Date of mailing of the international search report

12/08/2014

Name and mailing address of the ISA/

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 Fax: (+31-70) 340-3016

Authorized officer

Offermann, Stefanie

## INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/034570

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WIESER R ET AL: "SIGNALING ACTIVITY OF TRANSFORMING GROWTH FACTOR SS TYPE II RECEPTORS LACKING SPECIFIC DOMAINS IN THE CYTOPLASMIC REGION", MOLECULAR AND CELLULAR BIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 13, no. 12, 1 December 1993 (1993-12-01), pages 7239-7247, XP000892662, ISSN: 0270-7306 the whole document	1,9-11, 13, 16-18, 22,58
X	WO 2012/138858 A1 (BAYLOR COLLEGE MEDICINE [US]; LEEN ANN MARIE [US]; VERA JUAN F [US]) 11 October 2012 (2012-10-11)	1,9-11, 13-19, 22-27, 29,31, 32, 34-36, 40-42, 46,49,51
Y	claims 1, 7, 1-13; example 2	1,2,4-58
Y	L ZHANG ET AL: "Inhibition of TGF-[beta] signaling in genetically engineered tumor antigen-reactive T cells significantly enhances tumor treatment efficacy", GENE THERAPY, vol. 20, no. 5, 13 September 2012 (2012-09-13), pages 575-580, XP055131633, ISSN: 0969-7128, DOI: 10.1038/gt.2012.75 the whole document	1,2,4-58
Y	LIYING GENG ET AL: "TGF-Beta Suppresses VEGFA-Mediated Angiogenesis in Colon Cancer Metastasis", PLOS ONE, vol. 8, no. 3, 25 March 2013 (2013-03-25), page e59918, XP055131625, DOI: 10.1371/journal.pone.0059918 the whole document	1,2,4-58
Y	S. WILKIE ET AL: "Selective Expansion of Chimeric Antigen Receptor-targeted T-cells with Potent Effector Function using Interleukin-4", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 285, no. 33, 18 June 2010 (2010-06-18), pages 25538-25544, XP055125366, ISSN: 0021-9258, DOI: 10.1074/jbc.M110.127951 the whole document	1,2,4-58
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## INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/034570

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	Norihiro Watanabe ET AL: "Transgenic Expression of a Novel Immunosuppressive Signal Converter on T Cells", Molecular Therapy Volume 21, Supplement 1, May 2013, 1 May 2013 (2013-05-01), pages S153-S153, XP055131645, Retrieved from the Internet: URL: <a href="http://www.nature.com/mt/journal/v21/n1s/pdf/mt201382a.pdf">http://www.nature.com/mt/journal/v21/n1s/pdf/mt201382a.pdf</a> [retrieved on 2014-07-25] -----	
T	ANN M LEEN ET AL: "Reversal of Tumor Immune Inhibition Using a Chimeric Cytokine Receptor", MOLECULAR THERAPY, vol. 22, no. 6, 20 March 2014 (2014-03-20), pages 1211-1220, XP055131822, ISSN: 1525-0016, DOI: 10.1038/mt.2014.47 -----	

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Information on patent family members

International application No

PCT/US2014/034570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2832569 A1	11-10-2012
		CN 103582699 A	12-02-2014
		EP 2694640 A1	12-02-2014
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