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Altering gene expression in CART cells and uses thereof

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ABSTRACT

The present invention relates to compositions and methods for generating a modified T cell with a nucleic acid capable of downregulating endogenous gene expression selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS and further comprising a nucleic acid encoding a modified T cell receptor (TCR) comprising affinity for a surface antigen on a target cell or an electroporated nucleic acid encoding a chimeric antigen receptor (CAR). Also included are methods and pharmaceutical compositions comprising the modified T cell for adoptive therapy and treating a condition, such as an autoimmune disease.

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ALTERING GENE EXPRESSION IN CART CELLS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

The present application is entitled to priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 62/073,651, filed October 31, 2014, which is hereby incorporated by reference in its entirety herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under CA120409 awarded by the National Institute of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Adoptive cell transfer (ACT) using chimeric antigen receptor (CAR) modified T cells has been shown to be a promising strategy for the treatment of cancers (Louis et al., 2011, Blood 118:6050-6056; Kochenderfer et al., 2010, Blood 116:3875-3886 and Porter et al., 2011, N Engl J Med 365:725-733).

Integration associated safety concerns using lentiviral or retroviral vectors are a major concern for modification of cells used for ACT. Some advances have been made to avoid on-target or off-target unwanted side effects, such as RNA transfection of T cells with T cell receptor (TCR) or CAR RNA electroporation (Zhao, 2006, Mol Ther 13:151-159; Mitchell et al., Smits et al., 2004, Leukemia 18:1898-1902). By minimizing dosage of both RNA and T cells, such methods efficiently permit the introduction of multiple genes into cells. However, the major constraint for transient expression of CARs is the suboptimal effector activity and functionality of RNA transfected T cells. Multiple T cell infusions and/or significant use of low dose chemotherapy have been used to improve CAR function (Barrett et al., 2013, Hum Gene Ther 24(8):717-27).

Various attempts have been made to improve effector activity and functionality of CARs while in order to avoid the need for combination therapies and additional treatments. Increasing RNA during the transfection process poses a negative impact on T cell function, especially in vivo anti-tumor activities (Barrett et al., 2011, Hum Gene Ther 22:1575-1586). Alternative constructs fusing an anti-CD3 antigen antibody fragment to an anti-tumor antigen antibody fragment have also been tested in clinical trials for cancer

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treatments (Bargou et al., 2008, Science 321:974-977; Klinger et al., 2012, Blood 119:6226-6233.). Unfortunately, these constructs were severely limited in functionality because of a short half-life, poor accessibility to target cell sites, and a lack of proper long term signaling function.

5 Clinical TCR studies have been hampered by low expression levels of the transduced TCR, as well as mispairing of α and β chains. Because four TCRs can potentially be expressed at the cell surface when a T cell transcribes the chains of two different TCRs (native alpha/beta, exogenous alpha/beta, and native/exogenous “mispaired” heterodimers), significant obstacles to the use of this approach are evident. In studies performed to date, preclinical studies have clearly demonstrated that TCR miss-
10 pairings have the potential to induce harmful recognition of self-antigens.

Although early TCR and CAR T cell clinical data obtained in treating cancers has shown promising results, the risk to the patient is high, and some patients’ T cells are not
15 potent enough for effective treatment even after TCR or CAR redirection, forcing modification of allogeneic donor-derived T cells. However, the endogenous $\alpha\beta$ T-cell receptor on infused allogeneic T cells may recognize major and minor histocompatibility antigens in the recipient, leading to graft- versus-host-disease (GVHD). As a result, the majority of current clinical trials using infusion of autologous CAR T cells rely on
20 immune tolerance to prevent TCR-mediated deleterious recognition of normal tissues after adoptive cell transfer. This approach has achieved early clinical successes but is limited by the time and expense to manufacture patient-specific T-cell products. Therefore a need exists for safer methods of modifying T cells, while circumventing the time and expense to manufacture patient-specific T-cell products.

25 SUMMARY OF THE INVENTION

As described herein, the present invention relates to compositions and methods for generating a modified T cell with a nucleic acid capable of altering gene expression of an endogenous gene selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS and further comprising a
30 nucleic acid encoding a chimeric antigen receptor (CAR).

One aspect of the invention includes a modified T cell comprising a nucleic acid capable of downregulating gene expression of an endogenous gene selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS; and a nucleic acid encoding a chimeric antigen receptor (CAR)

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comprising an antigen binding domain, a transmembrane domain and an intracellular domain of a co-stimulatory molecule.

5 In another aspect, the invention includes a method for generating a modified T cell comprising introducing a nucleic acid capable of downregulating gene expression of an endogenous gene selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS into a T cell; and introducing a nucleic acid encoding a chimeric antigen receptor (CAR) comprising an antigen binding domain, a transmembrane domain.

10 In yet another aspect, the invention includes a method of treating a disease or condition associated with enhanced immunity in a subject comprising administering an effective amount of a pharmaceutical composition comprising the modified T cell described herein to a subject in need thereof.

15 In still another aspect, the invention includes a method of treating a condition in a subject, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising the modified T cell described herein.

In another aspect, the invention includes a method for stimulating a T cell-mediated immune response to a target cell or tissue in a subject comprising administering to a subject an effective amount of a pharmaceutical composition comprising the modified T cell described herein.

20 In yet another aspect, the invention includes a method for adoptive cell transfer therapy comprising administering an effective amount of a pharmaceutical composition comprising the modified T cell described herein to a subject in need thereof to prevent or treat an immune reaction that is adverse to the subject.

25 In still another aspect, the invention includes use of the modified T cell described herein in the manufacture of a medicament for the treatment of an immune response in a subject in need thereof.

In another aspect, the invention includes a composition comprising the modified T cell generated according to the method described herein.

30 In yet another aspect, the invention includes a pharmaceutical composition comprising the modified T cell generated according to the method described herein.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the nucleic acid capable of downregulating gene expression is selected from the group consisting of an antisense RNA, antigomer RNA, siRNA, shRNA, and a CRISPR system, such as an pAd5/F35-CRISPR vector.

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In one embodiment, the the antigen binding domain of the CAR comprises an antibody selected from the group consisting of a monoclonal antibody, a polyclonal antibody, a synthetic antibody, human antibody, humanized antibody, single domain antibody, single chain variable fragment, and antigen-binding fragments thereof. In another embodiment, the antigen binding domain of the CAR specifically binds an antigen on a target cell. In yet another embodiment, the intracellular domain of the CAR comprises dual signaling domains.

In another embodiment, modified T cell described herein further comprises an exogenous nucleic acid encoding a costimulatory molecule, such as CD3, CD27, CD28, CD83, CD86, CD127, 4-1BB, 4-1BBL, PD1 and PD1L. In one embodiment, the method of generating the modified T cell described herein further comprises electroporating a RNA encoding a co-stimulatory molecule into the T cell. In some embodiments where the costimulatory molecule is CD3, the CD3 comprises at least two different CD3 chains, such as CD3 zeta and CD3 epsilon chains.

In another embodiment, the T cell is obtained from the group consisting of peripheral blood mononuclear cells, cord blood cells, a purified population of T cells, and a T cell line.

In yet another embodiment, the method of generating the modified T cell as described herein further comprises expanding the T cell. In one embodiment, expanding the T cell comprises culturing the T cell with a factor selected from the group consisting of flt3-L, IL-1, IL-3 and c-kit ligand.

In still another embodiment, the method of generating the modified T cell as described herein further comprising cryopreserving the T cell. In another embodiment, the method described herein further comprises thawing the cryopreserved T cell prior to introducing the nucleic acid into the T cell.

In one embodiment, introducing the nucleic acid is selected from the group consisting of transducing the expanded T cells, transfecting the expanded T cells, and electroporating the expanded T cells.

In yet another embodiment, the method described herein further comprises expressing Klf4, Oct3/4 and Sox2 in the T cells to induce pluripotency of the T cell.

In various embodiments of the above aspects or any other aspect of the invention delineated herein, the invention includes administering the modified T cell to a subject. In one embodiment, the subject has a condition, such as an autoimmune disease. In some embodiments, the autoimmune disease is selected from the group consisting of Acquired

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5 Immunodeficiency Syndrome (AIDS), alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease (AIED), autoimmune lymphoproliferative syndrome (ALPS), autoimmune thrombocytopenic purpura (ATP), Behcet's disease, cardiomyopathy, celiac sprue-dermatitis hepeticiformis; chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy (CIPD), cicatricial pemphigoid, cold agglutinin disease, crest syndrome, Crohn's disease, Degos' disease, dermatomyositis-juvenile, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, Graves' disease, Guillain-Barre syndrome, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin-dependent diabetes mellitus, juvenile chronic arthritis (Still's disease), juvenile rheumatoid arthritis, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, pernicious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynaud's phenomena, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma (progressive systemic sclerosis (PSS), also known as systemic sclerosis (SS)), Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, Takayasu arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis, vitiligo, Wegener's granulomatosis, and any combination thereof.

15 In another embodiment, the condition is a cancer, such as a cancer selected from the group consisting of breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, and any combination thereof.

20 In another embodiment, the method described herein further comprises inducing lysis, such as antibody-dependent cell-mediated cytotoxicity (ADCC), of the target cell or tissue.

30 **BRIEF DESCRIPTION OF THE DRAWINGS**

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not

limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figures 1A-1C, is an illustration of the CRISPR design and targeting of the TCR $\alpha\beta$ -CD3 complex in 293T cells. Figure 1A shows the CRISPR gRNA targeting sites within the genomic locus of TCR- α and β constant region. Each exon is shown by a block. Black blocks represent coding regions. Grey columns represent non-coding regions. Thirteen gRNAs were designed to target exon 1 of the TCR α constant region (TRAC), 10 gRNAs target a conserved sequence on exon 1 of the TCR β constant regions 1 (TRBC1) and 2 (TRBC2), and 10 gRNAs target exon 1 of the beta-2 microglobulin gene. Figure 1B shows a typical gRNA scaffold sequence. gRNA PCR products were generated by overlap PCR and cloned into MSGV vector with a T7 promoter. Figure 1C shows Sanger sequencing results showing that multiple peaks exist in 293T TCR TRAC and TRBC genomic PCR products after transfection of CAS9 mRNA and gRNAs into the cells.

Figure 2, comprising Figures 2A-2E, shows the disruption of the TCR $\alpha\beta$ -CD3 complex in primary T cells. Figure 2A is a table showing the parameters used for electroporating CAS9 mRNA and gRNA into primary T cells with BTX830. 360V 1ms with 2mm cuvettes yielded the best mean fluorescent intensity (MFI) and efficiency for electroporating day3 beads stimulated primary T cells. Figure 2B is a panel of graphs showing T cells incubated at 32°C 5% CO₂ having a much higher MFI than normal 37°C 5% CO₂ condition. Figure 2C is a schematic illustration of the CRISPR system transferred into primary T cells. CAS9 mRNA and gRNA were electro-transferred into T cells three days after bead stimulation of primary T cells. T cells were then cultured with 100 IU/mL of IL-2 and some cells were incubated at 32°C 5% CO₂ for 1 day and then for another 7 to 9 days. CD3 expression was analyzed on days 7-9 after electroporation by flow cytometry. Figure 2D is a panel of graphs showing that the targeting efficiency at 37°C was about 2.5 times higher than at 32°C. Figure 2E is a panel of graphs showing down regulation of CD3 on day 6 after electro-transfer of varying amounts and ratios of CAS9 and gRNA targeting TCR β . CD3 expression was analyzed by staining for CD3. The representative flow data at day 6 after electroporation is shown. Quadrant represent the percentage of CD3 negative cells in T-cell populations.

Figure 3, comprising Figures 3A-3D, shows that TCR^{neg} alpha or beta knock out in T cells can be enriched by depletion of TCR^{pos} T cells. Figure 3A is a panel of graphs showing CD3 expression before and after micro-bead depletion of TCR^{neg} alpha or beta

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knock out in T cells. Flow cytometry illustrates expression of CD3. Numbers in the lower right quadrant represent the percentage of CD3 negative cells in T-cell populations. Figure 3B is a panel of sequencing graphs showing that multiple peaks were observed in CD3^{neg} enriched T cell genomic PCR products. Figure 3C is a panel of graphs showing the CD4 and CD8 T cell repertoire analysis after CD3 micro-bead enrichment in single alpha chain, beta chain, and alpha beta double knock out T cells modified with CRISPR. Data shows the ratio of CD8 T cell population was enriched by CRISPR modification, suggesting CD8 T cell may be more easily modified than CD4 T cells. Figure 3D shows sequencing results of deletions and insertions introduced to the TCR alpha and beta locus after CRISPR modification.

Figure 4, comprising Figures 4A-4C, shows that multiple electro-transfers of gRNA greatly improved the targeting efficiency of CRISPR system in primary T cells. Figure 4A is a panel of graphs showing that multiple electroporations of gRNAs greatly improved the targeting efficiency. Electroporating T cells up to three times within 24 hours gave the highest targeting efficiency, nearly 80%. In the initial experiment, only a 15% percent of TCR targeting efficiency was achieved in T cells. Sustained expression of CAS9 was observed after electro-transfer of CAS9 mRNA into the T cells. A likely reason for low cleavage efficiency may be due to rapid degradation of gRNAs. A higher CD3 negative population was obtained. Figure 4B is a panel of graphs showing that capping impairs the function of gRNA, while early introduction of gRNAs in a second round yielded higher efficiencies. Figure 4C is a panel of graphs showing that multiple electro-transfers of gRNA targeting TRAC and TRBC in ND221 gave a cleavage rate of approximately 64.5% and 57.5%, respectively.

Figure 5, comprising Figures 5A and 5B, shows TCR^{neg} T cells could be expanded under different stimulating conditions. Figure 5A is a panel of graphs showing that TCR^{neg} T cells restored CD3 expression after re-introduction of TCR alpha and beta chains into TCR^{neg} T cells. CD3 and Vb13.1 were detected after electroporating TCR alpha and beta chain into TCR^{neg} T cells. CD3 expression level was comparable to TCR^{pos} T cells. Figure 5B is a panel of graphs showing fold of expansion after different conditions used to stimulate TCR^{neg} T cells. PBMC REP yielded approximately 500 fold expansion, while CD3/CD28 beads, or K562 aAPC re-stimulation yielded about 25-58 fold expansion.

Figure 6, comprising Figures 6A and 6B, shows TCR^{neg} T cell characteristics after expansion under different conditions. Figure 6A is a panel of graphs showing TCR^{neg} T

cell phenotype characteristics after expansion under different conditions. Figure 6B is a panel of graphs showing TCR^{neg} T cell phenotype characteristics after expansion under different conditions.

Figure 7, comprising Figures 7A-7C, shows expanded TCR^{neg} T cells with potent anti-tumor activity after re-direction in vitro. Figure 7A is a panel of graphs showing that TCR^{neg} T cells could be re-directed by introduction of an anti NY-ESO 1G4 TCR in the cells. Compared with CAS9 MOCK group, when re-directed by 1G4 TCR, TCR^{neg} T cells showed a higher level of Vb13.1 expression due to less miss-pairing of exo and endogenous TCR alpha and beta chains. Figure 7B is a panel of graphs showing that TCR^{neg} T cells re-directed with 1G4 TCR had high de-granulation activity when cocultured with a tumor (Nalm6-ESO) cell line. Figure 7C is a graph showing that TCR^{neg} T cells re-directed with 1G4 TCR had high cytotoxicity against a tumor cell line.

Figure 8 is a panel of illustrations showing that directed TCR^{neg} T cells control the growth of tumor in NSG mice after re-direction.

Figure 9, comprising Figure 9A-9D, shows that HLA-CLASS I elimination was obtained by disruption of beta-2 microglobin. Figure 9A shows sequencing data of CRISPRs able to disrupt the beta-2 microglobin locus in HEK293 cells. Figure 9B is a panel of graphs showing that bHLA-CLASS I negative T cell population was generated by disruption of beta-2 microglobin. Figure 9C is a panel of graphs showing that cIFN γ improved the targeting efficiency of beta-2 microglobin in primary T cells. Figure 9D is a panel of graphs showing that HLA-CLASS I^{neg} T cells were enriched by microbead depletion.

Figure 10 is a panel of graphs showing simultaneous knock out of HLA-CLASS I and TCR in primary T cells. CD4 and CD8 T cells were stimulated with CD3/CD28 dynabeads. Three days after stimulation, expanded T cells were electroporated with CAS9 mRNA together with TCR β constant region (TRBC) and beta-2 microglobin targeting gRNAs. Both TCR expression and beta-2 microglobin expression were evaluated using anti-CD3 monoclonal antibody (mAb) and anti-beta-2 microglobin mAb six days after electroporation. Numbers represent the percentage of population in each quadrant.

Figure 11, comprising Figures 11A-11D, shows triple knock out of HLA-CLASS I and TCR alpha and beta chain in primary T cells. Figure 11A is a panel of graphs showing that CD4 and CD8 T cells were stimulated with CD3/CD28 dynabeads. Three days after stimulation, expanded T cells were electroporated with CAS9 mRNA, together

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with TCR alpha, beta constant region (TRAC,TRBC) and beta-2 microglobulin targeting gRNAs. Both TCR expression and HLA-CLASS I expression were evaluated using anti-CD3 monoclonal antibody (mAb) and anti-beta-2 microglobulin mAb six days after electroporation. Numbers represent the percentage of population in each quadrant. Figure 11B is a schematic illustrating isolation of HLA-CLASS I and TCR alpha and beta chain triple knock out T cells. Figure 11C is a panel of graphs showing electroporation efficiency tested by GFP expression. Figure 11D is a panel of graphs showing re-introduction of TCR alpha and beta chains into TCR^{neg} T cells measured by flow cytometry. About 64% of alpha negative and about 14% beta negative population was observed in total TCR^{neg} T cells.

Figure 12, comprising Figures 12A-12D, shows knock out of FAS in 293T cells. Figure 12A is an image showing Sanger sequencing results of multiple peaks when FAS was knocked out in 293T cells. Figure 12B is a panel of graphs showing FACS data revealing surface expression of FAS protein was disrupted by CRISPRs. Figure 12C is a panel of images showing FAS protein was replaced by GFP after homologous recombination with CRISPRs. Figure 12D is a panel of graphs of FACS data showing the percentage of homologous recombinations with CRISPRs.

Figure 13 shows knock out of FAS in primary T cells. FACS data illustrated that surface FAS protein expression was abolished by CRISPRs.

Figure 14, comprising Figures 14A and 14B, shows knock out of PD1 in 293T and primary T cells. Figure 14A is an image showing Sanger sequencing results of multiple peaks when PD1 were targeted in 293T cells. Figure 14B is a panel of graphs showing FACS data of surface expression of PD1 protein disrupted by CRISPRs.

Figure 15, comprising Figures 15A and 15B, shows knock out of CTLA4 in 293T and primary cells, such as CCD1079-SK. Figure 15A is an image showing Sanger sequencing results of multiple peaks when CTLA4 were targeted in 293T cells. Figure 15B is an image showing sequence data after limiting dilution and single cell expansion. Sanger sequencing results identified the deletions and insertions at the CTLA4 genomic locus.

Figure 16 shows knock out of PPP2r2d in 293T. Sanger sequencing data indicated PPP2r2d was targeted in 293T cells by CRISPRs.

Figure 17, comprising Figures 17A and 17B, shows generation of iPSCs from FAS knock out T cells. Figure 17A is a panel of images showing morphological change during the process of reprogramming FAS^{neg} T cells to iPSCs. Typical embryonic stem

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cell morphology formation indicating FAS^{neg} T cells can be induced to pluripotent state. Figure 17B is a graph showing that FAS^{neg} T cells were reprogrammed to iPSCs at an efficiency of about 5 times of the wild type counterparts. p53 deficient cell lines have been reported as easier to reprogram due to the hinderance of the apoptosis pathway. FAS knock out may facilitate the reprogramming process by a similar mechanism.

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Figure 18, comprising Figure 18A and 18B, show the generation of iPSCs from CD3^{neg} T cells. Figure 18A is a panel of images showing ES-like morphology formed by CD3^{neg} TCR alpha or beta chain knock out T cells under defined reprogramming conditions. The morphology remains constant after several passages. Figure 18B is a series of graphs showing that reprogramming CD3^{neg} T cells was about 5 time more efficient than the wild type counterparts, suggesting that TCR knock-out may play a role in the process of T cell reprogramming or affect the cell viability after Sendai virus infection.

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Figure 19 is a graph showing knockdown of endogenous T cell receptors (TCRs) with siRNA and adding a second disulfide bond and de-N-glycosylation to the beta chain.

Figure 20, comprising Figures 20A and 20B, shows TCR knockout by CAS9 RNA and gRNA. Six days after electroporation, cells were analyzed for TCR expression by assessing CD3.

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Figure 21 is an illustration showing PCR sequencing results after CD3 micro-bead depletion.

Figure 22 is a panel of graphs showing re-expression of CD3 four hours after NY-ESO-1 TCR RNA electroporation.

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Figure 23, comprising Figures 23A-23D, is a panel of graphs showing that knocking down endogenous TCR enhanced both transgene expression and function of TCR RNA electroporated T cells. Figure 23A shows TCR expression of T cells electroporated with TCR siRNA (solid open histogram), control siRNA (dotted open histogram) and T cells without any siRNA (filled histogram). Figure 23B shows transgene (TCR vb13.1) expression of wild type NY-ESO-1 TCR (wt) or modified TCR (SD) RNA electroporated T cells with TCR siRNA, control siRNA, or no siRNA. Figure 23C shows NY-ESO-1 tetramer staining of wild type NY-ESO-1 TCR (wt) or modified TCR (SD) RNA electroporated T cells with TCR siRNA, control siRNA, or no siRNA. Figure 23D shows specific lysis of a HLA-A2/NY-ESO-1 positive tumor line by TCR siRNA knockdown, wildtype NY-ESO-1 TCR RNA electroporated T cells.

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5 Figure 24 is a graph showing fluorescence of tumor cells after injection of T cells into a mouse model. Ten million Nalm6-CBG-ESO-GFP (click beetle green) tumor cells that expressed both NY-ESO-1 and GFP were intravenously injected into NOD/SCID mice. Five days after tumor inoculation, CBR transduced and RNA electroporated T cells were injected as indicated in the different groups and tumor cells were detected by fluorescence.

Figure 25 is a panel of images showing fluorescence of injected tumor and hybrid TCR T cells in mouse models over time.

10 Figure 26 is a panel of images showing the generation of universal CAR19 T cells. The top of the figure is an illustration of the protocol to generate the universal CAR19 T cells. The graph on the left shows the percentage of CAR19 positive T cells after lentiviral-CAR19 gene transduction. The right panel of graphs shows the percentage of TCR single negative and TCR/HLA-A double negative T cells before and after sorting.

15 Figure 27 is a panel of graphs and a table showing fold expansion of CD19 positive cells after stimulation with irradiated CD19 presenting K562 cells.

Figure 28A is a panel of graphs showing the endogenous and transgenic gene expression of K562-CD19 expanded cells.

20 Figure 28B is a panel of graphs showing that endogenous TCR expression remained negative in TCR single negative cells, while TCR and HLA-A expression remained negative in TCR/HLA-A double negative T cells after K562-CD19 stimulated expansion

Figure 29A is a panel of graphs showing that the majority of expanded universal CAR19 T cells are CD45RO positive and expressed medium levels of CD28 expression.

25 Figure 29B is a panel of graphs showing that the majority of expanded universal CAR19 T cells retained high levels of CD62L expression and low levels of CCR7 expression.

Figure 30A is a graph showing that CRISPR gene editing did not affect the anti-tumor activity of universal CAR19 T cells in vitro.

30 Figure 30B is a panel of graphs showing that the TCR single and TCR/HLA-A double negative CAR19 T cells showed robust lytic capacity when challenged with Nalm6 tumor cells.

Figure 30C is a panel of graphs showing cytokine secretion as part of the potent anti-tumor activity of these cells.

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Figure 30D is a panel of graphs showing TCR single ablation or TCR and HLA-A double ablation in CAR19 T cells that exhibited similar proliferation kinetics after challenge with Nalm6 tumor cells.

Figure 31 is a panel of images showing that CRISPR gene editing did not affect the anti-tumor activity of universal CAR19 T cells *in vivo*. All the mice receiving unmanipulated T cells and mice infused with lentiviral GFP transduced wild type T cells died within 3 weeks after tumor cell infusion. Objective tumor regression was observed for mice receiving CAR19 T cells. CRISPR edited TCR single or TCR/HLA-A double negative universal CAR19 T cells showed the same anti-tumor activity.

Figure 32A is a panel of graphs showing TCR single or TCR and HLA-A double ablation in T cells sharply reduced alloreactivity.

Figure 32B is a panel of graphs showing elimination of HLA-A molecule activated NK cells with a long period of co-culture (5 days).

Figure 32C is a graph showing that no off-target activity was observed when the cells were challenged by allogeneic whole blood PBMC for 24 hours in an IFN γ Eispot assay.

Figure 33 is a panel of graphs showing that FAS ablation enhanced the anti-tumor activity of CAR19 T cells. FAS negative CAR19 T cells were generated. FAS ablation was confirmed by flow cytometry analysis. CAR19 gene expression of FASneg T cells was comparable to the wild type. Even after incubation with Nalm6 tumor cells for a short period of 4 hours, CD107a expression was greatly enhanced in FASneg CAR19 T cells compared the wild type counterpart.

Figure 34A is a graph showing that FAS ablation in CAR19 T cells enhanced CART cell survival and proliferation under *in vitro* antigenic conditions. FASneg CAR19 T cells expanded faster than wild type CAR19 T cells when the cells were stimulated with high levels of CD19+ K562 cells.

Figure 34B is a panel of graphs showing FASneg CAR19 T cells had reduced apoptosis levels as measured by Annexin V staining.

Figure 35A is a graph showing that FAS ablation in CAR19 T cells enhanced CART cell function in an animal model. As had been observed *in vitro*, FASneg T cells showed enhanced proliferation as compared to the wild type T cells.

Figure 35B is a panel of images showing FASneg CAR19 group demonstrated superior anti-tumor activity when compared to wild type group.

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Figure 35C is a graph showing significant differences in bioluminescence data between FASneg CAR19 group and wild type group.

Figure 36 is a panel of graphs showing the generation of PD1 negative PSCA-CAR T cells. PD1 ablation was confirmed by flow cytometric analysis. PD1 negative cells were enriched by microbead depletion. Wild type or PD1 negative PSCA-CAR T cells were expanded by stimulation with irradiated PSCA antigen presenting PC3 tumor cells. PSCA-CAR positive cells were enriched after expansion.

Figure 37 is a panel of graphs showing that PD1 ablation and CD137 expression in PSCA-CAR T cells enhanced CART cell activation under in vitro antigenic conditions.

Figure 38A is a panel of images showing PD1 ablation in an in vivo PC3-PSCA-PDL1 NSG model. The PSCA-CAR T cells demonstrated enhanced CART cell in vivo anti-tumor activity as compared to wild type group.

Figure 38B is a graph showing the difference in tumor burden between the PD1 negative and the wild type group.

Figure 39 is a panel of histological images showing that T cells with TCR or TCR/HLA-I ablated did not cause graft versus host disease (GVHD). The mice treated with the double or triple knock out CART cells did not develop any signs of GVHD. By contrast, 3 out of 4 mice from the wild-type CD19 CART group developed GVHD by day 65, which was confirmed by histological examination of different organs.

Figure 40A is a graph showing the percent survival of animals injected with T cells with TCR or TCR/HLA-I ablated. Mice were sub-lethally irradiated and injected. Four out 5 mice receiving wild type T cells died of GVHD during the 60 day study. PBS treated, TCR single and TCR/HLA-I double ablated T cell treated groups did not show any signs of GVHD.

Figure 40B is a panel of graphs showing the body weight of mice receiving wild type T cells, PBS treated, TCR single or TCR/HLA-I double-ablated T cells.

Figure 41A is a panel of images showing improved anti-tumor activity of universal CART cells after blocking PD1 and Fas pathways with CRISPR/Cas9. Superior anti-tumor activity was detected in PD1 knock out universal CD19-CART cells when injected into Nalm6-PDL1 bearing mice.

Figure 41B is a graph showing quantitative bioluminescence data of mice receiving different CRISPR/Cas9 edited T cells.

Figure 42 is a panel of illustrations showing a one-shot system to generate universal CART cells. As gRNAs are prone to degrade, a simplified one-shot method

was developed to constitutively express gRNAs together with CAR in a single lentiviral vector.

Figure 43 is a panel of graphs showing efficient gene ablation with the one-shot system. Different amounts of CD3 ablation were observed after electro-transfer of Cas9 mRNA.

Figure 44A is a panel of images showing the morphological changes during the process of reprogramming of iPSCs from Fas knock out T cells. Typical embryonic stem cell morphology formation indicating FASneg Tcells can be induced to pluripotent state.

Figure 44B is a graph showing FASneg T cells reprogrammed to iPSCs at an efficiency of about 5 times of the wild type counterparts. p53 deficient cell lines have been reported as easier to reprogram due to the hindrance of the apoptosis pathway. FAS knock out may facilitate the reprogramming process using a similar mechanism.

Figure 45A is a panel of images showing the ES-like morphology of iPSCs from CD3neg TCR alpha or beta chain knock out T cells under defined reprogramming conditions. The morphology remained constant after several passages.

Figure 45B is a graph showing that reprogramming of CD3neg T cells was about 5 times less efficient than the wild type counterparts, suggesting that TCR knock-out may play a role in the process of T cell reprogramming or affect the cell viability after Sendai virus infection.

Figure 45C is a panel of images showing phosphatase staining of CD3neg iPSC cells.

Figure 46 is a panel of graphs showing induction of endogenous pluripotent stem cell genes in different T-iPSC cell lines.

Figure 47A is a panel of images showing immunostaining for Tra-1-60 and SSEA4 expression.

Figure 47B is an image showing the confirmation of Fas knock out of T-iPSC by Sanger sequencing.

Figure 48A is a panel of graphs showing gene ablation in naïve T cells with a different version of Cas9. CD3 was knocked out with dCas9 and FokI-Cas9.

Figure 48B is a panel of graphs showing that two gRNAs were needed for gene ablation of dCas9 and FokI-Cas9.

Figure 48C is in image showing rare off-target events in gene modified T cells with CRISPR/cas9.

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Figure 49 is a panel of images showing the strategy of introducing CRISPR/Cas9 into T cells. Schematic representation of gRNAs driven by the T7 promoter is shown on the left. Schematic representation of the generation of gene-edited antigen-specific T cells using the CRISPR system is shown on the right. T cells were electroporated with Cas9 mRNA and gRNAs targeting a specific gene 3 days after CD3/CD28 bead stimulation and then cultured for 24 hours at 32°C in the presence of IL2 before being returned to the normal 37°C culture condition. Specific gene-disrupted T cells were sorted on day 8 and redirected with CAR or TCR by lentiviral transduction or mRNA electroporation gene transfer.

Figure 50A is a panel of graphs showing CRISPR/Cas9 mediated efficient TCR disruption in T cells. CD3 expression of CRISPR/Cas9 edited T cells cultured at 37°C or 32°C.

Figure 50B is a panel of graphs showing CD3 expression of CRISPR/Cas9 edited T cells cultured after sequential CRISPR RNA electroporation.

Figure 51A is a panel of graphs showing the efficient CRISPR gene disruption that occurred in T cells. CD3 expression of T cells transferred with CRISPR using different Cas9:gRNA ratios (upper and middle panel) and amount of total CRISPR RNA (lower panel).

Figure 51B is a table showing the targeting efficiency calculated by both flow cytometry and clonal sequencing.

Figure 52 is an image showing the amount of TCR-targeted gene disruption measured by a mismatch-selective T7 surveyor nuclease assay on DNA amplified from the cells. The calculated amount of targeted gene disruption in TRAC and TRBC is shown at the bottom. Arrows indicate expected bands.

Figure 53A is an image of indels (in gene disruption) observed by clonal sequence analysis of PCR amplicons after CRISPR-mediated recombination of the TCR α and β locus.

Figure 53B is an image of a diagram of the human locus encoding the TCR α and β CRISPR gRNA targeting sites within the genomic locus of the TCR α and β constant region. Each exon is shown by a block. Arrow: sense strand gRNA targeting site; blue arrow: anti-sense strand gRNA targeting site. Multiple peaks in the Sanger sequencing results show the CRISPR-mediated events of NHEJ at the TRAC and TRBC genomic loci.

Figure 54 is a panel of graphs showing CD3 expression in purified TCR^{neg} cells.

Figure 55 is a panel of graphs showing redirection of TCR/CD3^{neg} cells via the electrotransfer of 1G4 TCR (α and β) or CAR19 mRNA.

Figure 56 is a graph showing TCR/CD3^{neg} cell expansion after 10 days using different stimulation conditions.

Figure 57 is a panel of graphs showing that CRISPR/Cas9 editing did not impair antitumor efficacy of primary T cells. The phenotypes of TCR/CD3^{neg} cells after the four different expansion techniques are shown.

Figure 58 is a panel of graphs showing the relative CD19-CAR expression after electrotransfer of CD19-CAR RNA into Cas9 MOCK and TCR/CD3^{neg} cells.

Figure 59A is a panel of graphs showing that no significant functional difference was observed between CD19-CAR redirected Cas9 MOCK and TCR/CD3^{neg} cells as confirmed by CD107 release assay after incubation with Nalm6 target cells.

Representative data from 3 independent experiments are shown. Bars, standard error.

Figure 59B is a graph showing that no significant functional difference was observed between CD19-CAR redirected Cas9 MOCK and TCR/CD3^{neg} cells as confirmed by cytotoxicity assay after incubation with Nalm6 target cells. Representative data from 3 independent experiments are shown. Bars, SE=standard error.

Figure 59C is a panel of graphs showing that no significant functional difference was observed between CD19-CAR redirected Cas9 MOCK and TCR/CD3^{neg} cells as confirmed by IL2 and IFN γ secretion after incubation with the Nalm6 target cells. Representative data from 3 independent experiments are shown. Bars, SE=standard error.

Figure 59D is a panel of images of NOD/scid/ $\gamma c(-/-)$ mice ($n=12$) injected with 1×10^6 Nalm6 tumor cells (i.v.) the mice were randomly sorted into three groups. Cas9 MOCK and TCR/CD3^{neg} T cells (10×10^6) expressing the CD19-CAR after electroporation were injected i.v. every 4 days for a total of three injections (arrows). Mice treated with T cells electroporated with no RNA served as controls. Images were obtained from the surviving animals as indicated. Imaging commenced 1 day before the start of T cell treatment. Bars, SE=standard error, EP=electroporation; E:T=effector to tumor ratio; arrow, time point of T cell infusion; ns, not significant. **** $P < 0.0001$, ns by two-way ANOVA plus the Bonferroni post test.

Figure 59E is a graph showing the radiance of the fluorescent cells.

Figure 60 is a panel of graphs showing double and triple gene ablation by CRISPR/Cas9 to generate universal effector cells. HLA-I disruption with gRNA targeting B2M.

Figure 61 is a flow chart of the protocol to generate universal effector cells as described herein.

Figure 62 is a panel of graphs showing that TCR ablation abrogated non-specific killing activity. 624mel-CBG and PC3-CBG tumor cell lines were incubated with T cells pre-treated with or without PHA at an effector to target ratio of 20:1 for 24 hours and cytotoxicity was calculated based on a luciferase assay. Data are the means \pm SD; n=3.

Figure 63 is a panel of graphs showing an IFN γ Elispot assay to measure allo-reactivity of TCR and TCR/HLA disruption by challenging the gene-ablated T cells with irradiated allogenic PBMCs (left panel) or co-culturing allogenic PBMCs with irradiated gene-ablated T cells. Specific spots are shown on the y axis as the spots produced in the presence of stimulators minus the spots produced by the effectors alone. **P<0.01 by Mann-Whitney test.

Figure 64 is a panel of graphs showing that the disruption of the endogenous TCR by CRISPR/Cas9 improved TCR-redirected T cell function. Vb13.1 and CD3 expression is shown in T cells transfected with Cas9 mRNA alone (Cas9 Mock) or CD3^{neg} T cells with disrupted endogenous TCR α alone (α KO), β alone (β KO), α and β double ($\alpha+\beta$ KO) that were electroporated with NY-ESO-1 TCR α (1G4 α ,2ug), β (1G4 β ,2ug) or $\alpha+\beta$ RNA (1G4 $\alpha+\beta$,2+2ug) RNA.

Figure 65A is a panel of graphs showing CD107a up-regulation of the TCR (1G4) α/β RNA electroporated TCR α or β single knockout or $\alpha+\beta$ double knockout T cells stimulated with a HLA-A2/NY-ESO-1-positive cell line (Nalm6-ESO) or the control cell line Nalm6.

Figure 65B is a graphs showing the lytic ability of TCR $\alpha+\beta$ RNA (1G4 TCR) electroporated TCR α or β single-knockout or $\alpha+\beta$ double-knockout T cells shown in (a) in a luciferase-based CTL assay against Nalm6-ESO.

Figure 66 is a panel of graphs showing Vbeta and CD3 expression in TCR $\alpha+\beta$ double-disrupted T cells (TCR^{neg} T cells) electroporated with two different NY-ESO-1 TCR RNA (1G4 TCR,10ug or 8F TCR,10ug) compared with control Cas9 Mock T cells.

Figure 67A is a panel of graphs showing CD107a up-regulation in NY-ESO-1 TCR (1G4 TCR or 8F TCR) RNA electroporated TCR double-knockout CD8⁺ T cells stimulated with the HLA-A2/NY-ESO-1-positive cell lines Nalm6-ESO, 624-mel or U266. Nalm6 was used as the negative control.

Figure 67B is a panel of graphs showing cytokine production (IL-2 and TNF- α) of NY-ESO-1 TCR (1G4 TCR or 8F TCR) RNA electroporated TCR double-knockout T

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cells after stimulation with the HLA-A2/NY-ESO-1-positive cell lines Nalm6-ESO or U266; 888mel melanoma cells were used as a negative control. * $P < 0.05$, ** $P < 0.01$ **** $P < 0.0001$, by two-way ANOVA plus the Bonferroni post test.

Figure 68 is a panel of images showing the generation of universal CART cells with a combination of lentiviral gene transfer and CRISPR/Cas9 electroporation. A flow chart of the generation of universal CD19-CART cells is shown. T cells were transduced with lentiviral CD19-CAR on day 1 after stimulation, and Cas9 mRNA and gRNAs targeting the TCR α and TCR β chains and B2M were electroporated in the T cells 2 days later. The TCR and HLA-I double-negative cell population was enriched before re-simulation for expansion.

Figure 69 is a panel of graphs showing CD19-CAR expression in gene-modified lenti-CD19-CAR T cells expanded by CD3/CD28 bead stimulation after 1G4 TCR electroporation.

Figure 70 is a panel of graphs showing the phenotype of CD19-CAR T cells.

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Figure 71 is a graph showing CD107a release in TCR-negative and TCR/HLA-I double-negative CD19-CAR T cells. Representative data from 3 independent experiments are shown. Bars, SE=standard error.

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Figure 72 is a panel of graphs showing cytokine secretion of TCR-negative and TCR/HLA-I double-negative CD19-CAR T cells. Representative data from 3 independent experiments are shown. Bars, SE=standard error.

Figure 73 is a graph showing tumor lytic capability of TCR-negative and TCR/HLA-I double-negative CD19-CAR T cells. Representative data from 3 independent experiments are shown. Bars, SE=standard error.

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Figure 74 a panel of graphs showing CFSE-labeled CD19-CAR and non-transduced T cells incubated with K562 and target K562-CD19 tumor cells at a ratio of 1 to 10 for 72 hours.

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Figure 75A is a graph showing BLI from mice treated with a single injection on day 7 expressing CD19-CAR and GFP using a lentiviral vector. ns, no difference by two-way ANOVA plus the Bonferroni post test. Tumors were established in NSG mice ($n=4$ per group) by i.v. injection of 1×10^6 Nalm6 cells. Beginning on day 7, T cells (1×10^7) expressing lentiviral (LV) transduced CD19-CAR were infused with a single injection. T cells expressing LV GFP protein were injected as controls.

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Figure 75B is a graph showing the overall survival of mice receiving LV-GFP, LV-CD19-CAR, LV-CD19-CAR-TCR/CD3^{neg} and LV-CD19-CAR-TCR/HLA-I^{neg} T cells. ns, no difference by the log-rank Mantel-Cox test.

Figure 76 is a panel of images showing that gene-modified CAR T cells retained antitumor efficacy and did not induce GVHD. Tumors were established in NSG mice ($n=4$ per group) by i.v. injection with 1×10^6 Nalm6 cells. Beginning on day 7, T cells (2×10^7) expressing LV-CD19-CAR were infused with a single injection. T cells expressing LV GFP protein were injected as controls. Imaging commenced 1 day before T cell treatment. Organs of randomly chosen mice from different treatment groups were collected on day 65 and used for CD3 immunohistochemistry staining. Figure 77 is a series of schematics of vectors showing the design of pAd5F35-CRISPR targeting PD1, Fas and TCR alpha chain.

Figure 78 is an illustration showing the design of penton modified pAd5F35-CRISPR with anti-CD3 ScFv, and schematic delivery of pAd5F35-CRISPR for knock in/out chimeric antigen receptor into T cells in vitro and in vivo.

Figure 79A is a graph showing Sanger sequencing of PCR products flanking PD1-gRNA targeting site. Adenoviral-pAd5F35-CRISPR-PD1 virus was transduced into MD231 cells. 3 days later, genomic DNA was extracted and performed PCR.

Figure 79B shows the sequences of the targeting events in MDA231 cells after Adenoviral-CRISPR manipulation. PD1 PCR products were cloned into TOPO vector and sequenced.

Figure 80 is a chart showing that a decrease in gRNA use improved T cell fold expansion and only slightly decreased knockout efficiency.

Figure 81 is a chart showing the parameters used for optimizing electroporation conditions to obtain high CD3/B2M knockout efficiency with improved T cell fold expansion. Compared with standard electroporation (EP) conditions in a 2mm cuvette (EP#10-13) or 4mm cuvette. High CD3/B2M knockout efficiency was observed with improved T cell fold expansion (EP#1 and 5).

Figure 82 is a chart showing optimization of EP conditions to achieve maximum fold expansion with tolerable knockout efficiency.

Figure 83 is a chart showing additional optimization of EP conditions to achieve maximum fold expansion with tolerable knockout efficiency.

Figure 84 diagrams the T cell stimulation, lentiviral transduction and CRISPR electroporation procedure.

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Figure 85 is a chart showing T cell numbers (upper chart) and fold expansion (lower chart) after the electroporation and culturing procedure.

Figure 86 is a panel of graphs showing the average expansion of T cells. Fold expansion of the T cells transduced with CD19 CAR alone (TD alone) or transduced with CD19 CAR and edited with CRISPR (TD/KO) (left graph). Fold expansion of the T cells on day 10 is shown in the right graph.

Figure 87 is a panel of flow graphs showing CD3/B2M/CAR expression at day 8 of expanded T cells.

Figure 88 is a panel of graphs showing CD3/B2M expression after CD3+ T cell depletion.

Figure 89 is a panel of graphs showing CD3/B2M expression on day 11 in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells. ND463 non-transduced (NOTD) were used as a negative control. Figure 90 is a panel of graphs showing CD19 CAR expression on day 11 in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells. ND463 non-transduced (NOTD) were used as a negative control.

Figure 91 is a panel of graphs showing CD3/B2M/CAR expression on day 11 in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells. ND463 non-transduced (NOTD) were used as a negative control.

Figure 92 is a chart summarizing CD3/B2M/CAR expression in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells.

Figure 93 is a panel of graphs showing CD107a up-regulation in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells.

Figure 94 is a panel of graphs showing lytic activity of the T cells on day 11.

Figure 95 is a panel of graphs showing cytokine production of the T cells on day 11.

Figure 96 is a panel of graphs showing T cell expansion. No abnormal T cell growth was observed.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

“About” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

“Activation,” as used herein, refers to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. Activation can also be associated with induced cytokine production, and detectable effector functions. The term “activated T cells” refers to, among other things, T cells that are undergoing cell division.

The term “antibody,” as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)₂, as well as single chain antibodies (scFv) and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

The term “antibody fragment” refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody

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fragments include, but are not limited to, Fab, Fab', F(ab')₂, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

An “antibody heavy chain,” as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

An “antibody light chain,” as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations. α and β light chains refer to the two major antibody light chain isotypes.

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By the term “synthetic antibody” as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

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The term “antigen” or “Ag” as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an “antigen” as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a “gene” at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

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5 The term “anti-tumor effect” as used herein, refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An “anti-tumor effect” can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies of the invention in prevention of the occurrence of tumor in the first place.

10 The term “auto-antigen” means, in accordance with the present invention, any self-antigen which is recognized by the immune system as being foreign. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors.

15 The term “autoimmune disease” as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases include but are not limited to, Addison's disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barr syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

20 As used herein, the term “autologous” is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

25 “Allogeneic” refers to a graft derived from a different animal of the same species.

“Xenogeneic” refers to a graft derived from an animal of a different species.

30 The term “cancer” as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like. In certain embodiments, the cancer is medullary thyroid carcinoma.

The term “chimeric antigen receptor” or “CAR,” as used herein, refers to an artificial T cell receptor that is engineered to be expressed on an immune effector cell and

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specifically bind an antigen. CARs may be used as a therapy with adoptive cell transfer. T cells are removed from a patient and modified so that they express the receptors specific to a particular form of antigen. In some embodiments, the CARs have been expressed with specificity to a tumor associated antigen, for example. CARs may also comprise an intracellular activation domain, a transmembrane domain and an extracellular domain comprising a tumor associated antigen binding region. In some aspects, CARs comprise fusions of single-chain variable fragments (scFv) derived monoclonal antibodies, fused to CD3-zeta transmembrane and intracellular domain. The specificity of CAR designs may be derived from ligands of receptors (e.g., peptides). In some embodiments, a CAR can target cancers by redirecting the specificity of a T cell expressing the CAR specific for tumor associated antigens.

The term “cleavage” refers to the breakage of covalent bonds, such as in the backbone of a nucleic acid molecule. Cleavage can be initiated by a variety of methods, including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible. Double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides may be used for targeting cleaved double-stranded DNA.

As used herein, the term “conservative sequence modifications” is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of

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an antibody can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for the ability to bind antigens using the functional assays described herein.

“Co-stimulatory ligand,” as the term is used herein, includes a molecule on an antigen presenting cell (e.g., an aAPC, dendritic cell, B cell, and the like) that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A co-stimulatory ligand can include, but is not limited to, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, HVEM, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

A “co-stimulatory molecule” refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the T cell, such as, but not limited to, proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and a Toll ligand receptor.

A “co-stimulatory signal”, as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation and/or upregulation or downregulation of key molecules.

The term “CRISPR/CAS,” “clustered regularly interspaced short palindromic repeats system,” or “CRISPR” refers to DNA loci containing short repetitions of base sequences. Each repetition is followed by short segments of spacer DNA from previous exposures to a virus. Bacteria and archaea have evolved adaptive immune defenses termed CRISPR-CRISPR-associated (Cas) systems that use short RNA to direct degradation of foreign nucleic acids. In bacteria, the CRISPR system provides acquired immunity against invading foreign DNA via RNA-guided DNA cleavage.

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In the type II CRISPR/Cas system, short segments of foreign DNA, termed “spacers” are integrated within the CRISPR genomic loci and transcribed and processed into short CRISPR RNA (crRNA). These crRNAs anneal to trans-activating crRNAs (tracrRNAs) and direct sequence-specific cleavage and silencing of pathogenic DNA by Cas proteins. Recent work has shown that target recognition by the Cas9 protein requires a “seed” sequence within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA-binding region.

To direct Cas9 to cleave sequences of interest, crRNA-tracrRNA fusion transcripts, hereafter referred to as “guide RNAs” or “gRNAs” may be designed, from human U6 polymerase III promoter. CRISPR/CAS mediated genome editing and regulation, highlighted its transformative potential for basic science, cellular engineering and therapeutics.

The term “CRISPRi” refers to a CRISPR system for sequence specific gene repression or inhibition of gene expression, such as at the transcriptional level.

A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal’s health continues to deteriorate. In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

The term “downregulation” as used herein refers to the decrease or elimination of gene expression of one or more genes.

“Effective amount” or “therapeutically effective amount” are used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result or provides a therapeutic or prophylactic benefit. Such results may include, but are not limited to, anti-tumor activity as determined by any means suitable in the art.

“Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide

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sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

As used herein “endogenous” refers to any material from or produced inside an organism, cell, tissue or system.

As used herein, the term “exogenous” refers to any material introduced from or produced outside an organism, cell, tissue or system.

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The term “expand” as used herein refers to increasing in number, as in an increase in the number of T cells. In one embodiment, the T cells that are expanded ex vivo increase in number relative to the number originally present in the culture. In another embodiment, the T cells that are expanded ex vivo increase in number relative to other cell types in the culture. The term “ex vivo,” as used herein, refers to cells that have been removed from a living organism, (e.g., a human) and propagated outside the organism (e.g., in a culture dish, test tube, or bioreactor).

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The term “expression” as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

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“Expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., Sendai viruses, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

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“Homologous” as used herein, refers to the subunit sequence identity between two polymeric molecules, e.g., between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit; e.g., if a position in each of two DNA molecules is occupied by adenine, then
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they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (e.g., 9 of 10), are matched or homologous, the two sequences are 90% homologous.

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“Humanized” forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321: 522-525, 1986; Reichmann et al., *Nature*, 332: 323-329, 1988; Presta, *Curr. Op. Struct. Biol.*, 2: 593-596, 1992.

“Fully human” refers to an immunoglobulin, such as an antibody, where the whole molecule is of human origin or consists of an amino acid sequence identical to a human form of the antibody.

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“Identity” as used herein refers to the subunit sequence identity between two polymeric molecules particularly between two amino acid molecules, such as, between two polypeptide molecules. When two amino acid sequences have the same residues at the same positions; e.g., if a position in each of two polypeptide molecules is occupied by an Arginine, then they are identical at that position. The identity or extent to which two amino acid sequences have the same residues at the same positions in an alignment is often expressed as a percentage. The identity between two amino acid sequences is a direct function of the number of matching or identical positions; e.g., if half (e.g., five positions in a polymer ten amino acids in length) of the positions in two sequences are identical, the two sequences are 50% identical; if 90% of the positions (e.g., 9 of 10), are matched or identical, the two amino acids sequences are 90% identical.

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5 The term “immunoglobulin” or “Ig,” as used herein is defined as a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of proteins are IgA, IgG, IgM, IgD, and IgE. IgA is the primary antibody that is present in body secretions, such as saliva, tears, breast milk, gastrointestinal secretions and mucus secretions of the respiratory and genitourinary tracts. IgG is the most common circulating antibody. IgM is the main immunoglobulin produced in the primary immune response in most subjects. It is the most efficient immunoglobulin in agglutination, complement fixation, and other antibody responses, and is important in defense against bacteria and viruses. IgD is the immunoglobulin that has no known antibody function, but may serve as an antigen receptor. IgE is the immunoglobulin that mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to allergen.

15 The term “immune response” as used herein is defined as a cellular response to an antigen that occurs when lymphocytes identify antigenic molecules as foreign and induce the formation of antibodies and/or activate lymphocytes to remove the antigen.

20 As used here, “induced pluripotent stem cell” or “iPS cell” refers to a pluripotent stem cell that is generated from adult cells, such as T cells. The expression of reprogramming factors, such as Klf4, Oct3/4 and Sox2, in adult cells convert the cells into pluripotent cells capable of propagation and differentiation into multiple cell types.

25 As used herein, an “instructional material” includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the nucleic acid, peptide, and/or composition of the invention or be shipped together with a container which contains the nucleic acid, peptide, and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

30 “Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

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The term “knockdown” as used herein refers to a decrease in gene expression of one or more genes.

The term “knockout” as used herein refers to the ablation of gene expression of one or more genes.

5 A “lentivirus” as used herein refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer in vivo.

10 By the term “modified” as used herein, is meant a changed state or structure of a molecule or cell of the invention. Molecules may be modified in many ways, including chemically, structurally, and functionally. Cells may be modified through the introduction of nucleic acids.

15 By the term “modulating,” as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

20 In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. “A” refers to adenosine, “C” refers to cytosine, “G” refers to guanosine, “T” refers to thymidine, and “U” refers to uridine.

25 Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

30 The term “operably linked” refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence.

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Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The term “overexpressed” tumor antigen or “overexpression” of a tumor antigen is intended to indicate an abnormal level of expression of a tumor antigen in a cell from a disease area like a solid tumor within a specific tissue or organ of the patient relative to the level of expression in a normal cell from that tissue or organ. Patients having solid tumors or a hematological malignancy characterized by overexpression of the tumor antigen can be determined by standard assays known in the art.

“Parenteral” administration of an immunogenic composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The term “polynucleotide” as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric “nucleotides.” The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, *i.e.*, the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCRTM, and the like, and by synthetic means.

As used herein, the terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein’s or peptide’s sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. “Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination

thereof.

The term “promoter” as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term “promoter/regulatory sequence” means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A “constitutive” promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An “inducible” promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A “tissue-specific” promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

A “Sendai virus” refers to a genus of the Paramyxoviridae family. Sendai viruses are negative, single stranded RNA viruses that do not integrate into the host genome or alter the genetic information of the host cell. Sendai viruses have an exceptionally broad host range and are not pathogenic to humans. Used as a recombinant viral vector, Sendai viruses are capable of transient but strong gene expression.

A “signal transduction pathway” refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. The phrase “cell surface receptor” includes molecules and complexes of molecules capable of receiving a signal and transmitting signal across the plasma membrane of a cell.

“Single chain antibodies” refer to antibodies formed by recombinant DNA techniques in which immunoglobulin heavy and light chain fragments are linked to the Fv

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region via an engineered span of amino acids. Various methods of generating single chain antibodies are known, including those described in U.S. Pat. No. 4,694,778; Bird (1988) Science 242:423-442; Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883; Ward et al. (1989) Nature 334:54454; Skerra et al. (1988) Science 242:1038-1041.

5 By the term “specifically binds,” as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms “specific binding” or “specifically binding,” can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope “A”, the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled “A” and the antibody, will reduce the amount of labeled A bound to the antibody.

15 By the term “stimulation,” is meant a primary response induced by binding of a stimulatory molecule (e.g., a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex. Stimulation can mediate altered expression of certain molecules, such as downregulation of TGF-beta, and/or reorganization of cytoskeletal structures, and the like.

20 A “stimulatory molecule,” as the term is used herein, means a molecule on a T cell that specifically binds with a cognate stimulatory ligand present on an antigen presenting cell.

25 A “stimulatory ligand,” as used herein, means a ligand that when present on an antigen presenting cell (e.g., an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a “stimulatory molecule”) on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like.

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Stimulatory ligands are well-known in the art and encompass, *inter alia*, an MHC Class I molecule loaded with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

5 The term “subject” is intended to include living organisms in which an immune response can be elicited (e.g., mammals). A “subject” or “patient,” as used therein, may be a human or non-human mammal. Non-human mammals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and murine mammals. Preferably, the subject is human.

10 As used herein, a “substantially purified” cell is a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their
15 natural state. In some embodiments, the cells are cultured *in vitro*. In other embodiments, the cells are not cultured *in vitro*.

A “target site” or “target sequence” refers to a genomic nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule may specifically bind under conditions sufficient for binding to occur.

20 As used herein, the term “T cell receptor” or “TCR” refers to a complex of membrane proteins that participate in the activation of T cells in response to the presentation of antigen. The TCR is responsible for recognizing antigens bound to major histocompatibility complex molecules. TCR is composed of a heterodimer of an alpha (α) and beta (β) chain, although in some cells the TCR consists of gamma and delta (γ/δ)
25 chains. TCRs may exist in alpha/beta and gamma/delta forms, which are structurally similar but have distinct anatomical locations and functions. Each chain is composed of two extracellular domains, a variable and constant domain. In some embodiments, the TCR may be modified on any cell comprising a TCR, including, for example, a helper T cell, a cytotoxic T cell, a memory T cell, regulatory T cell, natural killer T cell, and
30 gamma delta T cell.

The term “therapeutic” as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

The term “transfected” or “transformed” or “transduced” as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A

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“transfected” or “transformed” or “transduced” cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

To “treat” a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

The phrase “under transcriptional control” or “operatively linked” as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

A “vector” is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, Sendai viral vectors, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

Universal T cells that avoid graft vs host disease (GVHD) are highly desired in the clinical setting. However, use of allogeneic T cells is a risk because of rejection by the host’s immune system through the recognition of HLA-A molecules. Targeting strategies

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to manipulate multiple genes are complicated and efforts have yielded low efficiency in T cells without preventing GVHD and host vs graft reactions simultaneously.

The FAS receptor/FAS ligand (FAS/FASL) apoptosis signaling pathway negatively regulates T cell function. PD1 and CTLA4 are two major inhibitory signaling pathways in T cells. The enhanced anti-tumor immunity that results from antibody-mediated blockade of CTLA-4, PD-1 or PD-L1 suggests the potential to improve efficiency of immunotherapies by inhibiting these pathways. The invention includes the generation of modified T cells where the TCR α and β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD-1, and/or FAS are depleted as a means to generate modified T cells with reduced immunogenicity.

The present invention includes methods and compositions for generating a modified T cell by knocking down endogenous gene expression and expressing either a modified T cell receptor or a chimeric antigen receptor. In some embodiments, the invention includes a method for generating the modified T cell. Such a modified T cell can be included in a therapeutic composition and administered to a patient in need thereof.

Knockdown of Endogenous Gene Expression

The present invention includes downregulation of endogenous gene expression in a T cell, such as downregulating an alpha and/or beta chain of the T cell receptor (TCR), beta-2 microglobulin, CTLA-4, FAS, PD1, or a major histocompatibility complex protein such as a HLA molecule. In one embodiment, the T cell with downregulated gene expression has reduced immunogenicity in an allogeneic environment. In another embodiment, the T cell with reduced immunogenicity expresses a modified TCR or a CAR for targeted effector activity.

In one aspect, the invention includes a method for generating a modified T cell comprising introducing a nucleic acid into a T cell capable of downregulating endogenous gene expression, where the gene is selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS. Downregulating expression of an endogenous gene that is involved in producing an immune response to a cell, such as TCR α chain, TCR β chain, beta-2 microglobulin, or a HLA molecule, reduces immune-mediated rejection of the modified T cell. For example, downregulating expression of endogenous TCR, MHC or beta-2 microglobulin genes removes surface presentation of alloantigens on the T cell that could cause rejection by

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the host immune system. Also, downregulating an endogenous gene that regulates inhibitory signaling pathways in T cells, such as CTLA-4, PD1, and/or FAS, enhances anti-tumor efficacy of the modified T cell when exposed to an immunosuppressive microenvironment.

5 In one aspect, a nucleic acid capable of downregulating endogenous gene expression is introduced, such as by electroporation, transfection, or lenti- or other viral transduction, into the T cell. In another aspect, the invention includes a modified T cell comprising an electroporated nucleic acid capable of downregulating endogenous gene expression. In yet another aspect, a modified T cell includes an electroporated nucleic acid capable of downregulating endogenous TCR gene expression. In another aspect, the composition comprising the modified T cell is generated according to a method described herein. In yet another aspect, the invention includes a pharmaceutical composition comprising the modified T cell or a modified T cell generated according to the method described herein and a pharmaceutically acceptable carrier.

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15 The nucleic acid capable of regulating endogenous gene expression may downregulate the endogenous gene expression. In one embodiment, the nucleic acid capable of downregulating endogenous gene expression is selected from the group consisting of an antisense RNA, antigomer RNA, siRNA, shRNA, and a CRISPR system. Endogenous gene expression may be downregulated, knocked-down, decreased, and/or inhibited by, for example, an antisense RNA, antigomer RNA, siRNA, shRNA, a CRISPR system, etc.

CRISPR/Cas

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25 The CRISPR/Cas system is a facile and efficient system for inducing targeted genetic alterations. Target recognition by the Cas9 protein requires a 'seed' sequence within the guide RNA (gRNA) and a conserved di-nucleotide containing protospacer adjacent motif (PAM) sequence upstream of the gRNA-binding region. The CRISPR/CAS system can thereby be engineered to cleave virtually any DNA sequence by redesigning the gRNA in cell lines (such as 293T cells), primary cells, and CAR T cells. The CRISPR/CAS system can simultaneously target multiple genomic loci by co-expressing a single CAS9 protein with two or more gRNAs, making this system uniquely suited for multiple gene editing or synergistic activation of target genes.

30 One example of a CRISPR/Cas system used to inhibit gene expression, CRISPRi, is described in U.S. Publication No.: 2014/0068797. CRISPRi induces permanent gene

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5 disruption that utilizes the RNA-guided Cas9 endonuclease to introduce DNA double stranded breaks which trigger error-prone repair pathways to result in frame shift mutations. A catalytically dead Cas9 lacks endonuclease activity. When coexpressed with a guide RNA, a DNA recognition complex is generated that specifically interferes with transcriptional elongation, RNA polymerase binding, or transcription factor binding. This CRISPRi system efficiently represses expression of targeted genes.

10 CRISPR/Cas gene disruption occurs when a guide nucleic acid sequence specific for a target gene and a Cas endonuclease are introduced into a cell and form a complex that enables the Cas endonuclease to introduce a double strand break at the target gene. In one embodiment, the CRISPR system comprises an expression vector, such as, but not limited to, an pAd5F35-CRISPR vector. In one embodiment, a modified T cell is generated by introducing a Cas expression vector and a guide nucleic acid sequence specific for a gene into a T cell. In another embodiment, the Cas expression vector induces expression of Cas9 endonuclease. Other endonucleases may also be used, including but not limited to, T7, Cas3, Cas8a, Cas8b, Cas10d, Cse1, Csy1, Csn2, Cas4, Cas10, Csm2, Cmr5, Fok1, other nucleases known in the art, and any combination thereof.

15 In one embodiment, inducing the Cas expression vector comprises exposing the T cell to an agent that activates an inducible promoter in the Cas expression vector. In such an embodiment, the Cas expression vector includes an inducible promoter, such as one that is inducible by exposure to an antibiotic (e.g., by tetracycline or a derivative of tetracycline, for example doxycycline). However, it should be appreciated that other inducible promoters can be used. The inducing agent can be a selective condition (e.g., exposure to an agent, for example an antibiotic) that results in induction of the inducible promoter. This results in expression of the Cas expression vector.

20 The guide nucleic acid sequence is specific for a gene and targets that gene for Cas endonuclease-induced double strand breaks. The sequence of the guide nucleic acid sequence may be within a loci of the gene. In one embodiment, the guide nucleic acid sequence is at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more nucleotides in length.

25 The guide nucleic acid sequence may be specific for any gene, such as a gene that would reduce immunogenicity or reduce sensitivity to an immunosuppressive microenvironment. In one embodiment, the gene may include a sequence specific for a T cell receptor (TCR) chain (such as an alpha, beta, gamma and/or delta chain), beta-2

microglobulin, FAS, PD1, a major histocompatibility complex protein (such as a HLA class I molecule and/or HLA class II molecule), CTLA-4, or any combination thereof.

The guide nucleic acid sequence includes a RNA sequence, a DNA sequence, a combination thereof (a RNA-DNA combination sequence), or a sequence with synthetic nucleotides. The guide nucleic acid sequence can be a single molecule or a double molecule. In one embodiment, the guide nucleic acid sequence comprises a single guide RNA.

T Cell Receptor

Adoptive immunotherapy with T cells harboring antigen-specific TCRs have therapeutic potential in the treatment of cancer and certain chronic viral infections. Gene-engineering of T cells with a specific TCR has the advantage of redirecting the T cell to an intracellular antigen. Given that most oncogenic proteins are intracellular, development of a panel of TCRs specific to an oncogenic driver protein has great appeal.

The present invention also includes a modified T cell with downregulated gene expression as described herein and an exogenous T cell receptor (TCR). In one aspect, the invention includes a method for generating a modified T cell comprising introducing a nucleic acid encoding a modified T cell receptor (TCR) comprising affinity for a surface antigen on a target cell into the T cell and a nucleic acid capable of regulating endogenous gene expression selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, PD1, and FAS, wherein the T cells are capable of expressing the modified TCR.

In another aspect, the invention includes a modified T cell comprising an exogenous nucleic acid encoding a modified T cell receptor (TCR) comprising affinity for a surface antigen on a target cell and a nucleic acid capable of downregulating endogenous gene expression selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, PD1, and FAS, wherein the T cell expresses the modified TCR and wherein the endogenous gene expression is downregulated in the T cell. The invention also includes a population of cells comprising the modified T cell described herein.

A T cell receptor is a complex of membrane proteins that participate in the activation of T cells in response to the presentation of antigen. Stimulation of the TCR is triggered by major histocompatibility complex molecules (MHC) on antigen presenting

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cells that present antigen peptides to the T cells and bind to the TCR complexes to induce a series of intracellular signaling cascades.

The TCR is generally composed of six different membrane bound chains that form the TCR heterodimer responsible for ligand recognition. TCRs exist in alpha/beta and gamma/delta forms, which are structurally similar but have distinct anatomical locations and functions. In one embodiment, the TCR comprises a TCR alpha and TCR beta chain, such as the nucleic acid encoding the TCR comprises a nucleic acid encoding a TCR alpha and a TCR beta chain. In another embodiment, a TCR alpha chain or a TCR beta chain or both chains comprise at least one N-deglycosylation.

Each chain is composed of two extracellular domains, a variable and constant domain. In one embodiment, the TCR comprises at least one murine constant region. The constant domain is proximal to the cell membrane, followed by a transmembrane domain and a short cytoplasmic tail. In one embodiment, the modified TCR comprises a cytoplasmic domain including a co-stimulatory signaling domain, such as a 4-1BB co-stimulatory signaling domain. The variable domain contributes to the determination of the particular antigen and MHC molecule to which the TCR has binding specificity. In turn, the specificity of a T cell for a unique antigen-MHC complex resides in the particular TCR expressed by the T cell.

Each of the constant and variable domains may include an intra-chain disulfide bond. In one embodiment, TCR comprises at least one disulfide bond. The variable domains include the highly polymorphic loops analogous to the complementarity determining regions (CDRs) of antibodies. The diversity of TCR sequences is generated via somatic rearrangement of linked variable (V), diversity (D), joining (J), and constant genes.

Functional alpha and gamma chain polypeptides are formed by rearranged V-J-C regions, whereas beta and delta chains consist of V-D-J-C regions. The extracellular constant domain includes a membrane proximal region and an immunoglobulin region.

In one embodiment, the TCR includes a wildtype TCR, a high affinity TCR, and a chimeric TCR. When the TCR is modified, it may have higher affinity for the target cell surface antigen than a wildtype TCR. In embodiments where the TCR is a chimeric TCR, the TCR may include chimeric domains, such as the TCR comprises a co-stimulatory signaling domain at a C' terminal of at least one of the chains. In other embodiment, the TCR may include a modified chain, such as a modified alpha or beta chain. Such modifications may include, but are not limited to, N-deglycosylation, altered domain

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(such as an engineered variable region to target a specific antigen or increase affinity), addition of one or more disulfide bonds, entire or fragment of a chain derived from a different species, and any combination thereof.

In one embodiment, the TCR comprises specificity to a target cell antigen. The target cell surface antigen may include any type of ligand that defines the surface of a target cell. For example, the target cell surface antigen may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands for the antigen binding domain of the TCR including those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. In one embodiment, the target cell surface antigen includes any tumor associated antigen (TAA) and viral antigen, disease cell associated antigen, or any fragment thereof.

The target cell antigen may include any protein that can be processed and presented by major histocompatibility complexes. For example, the target antigen may be associated with a particular disease state. Thus examples of cell markers that may act as targets of the TCR include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. In one embodiment, the target antigen includes any of tumor associated antigens (TAA) and viral antigens, or any fragment thereof.

In one aspect, the invention includes a population of modified T cells comprising a nucleic acid encoding a modified T cell receptor (TCR) comprising affinity for a surface antigen on a target cell and a nucleic acid capable of downregulating endogenous gene expression selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS, wherein the T cells are capable of expressing the modified TCR.

Techniques for engineering and expressing T cell receptors include, but are not limited to, the production of TCR heterodimers which include the native disulphide bridge which connects the respective subunits (Garboczi, et al., (1996), Nature 384(6605): 134-41; Garboczi, et al., (1996), J Immunol 157(12): 5403-10; Chang et al., (1994), PNAS USA 91: 11408-11412; Davodeau et al., (1993), J. Biol. Chem. 268(21): 15455-15460; Golden et al., (1997), J. Imm. Meth. 206: 163-169; U.S. Pat. No. 6,080,840).

Chimeric Antigen Receptor (CAR)

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5 The present invention also includes a modified T cell with downregulated gene expression as described herein and a CAR. Thus, the present invention encompasses the modified T cell comprising a CAR or a nucleic acid encoding a CAR, wherein the CAR includes an antigen binding domain, a transmembrane domain and an intracellular domain.

10 In one aspect, the invention includes a method of generating a modified T cell comprising introducing a nucleic acid capable of downregulating endogenous gene expression selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS into a T cell and a nucleic acid encoding a chimeric antigen receptor (CAR) into the T cell, wherein the CAR comprises an antigen binding domain, a transmembrane domain and an intracellular domain of a co-stimulatory molecule.

15 In another aspect, the invention includes a modified T cell comprising a nucleic acid capable of downregulating endogenous gene expression and a nucleic acid encoding a chimeric antigen receptor (CAR), wherein the downregulated gene expression is selected from the group consisting of TCR α chain, TCR β chain, beta-2 microglobulin, a HLA molecule, CTLA-4, PD1, and FAS, and wherein the CAR comprises an antigen binding domain, a transmembrane domain and an intracellular domain of a co-stimulatory molecule. In one embodiment, the modified T cell further comprises an exogenous
20 nucleic acid encoding a modified TCR comprising affinity for a surface antigen on a target cell as described elsewhere herein. The invention also includes a population of cells comprising the modified T cell described herein.

25 One or more domains or a fragment of a domain of the CAR may be human. In one embodiment, the present invention includes a fully human CAR. The nucleic acid sequences coding for the desired domains can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than as a cloned
30 molecule.

Example of CARs are described in U.S. Patent Nos.: 8,911,993, 8,906,682, 8,975,071, 8,916,381, 9,102,760, 9,101,584, and 9,102,761, all of which are incorporated herein by reference in their entireties.

Antigen Binding Domain

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In one embodiment, the CAR comprises an antigen binding domain that binds to an antigen on a target cell. Examples of cell surface markers that may act as an antigen that binds to the antigen binding domain of the CAR include those associated with viral, bacterial and parasitic infections, autoimmune disease, and cancer cells.

The choice of antigen binding domain depends upon the type and number of antigens that are present on the surface of a target cell. For example, the antigen binding domain may be chosen to recognize an antigen that acts as a cell surface marker on a target cell associated with a particular disease state.

In one embodiment, the antigen binding domain binds to a tumor antigen, such as an antigen that is specific for a tumor or cancer of interest. In one embodiment, the tumor antigen of the present invention comprises one or more antigenic cancer epitopes.

The antigen binding domain can include any domain that binds to the antigen and may include, but is not limited to, a monoclonal antibody, a polyclonal antibody, a synthetic antibody, a human antibody, a humanized antibody, a non-human antibody, and any fragment thereof. Thus, in one embodiment, the antigen binding domain portion comprises a mammalian antibody or a fragment thereof.

The antigen binding domain may bind one or more antigens, such as but not limited to CD19; CD123; CD22; CD30; CD171; CS-1 (also referred to as CD2 subset 1, CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule-1 (CLL-1 or CLECL1); CD33; epidermal growth factor receptor variant III (EGFRvIII); ganglioside G2 (GD2); ganglioside GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(1-4)bDGlcp(1-1)Cer); TNF receptor family member B cell maturation (BCMA); Tn antigen ((Tn Ag) or (GalNAc α -Ser/Thr)); prostate-specific membrane antigen (PSMA); Receptor tyrosine kinase-like orphan receptor 1 (ROR1); Fms-Like Tyrosine Kinase 3 (FLT3); Tumor-associated glycoprotein 72 (TAG72); CD38; CD44v6; Carcinoembryonic antigen (CEA); Epithelial cell adhesion molecule (EPCAM); B7H3 (CD276); KIT (CD117); Interleukin-13 receptor subunit alpha-2 (IL-13Ra2 or CD213A2); Mesothelin; Interleukin 11 receptor alpha (IL-11Ra); prostate stem cell antigen (PSCA); Protease Serine 21 (Testisin or PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta (PDGFR-beta); Stage-specific embryonic antigen-4 (SSEA-4); CD20; Folate receptor alpha; Receptor tyrosine-protein kinase ERBB2 (Her2/neu); Mucin 1, cell surface associated (MUC1); epidermal growth factor receptor (EGFR); neural cell adhesion molecule (NCAM); Prostase; prostatic acid phosphatase (PAP); elongation factor 2 mutated (ELF2M); Ephrin B2; fibroblast

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5 activation protein alpha (FAP); insulin-like growth factor 1 receptor (IGF-I receptor),
 carbonic anhydrase IX (CAIX); Proteasome (Prosome, Macropain) Subunit, Beta Type, 9
 (LMP2); glycoprotein 100 (gp100); oncogene fusion protein consisting of breakpoint
 cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-
 10 abl); tyrosinase; ephrin type-A receptor 2 (EphA2); Fucosyl GM1; sialyl Lewis adhesion
 molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGalp(1-4)bDGlc(1-1)Cer);
 transglutaminase 5 (TGS5); high molecular weight-melanoma-associated antigen
 (HMWMAA); o-acetyl-GD2 ganglioside (OAcGD2); Folate receptor beta; tumor
 endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R);
 15 claudin 6 (CLDN6); thyroid stimulating hormone receptor (TSHR); G protein-coupled
 receptor class C group 5, member D (GPC5D); chromosome X open reading frame 61
 (CXORF61); CD97; CD179a; anaplastic lymphoma kinase (ALK); Polysialic acid;
 placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycosphingolipid
 (GloboH); mammary gland differentiation antigen (NY-BR-1); uroplakin 2 (UPK2);
 20 Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin
 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex,
 locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading
 Frame Protein (TARP); Wilms tumor protein (WT1); Cancer/testis antigen 1 (NY-ESO-
 1); Cancer/testis antigen 2 (LAGE-1a); Melanoma-associated antigen 1 (MAGE-A1);
 25 ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML); sperm
 protein 17 (SPA17); X Antigen Family, Member 1A (XAGE1); angiopoietin-binding cell
 surface receptor 2 (Tie 2); melanoma cancer testis antigen-1 (MAD-CT-1); melanoma
 cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1; tumor protein p53 (p53); p53
 mutant; prostein; surviving; telomerase; prostate carcinoma tumor antigen-1 (PCTA-1 or
 30 Galectin 8), melanoma antigen recognized by T cells 1 (MelanA or MART1); Rat
 sarcoma (Ras) mutant; human Telomerase reverse transcriptase (hTERT); sarcoma
 translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG
 (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N-Acetyl
 glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen
 receptor; Cyclin B1; v-myc avian myelocytomatosis viral oncogene neuroblastoma
 derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Tyrosinase-related
 protein 2 (TRP-2); Cytochrome P450 1B1 (CYP1B1); CCCTC-Binding Factor (Zinc
 Finger Protein)-Like (BORIS or Brother of the Regulator of Imprinted Sites), Squamous
 Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5

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(PAX5); proacrosin binding protein sp32 (OY-TES1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Receptor for Advanced Glycation Endproducts (RAGE-1); renal ubiquitous 1 (RU1); renal ubiquitous 2 (RU2); legumain; human papilloma virus E6 (HPV E6); human papilloma virus E7 (HPV E7); intestinal carboxyl esterase; heat shock protein 70-2 mutated (mut hsp70-2); CD79a; CD79b; CD72; Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR or CD89); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); and immunoglobulin lambda-like polypeptide 1 (IGLL1).

In some instances, it is beneficial for the antigen binding domain to be derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial for the antigen binding domain of the CAR to comprise a human antibody, humanized antibody as described elsewhere herein, or a fragment thereof.

It is also beneficial that the antigen binding domain is operably linked to another domain of the CAR, such as the transmembrane domain or the intracellular domain, both described elsewhere herein, for expression in the cell. In one embodiment, a nucleic acid encoding the antigen binding domain is operably linked to a nucleic acid encoding a transmembrane domain and a nucleic acid encoding an intracellular domain.

Transmembrane Domain

With respect to the transmembrane domain, the CAR can be designed to comprise a transmembrane domain that connects the antigen binding domain of the CAR to the intracellular domain. In one embodiment, the transmembrane domain is naturally associated with one or more of the domains in the CAR. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in

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5 this invention may be derived from (i.e. comprise at least the transmembrane region(s) of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154. In some instances, a variety of human hinges can be employed as well including the human Ig (immunoglobulin) hinge.

In one embodiment, the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.

10 Intracellular Domain

15 The intracellular domain or otherwise the cytoplasmic domain of the CAR is responsible for activation of the cell in which the CAR is expressed. The term “intracellular domain” is thus meant to include any portion of the intracellular domain sufficient to transduce the activation signal. In one embodiment, the intracellular domain includes a domain responsible for an effector function. The term “effector function” refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

20 In one embodiment, the intracellular domain of the CAR includes a domain responsible for signal activation and/or transduction. The intracellular domain may transmit signal activation via protein-protein interactions, biochemical changes or other response to alter the cell’s metabolism, shape, gene expression, or other cellular response to activation of the chimeric intracellular signaling molecule.

25 Examples of an intracellular domain for use in the invention include, but are not limited to, the cytoplasmic portion of the T cell receptor (TCR) and any co-stimulatory molecule that acts in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these elements and any synthetic sequence that has the same functional capability. In one embodiment, the intracellular domain of the CAR comprises dual signaling domains. The dual signaling domains may include a fragment or domain from any of the molecules described herein.

30 Examples of the intracellular domain include a fragment or domain from one or more molecules or receptors including, but are not limited to, TCR, CD3 zeta, CD3 gamma, CD3 delta, CD3 epsilon, CD86, common FcR gamma, FcR beta (Fc Epsilon R1b), CD79a, CD79b, Fc gamma RIIa, DAP10, DAP12, T cell receptor (TCR), CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-

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5 associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), CD127, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, other co-stimulatory molecules described herein, any derivative, variant, or fragment thereof, any synthetic sequence of a co-stimulatory molecule that has the same functional capability, and any combination thereof.

15 In one embodiment, the intracellular domain of the CAR includes any portion of a co-stimulatory molecule, such as at least one signaling domain from CD3, CD27, CD28, ICOS, 4-1BB, PD-1, T cell receptor (TCR), any derivative or variant thereof, any synthetic sequence thereof that has the same functional capability, and any combination thereof.

20 Between the antigen binding domain and the transmembrane domain of the CAR, or between the intracellular domain and the transmembrane domain of the CAR, a spacer domain may be incorporated. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the antigen binding domain or, the intracellular domain in the polypeptide chain. In one embodiment, the spacer domain may comprise up to 300 amino acids, preferably 10 to 25 100 amino acids and most preferably 25 to 50 amino acids. In another embodiment, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the intracellular domain of the CAR. An example of a linker includes a glycine-serine doublet.

30 Human Antibodies

It may be preferable to use human antibodies or fragments thereof when using bispecific antibodies or the antigen binding domains of a CAR. Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Human antibodies can be made by a variety of methods known in the art including phage display

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5 methods using antibody libraries derived from human immunoglobulin sequences, including improvements to these techniques. See, also, U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety. The bispecific antibody can also include an antibody wherein the heavy and light chains are encoded by a nucleotide sequence derived from one or more sources of human DNA.

10 Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Antibodies directed against the target of choice can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies, including, but not limited to, IgG1 (gamma 1) and IgG3. For an overview of this technology for producing human antibodies, see, Lonberg and Huszar (Int. Rev. Immunol., 13:65-93 (1995)). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT Publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; and

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5,939,598, each of which is incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.) and Genpharm (San Jose, Calif.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above. For a specific discussion of transfer of a human germ-line immunoglobulin gene array in germ-line mutant mice that will result in the production of human antibodies upon antigen challenge see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immunol., 7:33 (1993); and Duchosal et al., Nature, 355:258 (1992).

Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581-597 (1991); Vaughan et al., Nature Biotech., 14:309 (1996)). Phage display technology (McCafferty et al., Nature, 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S, and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of unimmunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol., 222:581-597 (1991), or Griffith et al., EMBO J., 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905, each of which is incorporated herein by reference in its entirety.

Human antibodies may also be generated by in vitro activated B cells (see, U.S. Pat. Nos. 5,567,610 and 5,229,275, each of which is incorporated herein by reference in its entirety). Human antibodies may also be generated in vitro using hybridoma

techniques such as, but not limited to, that described by Roder et al. (Methods Enzymol., 121:140-167 (1986)).

Humanized Antibodies

Alternatively, in some embodiments, a non-human antibody can be humanized, where specific sequences or regions of the antibody are modified to increase similarity to an antibody naturally produced in a human. For instance, in the present invention, the antibody or fragment thereof may comprise a non-human mammalian scFv. In one embodiment, the antigen binding domain portion is humanized.

A humanized antibody can be produced using a variety of techniques known in the art, including but not limited to, CDR-grafting (see, e.g., European Patent No. EP 239,400; International Publication No. WO 91/09967; and U.S. Pat. Nos. 5,225,539, 5,530,101, and 5,585,089, each of which is incorporated herein in its entirety by reference), veneering or resurfacing (see, e.g., European Patent Nos. EP 592,106 and EP 519,596; Padlan, 1991, Molecular Immunology, 28(4/5):489-498; Studnicka et al., 1994, Protein Engineering, 7(6):805-814; and Roguska et al., 1994, PNAS, 91:969-973, each of which is incorporated herein by its entirety by reference), chain shuffling (see, e.g., U.S. Pat. No. 5,565,332, which is incorporated herein in its entirety by reference), and techniques disclosed in, e.g., U.S. Patent Application Publication No. US2005/0042664, U.S. Patent Application Publication No. US2005/0048617, U.S. Pat. No. 6,407,213, U.S. Pat. No. 5,766,886, International Publication No. WO 9317105, Tan et al., J. Immunol., 169:1119-25 (2002), Caldas et al., Protein Eng., 13(5):353-60 (2000), Morea et al., Methods, 20(3):267-79 (2000), Baca et al., J. Biol. Chem., 272(16):10678-84 (1997), Roguska et al., Protein Eng., 9(10):895-904 (1996), Couto et al., Cancer Res., 55 (23 Supp):5973s-5977s (1995), Couto et al., Cancer Res., 55(8):1717-22 (1995), Sandhu J S, Gene, 150(2):409-10 (1994), and Pedersen et al., J. Mol. Biol., 235(3):959-73 (1994), each of which is incorporated herein in its entirety by reference. Often, framework residues in the framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well-known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Pat. No. 5,585,089; and Riechmann et al., 1988, Nature, 332:323, which are incorporated herein by reference in their entireties.)

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A humanized antibody has one or more amino acid residues introduced into it from a source which is nonhuman. These nonhuman amino acid residues are often referred to as “import” residues, which are typically taken from an “import” variable domain. Thus, humanized antibodies comprise one or more CDRs from nonhuman immunoglobulin molecules and framework regions from human. Humanization of antibodies is well-known in the art and can essentially be performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody, i.e., CDR-grafting (EP 239,400; PCT Publication No. WO 91/09967; and U.S. Pat. Nos. 4,816,567; 6,331,415; 5,225,539; 5,530,101; 5,585,089; 6,548,640, the contents of which are incorporated herein by reference herein in their entirety). In such humanized chimeric antibodies, substantially less than an intact human variable domain has been substituted by the corresponding sequence from a nonhuman species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework (FR) residues are substituted by residues from analogous sites in rodent antibodies. Humanization of antibodies can also be achieved by veneering or resurfacing (EP 592,106; EP 519,596; Padlan, 1991, *Molecular Immunology*, 28(4/5):489-498; Studnicka et al., *Protein Engineering*, 7(6):805-814 (1994); and Roguska et al., *PNAS*, 91:969-973 (1994)) or chain shuffling (U.S. Pat. No. 5,565,332), the contents of which are incorporated herein by reference herein in their entirety).

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is to reduce antigenicity. According to the so-called “best-fit” method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987), the contents of which are incorporated herein by reference herein in their entirety). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993), the contents of which are incorporated herein by reference herein in their entirety).

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5 Antibodies can be humanized with retention of high affinity for the target antigen and other favorable biological properties. According to one aspect of the invention, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind the target antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen, is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

15 A humanized antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody to the target antigen may be increased using methods of “directed evolution,” as described by Wu et al., J. Mol. Biol., 294:151 (1999), the contents of which are incorporated herein by reference herein in their entirety.

Other Molecules

25 The present invention also includes the modified T cell described herein further comprising a co-stimulatory molecule or a nucleic acid encoding the co-stimulatory molecule. In one embodiment, the modified T cell of the invention further includes an exogenous nucleic acid encoding a co-stimulatory molecule, such that the modified T cell expresses the co-stimulatory molecule. The nucleic acid may be introduced into the T cell by transducing the T cell, transfecting the T cell, or electroporating the T cell. In another embodiment, the co-stimulatory molecule is selected from CD3, CD27, CD28, CD83, CD86, CD127, 4-1BB, 4-1BBL, PD1 and PD1L. In another embodiment, the so-stimulatory molecule includes CD3 and comprises at least two different CD3 chains, such as CD3 zeta and CD3 epsilon chains.

30 In another embodiment, the modified T cell further comprises Klf4, Oct3/4, and/or Sox2 or a nucleic acid encoding Klf4, Oct3/4, and/or Sox2 to induce pluripotency of the

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5 T cell. The T cell can be induced to pluripotency by expressing Klf4, Oct3/4 and Sox2. Klf4, Oct3/4 and Sox2 may be expressed from a nucleic acid, viral vector(s) or RNA molecule(s). In one embodiment, a viral vector encoding for Klf4, Oct3/4 and Sox2 is introduced into the T cell to induce pluripotency. In another embodiment, a Sendai viral vector is introduced into the T cells to induce pluripotency, wherein the Sendai viral vector encodes Klf4, Oct3/4 and Sox2.

Introduction of Nucleic Acids

10 Methods of introducing nucleic acids into a cell include physical, biological and chemical methods. Physical methods for introducing a polynucleotide, such as RNA, into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. RNA can be introduced into target cells using commercially available methods which include electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, Mass.) or the Gene Pulser II (BioRad, Denver, Colo.), Multiporator (Eppendorf, Hamburg Germany). RNA can also be introduced into cells using cationic liposome mediated transfection using lipofection, using polymer encapsulation, using peptide mediated transfection, or using biolistic particle delivery systems such as “gene guns” (see, for example, Nishikawa, et al. Hum Gene Ther., 12(8):861-70 (2001).

20 Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

25 Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

30 Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine (“DMPC”) can be obtained from Sigma, St. Louis, MO; dicetyl phosphate (“DCP”) can be obtained from K & K Laboratories (Plainview, NY); cholesterol (“Choi”) can be obtained from Calbiochem-Behring; dimyristyl

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phosphatidylglycerol (“DMPG”) and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. “Liposome” is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 *Glycobiology* 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the nucleic acids in the host cell, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; “biochemical” assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

In one embodiment, a nucleic acid encoding a T cell receptor (TCR) comprising affinity for a surface antigen on a target cell is introduced into the expanded T cells. The nucleic acid encoding the TCR may be the same or separate nucleic acid that is capable of downregulating endogenous TCR gene expression. The nucleic acid encoding the TCR may be introduced into the T cell at the same time or sequentially with the nucleic acid capable of downregulating endogenous TCR gene expression. In one embodiment, the nucleic acid encoding the TCR is introduced prior to the nucleic acid capable of downregulating endogenous TCR gene expression.

Moreover, the nucleic acids may be introduced by any means, such as transducing the expanded T cells, transfecting the expanded T cells, and electroporating the expanded T cells. One nucleic acid may be introduced by one method and another nucleic acid may be introduced into the T cell by a different method.

RNA

In one embodiment, the nucleic acids introduced into the T cell are RNA. In another embodiment, the RNA is mRNA that comprises in vitro transcribed RNA or synthetic RNA. The RNA is produced by in vitro transcription using a polymerase chain reaction (PCR)-generated template. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. The desired template for in vitro transcription is a chimeric membrane protein. By way of example, the template encodes an antibody, a fragment of an antibody or a portion of an antibody. By way of another example, the template comprises an extracellular domain comprising a single chain variable domain of an antibody, such as anti-CD3, and an intracellular domain of a co-stimulatory molecule. In one embodiment, the template for the RNA chimeric membrane protein encodes a chimeric membrane protein comprising an extracellular domain comprising an antigen binding domain derived from an antibody to a co-stimulatory molecule, and an intracellular domain derived from a portion of an intracellular domain of CD28 and 4-1BB.

PCR can be used to generate a template for in vitro transcription of mRNA which is then introduced into cells. Methods for performing PCR are well known in the art. Primers for use in PCR are designed to have regions that are substantially complementary to regions of the DNA to be used as a template for the PCR. “Substantially complementary”, as used herein, refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are complementary, or one or more bases are non-complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of a gene that is normally transcribed in cells (the open reading frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a gene that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the

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coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR are generated by synthetic methods that are well known in the art.

“Forward primers” are primers that contain a region of nucleotides that are substantially complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. “Upstream” is used herein to refer to a location 5' to the DNA sequence to be amplified relative to the coding strand. “Reverse primers” are primers that contain a region of nucleotides that are substantially complementary to a double-stranded DNA template that are downstream of the DNA sequence that is to be amplified. “Downstream” is used herein to refer to a location 3' to the DNA sequence to be amplified relative to the coding strand.

Chemical structures that have the ability to promote stability and/or translation efficiency of the RNA may also be used. The RNA preferably has 5' and 3' UTRs. In one embodiment, the 5' UTR is between zero and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to, designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the gene of interest. Alternatively, UTR sequences that are not endogenous to the gene of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the gene of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of mRNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art.

In one embodiment, the 5' UTR can contain the Kozak sequence of the endogenous gene. Alternatively, when a 5' UTR that is not endogenous to the gene of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many mRNAs is known in the art. In other embodiments the 5' UTR can be derived from an RNA virus

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whose RNA genome is stable in cells. In other embodiments various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the mRNA.

To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In one embodiment, the promoter is a T7 polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters. Consensus nucleotide sequences for T7, T3 and SP6 promoters are known in the art.

In one embodiment, the mRNA has both a cap on the 5' end and a 3' poly(A) tail which determine ribosome binding, initiation of translation and stability mRNA in the cell. On a circular DNA template, for instance, plasmid DNA, RNA polymerase produces a long concatameric product which is not suitable for expression in eukaryotic cells. The transcription of plasmid DNA linearized at the end of the 3' UTR results in normal sized mRNA which is not effective in eukaryotic transfection even if it is polyadenylated after transcription.

On a linear DNA template, phage T7 RNA polymerase can extend the 3' end of the transcript beyond the last base of the template (Schenborn and Mierendorf, *Nuc Acids Res.*, 13:6223-36 (1985); Nacheva and Berzal-Herranz, *Eur. J. Biochem.*, 270:1485-65 (2003).

The conventional method of integration of polyA/T stretches into a DNA template is molecular cloning. However polyA/T sequence integrated into plasmid DNA can cause plasmid instability, which is why plasmid DNA templates obtained from bacterial cells are often highly contaminated with deletions and other aberrations. This makes cloning procedures not only laborious and time consuming but often not reliable. That is why a method which allows construction of DNA templates with polyA/T 3' stretch without cloning highly desirable.

The polyA/T segment of the transcriptional DNA template can be produced during PCR by using a reverse primer containing a polyT tail, such as 100T tail (size can be 50-5000 T), or after PCR by any other method, including, but not limited to, DNA ligation or in vitro recombination. Poly(A) tails also provide stability to RNAs and reduce

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their degradation. Generally, the length of a poly(A) tail positively correlates with the stability of the transcribed RNA. In one embodiment, the poly(A) tail is between 100 and 5000 adenosines.

Poly(A) tails of RNAs can be further extended following in vitro transcription with the use of a poly(A) polymerase, such as E. coli polyA polymerase (E-PAP). In one embodiment, increasing the length of a poly(A) tail from 100 nucleotides to between 300 and 400 nucleotides results in about a two-fold increase in the translation efficiency of the RNA. Additionally, the attachment of different chemical groups to the 3' end can increase mRNA stability. Such attachment can contain modified/artificial nucleotides, aptamers and other compounds. For example, ATP analogs can be incorporated into the poly(A) tail using poly(A) polymerase. ATP analogs can further increase the stability of the RNA.

5' caps also provide stability to RNA molecules. In a preferred embodiment, RNAs produced by the methods disclosed herein include a 5' cap. The 5' cap is provided using techniques known in the art and described herein (Cougot, et al., Trends in Biochem. Sci., 29:436-444 (2001); Stepinski, et al., RNA, 7:1468-95 (2001); Elango, et al., Biochim. Biophys. Res. Commun., 330:958-966 (2005)).

The RNAs produced by the methods disclosed herein can also contain an internal ribosome entry site (IRES) sequence. The IRES sequence may be any viral, chromosomal or artificially designed sequence which initiates cap-independent ribosome binding to mRNA and facilitates the initiation of translation. Any solutes suitable for cell electroporation, which can contain factors facilitating cellular permeability and viability such as sugars, peptides, lipids, proteins, antioxidants, and surfactants can be included.

RNA Transfection

In some embodiments, the RNA encoding a TCR is electroporated into the cells. In one embodiment, the RNA encoding the TCR is in vitro transcribed RNA.

The disclosed methods can be applied to the modulation of T cell activity in basic research and therapy, in the fields of cancer, stem cells, acute and chronic infections, and autoimmune diseases, including the assessment of the ability of the genetically modified T cell to kill a target cancer cell.

The methods also provide the ability to control the level of expression over a wide range by changing, for example, the promoter or the amount of input RNA, making it possible to individually regulate the expression level. Furthermore, the PCR-based technique of mRNA production greatly facilitates the design of the mRNAs with different structures and combination of their domains.

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5 One advantage of RNA transfection methods of the invention is that RNA transfection is essentially transient and a vector-free. A RNA transgene can be delivered to a lymphocyte and expressed therein following a brief in vitro cell activation, as a minimal expressing cassette without the need for any additional viral sequences. Under these conditions, integration of the transgene into the host cell genome is unlikely. Cloning of cells is not necessary because of the efficiency of transfection of the RNA and its ability to uniformly modify the entire lymphocyte population.

10 Genetic modification of T cells with in vitro-transcribed RNA (IVT-RNA) makes use of two different strategies both of which have been successively tested in various animal models. Cells are transfected with in vitro-transcribed RNA by means of lipofection or electroporation. It is desirable to stabilize IVT-RNA using various modifications in order to achieve prolonged expression of transferred IVT-RNA.

15 Some IVT vectors are known in the literature which are utilized in a standardized manner as template for in vitro transcription and which have been genetically modified in such a way that stabilized RNA transcripts are produced. Currently protocols used in the art are based on a plasmid vector with the following structure: a 5' RNA polymerase promoter enabling RNA transcription, followed by a gene of interest which is flanked either 3' and/or 5' by untranslated regions (UTR), and a 3' polyadenyl cassette containing 50-70 A nucleotides. Prior to in vitro transcription, the circular plasmid is linearized
20 downstream of the polyadenyl cassette by type II restriction enzymes (recognition sequence corresponds to cleavage site). The polyadenyl cassette thus corresponds to the later poly(A) sequence in the transcript. As a result of this procedure, some nucleotides remain as part of the enzyme cleavage site after linearization and extend or mask the poly(A) sequence at the 3' end. It is not clear, whether this nonphysiological overhang
25 affects the amount of protein produced intracellularly from such a construct.

30 RNA has several advantages over more traditional plasmid or viral approaches. Gene expression from an RNA source does not require transcription and the protein product is produced rapidly after the transfection. Further, since the RNA has to only gain access to the cytoplasm, rather than the nucleus, and therefore typical transfection methods result in an extremely high rate of transfection. In addition, plasmid based approaches require that the promoter driving the expression of the gene of interest be active in the cells under study.

In another aspect, the RNA construct is delivered into the cells by electroporation. See, e.g., the formulations and methodology of electroporation of nucleic acid constructs

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5 into mammalian cells as taught in US 2004/0014645, US 2005/0052630A1, US 2005/0070841A1, US 2004/0059285A1, US 2004/0092907A1. The various parameters including electric field strength required for electroporation of any known cell type are generally known in the relevant research literature as well as numerous patents and applications in the field. See e.g., U.S. Pat. No. 6,678,556, U.S. Pat. No. 7,171,264, and U.S. Pat. No. 7,173,116. Apparatus for therapeutic application of electroporation are available commercially, e.g., the MedPulser™ DNA Electroporation Therapy System (Inovio/Genetronics, San Diego, Calif.), and are described in patents such as U.S. Pat. No. 6,567,694; U.S. Pat. No. 6,516,223, U.S. Pat. No. 5,993,434, U.S. Pat. No. 6,181,964, U.S. Pat. No. 6,241,701, and U.S. Pat. No. 6,233,482; electroporation may also be used for transfection of cells in vitro as described e.g. in US20070128708A1. Electroporation may also be utilized to deliver nucleic acids into cells in vitro. Accordingly, electroporation-mediated administration into cells of nucleic acids including expression constructs utilizing any of the many available devices and electroporation systems known to those of skill in the art presents an exciting new means for delivering an RNA of interest to a target cell.

15 In one embodiment, the method includes electroporating a RNA encoding a TCR alpha and beta chain. The TCR alpha and beta chain can be encoded on the same or separate RNAs. When the alpha and beta are encoded by separate RNAs, the RNA may be co-electroporated.

20 In another embodiment, the method may further include electroporating a nucleic acid encoding a costimulatory molecule. The costimulatory molecule nucleic acid may be co-electroporated with the TCR RNA.

25 Sources of T Cells

Prior to expansion, a source of T cells is obtained from a subject. Non-limiting examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Preferably, the subject is a human. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, spleen tissue, umbilical cord, and tumors. In certain embodiments, any number of T cell lines available in the art, may be used. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll separation. In one embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The

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apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. The cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media, such as phosphate buffered saline (PBS) or wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations, for subsequent processing steps. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

In another embodiment, T cells are isolated from peripheral blood by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient. Alternatively, T cells can be isolated from umbilical cord. In any event, a specific subpopulation of T cells can be further isolated by positive or negative selection techniques.

The cord blood mononuclear cells so isolated can be depleted of cells expressing certain antigens, including, but not limited to, CD34, CD8, CD14, CD19 and CD56. Depletion of these cells can be accomplished using an isolated antibody, a biological sample comprising an antibody, such as ascites, an antibody bound to a physical support, and a cell bound antibody.

Enrichment of a T cell population by negative selection can be accomplished using a combination of antibodies directed to surface markers unique to the negatively selected cells. A preferred method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8.

For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (e.g., particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In

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yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion.

5 T cells can also be frozen after the washing step, which does not require the monocyte-removal step. While not wishing to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, 10 in a non-limiting example, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or other suitable cell freezing media. The cells are then frozen to -80°C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled 15 freezing immediately at -20°C or in liquid nitrogen.

In one embodiment, the population of T cells is comprised within cells such as peripheral blood mononuclear cells, cord blood cells, a purified population of T cells, and a T cell line. In another embodiment, peripheral blood mononuclear cells comprise the population of T cells. In yet another embodiment, purified T cells comprise the 20 population of T cells.

Chimeric Membrane Protein

Generally, T cells are expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that 25 stimulates a co-stimulatory molecule on the surface of the T cells. The present invention comprises a novel method of expanding a population of T cells comprising electroporating the T cells with RNA encoding a chimeric membrane protein and culturing the electroporated T cells, wherein the electroporated T cells within the population expand at least 10 fold. The chimeric membrane protein of the invention 30 comprises an extracellular and intracellular domain. The extracellular domain comprises a target-specific binding element, such as an antibody. In one embodiment, the chimeric membrane protein comprises a single chain variable fragment (scFv) against CD3 and an

intracellular domain derived from a portion of an intracellular domain of CD28 and 4-1BB.

Expression of the chimeric membrane protein allows interaction with other cells in the population, such as cells that express CD3, to stimulate and activate expansion of the electroporated T cells. Not wishing to be held to any particular theory, the cells that express CD3 may come into contact and bind with the chimeric membrane protein that is expressed on the surface of the electroporated T cells. At least one T cell expressing the chimeric membrane protein interacts with another cell expressing CD3. This interaction stimulates expansion of the electroporated T cells.

In one embodiment, the T cells are expanded prior to downregulation of an endogenous gene. In another embodiment, the modified T cells are expanded.

Extracellular Domain

The present invention includes an extracellular domain comprising an antigen binding domain derived from an antibody directed against a co-stimulatory molecule. The co-stimulatory molecule can include any molecule that co-stimulates T cells, such as, but not limited to, CD3, CD28, or a combination thereof. In one embodiment, the extracellular domain can include an antigen binding domain derived from anti-CD3, anti-CD28, or a combination thereof. In another embodiment, the extracellular domain comprises a single chain variable fragment (scFv) against CD3.

In another embodiment, the extracellular domain can include any portion of an antibody that binds to antigen including, but not limited to, the antigen binding domain of a synthetic antibody, human antibody, humanized antibody, single domain antibody, single chain variable fragments, and fragments thereof. In some instances, it is beneficial for the extracellular domain to be derived from the same species in which the chimeric membrane protein will ultimately be used in. For example, for use in humans, it may be beneficial for the extracellular domain of the chimeric membrane protein to comprise a human antibody or fragment thereof. Thus, in one embodiment, the extracellular domain portion comprises a human antibody or a fragment thereof as described elsewhere herein. Alternatively, in some embodiments, the extracellular domain portion comprises a non-human antibody that is humanized as described elsewhere herein.

Intracellular Domain

The intracellular domain or cytoplasmic domain comprises a costimulatory signaling region. The costimulatory signaling region refers to an intracellular domain of a

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costimulatory molecule. Costimulatory molecules are cell surface molecules other than antigen receptors or their ligands that are required for an efficient response of lymphocytes to antigen.

5 The cytoplasmic domain or the intracellular signaling domain of the chimeric membrane protein is responsible for activation of at least one of effector functions of the T cell. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The intracellular signaling domain includes any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

10 Nonlimiting examples of intracellular signaling domains for use in the chimeric membrane protein include any portion of the intracellular domain of CD28, 4-1BB, T cell receptor (TCR), co-stimulatory molecules, any derivative or variant of these sequences, any synthetic sequence that has the same functional capability, and any combination thereof. In one embodiment, the intracellular domain comprises a portion of an intracellular domain of CD28 and 4-1BB.

Other Domains of the Chimeric Membrane Protein

15 20 Between the extracellular domain and the transmembrane domain of the chimeric membrane protein, or between the cytoplasmic domain and the transmembrane domain of the chimeric membrane protein, there may be incorporated a spacer domain, such as an oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular domain or, the cytoplasmic domain in the polypeptide chain. The spacer domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids.

25 30 In some embodiments, the chimeric membrane protein further comprises a transmembrane domain. In some embodiment, the chimeric membrane protein further comprises a hinge domain. In one embodiment, the RNA encoding the chimeric membrane protein further comprises a transmembrane and hinge domain, such as a CD28 transmembrane domain and a CD8-alpha hinge domain.

Expansion of T Cells

As demonstrated by the data disclosed herein, expanding the T cells by the methods disclosed herein can be multiplied by about 10 fold, 20 fold, 30 fold, 40 fold, 50

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fold, 60 fold, 70 fold, 80 fold, 90 fold, 100 fold, 200 fold, 300 fold, 400 fold, 500 fold, 600 fold, 700 fold, 800 fold, 900 fold, 1000 fold, 2000 fold, 3000 fold, 4000 fold, 5000 fold, 6000 fold, 7000 fold, 8000 fold, 9000 fold, 10,000 fold, 100,000 fold, 1,000,000 fold, 10,000,000 fold, or greater, and any and all whole or partial intergers therebetween.

In one embodiment, the T cells expand in the range of about 20 fold to about 50 fold.

Following culturing, the T cells can be incubated in cell medium in a culture apparatus for a period of time or until the cells reach confluency or high cell density for optimal passage before passing the cells to another culture apparatus. The culturing apparatus can be of any culture apparatus commonly used for culturing cells in vitro. Preferably, the level of confluence is 70% or greater before passing the cells to another culture apparatus. More preferably, the level of confluence is 90% or greater. A period of time can be any time suitable for the culture of cells in vitro. The T cell medium may be replaced during the culture of the T cells at any time. Preferably, the T cell medium is replaced about every 2 to 3 days. The T cells are then harvested from the culture apparatus whereupon the T cells can be used immediately or cryopreserved to be stored for use at a later time. In one embodiment, the invention includes cryopreserving the expanded T cells. The cryopreserved T cells are thawed prior to introducing nucleic acids into the T cell.

In another embodiment, the method comprises isolating T cells and expanding the T cells. In another embodiment, the invention further comprises cryopreserving the T cells prior to expansion. In yet another embodiment, the cryopreserved T cells are thawed for electroporation with the RNA encoding the chimeric membrane protein.

Another procedure for ex vivo expansion cells is described in U.S. Pat. No. 5,199,942 (incorporated herein by reference). Expansion, such as described in U.S. Pat. No. 5,199,942 can be an alternative or in addition to other methods of expansion described herein. Briefly, ex vivo culture and expansion of T cells comprises the addition to the cellular growth factors, such as those described in U.S. Pat. No. 5,199,942, or other factors, such as flt3-L, IL-1, IL-3 and c-kit ligand. In one embodiment, expanding the T cells comprises culturing the T cells with a factor selected from the group consisting of flt3-L, IL-1, IL-3 and c-kit ligand.

The culturing step as described herein (contact with agents as described herein or after electroporation) can be very short, for example less than 24 hours such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 hours. The culturing

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step as described further herein (contact with agents as described herein) can be longer, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or more days.

Various terms are used to describe cells in culture. Cell culture refers generally to cells taken from a living organism and grown under controlled condition. A primary cell culture is a culture of cells, tissues or organs taken directly from an organism and before the first subculture. Cells are expanded in culture when they are placed in a growth medium under conditions that facilitate cell growth and/or division, resulting in a larger population of the cells. When cells are expanded in culture, the rate of cell proliferation is typically measured by the amount of time required for the cells to double in number, otherwise known as the doubling time.

Each round of subculturing is referred to as a passage. When cells are subcultured, they are referred to as having been passaged. A specific population of cells, or a cell line, is sometimes referred to or characterized by the number of times it has been passaged. For example, a cultured cell population that has been passaged ten times may be referred to as a P10 culture. The primary culture, i.e., the first culture following the isolation of cells from tissue, is designated P0. Following the first subculture, the cells are described as a secondary culture (P1 or passage 1). After the second subculture, the cells become a tertiary culture (P2 or passage 2), and so on. It will be understood by those of skill in the art that there may be many population doublings during the period of passaging; therefore the number of population doublings of a culture is greater than the passage number. The expansion of cells (i.e., the number of population doublings) during the period between passaging depends on many factors, including but is not limited to the seeding density, substrate, medium, and time between passaging.

In one embodiment, the cells may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. Conditions appropriate for T cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-gamma, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF-beta, and TNF- α . or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, α -MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented

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with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37° C) and atmosphere (*e.g.*, air plus 5% CO₂).

The medium used to culture the T cells may include an agent that can co-stimulate the T cells. For example, an agent that can stimulate CD3 is an antibody to CD3, and an agent that can stimulate CD28 is an antibody to CD28. This is because, as demonstrated by the data disclosed herein, a cell isolated by the methods disclosed herein can be expanded approximately 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold, 100 fold, 200 fold, 300 fold, 400 fold, 500 fold, 600 fold, 700 fold, 800 fold, 900 fold, 1000 fold, 2000 fold, 3000 fold, 4000 fold, 5000 fold, 6000 fold, 7000 fold, 8000 fold, 9000 fold, 10,000 fold, 100,000 fold, 1,000,000 fold, 10,000,000 fold, or greater. In one embodiment, the T cells expand in the range of about 20 fold to about 50 fold, or more by culturing the electroporated population.

In one embodiment, the method includes introducing a nucleic acid encoding a T cell receptor (TCR) comprising affinity for a surface antigen on a target cell into the expanded T cells, and electroporating a RNA encoding a co-stimulatory molecule into the T cells, wherein the electroporated T cells are capable of expressing the TCR and the co-stimulatory molecule.

In another embodiment, the method further comprises stimulating the expanded T cells with at least one co-stimulatory molecule selected from the group consisting of CD3, CD27, CD28, CD83, CD86, CD127, 4-1BB, 4-1BBL, PD1 and PD1L. The stimulation may include co-electroporation with RNA encoding the co-stimulatory molecule. In such an embodiment, the expanded T cells are further electroporated or co-electroporated with a RNA encoding CD3. The CD3 includes comprises at least two different CD3 chains, such as CD3 zeta and CD3 epsilon chains.

In another embodiment, the method of expanding the T cells can further comprise isolating the expanded T cells for further applications. In yet another embodiment, the method of expanding can further comprise a subsequent electroporation of the expanded T cells followed by culturing. The subsequent electroporation may include introducing a nucleic acid encoding an agent, such as a transducing the expanded T cells, transfecting the expanded T cells, or electroporating the expanded T cells with a nucleic acid encoding

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5 a TCR, into the expanded population of T cells, wherein the agent further stimulates the T cell. The agent may stimulate the T cells, such as by stimulating further expansion, effector function, or another T cell function. In one embodiment, the agent nucleic acid is co-electroporated with the chimeric membrane protein RNA. In another embodiment, the agent nucleic acid, such as a TCR RNA, is electroporated after culturing the electroporated population. In a further embodiment, the agent nucleic acid, such as a TCR RNA, is electroporated into expanded T cells that were cryopreserved.

Therapy

10 The modified T cells described herein may be included in a composition for therapy. The composition may include a pharmaceutical composition and further include a pharmaceutically acceptable carrier. A therapeutically effective amount of the pharmaceutical composition comprising the modified T cells may be administered.

15 In one aspect, the invention includes a method for stimulating a T cell-mediated immune response to a target cell or tissue in a subject comprising administering to a subject an effective amount of a modified T cell. In this embodiment, the T cell is modified as described elsewhere herein. The modified T cells may be administered to induce lysis of the target cell or tissue, such as where the induced lysis is antibody-dependent cell-mediated cytotoxicity (ADCC).

20 In another aspect, the invention includes a method for adoptive cell transfer therapy comprising administering an effective amount of pharmaceutical composition comprising the modified T cell described herein to a subject in need thereof to prevent or treat an immune reaction that is adverse to the subject.

25 In yet another embodiment, a method of treating a disease or condition associated with enhanced immunity in a subject comprising administering an effective amount of a pharmaceutical composition comprising the modified T cell described herein to a subject in need thereof.

30 The modified T cells generated as described herein can be uniform and possess T cell function. Further, the modified T cells can be administered to an animal, preferably a mammal, even more preferably a human, to suppress an immune reaction, such as those common to autoimmune diseases such as diabetes, psoriasis, rheumatoid arthritis, multiple sclerosis, GVHD, enhancing allograft tolerance induction, transplant rejection, and the like. In addition, the cells of the present invention can be used for the treatment of any condition in which a diminished or otherwise inhibited immune response,

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especially a cell-mediated immune response, is desirable to treat or alleviate the disease. In one aspect, the invention includes treating a condition, such as an autoimmune disease, in a subject, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising the modified T cell described herein.

5 Examples of autoimmune disease include but are not limited to, Acquired Immunodeficiency Syndrome (AIDS, which is a viral disease with an autoimmune component), alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease (AIED), autoimmune lymphoproliferative syndrome (ALPS), autoimmune thrombocytopenic purpura (ATP), Behcet's disease, cardiomyopathy, celiac sprue-dermatitis hepeticiformis; chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy (CIPD), cicatricial pemphigoid, cold agglutinin disease, crest syndrome, Crohn's disease, Degos' disease, dermatomyositis-juvenile, discoid lupus, essential mixed
15 cryoglobulinemia, fibromyalgia-fibromyositis, Graves' disease, Guillain-Barre syndrome, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin-dependent diabetes mellitus, juvenile chronic arthritis (Still's disease), juvenile rheumatoid arthritis, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, pernicious anemia,
20 polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynaud's phenomena, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma (progressive systemic sclerosis (PSS), also known as systemic sclerosis (SS)), Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, Takayasu arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis, vitiligo and Wegener's granulomatosis.

25 The T cells generated as described herein can also be modified and used to treat inflammatory disorders. Examples of inflammatory disorders include but are not limited to, chronic and acute inflammatory disorders. Examples of inflammatory disorders
30 include Alzheimer's disease, asthma, atopic allergy, allergy, atherosclerosis, bronchial asthma, eczema, glomerulonephritis, graft vs. host disease, hemolytic anemias, osteoarthritis, sepsis, stroke, transplantation of tissue and organs, vasculitis, diabetic retinopathy and ventilator induced lung injury.

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In another embodiment, the modified T cell described herein may be used for the manufacture of a medicament for the treatment of an immune response in a subject in need thereof.

Cells of the invention can be administered in dosages and routes and at times to be determined in appropriate pre-clinical and clinical experimentation and trials. Cell compositions may be administered multiple times at dosages within these ranges. Administration of the cells of the invention may be combined with other methods useful to treat the desired disease or condition as determined by those of skill in the art.

The cells of the invention to be administered may be autologous, allogeneic or xenogeneic with respect to the subject undergoing therapy.

The administration of the cells of the invention may be carried out in any convenient manner known to those of skill in the art. The cells of the present invention may be administered to a subject by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient transarterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (*i.v.*) injection, or intraperitoneally. In other instances, the cells of the invention are injected directly into a site of inflammation in the subject, a local disease site in the subject, a lymph node, an organ, a tumor, and the like.

The cells described herein can also be administered using any number of matrices. The present invention utilizes such matrices within the novel context of acting as an artificial lymphoid organ to support, maintain, or modulate the immune system, typically through modulation of T cells. Accordingly, the present invention can utilize those matrix compositions and formulations which have demonstrated utility in tissue engineering. Accordingly, the type of matrix that may be used in the compositions, devices and methods of the invention is virtually limitless and may include both biological and synthetic matrices. In one particular example, the compositions and devices set forth by U.S. Pat. Nos. 5,980,889; 5,913,998; 5,902,745; 5,843,069; 5,787,900; or 5,626,561 are utilized, as such these patents are incorporated herein by reference in their entirety. Matrices comprise features commonly associated with being biocompatible when administered to a mammalian host. Matrices may be formed from natural and/or synthetic materials. The matrices may be non-biodegradable in instances where it is desirable to leave permanent structures or removable structures in the body of an animal, such as an implant; or biodegradable. The matrices may take the form of

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sponges, implants, tubes, telfa pads, fibers, hollow fibers, lyophilized components, gels, powders, porous compositions, or nanoparticles. In addition, matrices can be designed to allow for sustained release of seeded cells or produced cytokine or other active agent. In certain embodiments, the matrix of the present invention is flexible and elastic, and may be described as a semisolid scaffold that is permeable to substances such as inorganic salts, aqueous fluids and dissolved gaseous agents including oxygen.

A matrix is used herein as an example of a biocompatible substance. However, the current invention is not limited to matrices and thus, wherever the term matrix or matrices appears these terms should be read to include devices and other substances which allow for cellular retention or cellular traversal, are biocompatible, and are capable of allowing traversal of macromolecules either directly through the substance such that the substance itself is a semi-permeable membrane or used in conjunction with a particular semi-permeable substance.

Pharmaceutical compositions

Pharmaceutical compositions of the present invention may comprise the modified T cell as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

When "an immunologically effective amount", "an anti-immune response effective amount", "an immune response-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, immune response, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the modified T cells

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described herein may be administered at a dosage of 10^4 to 10^9 cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. T cell compositions may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, *e.g.*, Rosenberg et al., *New Eng. J. of Med.* 319:1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

In certain embodiments, it may be desired to administer activated T cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate T cells therefrom according to the present invention, and reinfuse the patient with these activated and expanded T cells. This process can be carried out multiple times every few weeks. In certain embodiments, T cells can be activated from blood draws of from 10 ml to 400 ml. In certain embodiments, T cells are activated from blood draws of 20 ml, 30 ml, 40 ml, 50 ml, 60 ml, 70 ml, 80 ml, 90 ml, or 100 ml. Not to be bound by theory, using this multiple blood draw/multiple reinfusion protocol, may select out certain populations of T cells.

In certain embodiments of the present invention, cells expanded and modified using the methods described herein, or other methods known in the art where T cells are expanded to therapeutic levels, are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or natalizumab treatment for MS patients or efalizumab treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu et al., *Cell* 66:807-815, 1991; Henderson et al., *Immun.* 73:316-321, 1991; Bierer et al., *Curr. Opin. Immun.* 5:763-773, 1993). In a further embodiment, the cell compositions of the present

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invention are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. The dose for CAMPATH, for example, will generally be in the range 1 to about 100 mg for an adult patient, usually administered daily for a period between 1 and 30 days. The preferred daily dose is 1 to 10 mg per day although in some instances larger doses of up to 40 mg per day may be used (described in U.S. Patent No. 6,120,766).

It should be understood that the method and compositions that would be useful in the present invention are not limited to the particular formulations set forth in the examples. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the cells, expansion and culture methods, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, fourth edition (Sambrook, 2012); “Oligonucleotide Synthesis” (Gait, 1984); “Culture of Animal Cells” (Freshney, 2010); “Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1997); “Gene Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Short Protocols in Molecular Biology” (Ausubel, 2002); “Polymerase Chain Reaction: Principles, Applications and Troubleshooting”, (Babar, 2011); “Current Protocols in Immunology”

(Coligan, 2002). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

The materials and methods employed in these experiments are now described.

Primary human lymphocytes. Primary lymphocytes were stimulated with microbeads coated with CD3 and CD28 stimulatory antibodies (Life Technologies, Grand Island, NY, Catalog) as described (*Human gene therapy* 2011, 22(12):1575-1586). T cells were cryopreserved at day 10 in a solution of 90% fetal calf serum and 10% dimethylsulfoxide (DMSO) at 1×10^8 cells/vial.

NALM-6 was purchased from the German DSMZ Cell Collection (DSMZ catalog code: ACC 128). K562 and PC3 were purchased from American Type Culture Collection. 624mel melanoma line was obtained from the Surgery Branch (NCI/NIH). All the cell lines were cultured as instructed and routinely tested for mycoplasma contamination and confirmed as being negative.

Generation of TCR constructs for mRNA electroporation and lentiviral transduction. 1G4 NY-ESO-1 TCR with different mutations (1G4 and 8F) and CARs (PSCA or CD19) were synthesized and/or amplified by PCR, based on sequencing information provided by the relevant publications (*The Journal of experimental medicine*

2005, 201(8):1243-1255; *J Immunol* 2008, 180(9):6116-6131), and subcloned into pGEM.64A RNA based vector or pTRPE lentiviral vectors.

Human primary T cell preparation. Primary human CD4 and CD8 T cells were isolated from healthy volunteer donors following leukapheresis by negative selection using RosetteSep kits (Stem Cell Technologies, Vancouver BC, Canada). All specimens were collected under a University Institutional Review Board-approved protocol, and written informed consent was obtained from each donor.

Design and construction of CRISPRs. Cas9 DNA was synthesized by PCR then inserted to PGEM vector. gRNAs were selected by GN19 with a NGG PAM site, with some selected from N20 with a NGG PAM site. All gRNAs contained complementary sequences comprised of more than 13 base pair mismatches, with potential off-target mRNA sites excluded (Table 1). GRNAs were designed, as shown in Figure 1A, and synthesized by overlap PCR. All gRNA PCR products were ligated into the MSGV vector. In vitro transcribed CAS9 and gRNA targeted constant regions of TCR α , β chains and beta-2 microglobulin. gRNAs were designed to target either a sequence within exon 1 of the TCR α constant region, a consensus sequence common to exon 1 of both TCR β constant regions 1 and 2, beta-2 microglobulin or PD1. Sequences encoding the gRNAs were assembled using overlap PCR and cloned into the MSGV vector containing a T7 promoter. These plasmids were linearized with EcoRI. gRNA was in vitro transcribed. Cas9 mRNA was in vitro transcribed using mMACHINE T7 ULTRA kit (Life Technologies, Carlsbad, CA). The mRNA was stored at -80°C in nuclease-free vials for single use. The gRNA targeting sequences used for the animal study were as follows:

TRAC-gRNA: TGTGCTAGACATGAGGTCTA, SEQ ID NO:1

TRBC-gRNA: GCAGTATCTGGAGTCATTGA, SEQ ID NO:2

B2M-gRNA: CGCGAGCACAGCTAAGGCCA, SEQ ID NO:3

PD1-gRNA: GGCGCCCTGGCCAGTCGTCT, SEQ ID NO:4

FAS-gRNA: GAGGGTCCAGATGCCAGCA, SEQ ID NO:5

Flow cytometry. The following monoclonal antibodies and reagents were used with indicated specificity and the appropriate isotype controls. From BD Biosciences (San Jose, CA): APC-conjugated anti-CD3 (555335), FITC-anti-CD8(555366), PE-anti-CD8(555635), FITC-anti-CD27 (555440), PE-anti-CD107(555801), PE-anti-beta-2 microglobulin (551337), FITC-anti-HLA(555552); Biolegend (San Diego, CA): FITC-anti-CD45RO(304204), APC-anti-CD62L(304814), APC-anti-CCR7(353214); and Beckman

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Coulter (Pasadena, CA): PE-anti-Vb13.1(IM202IU). Data was acquired on a FACS Accuri (BD Biosciences, San Jose, CA) using CellQuest version 3.3 (BD Biosciences, San Jose, CA) and analyzed by FCS Express version 3.00 (De Novo Software, Los Angeles, CA) or FlowJo version 7.6.1 (Tree Star, Inc. Ashland, OR).

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Propagation of primary T cells. Primary human T cells were cultured in RPMI 1640 supplemented with 10% FCS, 100-U/ml penicillin, 100-g/ml streptomycin sulfate, 10-mM Hepes, and stimulated with magnetic beads coated with anti-CD3/anti-CD28 at a 1:3 cell to bead ratio. Cells were counted and fed every 2 days and once T cells appeared to rest down, as determined by both decreased growth kinetics and cell size, the T cells were either used for functional assays or cryopreserved.

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Generation of CD3^{neg} T cells. DNA supercoiled plasmids were linearized by SpeI and EcoRI, respectively. gRNA was in vitro transcribed by T7 mScript™ Standard mRNA Production System (Cambio, C-MS100625, Cambridge, England). All mRNA (Cas9, TCR α , TCR β and CARs) was in vitro transcribed using mMACHINE mMACHINE T7 ULTRA kit (Life Technologies, AM1345, Carlsbad, CA). T cells were stimulated by CD3/CD28 dynabeads for three days prior to electroporation. Ten million primary T cells were de-beaded prior to electro-transfer of 20 μ g Cas9, 10 μ g gRNA species into the cells with a 360V, 1ms parameter by BTX830, following a second and/or a third electro-transfer of 10 μ g gRNA. Also, T cells were washed three times with OPTI-MEM and re-suspended in OPTI-MEM (Invitrogen) at a final concentration of 1-3x10⁸ cells/ml. Subsequently, 0.1 ml of the cells was mixed with 10 μ g of IVT RNA (or as indicated) and electroporated in a 2 mm cuvette. Ten million primary T cells were de-beaded prior to the electrotransfer of 20 μ g of Cas9 and 10 μ g of gRNA species into the cells using a BTX830 (Harvard Apparatus BTX) at 360 V and 1 ms; this process was followed by a second and a third electrotransfer of 5 μ g of gRNA 12 to 24 hours later.

Following electroporation, cells were immediately placed in 2 mL of pre-warmed culture media and cultured at 37°C, 5% CO₂, or 32°C, 5% CO₂ for 1 day then returned to 37°C, 5% CO₂.

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TCR α and β double disruption or TRAC, TRBC and B2M triple disruption. To generate TCR α and β double-knockout T cells, Cas9 mRNA was co-electroporated with two different gRNAs targeting TCR α chain (TRAC), and TCR β chain (TRBC). The TCR α and β double-knockout T cells could be purified in 2 steps: 1) depletion of TCR-positive and α chain single-knockout cells with anti-CD3 microbeads after the electroporation of the 1G4 TCR α chain RNA, and 2) depletion of TCR β chain single-

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knockout cells with anti-CD3 microbeads after the electroporation of the TCR β chain RNA. For TRAC, TRBC and B2M triple disruption, T cells were electroporated with Cas9 mRNA and gRNAs targeting the TCR α and β chains and beta-2 microglobulin 3 days after anti-CD3/CD28 bead stimulation. The HLA-I-negative cell population was enriched on day 9 and electroporated with TCR α chain RNA. The TCR-negative population was enriched on day 10. Five days later, these cells were electroporated with TCR β chain RNA, and the TCR-negative cell population was sorted the next day to obtain universal T cells. On day 18, TCR or CAR RNA was electroporated into the universal T cells to generate universal effector cells. TCR and HLA-I molecule expression was confirmed at each step.

Generation of universal CART cells. Universal CART cells were generated by combing the lentiviral transduction of CD19 or PSCA CAR with the RNA electroporation of CRISPR/gRNAs. 1 day after anti-CD3/CD28 beads stimulation, T cells were transduced with lentiviral-CD19 or PSCA CAR. 2 days later, Cas9 and gRNAs targeting TCR α , β chain, B2M, PD1 were transferred into T cells by electroporation. 6 days after CRISPRs delivery, T cells negative for CD3, HLA-I, PD1 were sorted by microbeads depletion.

Enrichment of CD3^{neg} T cells. Cells washed with Auto MACS buffer were incubated for 30 minutes with CD3 microbeads (Miltenyi Biotec, 130-050-101, Auburn, CA) at 4°C. After washing twice, cells were passed through a LD column (MiltenyiBiotec, 130-042-901, Auburn, CA), and the flow-through fraction was collected for further use. The CD3 expression of CD3^{neg} T cells was restored by co-electroporation of 1G4TCR α and β mRNA, and the cells were expanded using a single Rapid Expansion Protocol (REP), CD3/CD28 Dynabeads or K562-based aAPC.

Generation and propagation of CD3^{neg} T cells. CD3^{neg} T cells had CD3 expression restored by electro-transfer of exogenous 1G4TCR alpha chain and TCR beta chain in vitro transcribed mRNA (5 μ g for each chain). These cells were expanded using a single Rapid Expansion Protocol (REP). PBMCs from three different donors: ND052 105x10⁶, ND405 83x10⁶, ND410 136x10⁶, were irradiated, then mixed together, to obtain a total of 324x10⁶ PBMCs. The PBMCs were re-suspended in a final volume of 90 ml then R10 were added to 300 ml, mixed, and divided into two T150 ml flasks. OKT were added to a final concentration of 30 ng/ml. On day 2, IL-2 was added to 50 CU/ml. From day 5, cells were counted and fed every 2 days and once T cells appeared to rest down, as

determined by both decreased growth kinetics and cell size, they were either used for functional assays or cryopreserved.

Sanger sequencing. The level of genomic disruption of TCR α chain (TRAC), TCR β chain 1 (TRBC1), and TCR β chain 2 (TRBC2) in T cells was determined by Surveyor Nuclease assay (Transgenomics, Omaha, NE). The percent target disruption was quantified by densitometry. The PCR primers used for the amplification of target locus were:

TRAC forward, 5'-TCATGTCCTAACCCTGATCCTCTT-3' SEQ ID NO:6

TRAC reverse, 5'-TTGGACTTTTCCCAGCTGACAGA-3' SEQ ID NO:7

TRBC total forward, 5'- TACCAGGACCAGACAGCTCTTAGA-3' SEQ ID NO:8

TRBC total reverse, 5'- TCTCACCTAATCTCCTCCAGGCAT-3' SEQ ID NO:9

PCR products were purified and ligated to TOPO cloning vector (Invitrogen) then transformed in E.coli. Single clone was picked and sequenced to calculate the indels.

Generation of siRNA and CRISPRi for electroporation. RNA duplex targeting TCR constant regions for either alpha (5'-rArGrGrArGrGrArUrUrCrGrGrArArCrCrCrArArUrCrArCrUrGrArC-3' SEQ ID NO:10 and 5'-rCrArGrUrGrArUrUrGrGrGrUrUrCrCrGrArArUrCrCrUrCCT-3' SEQ ID NO:11) or beta (5'-rArCrCrUrCrCrUrUrCrCrCrArUrUrCrArCrCrCrArCrCrArGrCrUrC-3' SEQ ID NO:12 and 5'-rGrCrUrGrGrUrGrGrUrGrArArUrGrGrGrArArGrGrArGGT-3' SEQ ID NO:13) were designed using Custom RNAi Design Tool (Integrated DNA Technologies, Coralville, IA) and the siRNA was synthesized (Integrated DNA Technologies, Coralville, IA). siRNA for both TCR alpha and beta was mixed and electroporated into stimulated T cells for endogenous TCR knockdown.

mRNA in vitro transcription and T cell electroporation. T7 mscript systems kit (CellScript) was used to generate in vitro transcribed (IVT) RNA. CD3/CD28 bead stimulated T cells were electroporated with IVT RNA using BTX EM830 (Harvard Apparatus BTX) as previously described (*Cancer research* 2010, 70(22):9053-9061). Briefly, T cells were washed three times and resuspended in OPTI-MEM (Invitrogen) at a final concentration of $1-3 \times 10^8$ cells/ml. Subsequently, 0.1 ml of cells were mixed with 10 ug IVT RNA (or as indicated) and electroporated in a 2 mm cuvette.

ELISA assays. Target cells, different tumor cell lines expressing CD19, were washed and suspended at 1×10^6 cells/ml in R10 medium (RPMI 1640 supplemented with

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10% fetal calf serum; Invitrogen). 100 ul of each target cell type was added in duplicate to a 96 well round bottom plate (Corning). Effector T cells were washed, and re-suspended at 1×10^6 cells/ml in R10 medium and then 100 ul of T cells were combined with the target cells in the indicated wells. In addition, wells containing T cells alone were prepared as a control. The plates were incubated at 37°C for 18 to 20 hours. After the incubation, supernatant was harvested and subjected to an ELISA assay (eBioscience).

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CD107a staining Cells were plated at an Effector cell:T cell ratio of 1:1 (1×10^5 effectors to 1×10^5 targets) in 160 μl of complete RPMI medium in a 96 well plate. 20 μl of phycoerythrin-labeled anti-CD107a antibody (BD Biosciences, 555801) was added and the plate was incubated at 37°C for 1 hour before adding Golgi Stop (2 ul Golgi Stop in 3 ml RPMI medium, 20 ul/well; BD Biosciences, 51-2092KZ) and incubating the plate for another 2.5 hours. Then 5 μl FITC-anti-CD8 and 5 ul APC-anti-CD3 were added and incubated at 37°C for 30 min. After incubation, the samples were washed with FACS buffer and analyzed by flow cytometry.

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Luciferase based CTL assay. Naml6-CBG tumor cells were generated and employed in a modified version of a luciferase based cytotoxic T lymphocyte assay. Briefly, click beetle green luciferase (CBG) was cloned into the pELNS vector, packaged into lentivirus, transduced into Naml6 tumor cells and sorted for CBG expression. The resulting Naml6-CBG cells were washed and resuspended at 1×10^5 cells/ml in R10 medium, and 100 ul of CBG-labeled cells were incubated with different ratios of T cells (e.g. 30:1, 15:1, etc) overnight at 37°C . 100 ul of the mixture was transferred to a 96 well white luminometerplate. 100 ul of substrate was added to the cells and luminescence was immediately determined. The results are reported as percent killing based on the luciferase activity in the wells with tumor cells but no T cells ($\% \text{ killing} = 100 - ((\text{RLU from well with effector and target cell coculture}) / (\text{RLU from well with target cells}) \times 100)$).

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Mouse xenograft studies. Studies were performed as previously described with certain modifications (*Human gene therapy* 2011, 22(12):1575-1586; *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106(9):3360-3365). Briefly, 6-10 week old NOD/SCID gamma (NSG) mice were injected subcutaneously with 1×10^6 PC3-CBG tumors cells on the right flank at day 0 and the same mice were given SK-OV3-CBG tumor cells (5×10^6 cells/mouse, subcutaneously.) on the left flank at day 5. The mice were treated with T cells via the tail vein at day 23 post PC3-CBG tumor

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inoculation, such that both tumors were approximately 200 mm³ in volume. Lentivirally transduced T cells were given at 1x10⁷ cells/mouse (10M), or 3x10⁶ cells/mouse (3M). Briefly, for the Nalm6 tumor model, 6- to 10-week-old NOD/SCID gamma (NSG) mice were injected with 1x10⁶ click beetle green (CBG) transduced Nalm6 (Nalm6-CBG) cells through the tail vein on day 0. The T cell treatment began on day 7 after the tumor inoculation. For the PC3-PDL1 solid tumor model, 6- to 10-week-old NOD/SCID gamma (NSG) mice were injected subcutaneously with 1x10⁶ PSCA, PD-L1 and CBG transduced PC3 (PC3-PSCA-PDL1-CBG) tumors cells in the right flank on day 0. The mice were treated with T cells via the tail vein at day 22 post PC3-PDL1-CBG tumor inoculation, such that the tumors were approximately 200 mm³ in volume. T cells were given at 2x10⁶ cells/mouse (2M). Animals were randomized and grouped based on baseline tumor size. All animals were included in the experiments and blinded tumor assessment was done for all the animal experiments conducted.

T cell stimulation, lentiviral transduction and CRISPR electroporation procedure.

Figure 84 shows the procedure used to stimulate, lentiviral transduce and CRISPR electroporate T cells. On day 0, T cells were obtained from 3 donors (100x10⁶ cells/donor). The cells were stimulated with anti-CD3/anti-CD28 beads at a T cell:bead ratio of 1:3. The concentration of cells was adjusted to 0.5x10⁶/ml with 100mL/flask. On day 1, stimulated T cells were transduced with CD19 CAR lentivirus at multiplicity of infection (MOI) of 2. 50mL (25x10⁶ cells) of T cells were reserved as unmodified T cells (Group 9). On Day 3, the beads were removed, the cells washed 2x in Opti-MEM media, and the transduced T cells from each donor were separated into two groups, CART/mock EP (10mL, 50x10⁶/mL) and CART/CRISPR (10mL, 50x10⁶/mL). The cells were then electroporated with CAS9 RNA (1st EP) at 500V/1ms with 120µg of CAS9 RNA/400µL of T cells. After electroporation, Groups 1, 3, 5 and 7 cells were then split by culturing T cells in half new medium and half cultured medium. On day 4, the cells were washed twice and resuspended in Opti-MEM at 50x10⁶/mL. 20µg TRBC4 and B2M gRNA was electroporated into the 400µL of T cells. After electroporation, the cells were cultured at 1x10⁶ cells/mL in half fresh medium and half cultured medium. On days 5 and 7, the cells were split and resuspended in half fresh medium and half cultured medium. On day 8, CD3+ cells were removed from Groups 2, 4 and 6 via a low-density column. The CD3- T cells were resuspended at 0.5-1x10⁶ cells/mL in half fresh medium and half cultured medium and cultured to expand the cells. On day 11, the T cells were harvested

and 25×10^5 cells from the three donors were sent for karyotyping. The remaining cells were aliquoted and frozen.

The results of the experiments are now described.

5 Example 1: Disruption of the TCR-CD3 complex on T cells using CRISPR.

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10 Thirteen gRNAs targeting the constant regions of TCR α chain, 10 gRNAs targeting the constant regions TCR β chain, and 10 RNAs targeting the beta-2 microglobin gene.(Figures 1A-1C and Figures 9A-9D) were developed and tested in 293T cells. Primary human T cells were propagated ex vivo for three days with anti-CD3/anti-CD28 dynabeads for three days. Since transient expression of CRISPR is sufficient to mediate gene knockout, a "hit-and-run" delivery strategy was developed to transiently express the CRISPRs by utilizing electro-transfer of in vitro transcribed RNA encoding CAS9 and gRNAs (Figure 2C).

15 To measure TCR expression, a mAb specific for CD3 was used, which is only present on the cell surface when TCR antibody is expressed. Six days after electro-transfer, flow cytometric analysis revealed that CRISPRs targeting TRBC eliminated CD3 expression on primary T cells at levels of 13.7 (Figure 2D) in donor ND147. The efficiency of TCR knockout correlated with the amount of electro-transferred mRNA (Figure 2D). Although the electro-transfer of RNA in primary T cells was well-tolerated,
20 a slight reduction in cell viability was observed that correlated with increasing amounts of introduced RNA. ZFN and TALEN mediated gene disruption has been reported to be more efficient when cells were transiently exposed to mild hypothermia. The same phenomenon was observed with this CRISPR system.

25 The T cells were cultured for 1 day at 32°C after electro-transfer. CRISPR-mediated disruption of CD3 was up to 2.5-fold better when electroporated T cells were cultured at 32°C versus 37°C . Using this approach, 5.43% and 16.7% of electroporated T-cells lost expression of CD3 using the CRISPRs targeting TRAC and TRBC, respectively, (Figure 2D, lower panel). No change in the levels of CD3 negative cells in the CAS9 MOCK samples and no appreciable decrease in viability (measured by Trypan
30 blue) were observed.

When gRNAs were electro-transferred for a second and a third time, the efficiency of eliminating CD3 expression on primary T cells at levels was greatly improved.

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- Targeting TRAC: at levels reaching 77% after three times electro-transfer of gRNA (Figure 4A),
- Targeting TRAC or TRBC at levels reaching 64.5% or 57.5%, respectively, after a second electro-transfer of gRNAs with a slight decreased viability (Figure 4C).

To confirm that electroporated T cells had been genetically modified at the intended gRNA target sites (TCR α or β loci), Sanger sequencing was performed using specific oligonucleotide primers flanking target sites within TRAC, TRBC1, or TRBC2. Multiple peaks at the indicated PCR products starting from the target sites were present only after electro-transfer of CRISPRs and the percent disruption correlated with loss of cell surface CD3 expression (Figure 1C and 3B). These experiments in primary T cells confirmed that CRISPRs designed to target TRAC or TRBC led to permanent disruption of $\alpha\beta$ TCR expression, as assessed by Sanger sequencing and confirmed by flow cytometric analysis of CD3.

Example 2: Enrichment of TCR $\alpha\beta$ negative T cells.

For future clinical applications, rapid and robust methods for isolating sources of TCR disrupted populations may be utilized. To begin to address this issue, the TCR/CD3^{neg} population was enriched by negative selection using clinically-approved paramagnetic beads and a depletion column. With a single depletion step, the CD3^{neg} population was enhanced to over 99% (Figure 3A). A CD3^{neg} population could not be enriched from untransfected control cells. Back-to-back depletion steps resulted in >99% enrichment, without skewing the CD4 or CD8 T cell subsets (Figure 3C). Sequencing results also showed deletions and insertions were introduced to TCR alpha and beta locus after CRISPR modification (Figure 3D).

Example 3: Generation of HLA-CLASS I^{neg} T cells by CRISPR.

To test the ability of CRISPR to knock out HLA-CLASS I expression from allogeneic T cells, gRNAs targeting beta-2 microglobin were designed. The beta-2 microglobin locus could be manipulated by CRISPR in 293 T cells (Figure 9A). Evidence showed disruption of beta-2 microglobin abolished T cell surface HLA-CLASS I expression (Figure 9B).

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IFN-gamma greatly improved, approximately 10 fold, targeting efficiency of beta-2 microglobulin in T cells (Figure 9C). Multiple electro-transfers of beta-2 microglobulin gRNAs gave a 66% beta-2 microglobulin negative population (Figure 11A).

For future allograft transplantation clinical applications, rapid and robust methods for isolating sources of HLA-CLASS I null populations will be needed. To begin to address this issue, the cells were labeled with PE-anti-beta-2 microglobulin antibody, and enriched for a HLA-CLASS I^{neg} population by negative selection using clinically-approved paramagnetic anti-PE microbeads and a depletion column. With a single depletion step, the HLA-CLASS I^{neg} population was enhanced to over 99%. A HLA-CLASS I^{neg} population could not be enriched from untransfected control cells. An analysis of HLA-CLASS I repertoire in enriched HLA-CLASS I^{neg} T cells via flow cytometry validated the elimination of HLA-CLASS I expression from the cell surface (Figure 9D).

15 Example 4: CD3^{neg} T cells can be propagated by different methods.

CD3^{neg} T cells restored CD3 expression after electro-transfer of exogenous IG4-TCR alpha and beta chain in vitro transcribed mRNA (5 µg each). These cells were expanded by: (1) a single Rapid Expansion Protocol (REP), then tested for activity and specificity. PBMCs were obtained from three different donors: ND052 105x10⁶, ND405 83x10⁶, ND410 136x10⁶. Cells were after irradiated, then mixed together, and a total 324x10⁶ PBMCs were obtained. 2x10⁶ cells were electro-transferred with RNA. CD3^{neg} T were re-suspended in a final volume of 90 ml and R10 media was added for a total volume of 300 ml. The cells were divided into 2 T150 ml flasks. OKT was added to a final concentration of 30 ng/ml. On day 2, IL-2 was added to 50 CU/ml. From day 5, cells were counted and fed every 2 days and once T cells appeared to rest down, as determined by both decreased growth kinetics and cell size, they were either used for functional assays or cryopreserved.

After a single REP, CD3^{neg} T cells were expanded for a 500 fold increase in number. These cells were expanded by: (2) stimulated with magnetic beads coated with anti-CD3/anti-CD28 at a 1:3 cell to bead ratio.

After a single REP, CD3^{neg} T cells were expanded for a 500 fold increase in number. These cells were expanded by: (3) co-cultured with irradiated K562-CD19 and K562/86/64/A2(2D11) in equal mixture at a concentration of 1x10⁶/ml.

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After a single REP, CD3^{neg} T cells were expanded for a 500 fold increase in number. These cells were expanded by: (4) co-cultured with irradiated K562-CD19 and K562/86/64/A2(2D11) in equal mixture at a concentration of 1×10^6 /ml with 30 ng/ml OKT

After a single REP, CD3^{neg} T cells were expanded for a 500 fold increase in number. These cells were expanded by: (5) co-cultured with irradiated K562-CD19 and K562/86/64/A2(2D11) in equal mixture at a concentration of 1×10^6 /ml with 1 mg/ml NY-ESO peptide.

Example 5: Re-direction of TCR^{neg} T cells by electro-transfer of TCR.

To test the function of TCR^{neg} T cells, these cells were re-directed by electro-transfer of TCR. By introducing TCR alpha chain and TCR beta chain, these cells expressed high levels of TCR. The expression of Vb13.1 was much higher in electro-transferred TCR^{neg} T cells compared to CAS9 MOCK control (Figure 7A). When the cells were co-cultured with the Nalm-6 NY-ESO leukemia cell line, positive for both HLA-A2 and NY-ESO, the cells showed high levels of 107a, indicating elevated degranulation activity (Figure 7B). The killing assay also showed potent toxicity towards this cell line (Figure 7C). This indicated that these cells are potentially safer than traditional clinical trials with T cell expressing CARs and TCRs, as these cells would not trigger GVHD and have less miss-pair toxicity than normal T cells with TCR treatment.

Some reports have shown that T cells can be genetically edited by ZFNs or TALEN to eliminate expression of the endogenous $\alpha\beta$ TCR. The methods and compositions described herein to selectively deplete T cells expressing undesired $\alpha\beta$ TCR also include incomplete knockout of the endogenous TCR to treat GVHD and inhibit endogenous TCR from adversely affecting CAR function (e.g., through competition for transcription factors). Therefore, a genetic approach was designed using designer ZFNs to permanently disrupt the α and β constant region sequences in T cells, thereby eliminating TCR expression.

ZFNs and TALENs are artificial restriction enzymes generated by fusing a DNA binding domain to a DNA cleavage domain. When ZFNs and TALENs do not work efficiently, it is often difficult to determine the cause. Failure could reflect a problem with the design, with accessibility of the target sequence, or a delivery issue. At the same time, ZFN targeting efficiency is usually low in T cells, making it difficult to manipulate multiple genes at one time.

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Distinct ZFNs and TALENs, the CRISPR/Cas system has recently emerged as a potentially facile and efficient alternative to ZFNs and TALENs for inducing targeted genetic alterations. Recent work has shown that target recognition by the Cas9 protein requires a 'seed' sequence within the crRNA and a conserved di-nucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA-binding region. The CRISPR/CAS system can thereby be retargeted to cleave virtually any DNA sequence by redesigning the crRNA. The data disclosed herein shows the potential for gene editing by CRISPR/CAS in 293T cells and primary T cells. The CRISPR/CAS system can simultaneously target multiple genomic loci by co-expressing a single CAS9 protein with two or more gRNAs, making this system uniquely suited for multiplex gene editing or synergistic activation of target genes. By administering different gRNAs together with CAS9, multiple genes can be simultaneously disrupted in T cells.

Example 6: HLA CLASS I and TCR α , β chain triple knockout by CRISPR.

To work toward "off- the-shelf" allogeneic t-cell therapies for malignancies and infectious diseases, cell therapy by infusion of T cells was designed to reconstitute immunity against pathogens and malignancies. The amount of time required to manufacture T cells with the desired properties and in sufficient numbers ex vivo is often incompatible with the treatment window for patients. Furthermore, autologous T cells from patients with advanced disease may have compromised function and be tolerant to desired antigens.

To address this, patients can be infused with allogeneic T cells to avoid immune-mediated rejection caused by host T cells recognizing disparate major or minor histocompatibility antigens on the infused cells. To broaden the application of T cell therapy, and for future allograft transplantation, rapid and robust methods for isolating sources of TCR and HLA-CLASS I disrupted populations can be generated.

ZFN and TALEN comprise a zinc finger DNA-binding domain designed to bind a specific DNA sequence fused to the cleavage domain of FokI endonuclease. The design and construction of ZFN and TALEN is very complicated and time consuming if there is more than one gene to be manipulated, because the genes must be targeted individually. With the CRISPR system described herein, the efficiency and shortened the time course of gene disruption can be obtained.

To address this issue, CAS9 was electro-transferred with three different gRNAs targeting TRAC, TRBC and beta-2 microglobin. Cells were labeled with PE-anti-beta-2

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microglobin antibody and enriched for a HLA-CLASS I^{neg} population by negative selection using clinically- approved paramagnetic anti-PE microbeads and a depletion column. With a single depletion step, the HLA-CLASS I^{neg} population was enhanced to over 99% (Figure 9D). Then the cells were re-introduced with TCR alpha chain, and HLA-CLASS I^{neg} CD3^{neg} population was enriched by microbeads (Figure 11). Five days later, the TCR beta chain was re-introduced into the cells, and a HLA-CLASS I^{neg} CD3^{neg} population was enriched by microbeads again. Two days later, TCR was electro-transferred into these triple knock out cells. On the day after electro-transformation, the cells were stimulated with CD3/CD28 dynabeads. Then, the cells underwent lentiviral delivery of antigen specific TCR the next day and culture expansion.

Example 7: FAS, PD1, CTLA4, PPP2R2D knockout by CRISPR.

The FAS receptor/FAS ligand (FAS/FASL) apoptosis signaling pathway has been widely studied and is well characterized in T cells. PD1 and CTLA4 are two major inhibitory signaling pathway in T cells that have also been extensively studied. Direct evidence for the potential therapeutic impact of targeting these pathways came from studies in preclinical murine tumor models demonstrating enhanced anti-tumor immunity after antibody-mediated blockade of CTLA-4, PD-1 or PD-L1. Similar antibodies for use in humans have been developed, and early clinical data showed promising results. Ppp2r2d knockdown may also inhibit T-cell apoptosis and enhance T-cell proliferation, as well as cytokine production. Ppp2r2d has potential as a target to improve the function of human T cells.

To address this issue, CAS9 and three different gRNAs targeting FAS, PD1, CTLA4, PPP2r2d were electro-transferred into T cells. Sanger sequencing data showed that the indicated locus of FAS, PD1, CTLA4, PPP2r2d had been modified by the CRISPRs. FAS was also replaced by GFP with homologous recombination triggered by CRISPR. FACS data showed the surface expression of FAS and PD1 was abolished.

Example 8: Generation of IPS cells with gene modified primary and T cells.

Progress in adoptive T-cell therapy for cancer and infectious diseases is hampered by the lack of readily available and antigen-specific human T lymphocytes. Pluripotent stem cells could provide an unlimited source of T lymphocytes. To address this issue, the expression of FAS, PD1, CTLA4, PPP2r2d were disrupted in primary cells and T cells.

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Sendai virus was used to reprogram primary cells and T cells. There are multiple methods to generate iPSCs, including virus-mediated gene transduction and chemical induction. While lentiviral and retroviral vectors require integration into host chromosomes to express reprogramming genes, DNA-based vectors, such as adenovirus, adeno-associated virus, and plasmid vectors, exist episomally and do not require integration. However, they may still be integrated into host chromosomes at certain frequencies, and the reprogramming efficiency is relatively low. Likewise, mRNA based reprogramming is complicated and shown to be extremely inefficient.

Unlike these methods, Sendai virus does not integrate into the host genome or alter the genetic information of the host cell. Sendai virus also has reprogramming potential comparable to lentiviral- and retroviral-based gene transduction.

Each well in a 24 well plate was seeded with 0.1 million wild type, FAS^{neg}, CD3^{neg} TCR alpha chain and TCR beta chain knock-out T cells. The cells were stimulated with CD3/CD28 beads. At day 3 post stimulation, the beads were removed, the cells resuspended in 1 mL of pre-warmed T cell complete medium, and then incubated with a calculated volume of CytoTune Sendai virus comprising a polycistronic vector for expression of hKlf4, hOct3/4 and hSox2 in the cells (Lifetechnologies, Carlsbad, CA). Treated T cells were seeded in 24 well plates, and centrifuged at 2250 rpm for 90 minutes at room temperature. An additional 1 mL of complete T cell medium was added to each well and the plate was incubated overnight at 37°C in a humidified atmosphere of 5% CO₂.

On the day after transduction, Sendai virus was removed by washing the T cells with fresh complete medium and culturing the cells for 2 days. Media was half changed every day. On day 3 after infection, cells were transferred to MEF feeder plates and cultured in T cell medium without any cytokines. Four days after infection, the cells were cultured in standard hES medium. Media was changed every day. ES-like colonies were observed around day 7. The cells were cultured in conditioned hES medium from day 15 and cultures continued for an additional 10 days. Colonies were picked at around 25 to 30 days after transduction.

At around day 4, cell clumps were formed on feeder cells, indicating that the initiation of the reprogramming process. T cells went through dramatic morphological changes during the process of reprogramming to iPSCs. At around day 12, large cell clumps with loose edges began to emerge. At around day 18, T cells were transformed to typical ES-like colonies with well defined edges. Typical embryonic stem cell

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morphology was observed indicating that the FAS^{neg}, CD3^{neg} TCR alpha chain and TCR beta chain knock-out T cells were induced to a pluripotent state under defined reprogramming conditions (Figure 17A and 18A).

5 FAS^{neg} T cells were easier to reprogram to iPSCs, at an efficiency of about 5 times of its wild type counterparts (Figure 17B). Likewise, reprogramming CD3^{neg} T cell was about 5 times more efficient than the wild type counterparts (Figure 18B). p53 deficient cell lines have been reported be easier to reprogram since the apoptosis pathway is hindered. FAS knock-out further induces apoptosis resistance. While loss of TCR expression makes T cells less healthy, an indication that apoptosis plays an important role in the process of reprogramming.

10 Example 9: Knockdown of TCR in T cells with siRNA.

15 Figure 19 is a graph showing IFN-gamma production of wild type NY-ESO-1 TCR (wt) or modified NY-ESO-1 TCR with a second disulfide bond and de-N-glycosylation to the beta chain(S/SD). RNA was electroporated into T cells with endogenous T cell receptors (TCRs) knocked down with siRNA. IFN-gamma was detected by ELISA after the T cells were stimulated with a HLA-A2 positive cell line pulsed with NY-ESO-1 specific peptide, p156-165, for 18h.

20 Figure 20, comprising Figures 20A and 20B, shows TCR alpha knockdown by CAS9 RNA and gRNA co-electroporation. Six days after electroporation, cells were analyzed for TCR expression by assessing CD3.

25 Figure 21 shows Sanger sequencing. Results show multiple peaks in CD3 negative enriched T cells, with either CAS9 mRNA and gRNAs electroporated to knockdown TCR alpha (TRAC-5) or TCR beta (TRBC-7).

30 Figure 22 is a panel of graphs showing CD3 negative T cells with endogenous TCR beta (TRB-7) knockdown re-expressed CD3 four hours after NY-ESO-1 TCR alpha and beta (1G4LY95 TCR) RNA electroporation. Normal T cells (ND424 Beads) were used as control, which showed nearly 100% CD3 positive with 5.25% endogenous TCR vb13.1 expression.

Figure 23, comprising Figures 23A-23D, is a panel of graphs showing knock down of endogenous TCR enhanced with both transgene expression and function of TCR RNA electroporated T cells. Figure 23A shows TCR expression of T cells electroporated with TCR siRNA (solid open histogram), control siRNA (dotted open histogram) and T cells without any siRNA (filled histogram). Figure 23B shows transgene (TCR vb13.1)

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expression of wild type NY-ESO-1 TCR (wt) or modified TCR (SD) RNA electroporated T cells with TCR siRNA, control siRNA, or no siRNA. Figure 23C shows NY-ESO-1 tetramer staining of wild type NY-ESO-1 TCR (wt) or modified TCR (SD) RNA electroporated T cells with TCR siRNA, control siRNA, or no siRNA. Figure 23D shows specific lysis of a HLA-A2/NY-ESO-1 positive tumor line by TCR siRNA knockdown, wildtype NY-ESO-1 TCR RNA electroporated T cells.

Figure 24 is a graph showing fluorescence of tumor cells after injection of T cells into a mouse model. Ten million Nalm6-CBG-ESO-GFP (click beetle green) tumor cells that expressed both NY-ESO-1 and GFP were intravenously injected into NOD/SCID mice. Five days after tumor inoculation, CBR (click beetle red) transduced and RNA electroporated T cells were injected as indicated in the different groups and tumor growth was monitored by bioluminescent image (BLI).

Figure 25 show bioluminescent images of the mice from two groups that had been treated by CD19BBZ CAR RNA T cells or modified NY-ESO-1 TCR RNA at different time points.

Example 10: Universal CAR19 T cells generated by combination of lentiviral transduction and disruption of the TCR-CD3 complex on T cells using CRISPR.

As shown in Figure 26, primary T cell were stimulated with anti-CD3/anti-CD28 beads at day 0, and then transduced with lenti-CAR19. Over 70% of the cells were CAR19 positive as detected by flow cytometry. Since transient expression of CRISPR is sufficient to mediate gene knockout, a “hit-and-run” delivery strategy was developed to transiently express CRISPR by utilizing electro-transfer of in vitro transcribed RNA encoding CAS9 and gRNAs targeting the constant regions of TCR α chain, TCR β chain, and beta-2 microglobulin gene on day 3. T cells were cultured for 24 hours at 32°C after electrotransfer, then returned to normal condition.

To measure TCR expression, a monoclonal antibody specific for CD3 was used. CD3 was chosen as CD3 is only present on the cell surface when TCRs are expressed. CRISPR constructs were electroporated into primary T cells (Figure 26). TCR single negative and TCR/HLA-A double negative cells were expanded by exposure to CD19 presenting K562 cells, which resulted in >100 fold expansion (Figure 27).

After expansion, the cells remained TCR single negative or TCR/HLA-A double negative, and the CAR19 positive population was enriched. Endogenous TCR expression remained negative in TCR single negative cells, while TCR and HLA-A expression

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remained negative in TCR/HLA-A double negative T cells after K562-CD19 stimulated expansion (Figure 28A). CAR19 positive cells were enriched by K562-CD19 stimulated expansion (Figure 28B).

The majority of expanded universal T cells were CD45RO positive (Figure 29A) and retained high levels of CD62L expression (Figure 29B), medium levels of CD28 expression (Figure 29A) and low levels of CCR7 expression (Figure 29B).

10
CRISPR gene editing did not affect the anti-tumor activity of universal CAR19 T cells in vitro (Figure 30A). Depletion of TCR or TCR/HLA-A had minimal effect on CAR19 expression and anti-tumor activity (Figures 30B and 30C). TCR single and TCR/HLA-A double negative CAR19 T showed robust lytic capacity when challenged with Nalm6 tumor cells (Figure 30B). CD107a release and cytokine secretion also showed potent anti-tumor activity in the universal cells (Figure 30C). TCR single ablation or TCR and HLA-A double ablation CAR19 T cells exhibited similar proliferation kinetics after challenge with CD19 expressing cells (Figure 30D).

15
To test the anti-tumor activity of CRISPR/CAS9 edited CAR19 T cells, TCR single negative, TCR and HLA-A double negative CAR19 T were infused into NSG mice bearing Nalm6 tumor cells. All the mice receiving unmanipulated T cells and mice infused with lentiviral GFP transduced wild type T cells died within 3 weeks after tumor cell infusion. Objective tumor regression was observed for mice receiving CAR19 T cells (Figure 6). CRISPR/CAS9 was found to not affect the *in vivo* tumor killing activity of
20
CAR19 T cells, thus, confirming the advantage of combining lentiviral gene transfer and CRISPR/CAS9 for T cell therapy.

25
Full ablation of TCR α and β chains and HLA-A molecule on T cells completely abrogated non-specific killing when the cells were challenged with HLA unmatched tumor cell lines (Figure 32A). Elimination of HLA-A molecules activated NK cells after a long period of co-culture (5 days). No off-target activity was observed when these cells were challenged by allogeneic whole blood PBMC after 24 hours in an IFN γ Elispot assay. The lack of off-target activity suggests T cells may play a dominant role in acute immune responses after encountering allogeneic cells. All of the results suggest that
30
CRISPR/CAS9 edited TCR α and β chains and HLA-A molecules (triple negative) T cells could serve as a source of universal effector donor cells.

CAS9 and different gRNAs targeting FAS were electro-transferred into T cells. FASneg cells were sorted and then transduced with lentiviral CAR19. Flow cytometry and Sanger sequencing data showed that FAS had been modified by the CRISPRs (Figure

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33). CAR19 gene expression of FASneg T cells was comparable to the wild type. Even after a short period of incubation with Nalm6 tumor cells, CD107a expression was greatly enhanced in FASneg CAR19 T cells compared to wild type counterpart cells even within 4 hours of co-culture.

5 Some reports showed that even weak antigenic stimuli can trigger FAS activation to promote T cell proliferation (Rethi, et al, Blood, vol. 112(4):1195-1204, 2008). Interestingly, FASneg CAR19 T cells expanded much quicker than the wild type CAR19 T cells when the cells were stimulated by high levels of CD19+ K562 cells. This suggests that FAS/FASL triggered apoptosis instead of activation under high level antigenic conditions (Figure 34A). FASneg CAR19 T cells further showed reduced apoptosis levels as measured by Annexin V staining (Figure 34B).

10 As had been observed *in vitro*, FASneg T cell showed enhanced proliferation as compared to wild type T cells. Similar proliferation results were observed when a True Count assay of CAR19 T cells was performed after infusion of the cells into Nalm6 bearing mice. The FASneg CAR19 group showed superior anti-tumor activity when compared to the wild type group (Figure 35B). This difference is illustrated in the graph of Figure 35C showing the bioilluminescence data between those two groups. These data indicate that FAS ablation in CART cells enhanced its anti-tumor activity.

15 20 CAS9 and different gRNAs targeting PD1 were electro-transferred into T cells after lentiviral transduction with PSCA-CAR. PD1 knock out cells were confirmed by surface PD1 expression after CD3/CD28 bead stimulation (Figure 36). PD1 negative cells were enriched by microbead depletion and then stimulated with PSCA antigen presenting PC3 tumor cells. PSCA-CAR positive cells were enriched both in the wild type and the PD1 negative groups. After incubation with PC3-PSCA-PDL1 tumor cells, PD1 expression was quickly upregulated on the surface of wild type PSCA-CAR T cells, with very low levels of PD1 expression detected on PD1 negative PSCA-CAR T cells (Figure 37). PD1 negative PSCA-CAR T cells also showed greatly enhanced and sustained high levels of CD137 expression (Figure 37), a marker of T cell activation, indicating that the PD1/PDL1 inhibitory signaling pathway was blocked.

25 30 When tested in an *in vivo* PC3-PSCA-PDL1 NSG model, significant enhanced anti-tumor activity was detected in the PD1 negative PSCA-CAR T cell group compared to the wild type group (Figures 38A and 38B) suggesting a therapeutic value of PD1 ablation for CART cell therapy.

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5 To test the graft vs host disease (GVHD) effect of CRISPR engineered universal CART cells, a high T cell dose was given to NSG mice with Nalm6 leukemia. The mice were treated with double or triple knock out CART cells and did not show any signs of developing GVHD. By contrast, 3 out of 4 mice from the wild-type CD19 CART group developed GVHD by day 65, which was confirmed by histological examination of different organs (Figure 39).

10 In another experiment, the cells were resuspended in FBS and infused intravenously into mice after a sub-lethal irradiation. Clinical GVHD was monitored 2 to 3 times per week. Four out 5 mice receiving wild type T cells died during the 60 day study, while PBS treated, TCR single and TCR/HLA-I double ablated T cell treated groups did not show any signs of GVHD. Mice receiving wild type T cells underwent body weight loss. However, PBS treated, TCR single and TCR/HLA-I double ablated T cell treated groups slightly gained weight during the study (Figures 40A and 40B).

15 T cells were treated with Cas9 and gRNAs targeting CD3, B2M and PD1 or Fas after lentiviral CD19-CAR transduction. Triple knock out universal CART cells were injected into mice bearing Nalm6-PDL1 tumors. Superior anti-tumor activity was observed in mice receiving PD1/CD3/HLA-I triple knock out cells as compared to CD3/HLA-I double knock out cells, further indicating the therapeutic value of blocking the PD1 signaling pathway (Figures 41A and 41B). These data supply a way to enhance
20 the treatment of universal CART cells with CRISPR/Cas9.

25 As gRNAs are prone to degrade, a simplified one-shot method was developed to generate universal CART cells. gRNAs were constitutively expressed together with CARs in a single lentiviral vector. Naïve T cells were transduced by lentivirus encoding gRNAs and CARs one day after stimulation with CD3/CD28 Dynabeads. The cells were electroporated with Cas9 mRNA at day 3(Figure 42). This system allows the manipulation of several genes with one vector (Figure 42). CD3 expression was confirmed by flow cytometry at day 6. T cells treated with the one-shot system showed consistent gene ablation as high as 90% in each of the different Cas9 mRNA groups (Figure 43).

30 Progress in adoptive T-cell therapy for cancer and infectious diseases has been hampered by the lack of readily available antigen-specific human T lymphocytes. Pluripotent stem cells could provide an unlimited source of T lymphocytes. To address this issue, expression of FAS, PD1, CTLA4, and PPP2r2d was disrupted in primary cells and T cells.

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Sendai virus was used to reprogram primary cells and T cells. There are multiple methods available for the generation of iPSCs, including virus-mediated gene transduction and chemical induction. While lentiviral and retroviral vectors require integration into host chromosomes to express reprogramming genes, DNA-based vectors, such as adenovirus, adeno-associated virus, and plasmid vectors, exist episomally and do not require integration, however, they may still be integrated into host chromosomes at certain frequencies, and the reprogramming efficiency is relatively low. Likewise, mRNA based reprogramming is complicated and has proven to be extremely inefficient.

In contrast, Sendai virus does not integrate into the host genome or alter the genetic information of the host cell. Sendai virus also has reprogramming potential comparable to lentiviral- and retroviral-based gene transduction.

Each well in a 24 well plate was seeded with 0.1 million wild type, FASneg, CD3neg TCR alpha and beta chain knock-out T cells. The cells were stimulated with CD3/CD28 beads. At day 3 post stimulation, the beads were removed, the cells were resuspended in 1 mL of pre-warmed T cell complete medium, and then incubated with a calculated volume of CytoTune Sendai virus comprising a polycistronic vector for expression of hKlf4, hOct3/4 and hSox2 in the cells (Lifetechnologies, Carlsbad, CA). Treated T cells were seeded in 24 well plates, and centrifuged at 2250 rpm for 90 minutes at room temperature. An additional 1 mL of complete T cell medium was added to each well and the plate was incubated overnight at 37°C in a humidified atmosphere of 5% CO₂.

On the day after transduction, Sendai virus was removed by washing the T cells with fresh complete medium and culturing the cells for 2 days. Media was half changed every day. On day 3 after infection, cells were transferred to MEF feeder plates and cultured in T cell medium without any cytokines. Four days after infection, the cells were cultured in standard hES medium. Media was changed every day. ES-like colonies were observed around day 7. The cells were cultured in conditioned hES medium from day 15 and cultures continued for an additional 10 days. Colonies were picked at around 25 to 30 days after transduction.

Around day 4, cell clumps were formed on feeder cells, indicating the initiation of the reprogramming process. T cells went through dramatic morphological changes during the reprogramming process to iPSCs (Figure 44A). Around day 12, large cell clumps with loose edges began to emerge. Around day 18, T cells were transformed to typical ES-like colonies with well-defined edges. FASneg T cells were reprogrammed to iPSCs

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at an efficiency of about 5 times of the wild type counterparts (Figure 44B). p53 deficient cell lines have been reported as easier to reprogram due to the hindrance of the apoptosis pathway. FAS knock out may facilitate the reprogramming process using a similar mechanism.

5 ES-like morphology of iPSCs reprogrammed from CD3neg TCR alpha or beta chain knock out T cells was observed (Figure 45A). The morphology remained constant after several passages. Reprogramming of CD3neg T cells was about 5 times less efficient than the wild type counterparts (Figure 45B), suggesting that TCR knock-out may play a role in the process of T cell reprogramming or affect the cell viability after Sendai virus infection. Figure 45C is a panel of images showing phosphatase staining of CD3neg iPSC cells.

10 Typical embryonic stem cell morphology was observed indicating that the FASneg, CD3neg TCR alpha and beta chain knock-out T cells were induced to a pluripotent state under defined reprogramming conditions. While loss of TCR expression makes T cells less healthy, the data described herein suggests that apoptosis plays an important role in the process of reprogramming.

15 Induction of endogenous pluripotent stem cell genes was also detected in the different T-iPSC cell lines (Figure 46). Immunostainings for Tra-1-60 and SSEA4 expression further indicated the stem cell phenotype of the T-iPSC cells (Figure 47A). Fas knock out was confirmed in the T-iPSCs by Sanger sequencing (Figure 47B).

20 dCas9 and FokI-Cas9 were reported to have less off-target activity. T cells were evaluated if they could be edited by a modified version of the CRISPR/dCAS9 and CRISPR/FokI-CAS9 system (Figure 48A). Flow cytometric data showed primary T cells were edited by both CRISPR/dCAS9 and CRISPR/FokI-CAS9 (Figure 48B). The CRISPR/dCAS9 gene knock out system exhibited enhanced specificity with at least one pair of gRNAs, rendering the knock out event more precise and more specific.

25 To test the off-target events of CRISPR/CAS9 in T cells, a surveyor assay was performed at off target sites. For the genes tested, no obvious cleavage was observed at the genomic loci (Figure 48C).

30 Example 11: Multiplex genome editing.

CART cells were generated by using a CRISPR/Cas9 system to simultaneously disrupt multiple genomic loci. The CART cells were deficient in the expression of endogenous TCR and HLA class I (HLA-I) molecules for use as allogeneic universal

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CART cells. T cell receptor (TCR) α chain, TCR β chain and beta-2 microglobulin (B2M) genes were disrupted with high efficiency through the co-electroporation of mRNA encoding Cas9 with gRNAs targeting these genes. Universal TCR or CART cells were generated by combining lentiviral (LV) delivery of CAR and CRISPR RNA electroporation to disrupt endogenous TCR and B2M genes simultaneously. In addition, disruption of endogenous PD1 enhanced the efficacy of CAR therapy in a solid tumor model.

10 Multiple deliveries of gRNAs disrupts genes in human primary T cells with high efficiency without impairing effector function

Efficient multiplex genomic editing is required to generate universal T cells that are deficient in TCR, HLA and other genes. CRISPR/gRNA RNA electroporation was optimized to achieve efficient gene disruption in T cells. First, Cas9 and gRNAs were co-electroporated with RNA generated using an in vitro transcription system (Figure 49, left), and a “hit-and-run” delivery strategy was developed to transiently deliver the Cas9 mRNA and gRNAs to T cells by electroporation (Figure 49, right).

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An initial experiment targeting the TCR α constant region (TRAC) or β constant region (TRBC) with single electroporation resulted in 1% to 3% CD3-negative (CD3^{neg}) T cells, respectively, (Figure 50A, upper graphs). To determine if transient exposure to mild hypothermia allowed more efficient gene disruption, cells were edited at 37°C or 32°C. CRISPR-mediated disruption of TRAC and TRB was increased up to 4-fold when T cells were cultured for 24h at 32°C after Cas9/gRNA co-electroporation (Figure 50A, lower graphs). The optimal molecular ratio of Cas9:gRNA for maximum disruption efficiency was 1:1 to 2:1, and the gene disruption efficiency was correlated with the amount of electro-transferred mRNA (Figure 51A).

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Compared with mRNA, gRNAs are more prone to rapid degradation, which potentially limits the targeting efficiency. Thus, multiple, sequential electroporations of gRNA were tested after the initial Cas9/gRNA electroporation. There was a marked increase in disruption frequency at the protein level, as 82.4% of cells were CD3^{neg} after the third gRNA electroporation (Figure 50B). Clonal sequencing showed that the genomic targeting efficiency reached 89.4% after the third gRNA electroporation (Figure 51B). A surveyor assay confirmed a cleavage rate of 81.7% and 49.3% at the genomic loci of TRAC and TRBC, respectively, after a third electroporation of gRNAs (Figure

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52). Multiple peaks in the Sanger sequencing data flanking the TRAC and TRBC target sites confirmed that the genomic reading frame shifted downstream of the target sites (Figure 53A). The occurrence of insertions or deletions (indels) caused by the NHEJ mediated by CRISPR/Cas9 was confirmed by clonal sequencing (Figure 53B). The TCR disrupted TCR/CD3^{neg} population was enriched to over 99% (99.70±0.20%) by a single step of CD3 negative selection (Figure 54).

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To develop methods to expand the TCR/CD3^{neg} T cells, TCR/CD3^{neg} T cells were co-electroporated with the HLA-A2 restricted 1G4 NY-ESO-1 TCR ($\alpha+\beta$) RNAs to restore CD3 expression (Figure 55, left panel). Following T cell stimulation/expansion methods, the following were compared: 1) a rapid T cell expansion protocol (REP) using PBMC as feeder cells, 2) anti-CD3/CD28 Dynabeads (Beads), or 3) OKT3 loaded K562-based artificial antigen-presenting cells expressing ligands for CD28 and 4-1BB (K562 aAPC). TCR/CD3^{neg} T cells were also electroporated with CD19 CAR RNA (Figure 55, right panel) and then stimulated by irradiated K562 aAPC that expressed CD19 (K562-CD19). Fold expansion values of 751.0±217.1, 35.7±9.3, 46.3±8.5 and 57.5±5.0 were achieved for REP, Beads, K562 aAPC and K562-CD19, respectively, after a single stimulation for 10 days (Figure 56).

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To test whether CRISPR/Cas9 gene editing would affect the phenotype and function of the T cells, the phenotype of TCR/CD3^{neg} T cells expanded by the different methods was examined and showed that all of the expanded cells remained CD3 negative and most retained a high level of CD27 (from 79.8% to 93.4%), consistent with a central memory cell phenotype (Figure 57). The expanded TCR/CD3^{neg} T cells were electroporated a second time with CD19 CAR mRNA to test their anti-tumor activities. The surface CAR expression of the TCR/CD3^{neg} T cells was equal to that of the control group (Figure 58). When the TCR/CD3^{neg} CD19 CAR T cells were stimulated with CD19⁺ Nalm6 leukemia cells, the CD107a up-regulation (Figure 59A), cytokine secretion (Figure 59C) and killing activity (Figure 59B) of CD19 CAR⁺ TCR/CD3^{neg} T cells was equivalent to those of the wild-type control cells. The CD19 CAR TCR/CD3^{neg} T cells were infused into Nalm6-bearing NSG mice to test their in vivo anti-tumor activity. Tumor regression was evident with an efficacy equivalent to that for the CART19 wild-type counterpart cells (Figures 59D and 59E). The results indicate that CRISPR/Cas9 editing of the endogenous TCR did not adversely affect the function of primary T cells for adoptive immunotherapy.

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Reduced alloreactivity of TCR α , β and B2M triple-disrupted T cells.

Disrupting both TCR α and β chains is required to prevent TCR miss-pairing-associated toxicity for TCR-redirection T cell adoptive immunotherapy and B2M is essential for the assembly and expression of HLA-I complex. In view of this, TCR α and β chains and B2M triple disruption was developed to generate universal T cells. First, the ability of eliminating HLA-I expression on the T cells by disrupting B2M was tested. T cells were electroporated with B2M-targeting Cas9/gRNA RNA. This resulted in a B2M and HLA-I double-negative population of 79.9%. The HLA-I^{neg} population could be further enriched by negative selection (Figure 60).

To generate triple-knockout T cells lacking the TCR α , β chains and B2M, Cas9 mRNA was co-electroporated with three different gRNAs targeting TRAC, TRBC and B2M. As a result, the CD3 and HLA-I double-negative cell population was 65.4% (Figure 61). After enrichment of the double and triple knockout cells, the TCR α and β chains and B2M triple-knockout T cells abrogated the non-specific killing of HLA unmatched tumor cell lines (Figure 62). No response was observed when these cells were challenged by allogeneic whole-blood irradiated PBMCs in an IFN γ Elispot assay (Figure 63, left panel). The ablation of HLA-I molecules also sharply reduced the allo-reactivity, as confirmed by co-culture of allogeneic PBMCs with irradiated B2M-disrupted cells (Figure 63, right panel). The results above suggest that triple-negative T cells that lack TCR α and β chains and B2M could potentially serve as a source of universal T cells for adoptive immunotherapy, resisting rejection by the host immune system while unable to cause graft versus host disease.

Improved anti-tumor activity of TCR redirected, endogenous TCR-disrupted T cells.

T cells with CRISPR/Cas9-disruption of TCR α and β chains showed elevated transgenic TCR expression on the cell surface after being redirected with an NY-ESO-1 TCR (1G4). Transgenic TCR expression was 67.6%, 78.8% or 94.3% for the TCR α or β chain single knockout or the α/β double knockout, respectively, compared with 46.8% for wild-type T cells. The improved transgenic TCR expression led to enhanced T cell function, as evidenced by increased antigen-specific CD107a expression (Figure 65A) and enhanced cytotoxicity (Figure 65B), especially for the α/β double-knockout T cells.

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In a separate experiment, α/β double-knockout T cells were transfected with a different NY-ESO-1 TCR (8F). Relative to 1G4 TCR, this 8F TCR exhibited a higher significant improvements in both transgenic TCR expression (Figure 66; 60.1% for TCR/CD3^{neg} versus 44.7% (with ~5% endogenous TCR V β 8 background) for wild-type T cells (Cas9 Mock T cells)) and function (CD107a expression in Figure 67A, and cytokine production in Figure 67B). These results highlight the differential influence of endogenous TCR on transgenic TCR expression and function.

Universal CART cells retain antitumor efficacy and do not cause GVHD.

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Universal CD19 CART cells were generated by combining LV transduction of CD19 CAR with RNA electroporation of Cas9/gRNAs (Figure 68). The cells were expanded and the remaining CD3^{neg} cells had high levels of CD19 CAR expression (Figure 69). The majority of the expanded T cells were CD45RO positive and retained a high level of CD62L expression and a medium level of CD28 expression, consistent with a central memory cell status (Figure 70). The expanded TCR/HLA-I double-negative CD19 CART showed robust in vitro anti-tumor activities, such as CD107a release (Figure 71), cytokine secretion (Figure 72), lytic capacity (Figure 73), and proliferation (Figure 74), that was as potent as those of the wild-type CD19 CART cells.

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The T cells were infused into NSG mice bearing disseminated Nalm6 leukemia. Mice treated with CART cells with a disrupted endogenous TCR (LV-CD19 CAR TCR^{neg}) or with a simultaneous disruption of TCR and HLA-I (LV-CD19 CAR TCR/HLA-I^{neg}) exhibited tumor regression similar to that of mice treated with wild-type CD19 CART cells (LV-CD19 CAR) (Figures 75A and 75B), suggesting that the disruption of TCR alone or together with B2M did not affect CART cell anti-tumor activity.

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To test the GVHD effect of the engineered T cells, a high T cell dose (20×10^6 /mouse) was given to NSG mice with Nalm6 leukemia. As shown in Figure 76, mice treated with CD19 CART cells with TCR disruption alone (LV-CD19 CAR TCR/CD3^{neg}) or the simultaneous disruption of TCR and B2M (LV-CD19 CAR TCR/HLA-I^{neg}) exhibited similar tumor regression compared with that of the wild-type CD19 CAR T cells (LV-CD19 CAR). Mice treated with the double or triple knock out CAR T cells did not develop any signs of GVHD. By contrast, 3 out of 4 mice from the wild-type CD19 CART (LV-CD19 CAR) group developed GVHD at day 65, which was

confirmed by histological examination of different organs. Thus, the disruption of TCR alone or together with HLA-I did not affect the in vivo anti-tumor activity of CART cells while eliminating alloreactivity.

Adenoviral CRISPR delivery into primary T cells.

The CRISPR/Cas9 system are rapidly being harnessed for gene regulation and gene editing purposes in model organisms and cell lines. Viral vectors may be particularly fit to broaden the applicability of CRISPR to other cell types, including dividing and quiescent primary cells. Adenovirus, namely second-generation fiber-modified adenovirus encoding Cas9 and single guide RNA (gRNA) molecules, were used to bring Cas9 nuclease to the PD1, Fas and TRAC loci (Figure 77). Adenoviral-mediated transduction of CRISPR into tumor cells (Figure 78) yielded high rates of targeted mutagenesis of up to about 71% (Figures 79A and 79B). Adenovirus appears to constitute a valuable platform for introducing CRISPR into human T cells regardless of their quiescent status. This approach will aid investigating the potential for CRISPR gene regulation and editing in numerous experimental settings.

Electroporation optimization.

CD3 and B2M knock-out efficiency and T cell expansion were assessed after Cas9 and gRNA electroporation (EP) in 4mm cuvettes and 2mm cuvettes. Standard EP conditions with a 2mm cuvette (360v/1ms, 1st EP - 20µg Cas9 RNA+ 10µg gRNA/100µl T cells, 2nd EP 5µg gRNA/100µl T cells) showed the highest CD3 and B2M knockout percentages, 81.8% and 88.6%, respectively, with T cell expansion at about 2.7 fold (EP#1), compared with about 18.8 fold expansion of control EP T cells (EP#12).

Decreasing the gRNA dose (EP#2-5) dramatically increased T cell expansion, but only slightly affected CD3 and B2M knock-out efficiency. See Figure 80. Standard EP conditions with a 4mm cuvette resulted in dramatically decreased CD3 and B2M knockout efficiency (EP#8), suggesting that the EP conditions (voltage or/and pulse length) need to be further optimized for use with 4mm cuvettes.

Compared with standard electroporation (EP) conditions in a 2mm cuvette (EP#10-13) or 4mm cuvette. High CD3/B2M knockout efficiency was observed with improved T cell fold expansion (EP#1 and 5). See Figure 81.

To further optimize EP conditions to achieve maximum T cell fold expansion with CD3/B2M knockout efficiency over 60%, different EP conditions and RNA amounts

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were tested. The results showed that fold expansion was improved with relatively high CD3/B2M knockout efficiency (63.5% for CD3 and 84.8% for B2M) for EP#4 (400v/2ms/120µg CAS9 RNA) for EP#1 and (500v/1ms/20µg gRNA) for EP#2. See Figure 82.

Additional experiments were performed to optimize EP conditions. Results showed that compared with the most favorable condition tested (EP#1 in Figure 82), using 500v/1ms/120µg CAS9 RNA (EP#1) and 500v/1ms/20µg gRNA (EP#2) produced increased CD3/B2M knockout efficiency and T cell expansion (EP#3). See Figure 83.

Large-scale electroporation and expansion.

Experiments were performed to determine if large-scale electroporations could yield high knock-out and expansion efficiencies. On day 0, anti-CD3/anti-CD28 beads were used to stimulate T cells obtained from 3 donors (100x10⁶ cells/donor, concentrated to 0.5x10⁶/ml). On day 1, stimulated T cells were transduced with CD19 CAR lentivirus. 50mL (25x10⁶ cells) of T cells were reserved as unmodified T cells (Group 9). On Day 3, the beads were removed and the transduced T cells from each donor were separated into two groups, CART/mock EP (10mL, 5x10⁶) and CART/CRISPR (10mL, 50x10⁶). The cells were then electroporated with CAS9 RNA (1st EP) and Groups 1, 3, 5 and 7 cells were split. On day 4, the gRNA was electroporated into the T cells and the cells were cultured at 1x10⁶ cells/mL. On days 5 and 7, the cells were split. On day 8, CD3+ cells were removed from Groups 2, 4 and 6. On day 11, the T cells were harvested and 25x10⁵ cells from the three donors were sent for karyotyping.

Table 1: Experimental groups.

Group #	Donor	T cells
1	ND391	CART/MOCK EP
2	ND391	CART/CRISPR
3	ND463	CART/MOCK EP
4	ND463	CART/CRISPR
5	ND463	UNMOD
6	ND469	CART/MOCK EP
7	ND469	CART/CRISPR

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5 T cell numbers (upper chart of Figure 85) and fold expansion (lower chart of Figure 85) were assessed after the electroporation and culturing procedure. Fold expansion of the T cells transduced with CD19 CAR alone (TD alone) or transduced with CD19 CAR and edited with CRISPR (TD/KO) is shown in the left graph of Figure 86 and fold expansion of the T cells on day 10 is shown in the right graph of Figure 86. By optimizing electroporation conditions and CAS9/gRNA doses, approx. 60-70% CD3/B2M knock-down efficiency and approx. 30 fold T cell expansion was observed after 10 days (Figure 87 shows CD3/B2M/CAR expression at day 10).

10 Eight days after CD3/CD28 bead stimulation and CRISPR RNA electroporation, CD3 positive T cells were removed. Figure 88 shows CD3/B2M expression in the three donor populations at day 8. On day 11, T cells were subjected to FACS staining to detect CD3, B2M and CAR expression. ND463 non-transduced (NOTD) were used as a negative control. Figure 89 shows CD3 and B2M expression in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells. Figure 90 shows CAR expression in CD19 CAR TD/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells. Figure 91 shows CD3/B2M/CAR expression on day 11 in CD19 CAR TD (transduced)/CRISPR electroporated, CD3 depleted T cells; CD19 CAR TD/CRISPR electroporated T cells; and CD19 CAR TD T cells. Figure 92 summarizes CD3/B2M/CAR expression in the different T cells groups.

15 On day 11 the different T cell groups, as indicated in Figure 93, were stimulated by a CD19 positive cell lines, Raji or Nalm6. K562 was used as a CD19 negative control. After 4hr of coculturing, CD107a up-regulation was detected in each of the T cell groups, except the negative controls.

20 On day 11, killing ability of the T cells, as indicated in Figure 94, were tested using a luminescent cytotoxic lymphocyte (CTL) assay after coculturing the T cells with CD19 positive target cells, Nalm6-CBG. Also on day 11, cytokine production of the T cells was analyzed by stimulating the T cell groups with Nalm6 target cells, see Figure 95.

25 The T cells were cultured in medium containing 100 U/ml of IL-2 for up to 26 days. The results shown in Figure 96 indicate no abnormal T cell growth was observed for the CRISPR edited T cells from the three donors.

30 As one of most attractive applications of the CRISPR/Cas9 system, multiplex genome editing holds great promise for advancing T cell-based adoptive immunotherapy.

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5 However, the low targeting efficiency of DNA transfection limits the use of multiplex genome engineering in primary T cells. A “hit-and-run” delivery strategy was developed to introduce CRISPRs to T cells via the co-electroporation of Cas9 mRNA and gRNA. Through a combination of up to three rounds of gRNA electroporation with transient exposure to mild hypothermia, a targeting efficiency of >80% at the protein level was routinely achieved for a single gene disruption. More encouragingly, triple gene disruption of TRAC, TRBC and B2M yielded double negative CD3 and HLA-I at about 65% without any purification and selection. The results also demonstrate that enrichment to >99% purity of gene-disrupted T cells was easily achieved using clinically approved paramagnetic beads and that the purified T cells were expanded up to 500-fold in 10 days. The expanded T cells maintained their gene disruption phenotype and displayed features consistent with central memory T cells. The disrupted T cells did not cause GVHD, suggesting that they may be used as allogeneic CAR T cells. Importantly, the gene-edited T cells showed anti-tumor activities both in vitro and in different tumor mouse models that were as potent or more potent than non-gene edited T cells. Thus, the process described herein to generate synthetic cells could be easily translated into current GMP-compliant manufacturing procedures.

15 The data described herein demonstrates that CRISPR/Cas9 is a powerful multiplex genome editing tool in primary human T cells. Previous reports have shown that T cells can be genetically edited by ZFNs or TALEN to eliminate the expression of the endogenous TCR α and β chains to avoid GVHD. Due to the complexity of the targeting strategies for manipulating multiple genes by zinc finger nucleases (ZFN) and TAL effector nuclease TALEN in T cells, previous studies have not been able to prevent GVHD and host-versus-graft reaction simultaneously in pre-clinical animal models. NK cell activation can also be aborted by the ablation of stimulatory NK ligands by CRISPR/Cas9 or by the expression of nonclassical HLA class I molecules such as HLA-E, which could potentially protect universal T cells from NK-cell-mediated rejection.

20 In summary clinical scale universal CART cells, with potent anti-tumor activity and reduced alloreactivity can be efficiently generated using multiplex CRISPR technology. This approach can be incorporated into current GMP-compliant manufacturing procedures and has a high potential for translation, given the successful translation of adoptive transfer therapy with ZFNs for HIV/AIDS. It is possible that universal CAR and TCR T cells will provide an alternative to autologous T cells. Indeed, it is conceivable that universal CAR and TCR T cells with disabled checkpoint molecules

may be more efficacious and have wider use than current CART therapy using autologous T cells against cancers and infectious diseases.

Other Embodiments

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

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CLAIMSWhat is claimed is:

1. A modified chimeric antigen receptor (CAR) T cell comprising:
 - (a) an insertion or a deletion in a Fas gene locus capable of downregulating gene expression of an endogenous Fas gene, wherein the insertion or deletion is introduced by a nucleic acid capable of downregulating gene expression of the endogenous Fas gene selected from the group consisting of an antisense RNA, antagomiR, siRNA, shRNA, and a CRISPR system;
 - (b) a nucleic acid encoding a chimeric antigen receptor (CAR) comprising an antigen binding domain, a transmembrane domain and an intracellular domain of a co-stimulatory molecule; and
 - (c) a recombinant viral vector comprising a nucleic acid encoding a reprogramming factor selected from the group consisting of a human Klf4, a human Oct3/4, a human Nanog, a human Rex1, a human Tert, a human GDF, a human Dppa4, and a human Sox2, or a combination thereof;wherein the expression of the endogenous Fas gene is decreased or eliminated in the modified CAR T cell.
2. The modified CAR T cell of claim 1, wherein the modified CAR T cell is an induced pluripotent CAR T cell.
3. The modified CAR T cell of claim 1, wherein the insertion or deletion is in the Fas gene nucleotide sequence of SEQ ID NO: 5.
4. The modified CAR T cell of claim 1, wherein the nucleic acid capable of downregulating gene expression is a CRISPR system.
5. The modified CAR T cell of claim 4, wherein the CRISPR system comprises an pAd5/F35-CRISPR vector.
6. The modified CAR T cell of claim 4, wherein the CRISPR system comprises a CRISPR nuclease and a guide RNA.

7. The modified CAR T cell of claim 6, wherein the guide RNA comprises a guide sequence that is sufficiently complementary to a target sequence of the Fas locus comprising the nucleic acid sequence of SEQ ID NO: 5.
8. The modified CAR T cell of claim 1, wherein:
 - (a) the modified CAR T cell exhibits superior anti-tumor activity when compared to a wild-type CAR T cell; or
 - (b) an expansion rate of the modified CAR T cell is at least 5-fold faster than the expansion rate of a wild-type CAR T cell.
9. The modified CAR T cell of claim 1, wherein the antigen binding domain of the CAR:
 - (a) comprises an antibody selected from the group consisting of a monoclonal antibody, a polyclonal antibody, a synthetic antibody, a human antibody, a humanized antibody, single domain antibody, and antigen-binding fragments thereof;
 - (b) comprises a single chain variable fragment; or
 - (c) specifically binds an antigen on a target cell.
10. A method for generating a modified chimeric antigen receptor (CAR) T cell, comprising:
 - (a) introducing a nucleic acid capable of downregulating gene expression of an endogenous FAS gene into a T cell to generate a T cell expressing decreased or eliminated FAS gene expression, wherein the nucleic acid capable of downregulating gene expression of the endogenous fas gene is selected from the group consisting of an antisense RNA, antagomiR, siRNA, shRNA, and a CRISPR system;
 - (b) exposing the T cell to mild hypothermia;
 - (c) isolating the T cell expressing decreased or eliminated Fas gene expression; and
 - (d) introducing a nucleic acid encoding a chimeric antigen receptor (CAR) comprising an antigen binding domain, a transmembrane domain, and an intracellular domain of a co-stimulatory molecule into the T cell to generate a modified CAR T cell expressing decreased or eliminated Fas gene expression.
11. The method of claim 10, wherein the nucleic acid capable of downregulating gene expression is a CRISPR system.

12. The method of claim 11, wherein:
 - (a) the CRISPR system comprises an pAd5/F35-CRISPR vector; or
 - (b) the CRISPR system comprises a CRISPR nuclease and a guide RNA.

13. The method of claim 12, wherein the guide RNA comprises a guide sequence that is sufficiently complementary to a target sequence of the Fas locus comprising the nucleic acid of SEQ ID NO: 5.

14. The method of claim 10, wherein the insertion or deletion is in the Fas gene nucleotide sequence of SEQ ID NO: 5.

15. The method of claim 12, wherein:
 - (a) introducing comprises one or more rounds of co-electroporation of the endonuclease and the gRNA into the T cell; or
 - (b) introducing comprises about 1 round, about 2 rounds, or about 3 rounds of co-electroporation of the endonuclease and the gRNA into the T cell.

16. The method of claim 15, wherein:
 - (a) exposing the co-electroporated T cell to mild hypothermia comprises incubating the co-electroporated T cell at a temperature of about 30 °C to about 32 °C; or
 - (b) exposing the co-electroporated T cell to mild hypothermia comprises incubating the co-electroporated T cell at about 32 °C for about 24 hours.

17. The method of claim 16, wherein the mild hypothermia enhances a targeting efficiency of the nucleic acid capable of downregulating gene expression of an endogenous Fas gene by at least about 4-fold when compared to the targeting efficiency of a co-electroporated T cell incubated at about 37°C.

18. The method of claim 10, further comprising reprogramming the modified CAR T cell into an induced pluripotent CAR T stem cell, wherein the reprogramming comprises transducing the modified CAR T cell with a recombinant viral vector.

19. The method of claim 18, wherein the recombinant viral vector comprises a nucleic acid encoding a reprogramming factor selected from the group consisting of a human Klf4, a human Oct3/4, a human Nanog, a human Rex1, a human Tert, a human GDF, a human Dppa4, a human Sox2, and any combination thereof.
20. The method of claim 18, wherein the recombinant viral vector is a Sendai viral vector.
21. The method of claim 20, wherein the Sendai viral vector comprises a polynucleotide encoding a human Klf4, a human Oct3/4, and a human Sox2 polypeptide.
22. The method of claim 18, wherein the reprogramming of the modified CAR T cell is at least five-times more efficient when compared to the reprogramming of a wild-type CAR T cell.

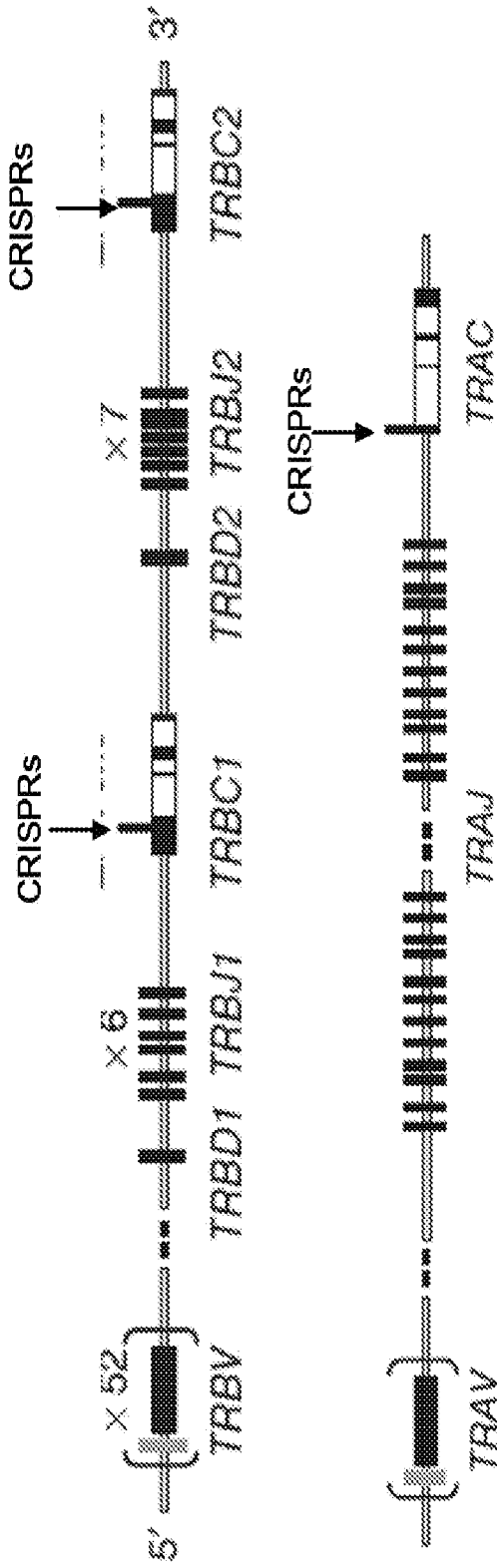


Figure 1A

T7 promoter + target RNA + guide RNA scaffold

TTAATACGAC TCAC TATAGCCACCAAGC TCGCC TTACC

GTTTTAGAGCTAGAAA TAGCAAGTTAAA TAAGGCTAGT

CCGTTATCAACTTGAAAAG TGGCACCGAG TCGGTGCTT

TTTTT

Figure 1B

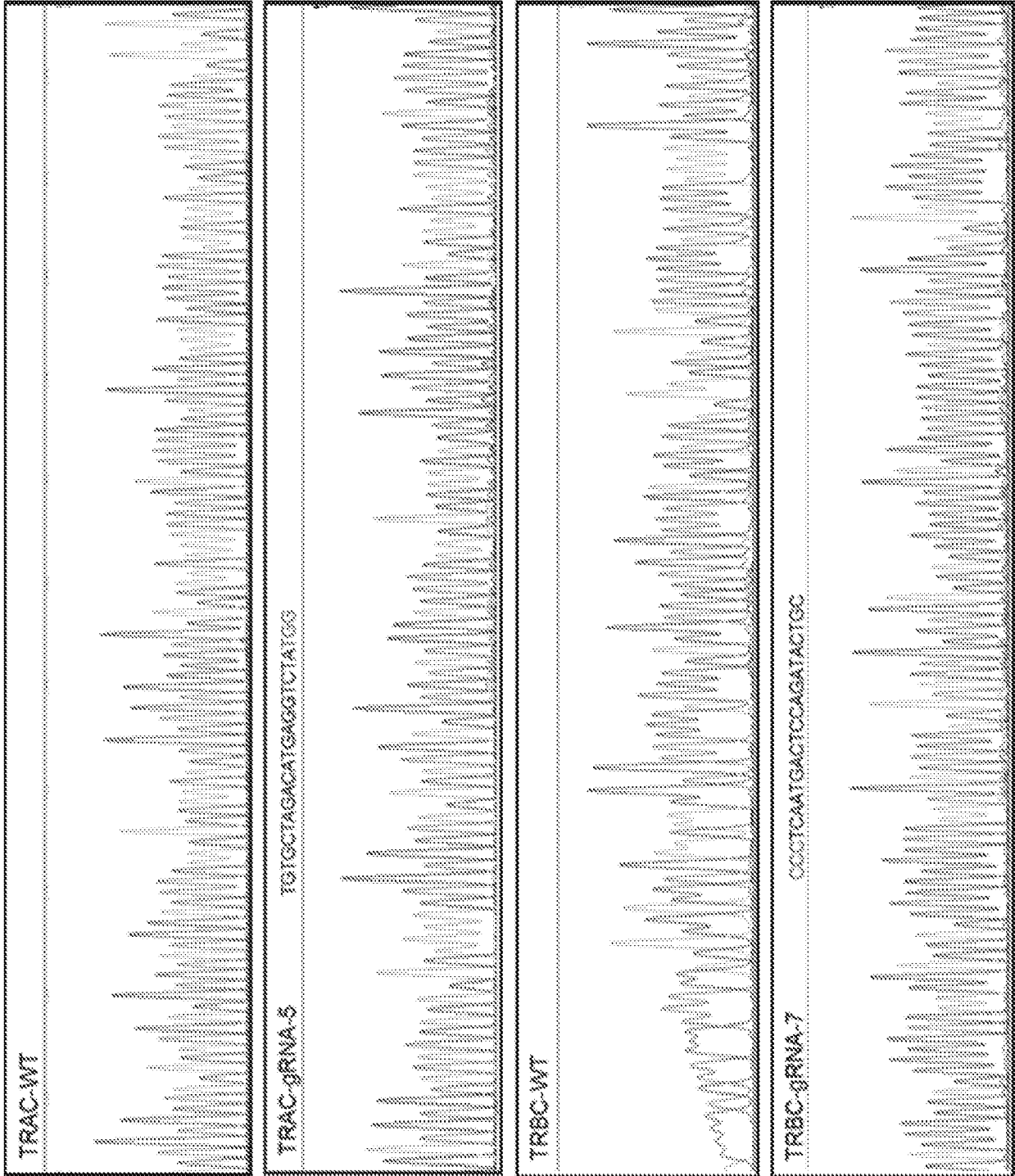


Figure 1C

	% GFP	MFI	% Viability	um3
200V/20ms	61.4	151806	31	300
200V/10ms	87.7	165419	37	280
300V/10ms	95.4	168023	23	250
360V/5ms	65.5	76453	40	320
360V/1ms	98.3	100755	82	600
400V/0.7ms	97.5	81368	87	610
500V/0.7ms	97.8	89314	80	600

Figure 2A

Sample Name	Subset Name	Count
E07 32C-NC day2 fcs	Lymphocytes	10549
E08 32C-1 ug day2 fcs	Lymphocytes	10467
E09 32C-2 ug day2 fcs	Lymphocytes	10527
D07 37C-NC day2 fcs	Lymphocytes	10508
D08 37C-1 ug day2 fcs	Lymphocytes	10414
D09 37C-2 ug day2 fcs	Lymphocytes	10569

Sample Name	Subset Name	Count
E01 32C-NC day1 fcs	Lymphocytes	10206
E02 32C-1 ug day1 fcs	Lymphocytes	10196
E03 32C-2 ug day1 fcs	Lymphocytes	10182
D01 37C-NC day1 fcs	Lymphocytes	10439
D02 37C-1 ug day1 fcs	Lymphocytes	10331
D03 37C-2 ug day1 fcs	Lymphocytes	10336

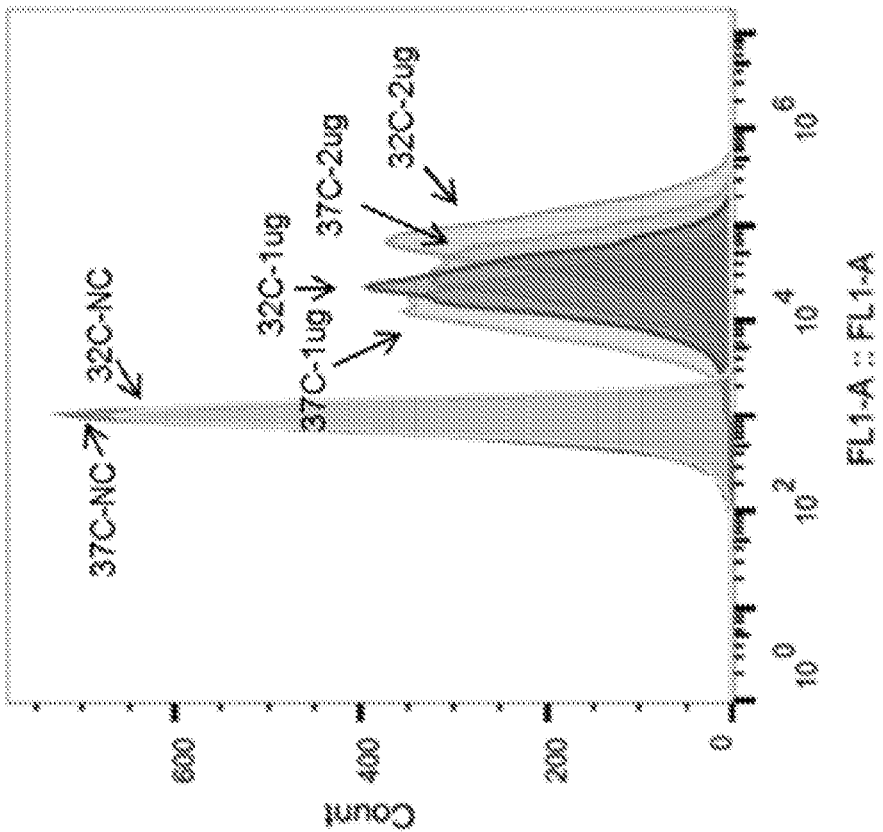
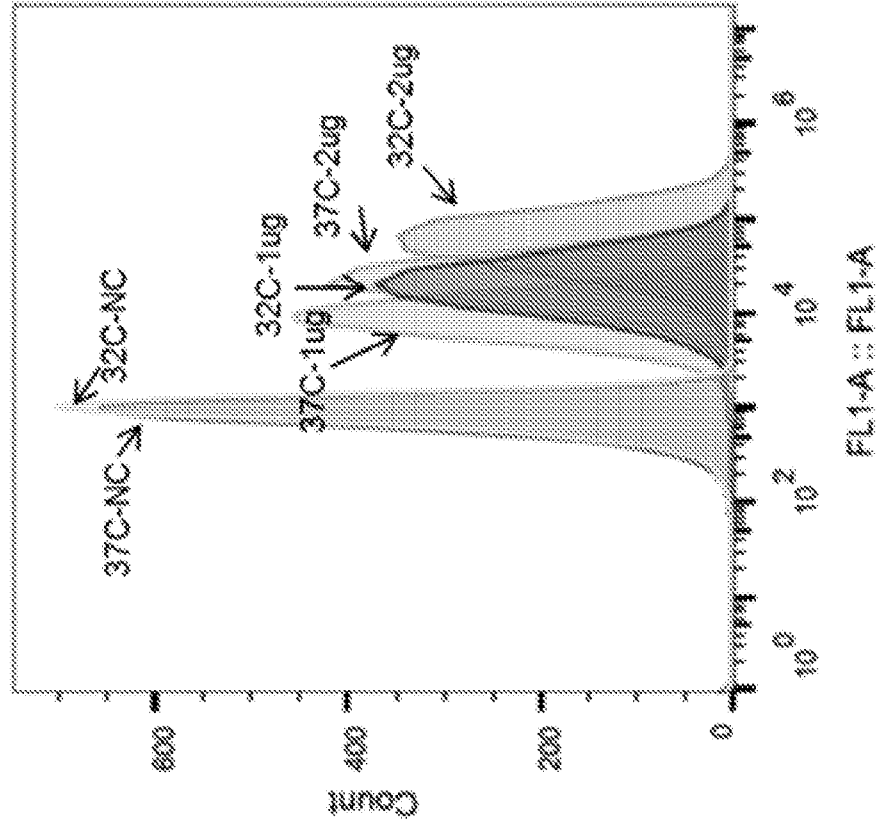


Figure 2B

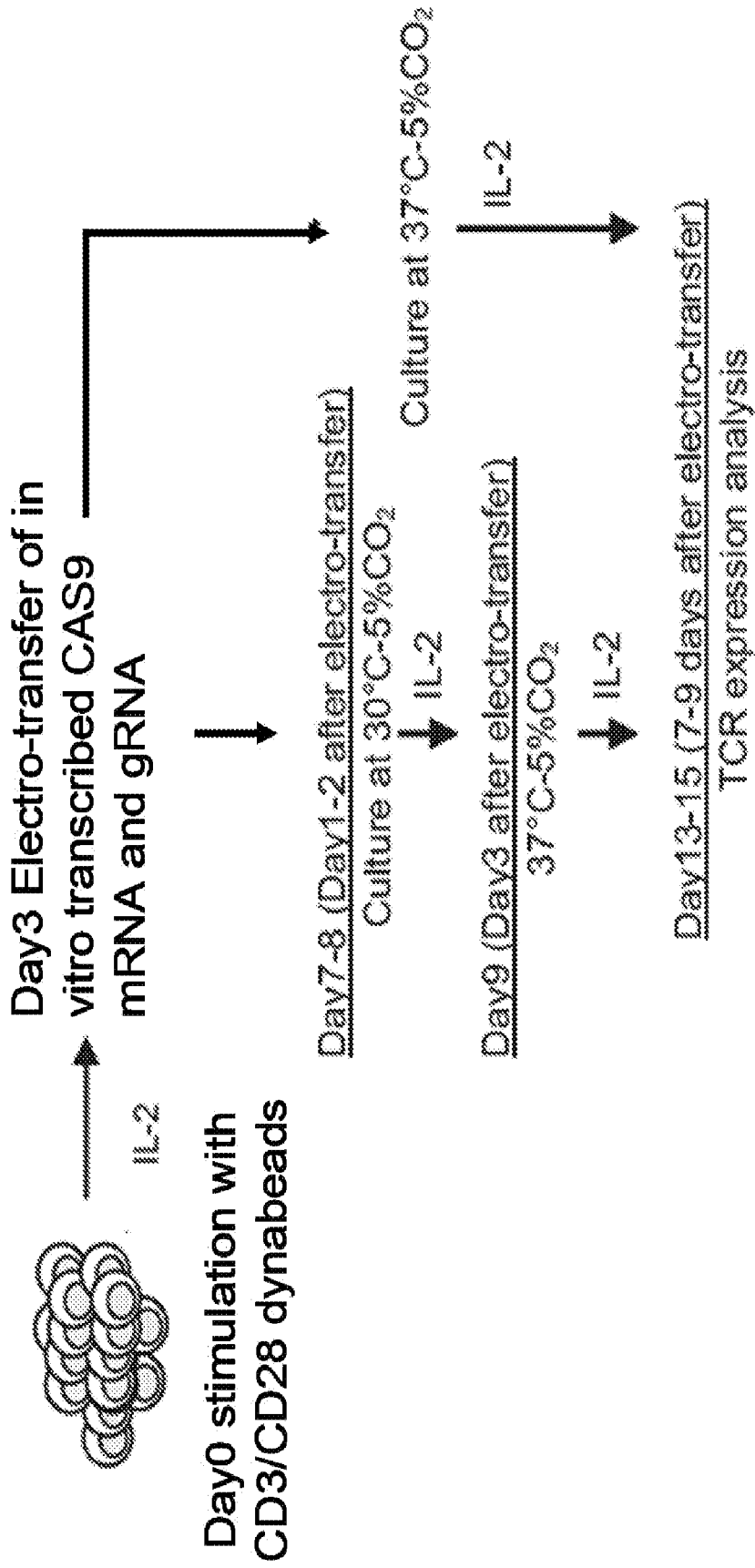


Figure 2C

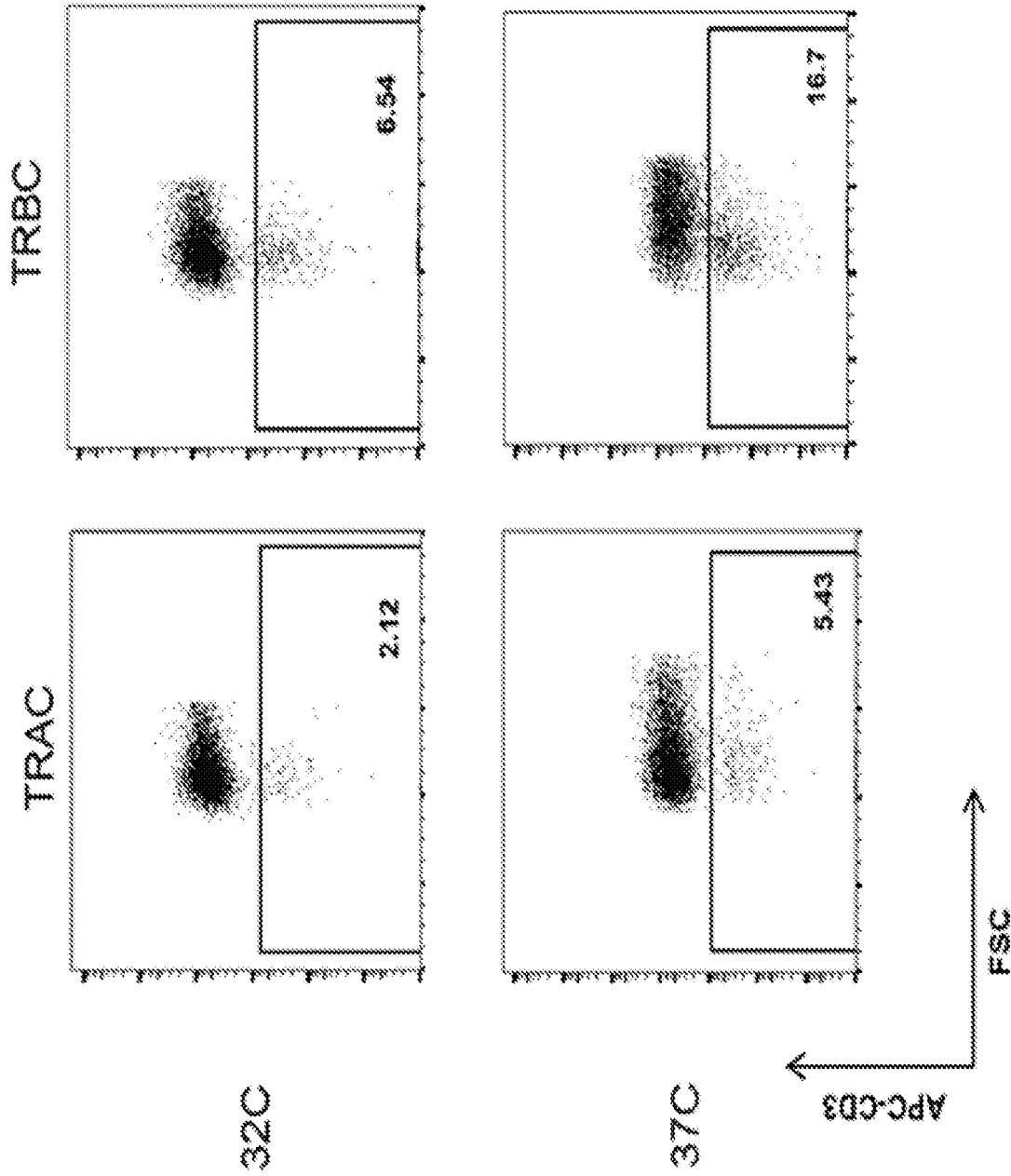


Figure 2D

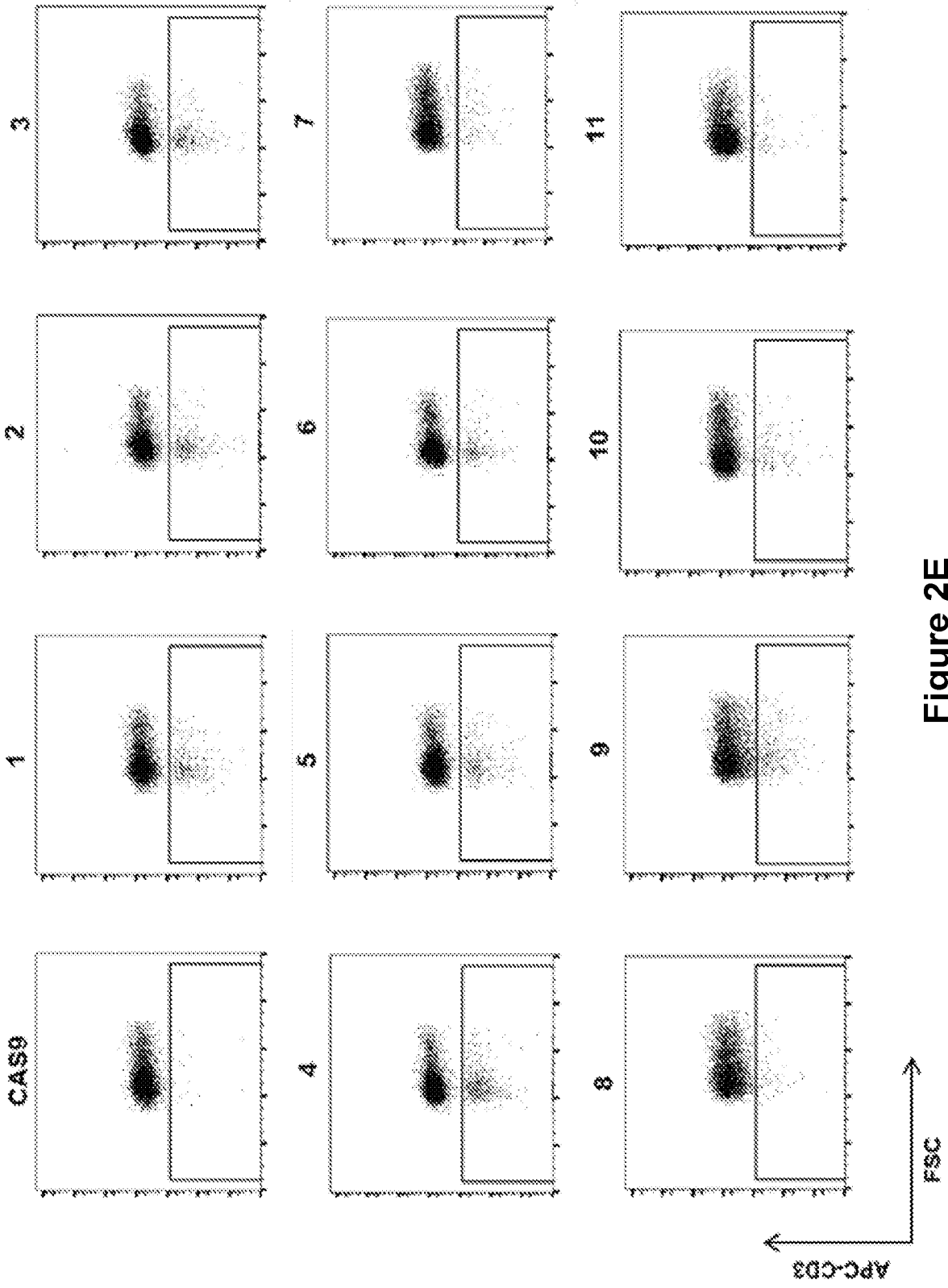


Figure 2E

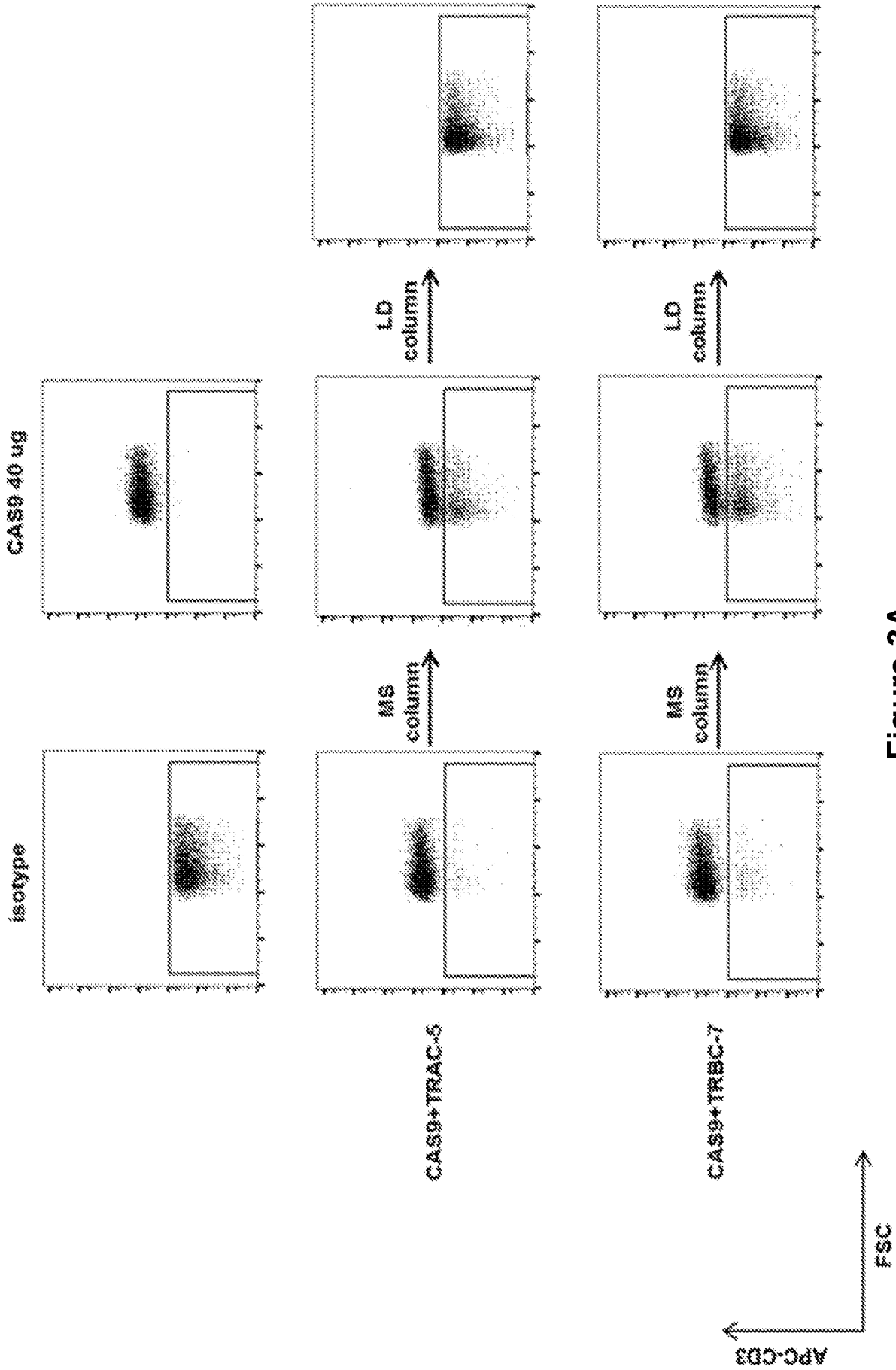


Figure 3A

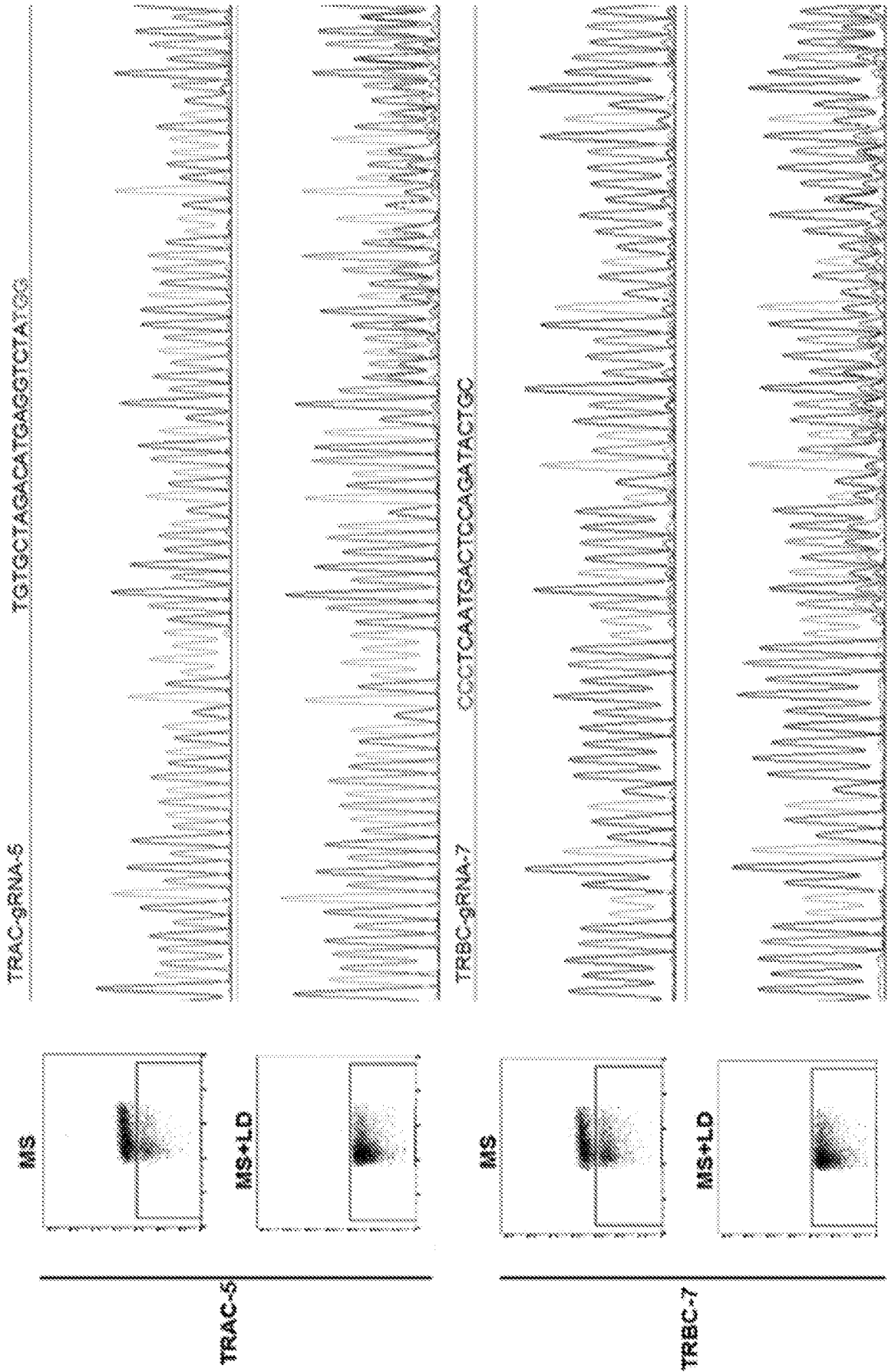


Figure 3B

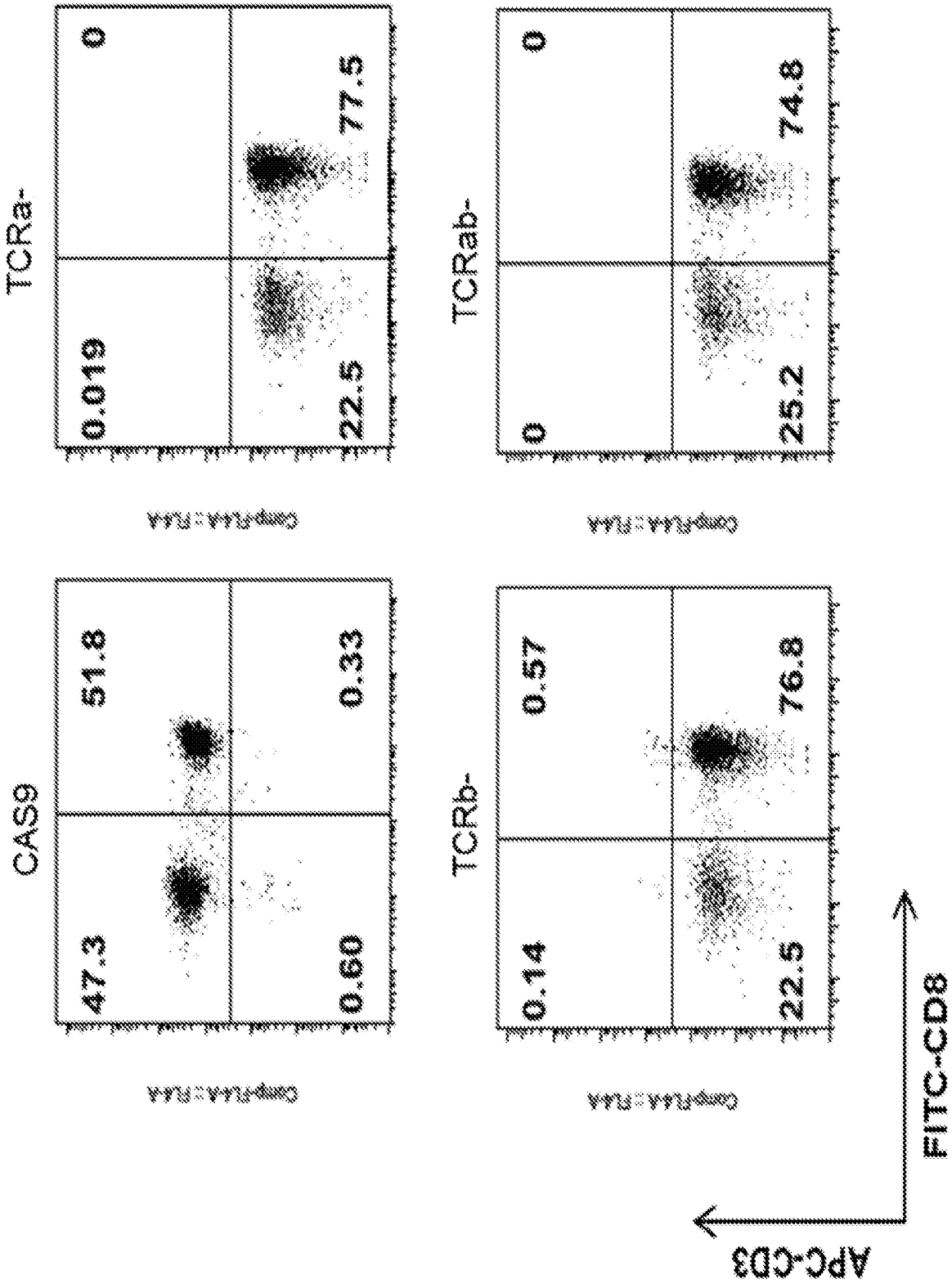


Figure 3C

```

TRAC WT  TATATCACAGACAAAACCTGTGCTAGACAATGAGGTCIATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X4)      TATATCACAGACAAAACCTGTGCTAGACATGAG-TCATATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X3)      TATATCACAGACAAAACCTGTGCTAGACATG--GTCTATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X1)      TATATCACAGACAAAACCTGTGCTAGACATGA-GTCTATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X1)      TATATCACAGACAAAACCTGTGCTAGACATG---TCATATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X1)      TATATCACAGACAAAACCTGTGCTAGAC-IGAGGTCIATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X1)      TATATCACAGACAAAACCTGTGCTAG---TGAGGTCIATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X1)      TATATCACAGACAAAACCTGTGCT- - - - -TGAGGTCIATGGACTTCAAGAGCAACAGTGTGTGGCCCT
(X1)      TATATCACAGACAAAACCTGTGCTAGACAIGAGGTCIATGGACTTCAAGAGCAACAGTGTGTGGCC
(X1)      TATATCACAGACAAAACCTGTGCTAGACATGAGGTCIATGGACTTCAAGAGCAACAGTGTGTGGCC

TRBC1 WT  AGCAGCCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X2)      AGCAGCCCCGCCCTC-ATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X2)      AGCAGCCCCGCC-TCATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X1)      AGCAGCCCCGCCCTCAATGACTC-AGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X1)      AGCAGCCCCGCCCTCAATGACT--AGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X1)      AGCAGCCCCGCC- - - - -GACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X1)      AGCAGCCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCT
(X1)      AGCAGCCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCT

TRBC2 WT  AGCAGCCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X3)      AGCAGCCCCGCCCTCAATGAC- - - - -AGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X2)      AGCAGCCCCGCCCTCAATGACTC-AGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X1)      AGCAGCCCCGCCCTCA- - - - - - - - - - -ACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCTG
(X1)      AGCAGCCCCGCC-TCATGACT-CAAGATACTG-CTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCT
(X1)      AGCAGCC- - - - -ACTG- - - - -C-G- - - - -TGAGCAGCCGGTGTGAGGGTCTCGGCCACCTTCT
(X1)      AGCAGCCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGGCCTGAGGGTCTCGGCCACCTTCT

```

Figure 3D

	0 hrs	18 hrs	24hrs	36hrs
1	CAS9 20ug, gRNA 10ug			
2	CAS9 20ug, gRNA 20ug			
3	CAS9 20ug, gRNA 40ug			
4	CAS9 20ug, gRNA 10ug			
5	CAS9 20ug, gRNA 10ug	gRNA 10ug		
6	CAS9 20ug, gRNA 10ug	gRNA 10ug	gRNA 10ug	
7	CAS9 20ug, gRNA 10ug		gRNA 10ug	gRNA 10ug
8	CAS9 20ug, gRNA 10ug	gRNA 10ug	gRNA 10ug	gRNA 10ug

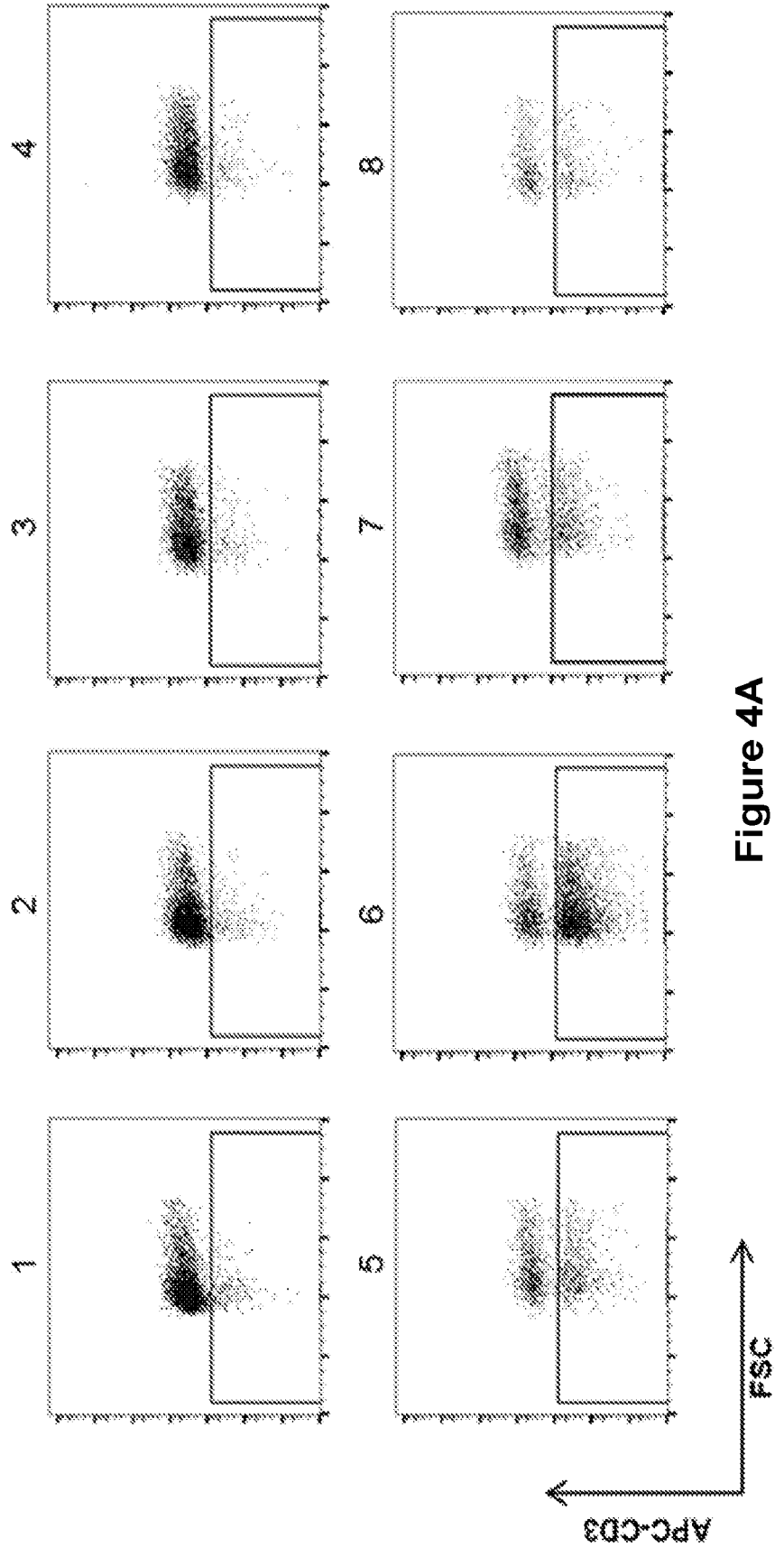


Figure 4A

	CAS9 20 ug	first time		second time	
		0 hrs	16 hrs	16 hrs	24 hrs
1	TRAC13	C AS9 20ug ,GRNA10 ug			
2	TRAC13	C AS9 20ug ,GRNA10 ug	GRNA 10 ug		
3	TRAC13	C AS9 20ug ,GRNA10 ug			GRNA 10 ug
4	TRBC4	C AS9 20ug ,GRNA10 ug			
5	TRBC4	C AS9 20ug ,GRNA10 ug	GRNA 10 ug		
6	TRBC4	C AS9 20ug ,GRNA10 ug			GRNA 10 ug
7	Capped TRAC13	C AS9 20ug ,GRNA10 ug			
8	Capped TRAC13	C AS9 20ug ,GRNA10 ug	GRNA 10 ug		
9	Capped TRAC13	C AS9 20ug ,GRNA10 ug			GRNA 10 ug
10	Capped TRBC4	C AS9 20ug ,GRNA10 ug			
11	Capped TRBC4	C AS9 20ug ,GRNA10 ug	GRNA 10 ug		
12	Capped TRBC4	C AS9 20ug ,GRNA10 ug			GRNA 10 ug

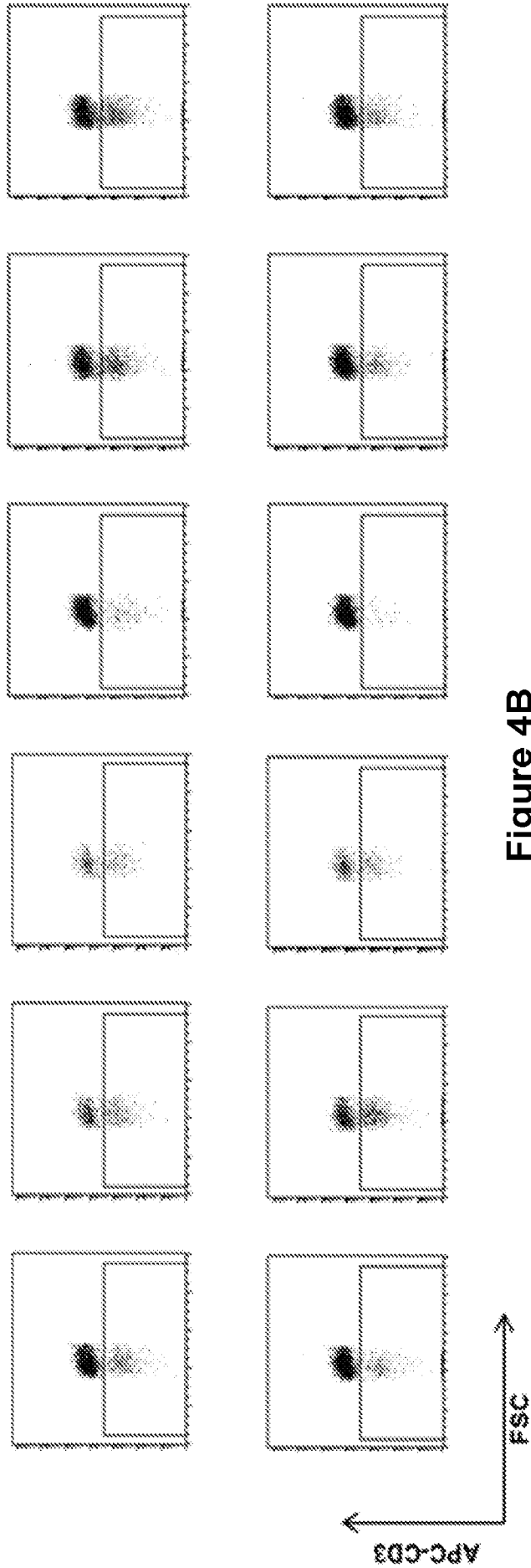


Figure 4B

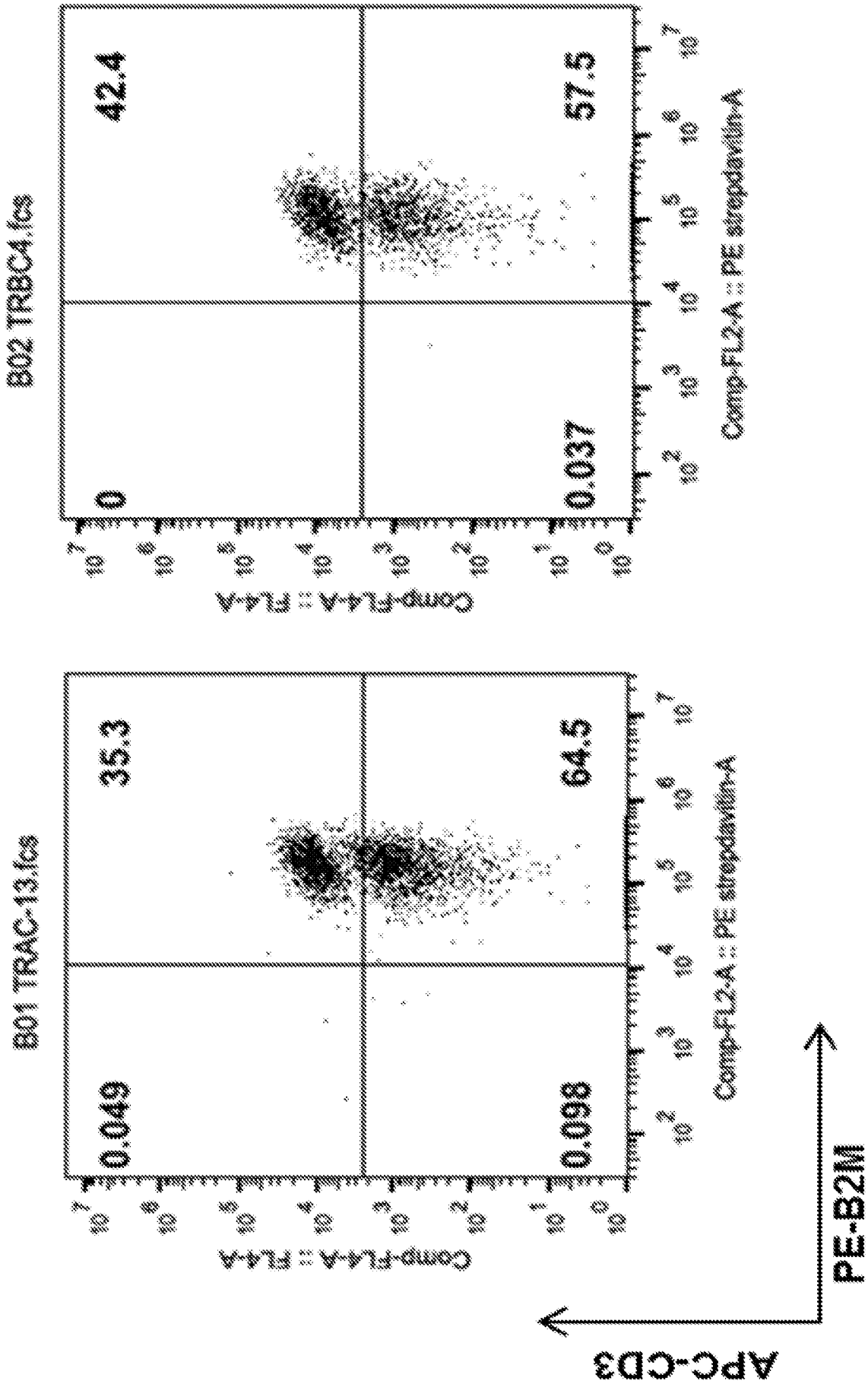


Figure 4C

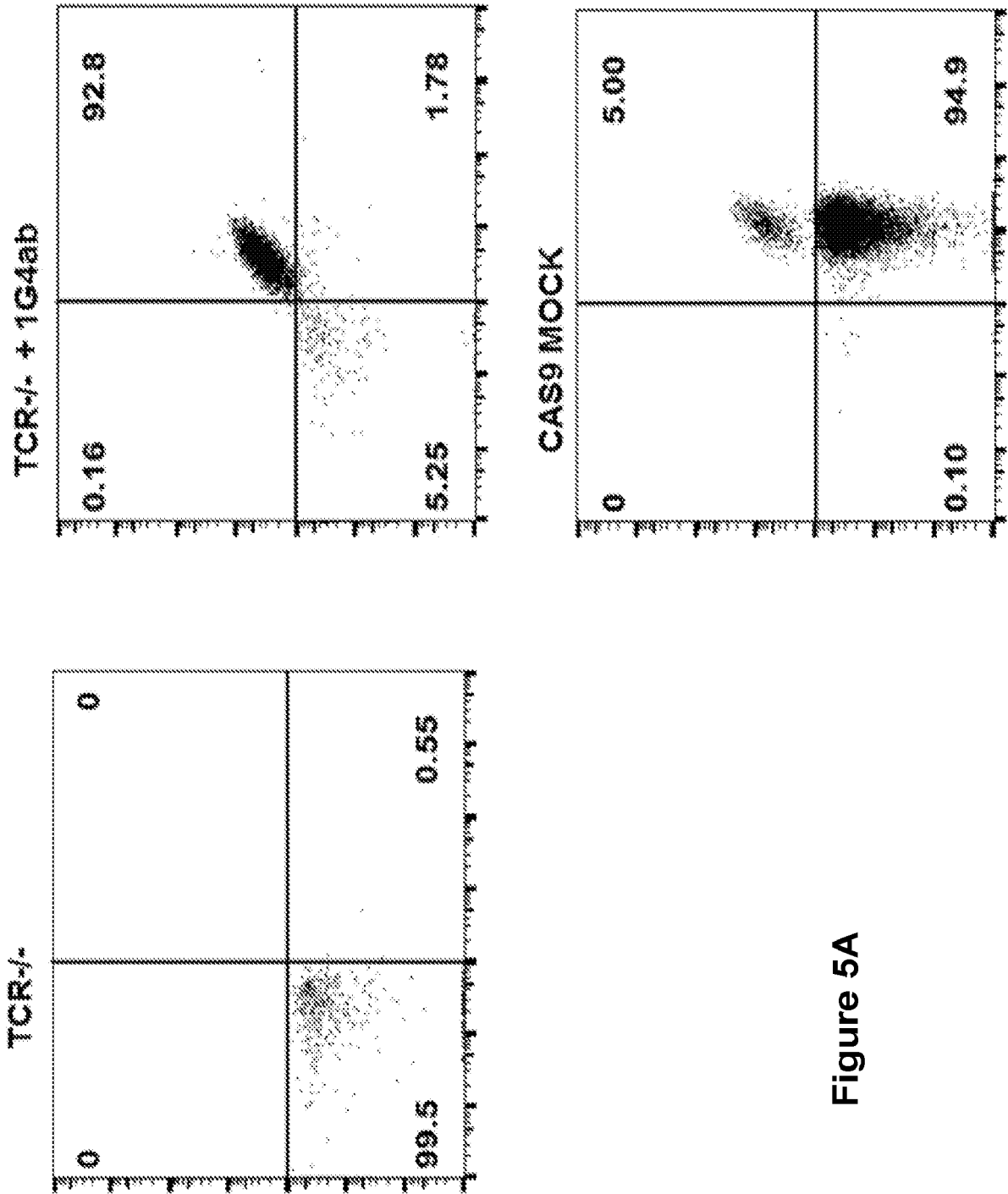


Figure 5A

Fold Expansion		Day 0	Day 3	Day 5	Day 7	Day 9	Day 11
Groups							
REP		1	15	100	198	335	507
CD3/CD28 Beads		1	2	7	14	17	25
KT/OKT3		1	2	5	18	29	40
KT/K19		1	3	7	24	36	52
KT/K19/TCR-CLEAR		1	3	8	25	42	58

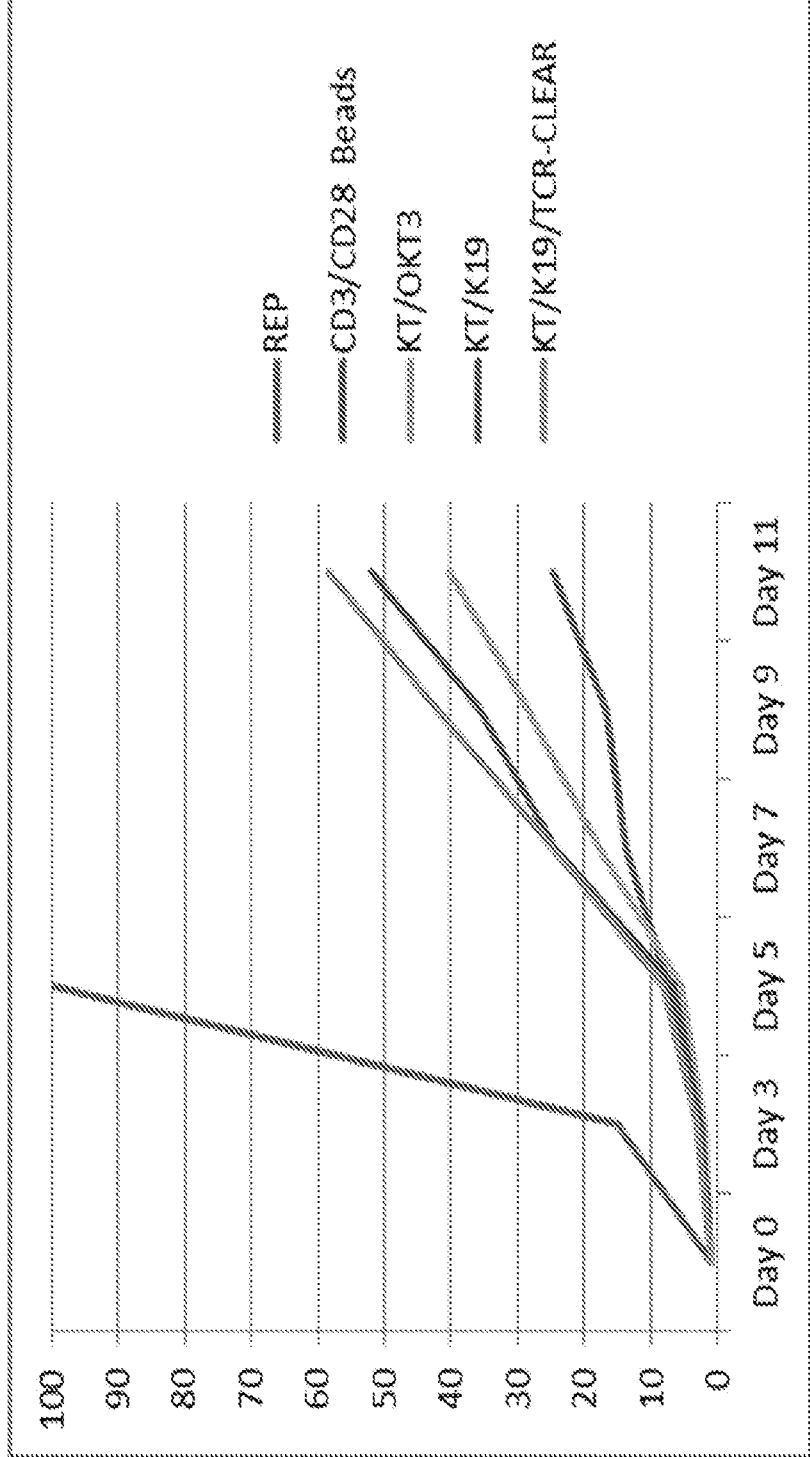


Figure 5B

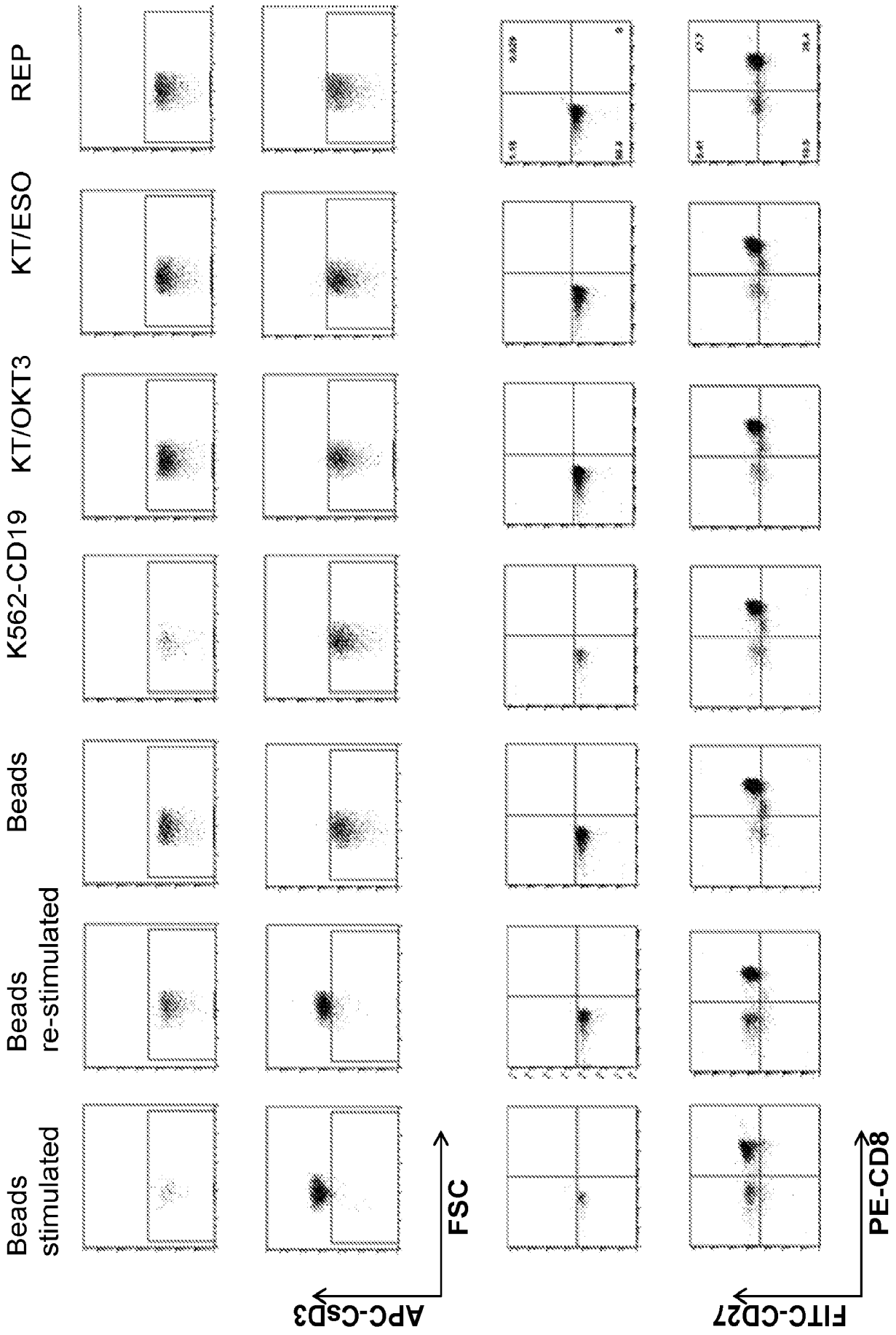


Figure 6A

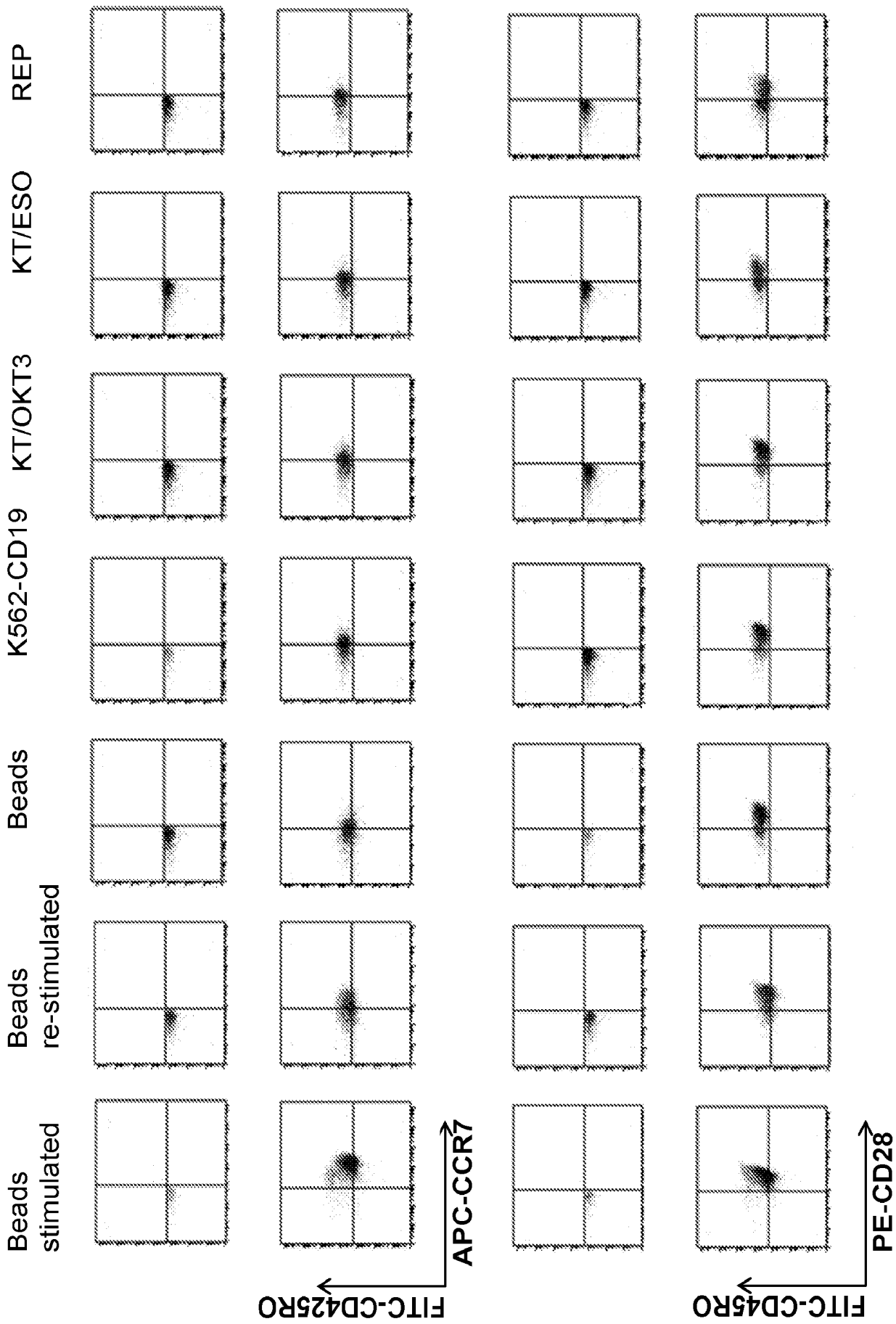


Figure 6B

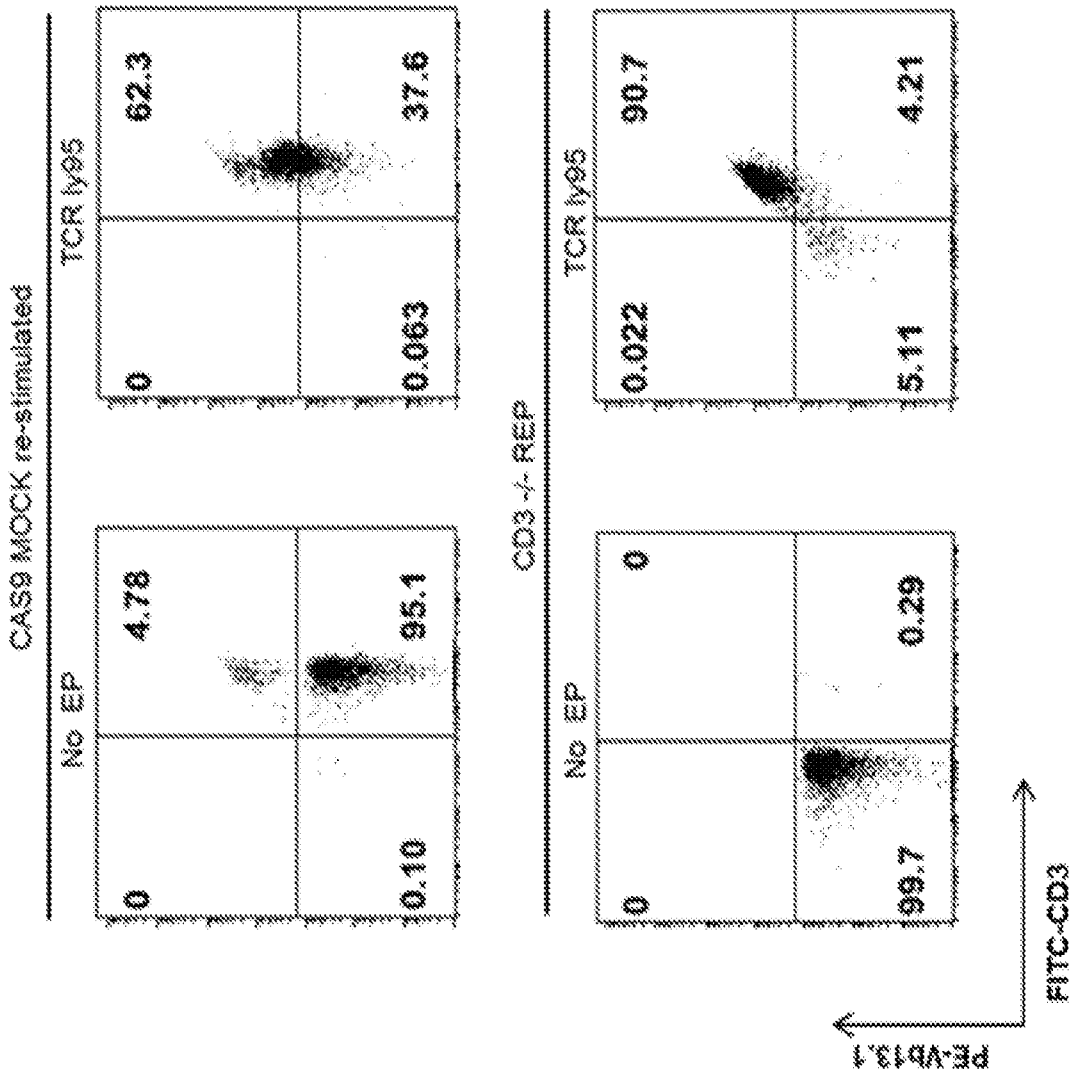


Figure 7A

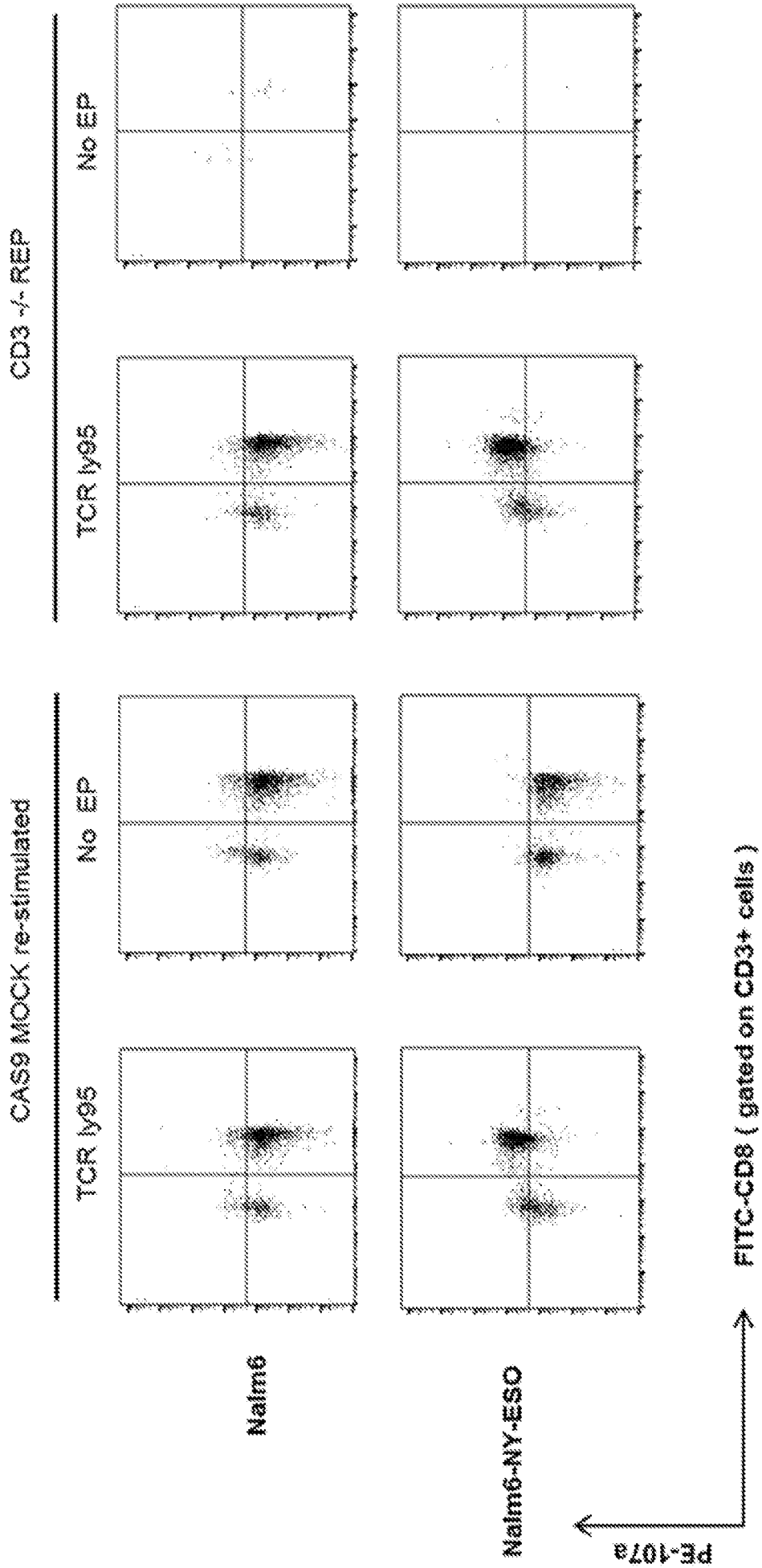


Figure 7B

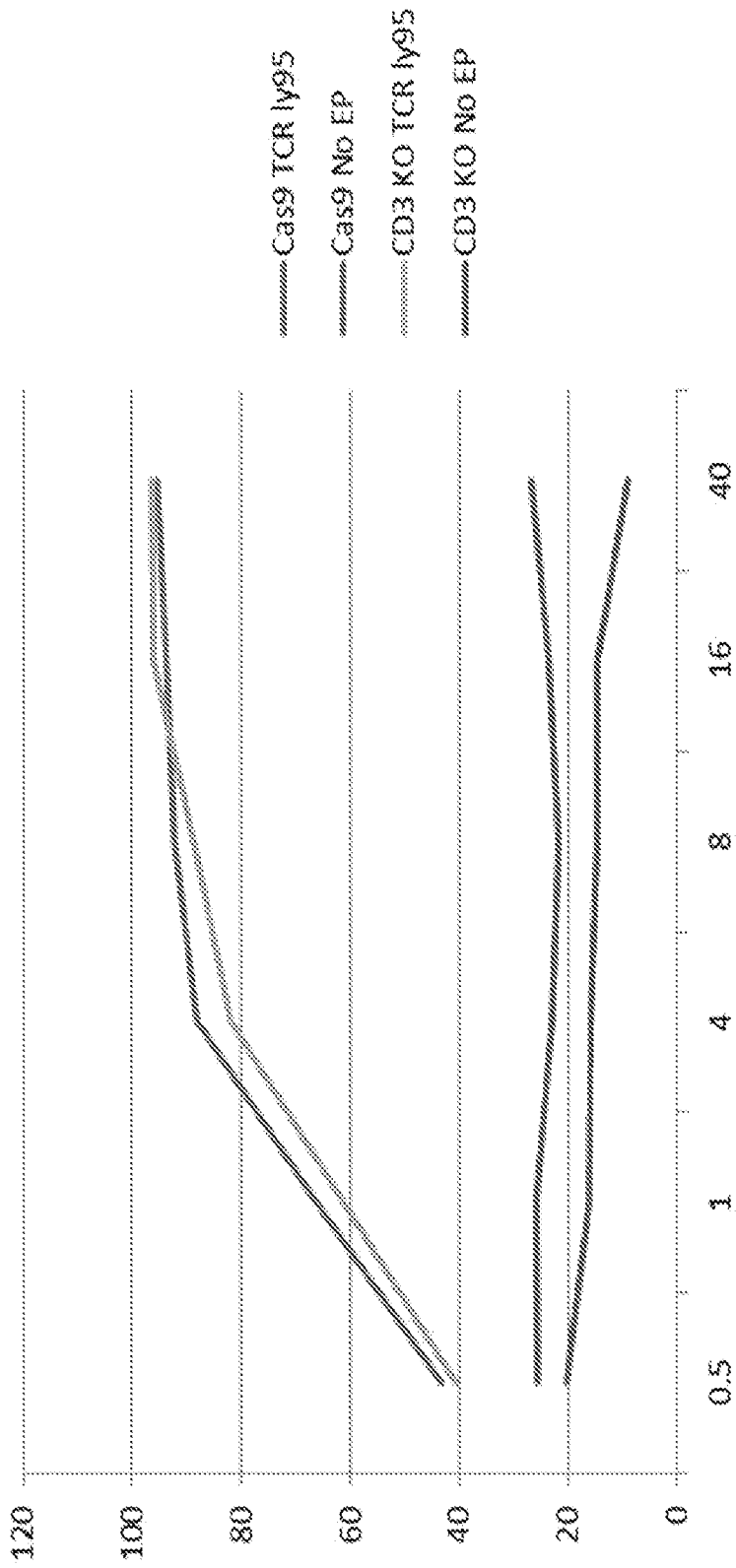


Figure 7C

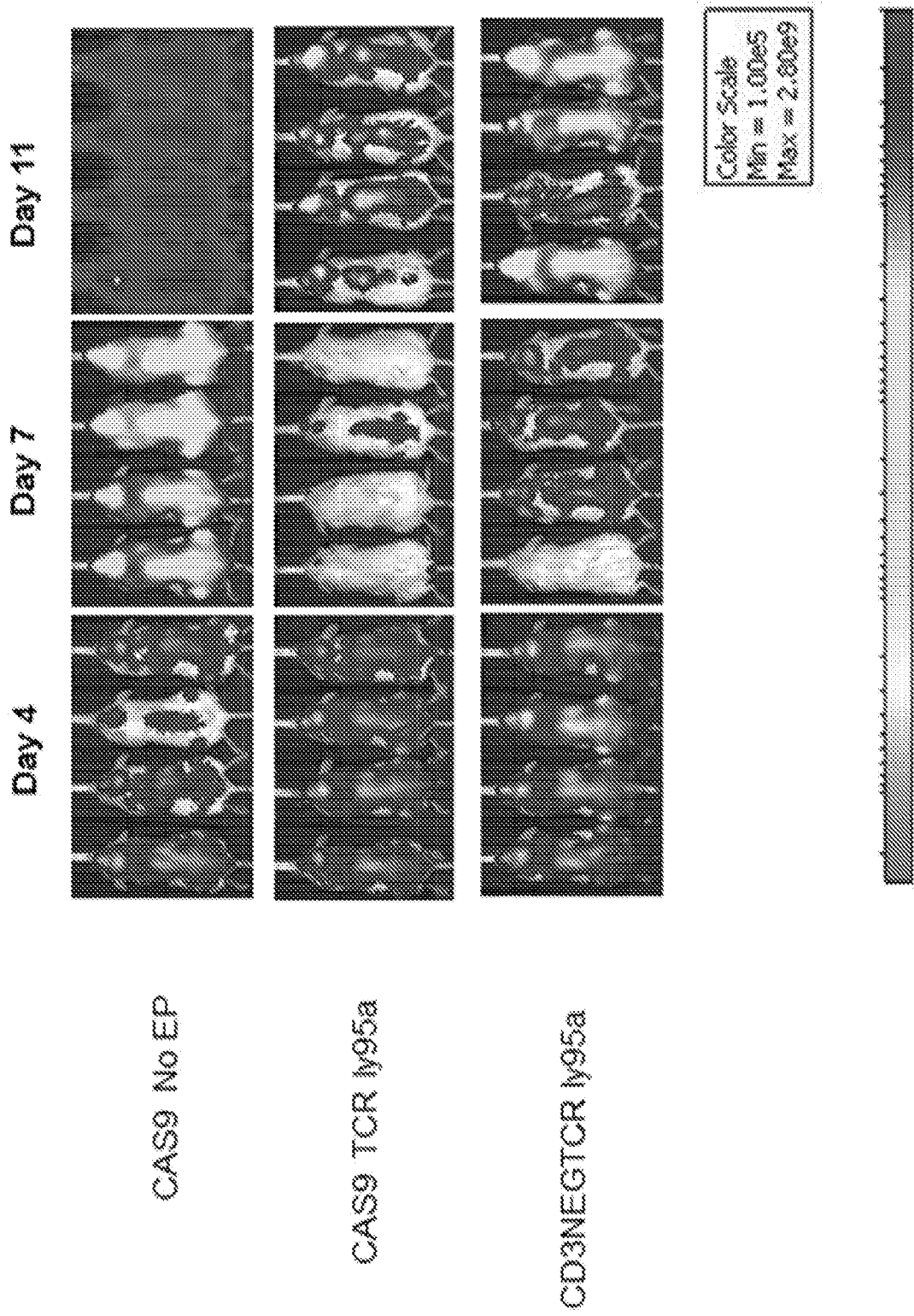


Figure 8

2024203330 20 May 2024

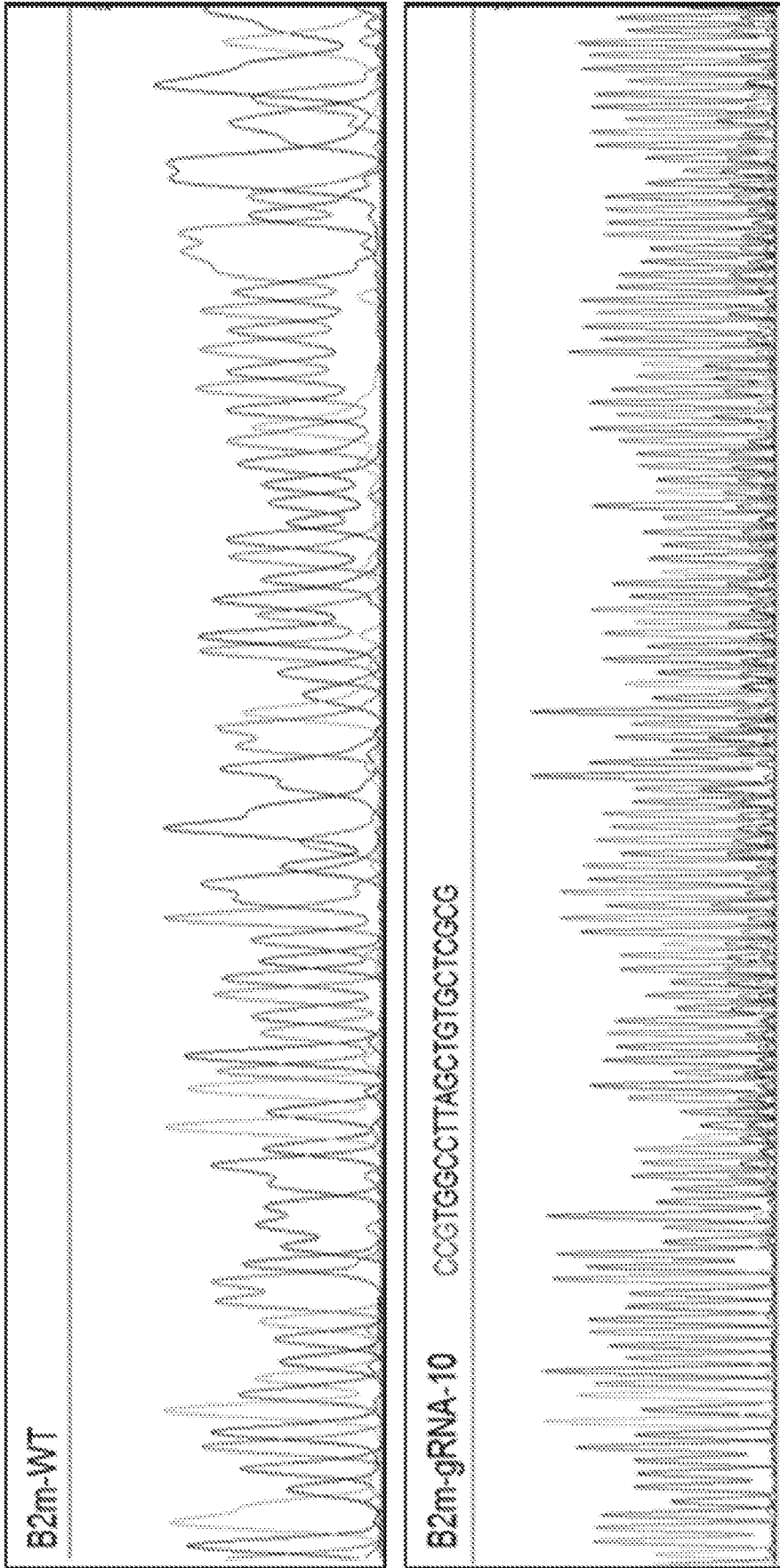


Figure 9A

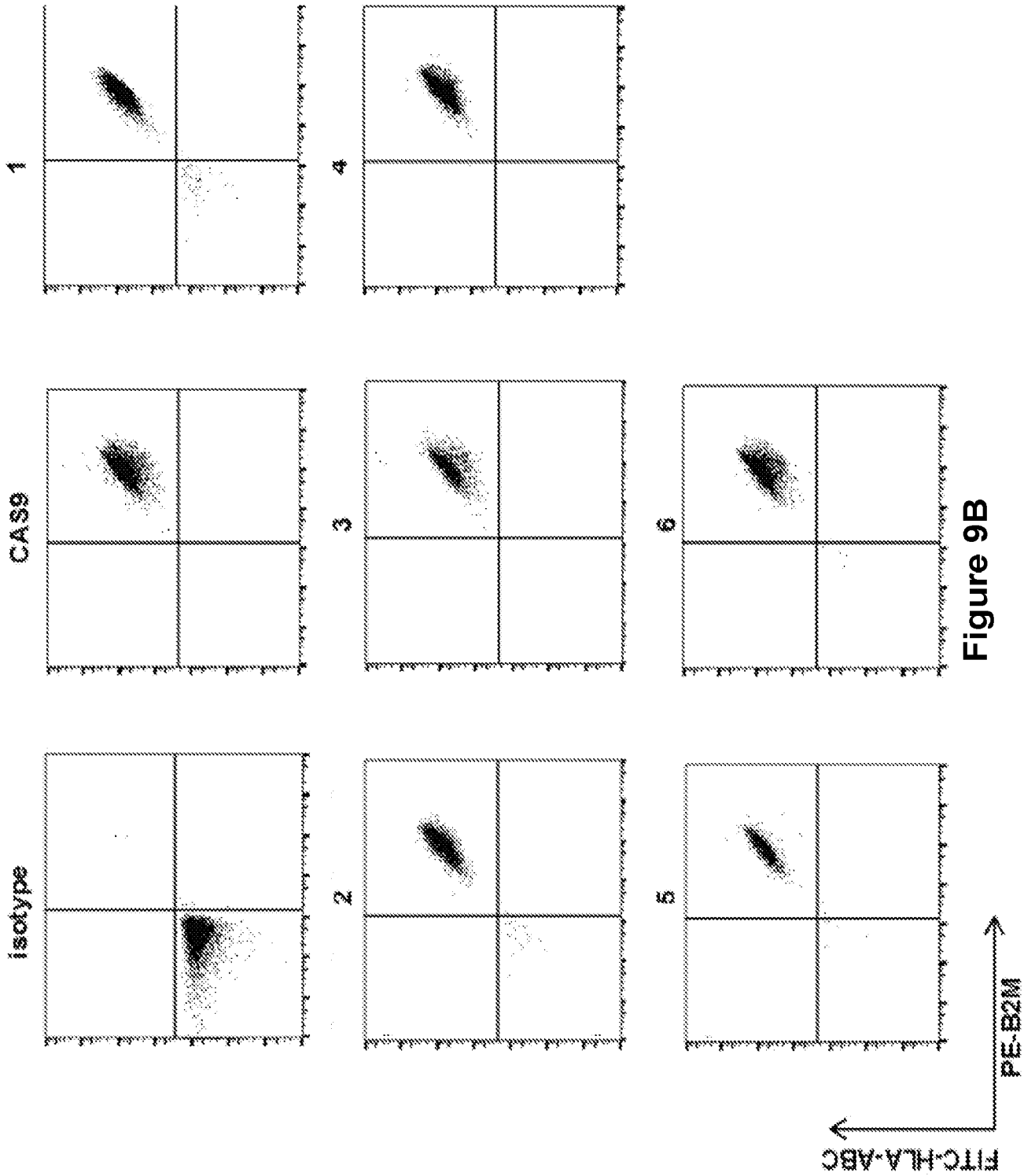


Figure 9B

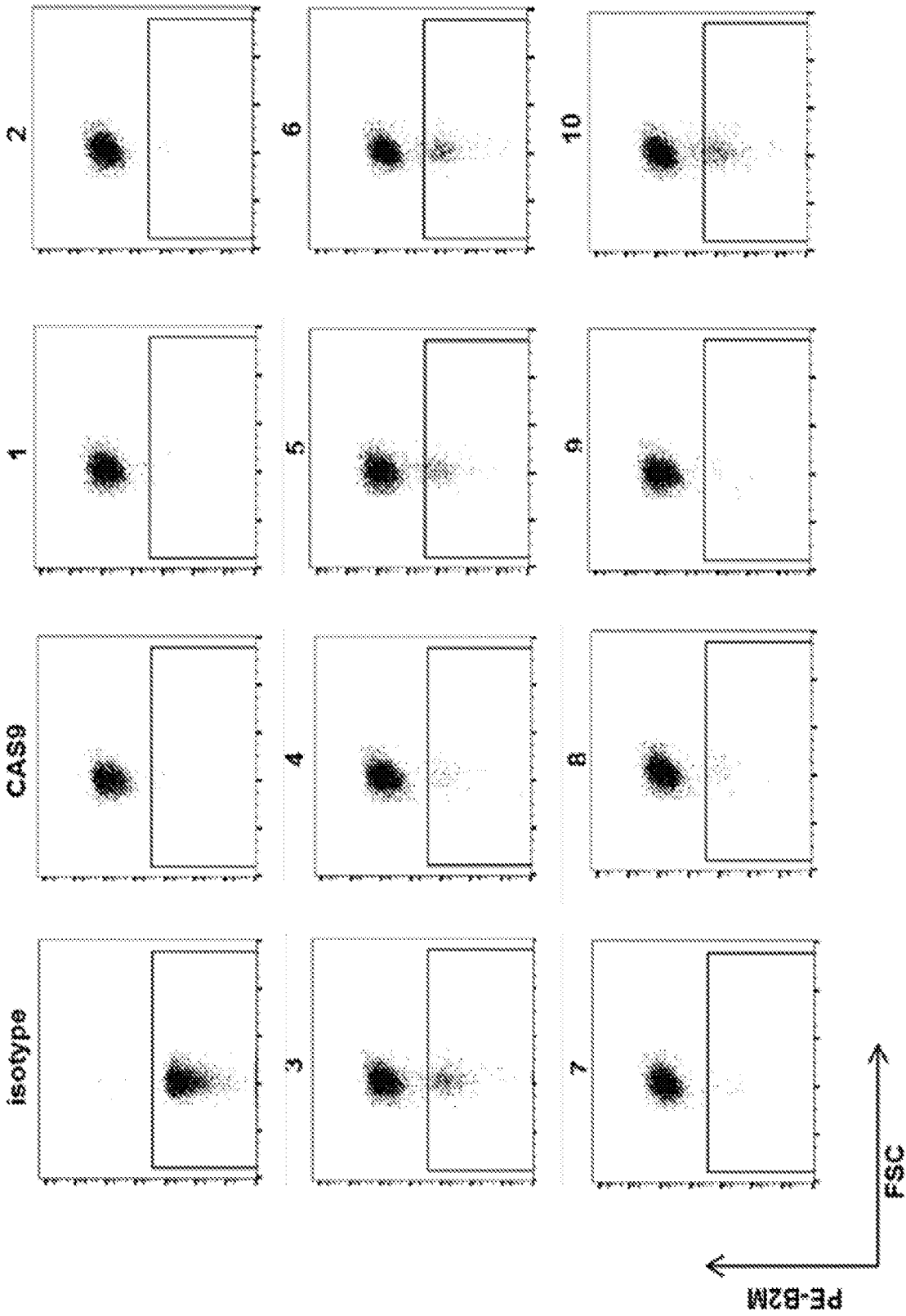


Figure 9C

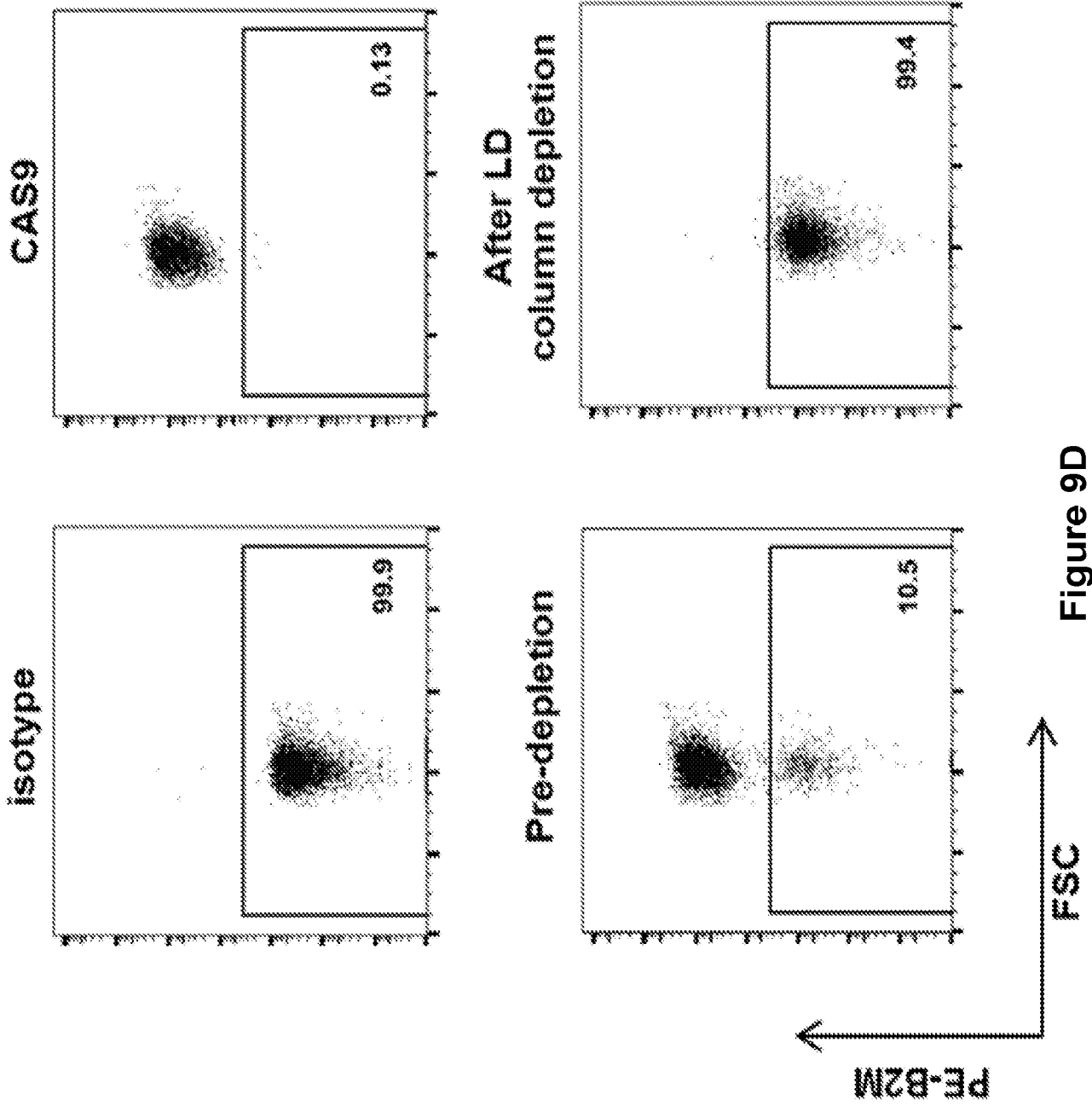


Figure 9D

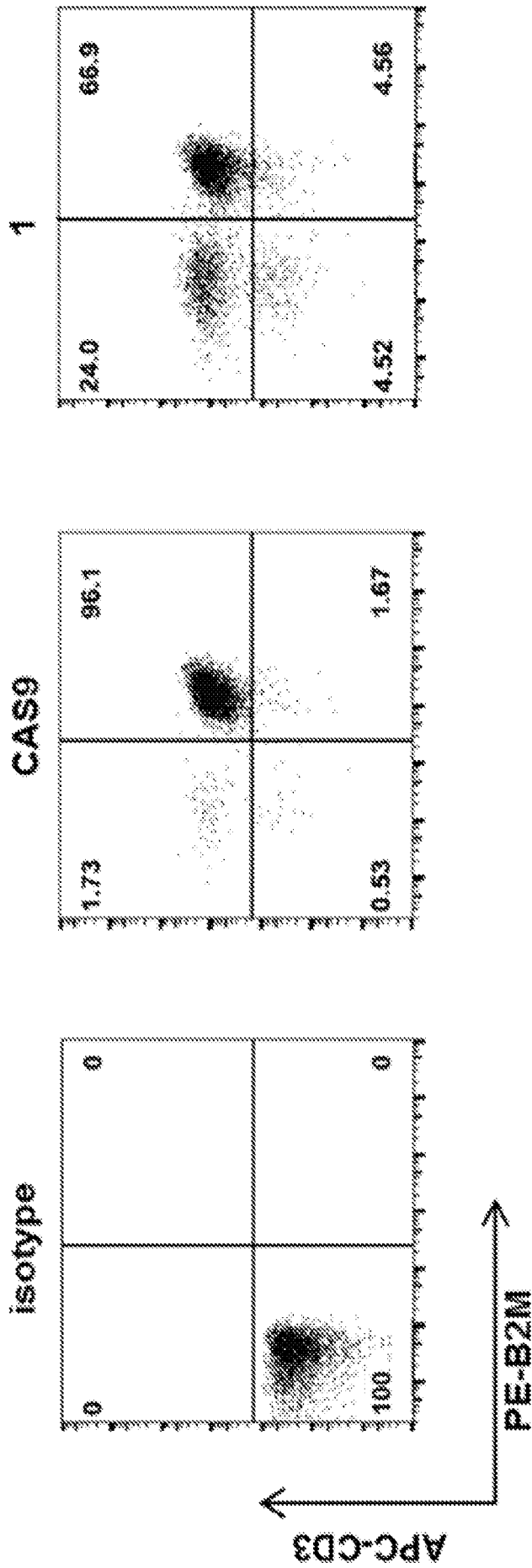


Figure 10

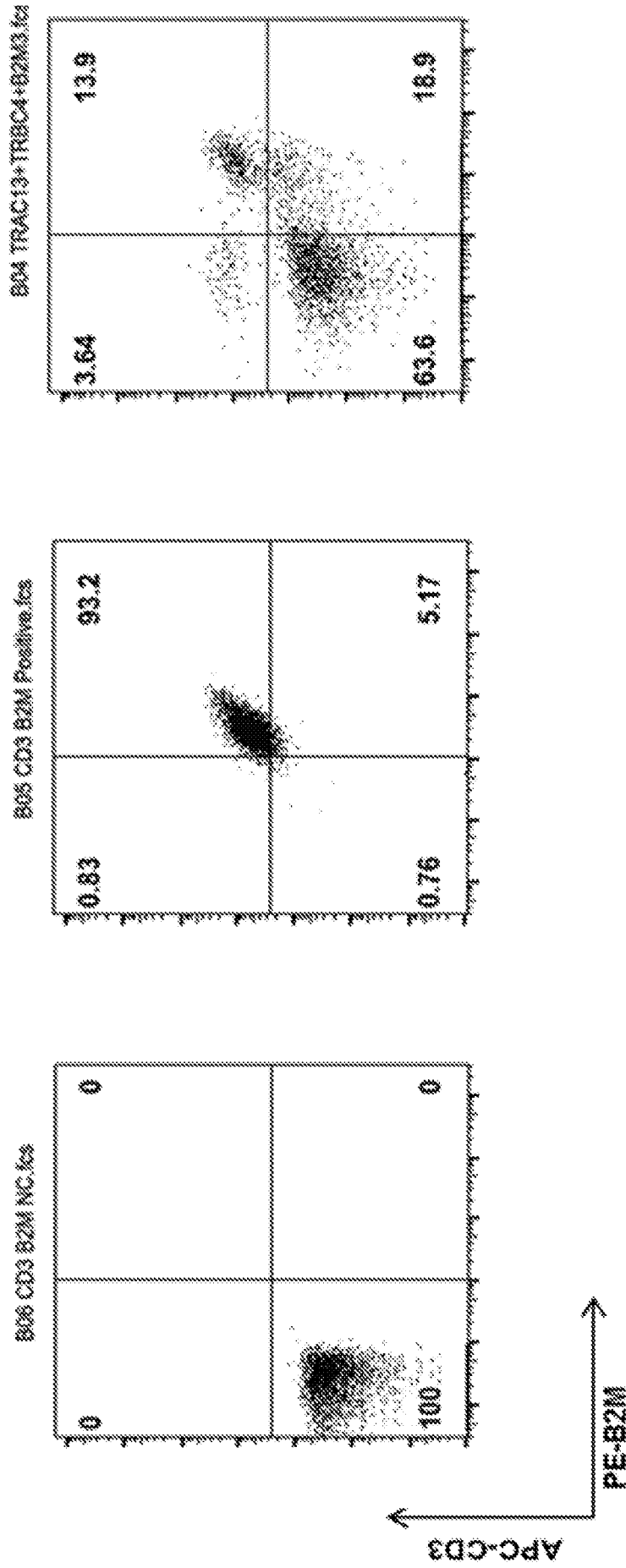


Figure 11A

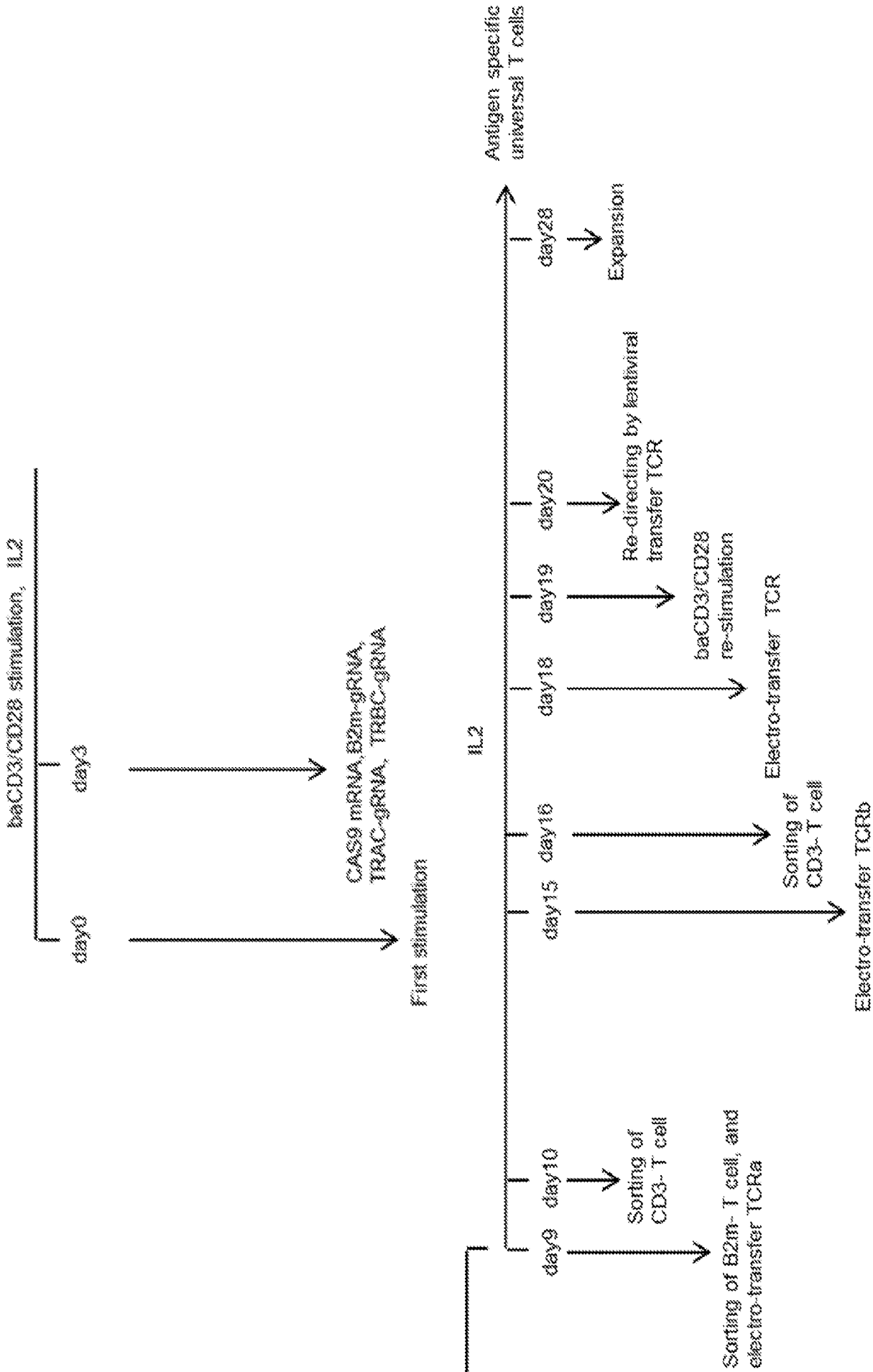
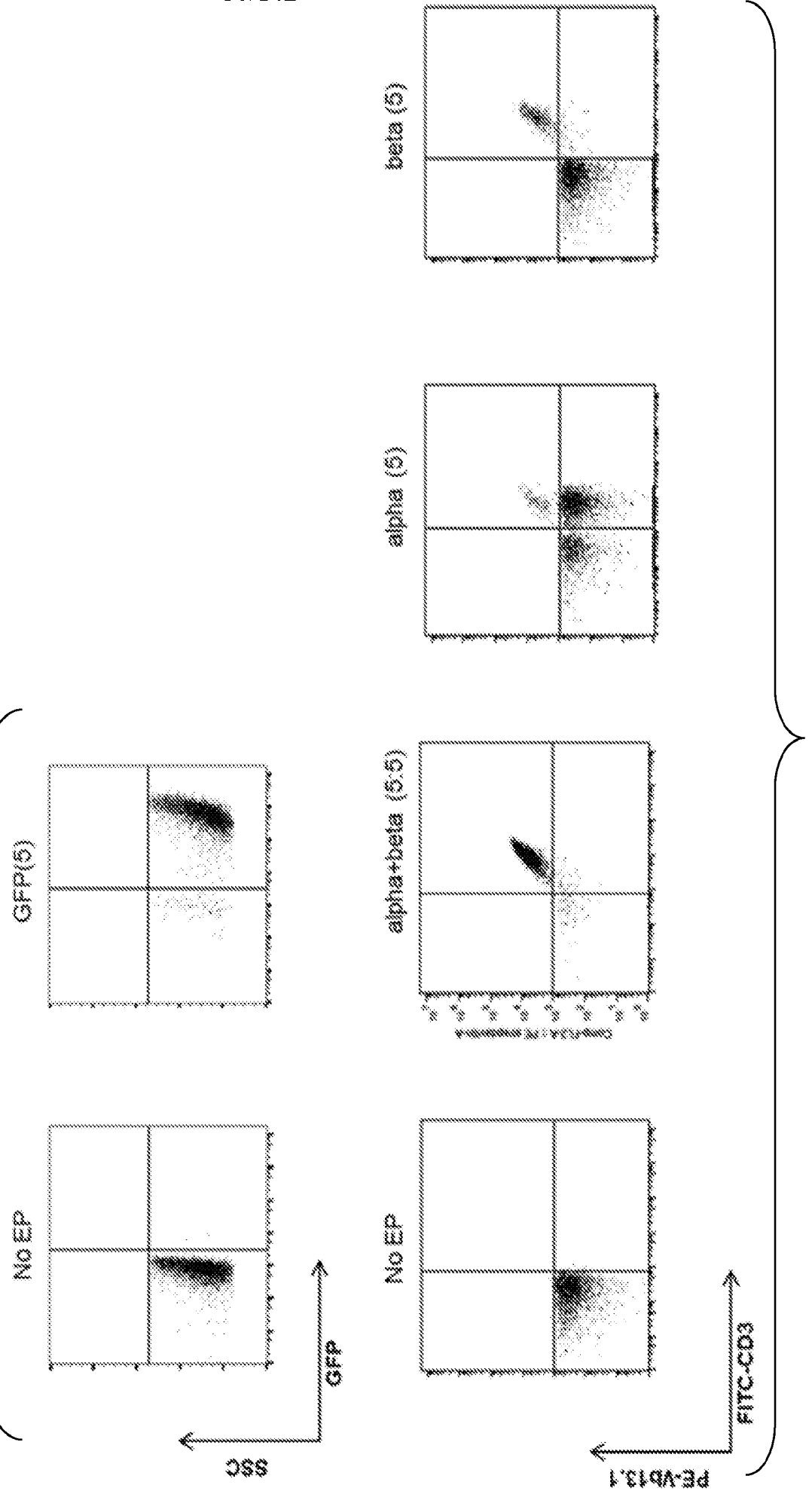


Figure 11B

Figure 11C



2024203330 20 May 2024

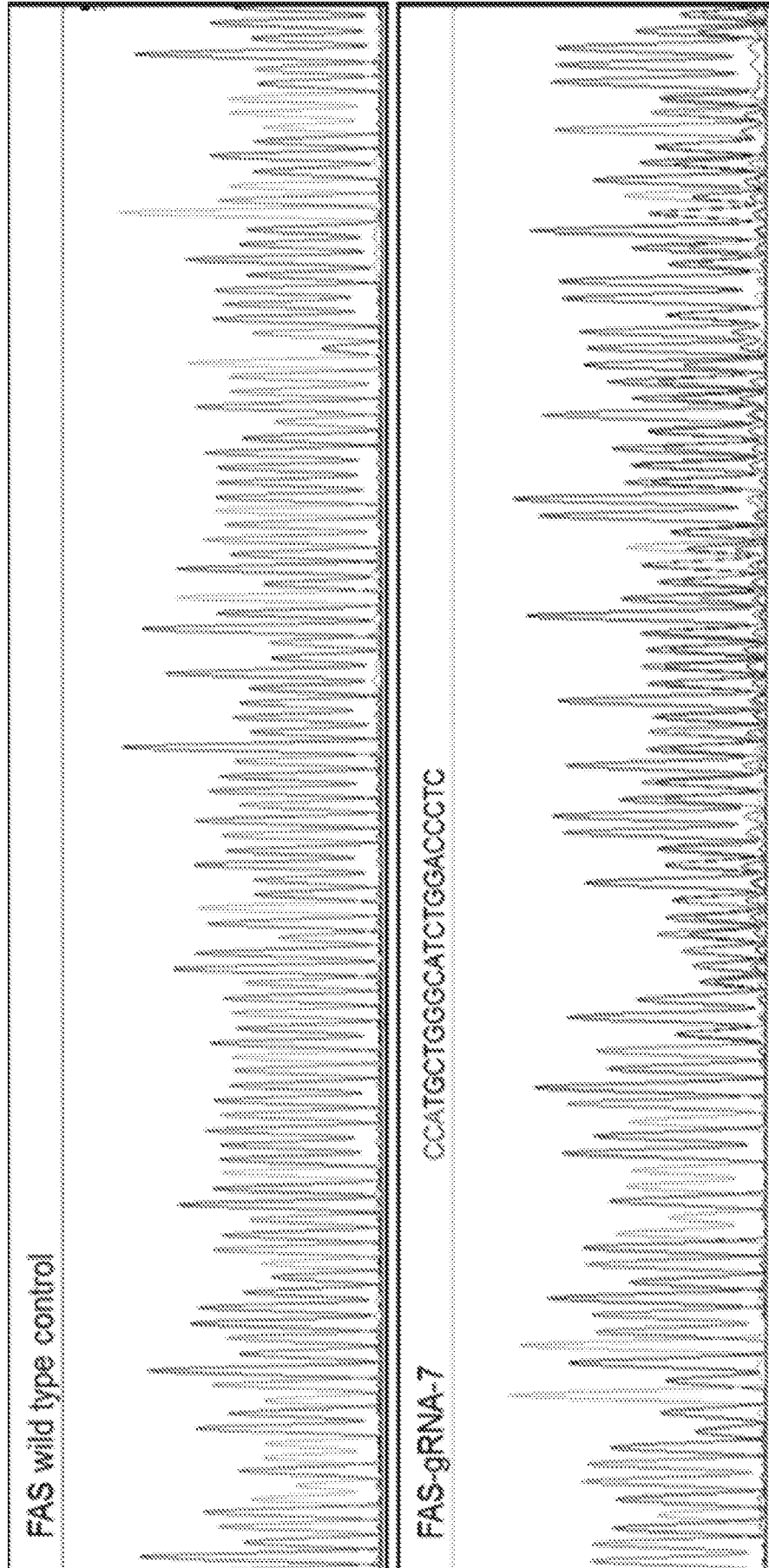


Figure 12A

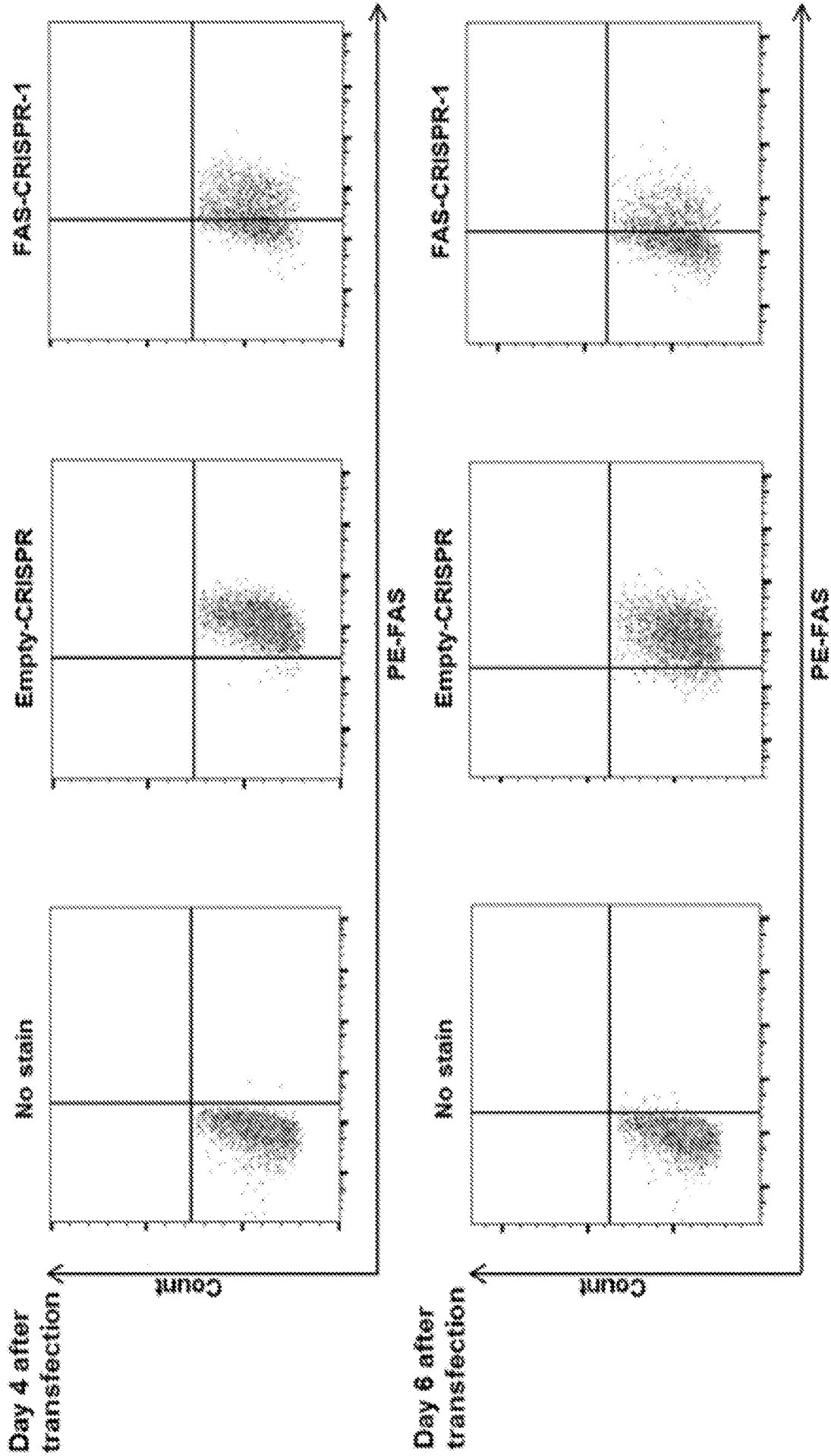
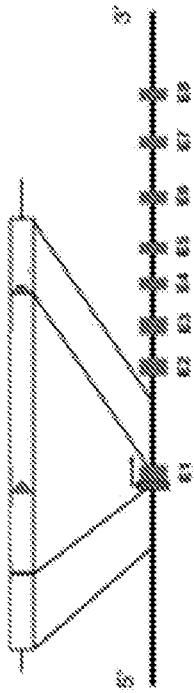


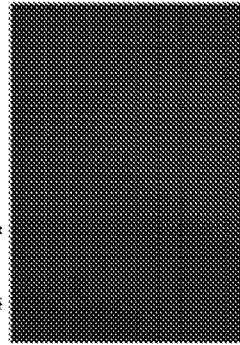
Figure 12B

Day 7 after selection



FAS targeting vector

Targeting vector alone, 4x



GFP



BF

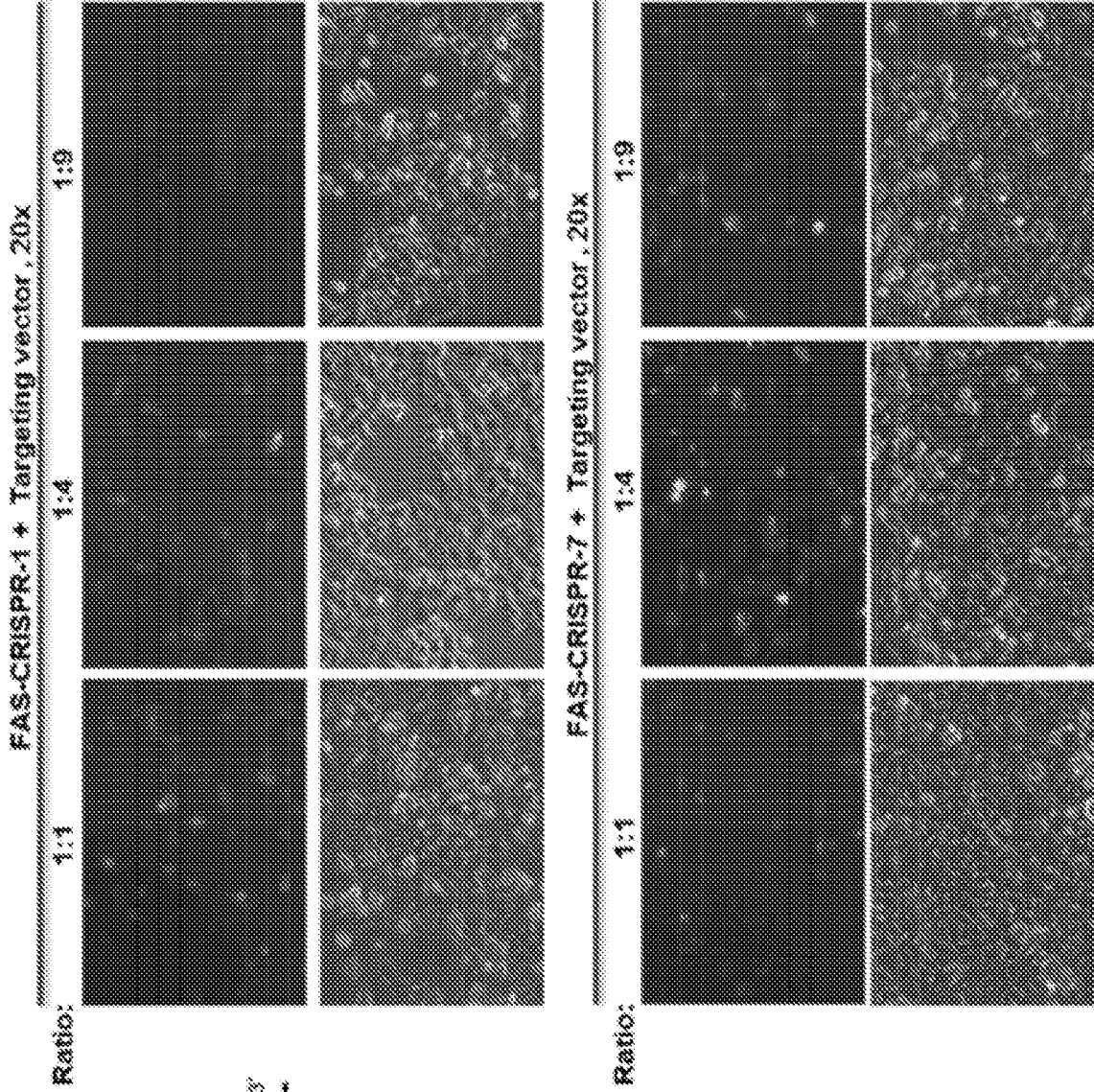


Figure 12C

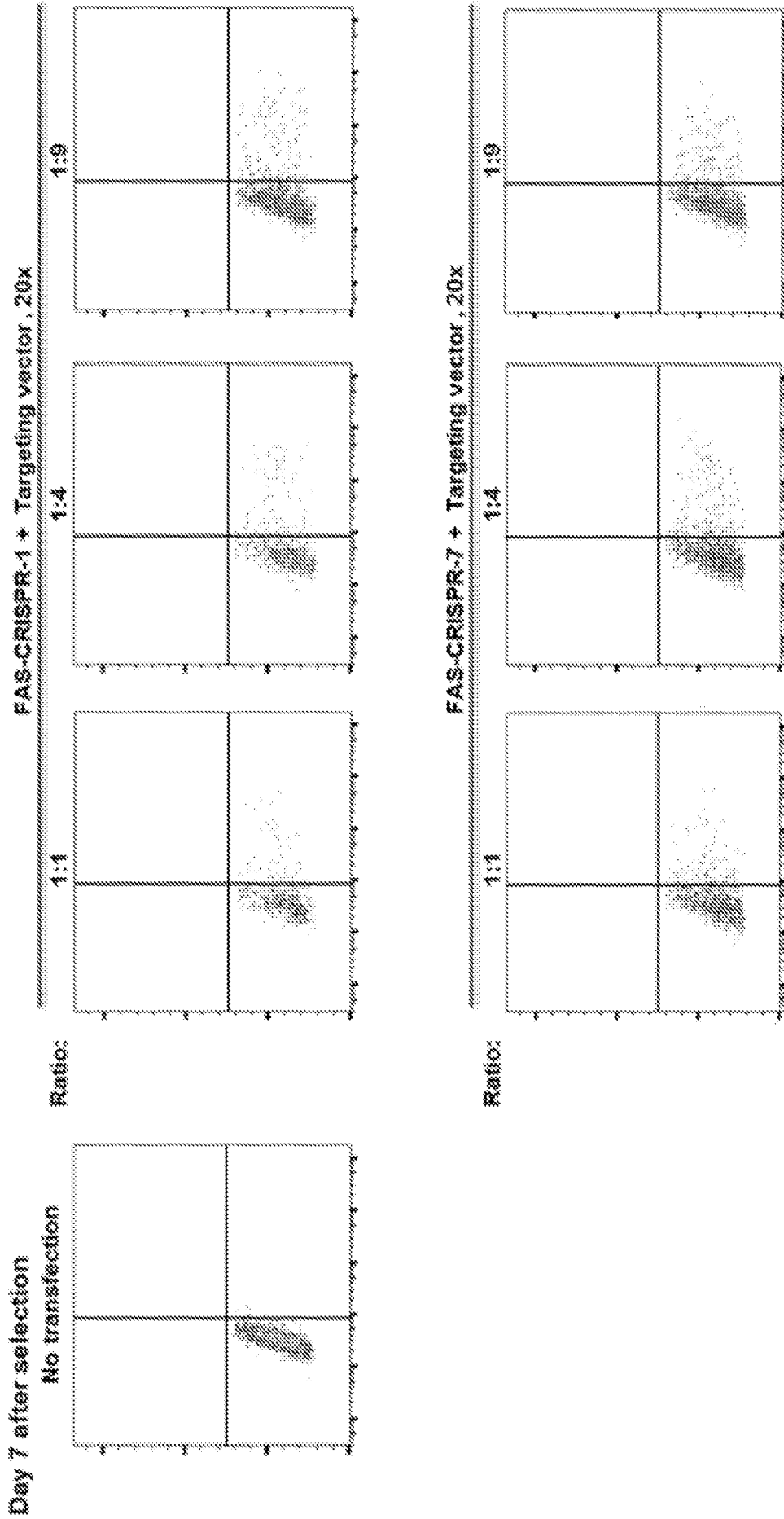


Figure 12D

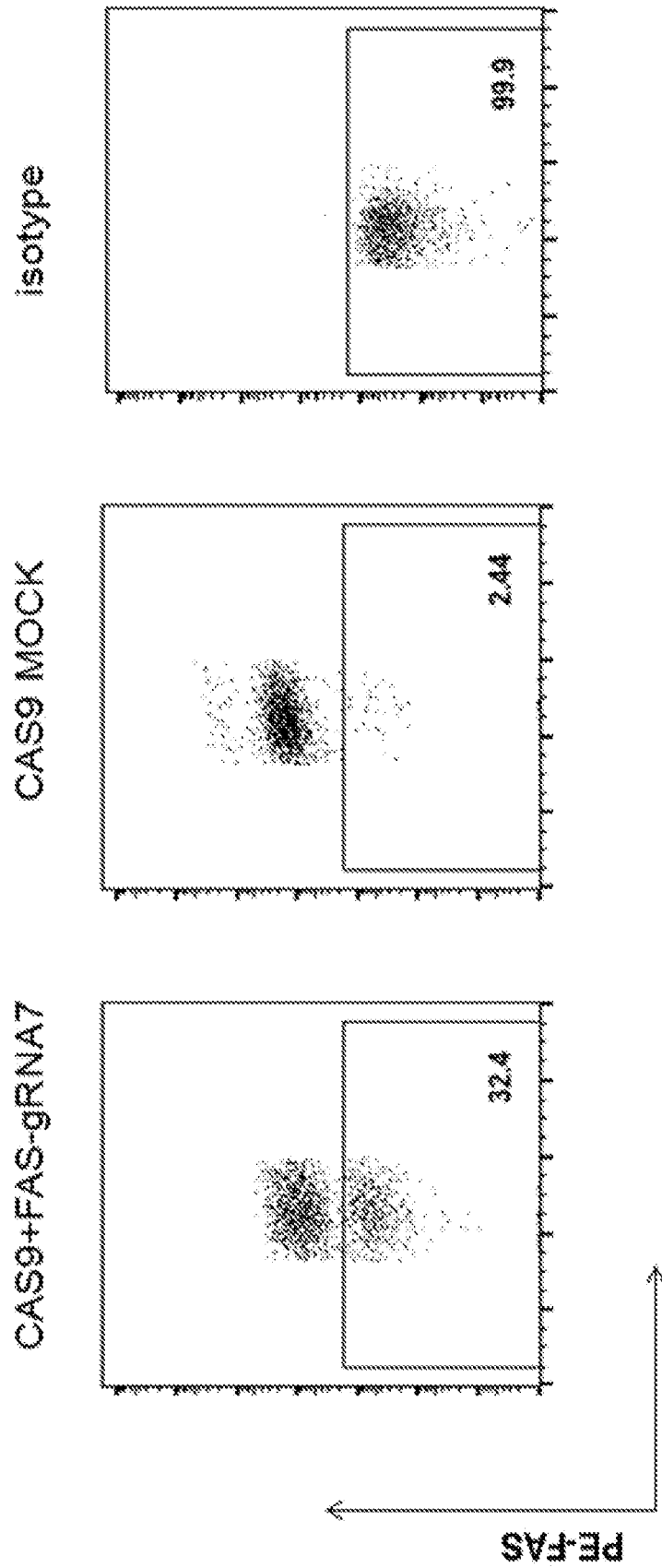


Figure 13

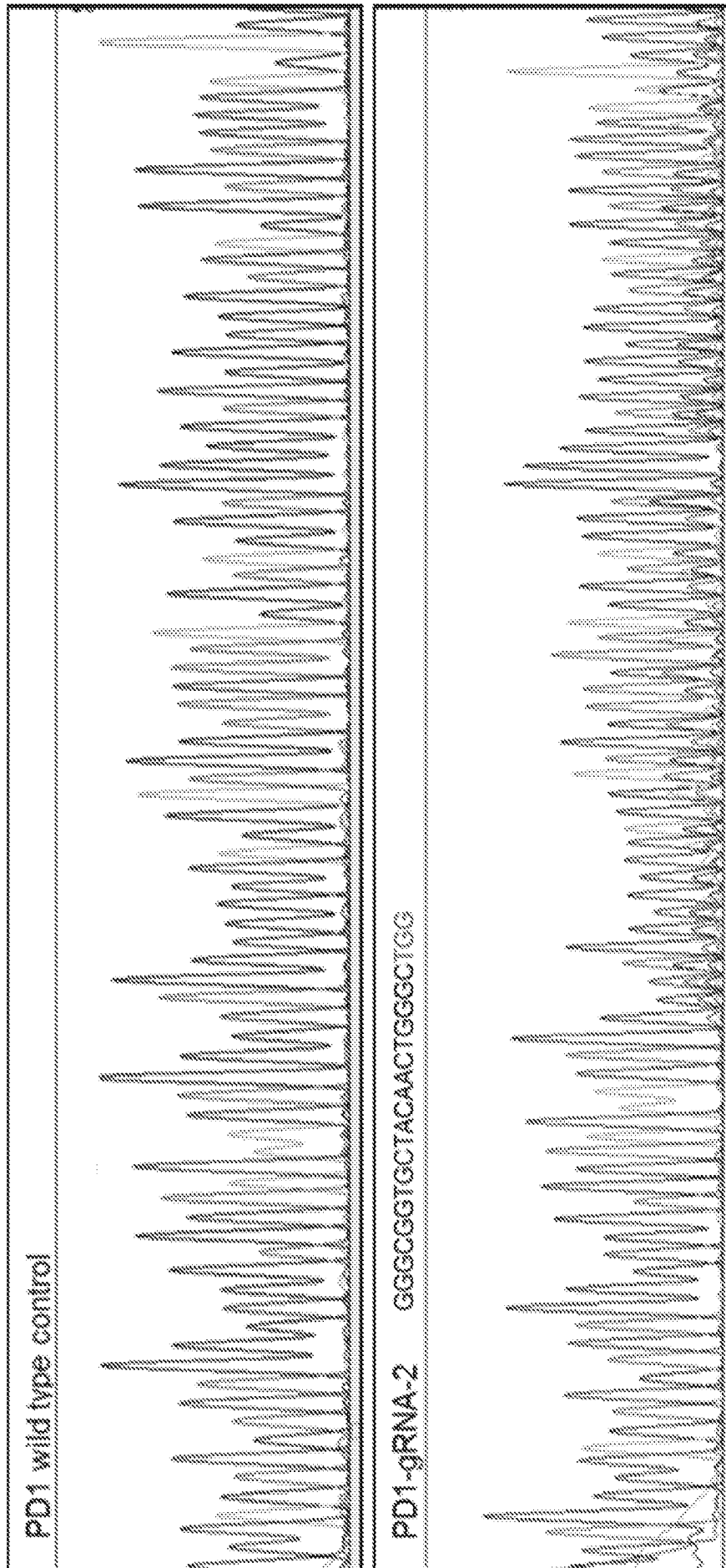
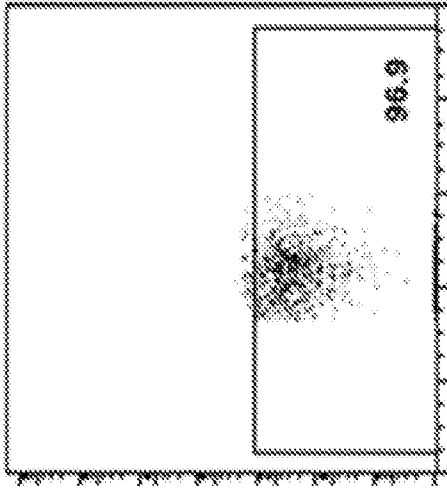


Figure 14A

CAS9+PD1-gRNA2



CAS9 MOCK

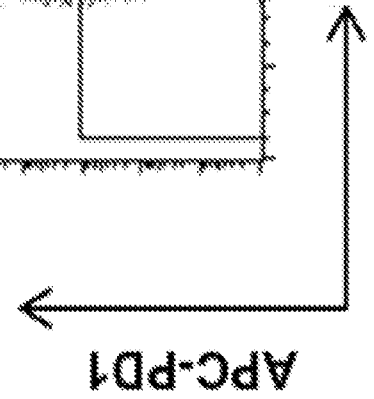
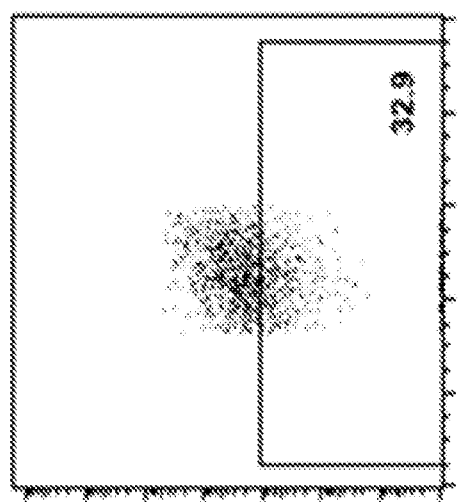
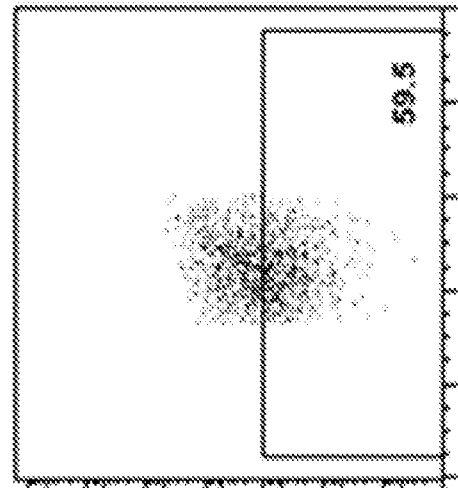
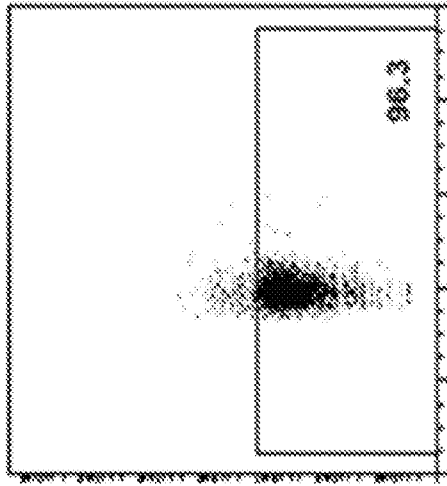


Figure 14B

2024203330 20 May 2024

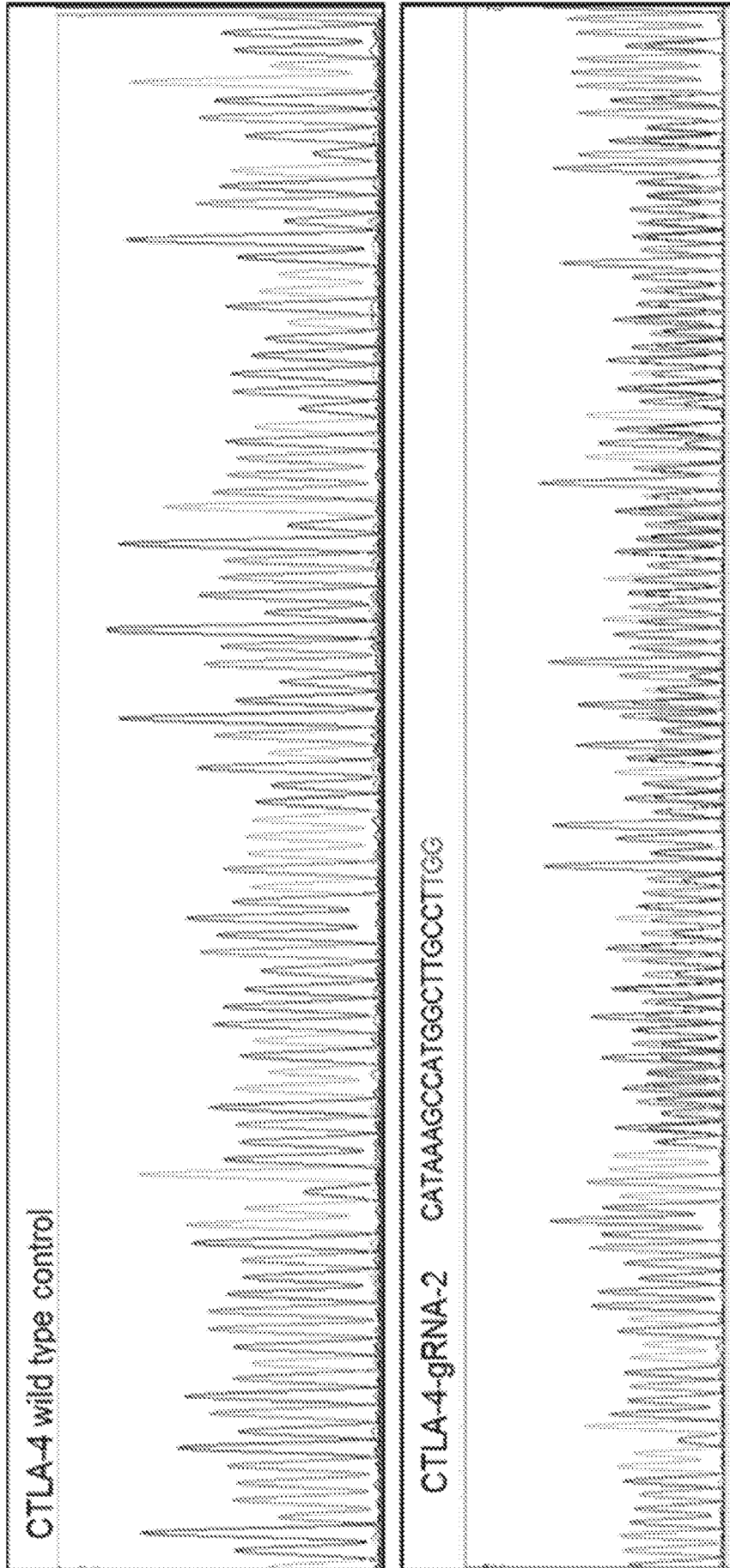


Figure 15A

```

WT AACACCGCTCCCATAAAGCCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
1 -5 AACACCGTCCCATAA-----TGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
1 -2 AACACCGTCCCATAA--CCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
2 -2 AACACCGTCCCATAA--CCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
2 +1 AACACCGTCCCATAAAGTCCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
3 -1 AACACCGTCCCATAA--CCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
3 -14 AACACCGTCCCATAAAG-----GGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
-7 -5 AACACCGTCCCATAA-----TGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
-7 -5 AACACCGTCCCATAA-----TGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
10 -1 AACACCGTCCCATAA--CCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
10 -3 AACACCGTCCCATAA--CCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
11 -5 AACACCGTCCCATAA-----TGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT
11 -0 AACACCGTCCCATAAAGCCATGGCTTGCCTTGGATTTTCAGCGGCCACAAAGGCTCAGCTGAACCT

```

Figure 15B

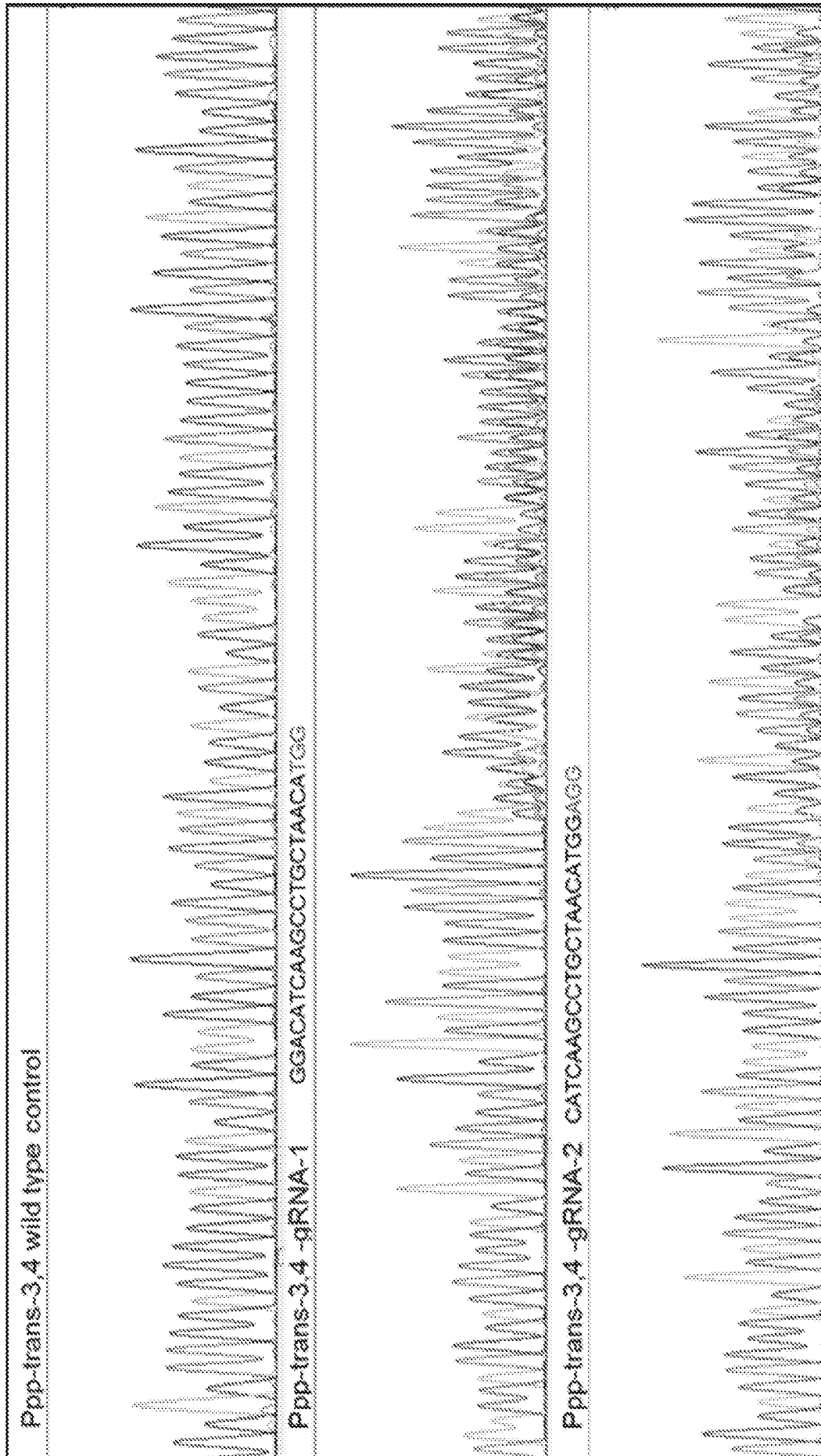
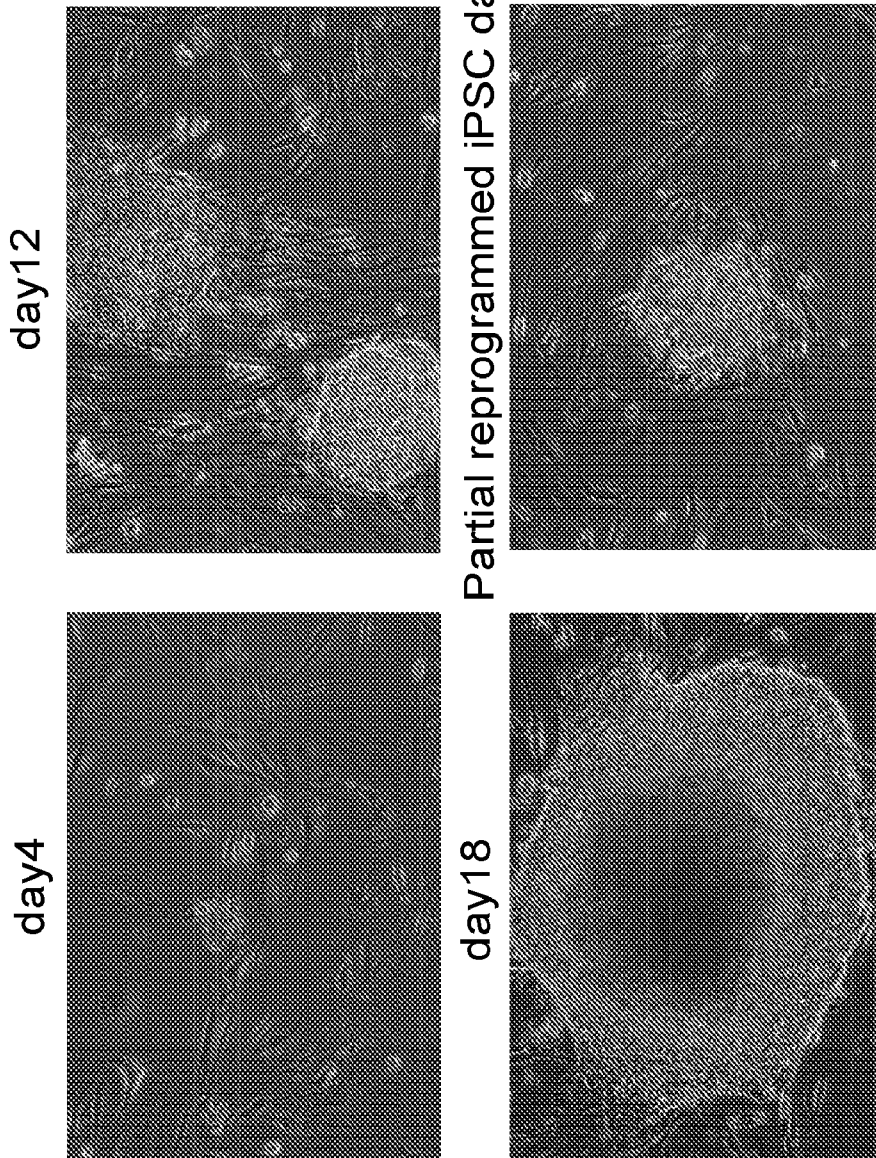


Figure 16



Passage 0

Figure 17A

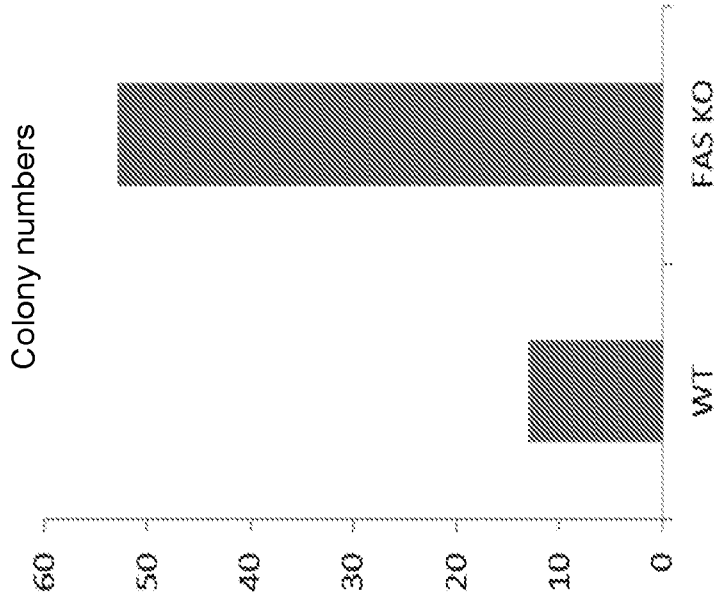


Figure 17B

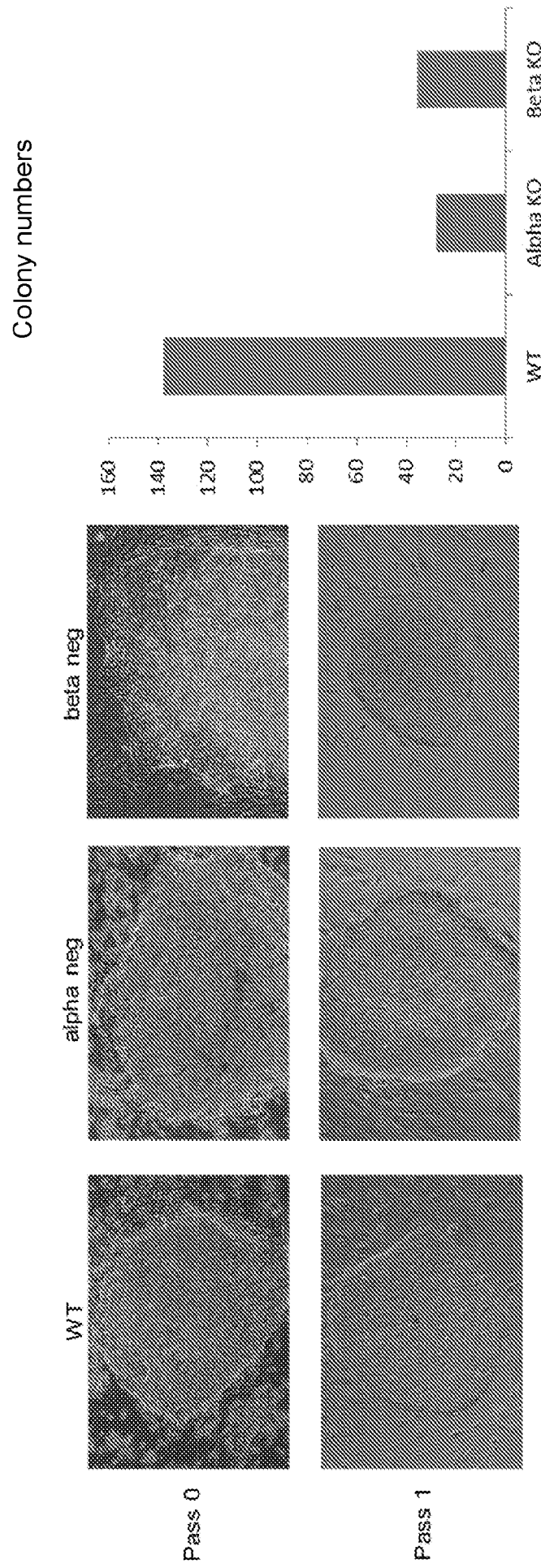
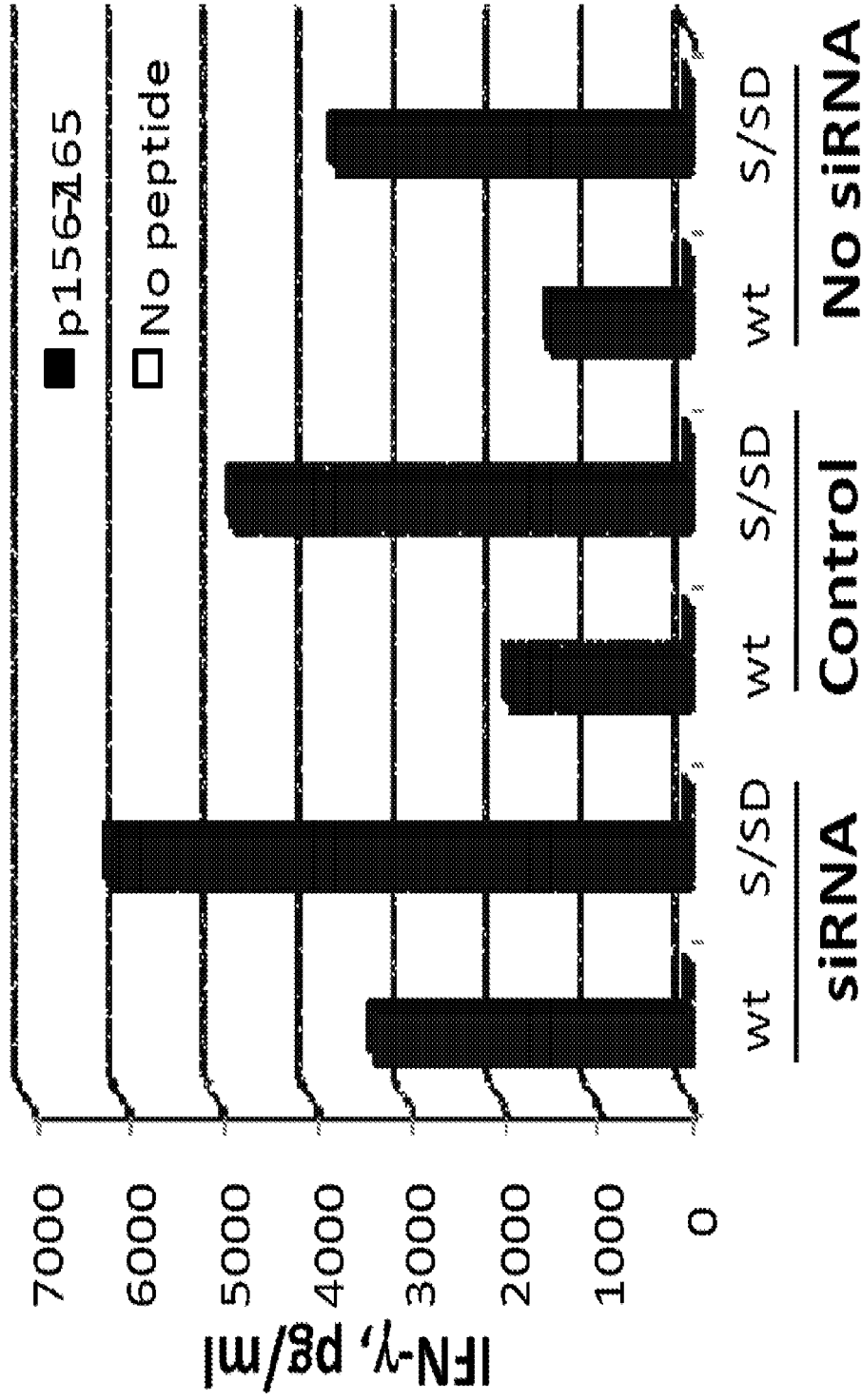


Figure 18B

Figure 18A



Wt: wildtype TCR

S/SD: Second disulfide bond alpha/ Second disulfide bond+ De-N-glycosylation beta

Figure 19

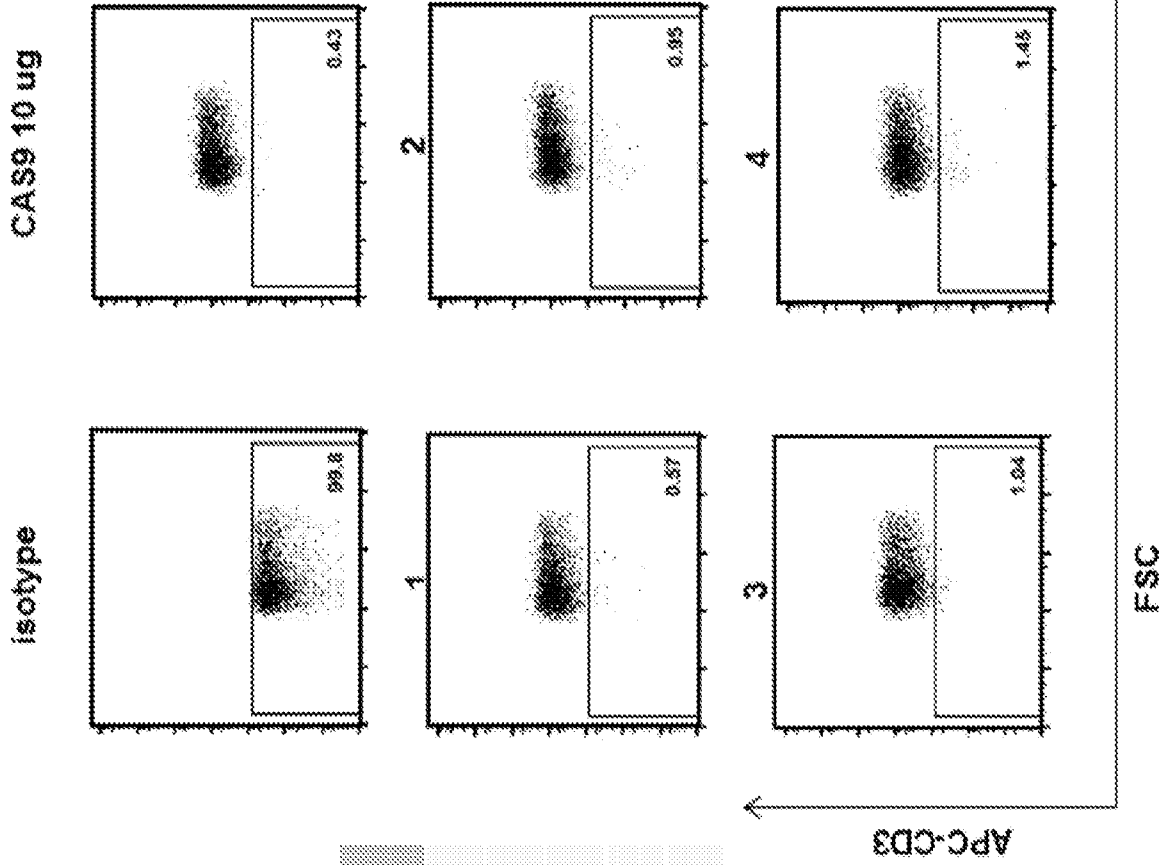


Figure 20B

	CAS9	Dose	gRNA	Dose
1	CAS9	2 ug	TRAC7	1 ug
2	CAS9	4 ug	TRAC7	2 ug
3	CAS9	8 ug	TRAC7	4 ug
4	CAS9	10 ug	TRAC7	5 ug
5	CAS9	20 ug	TRAC7	10 ug

Figure 20A

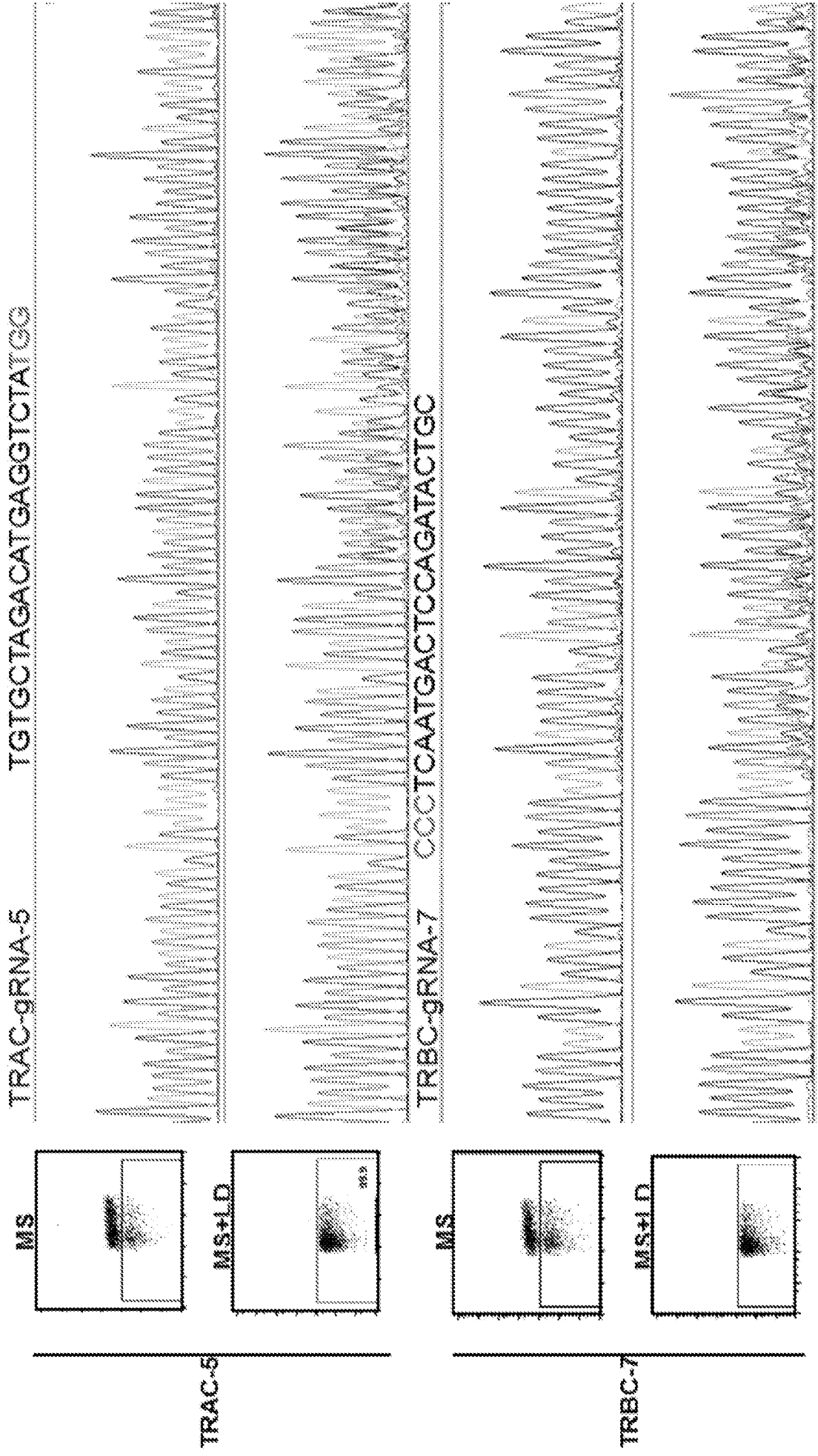


Figure 21

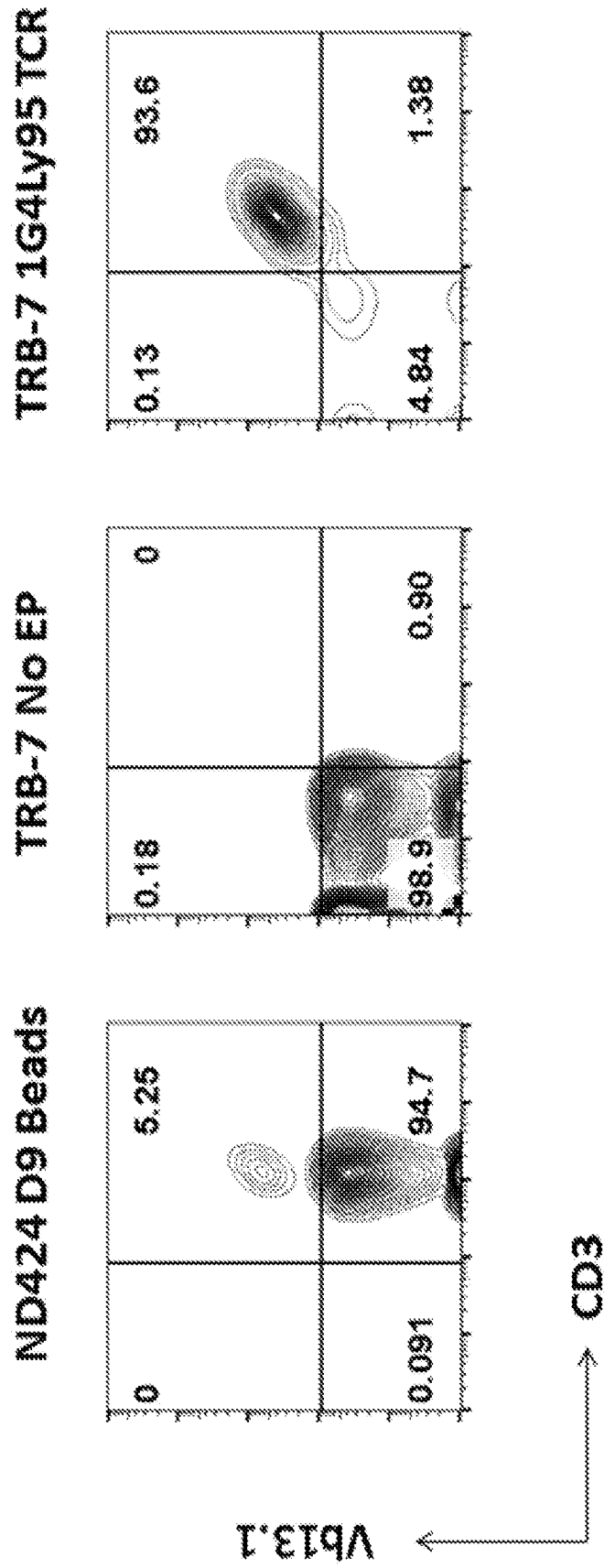
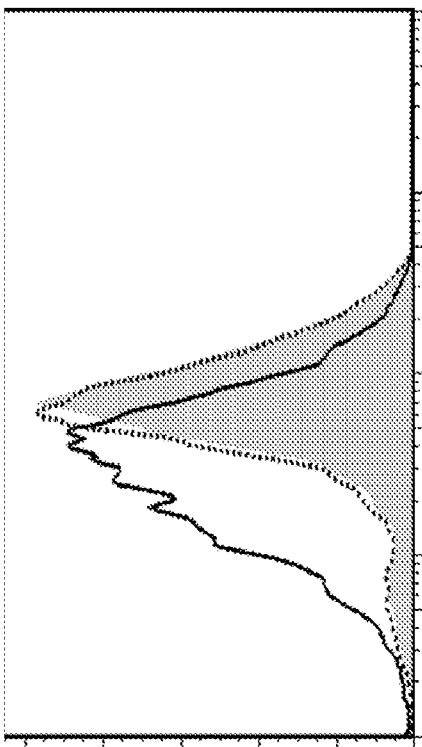
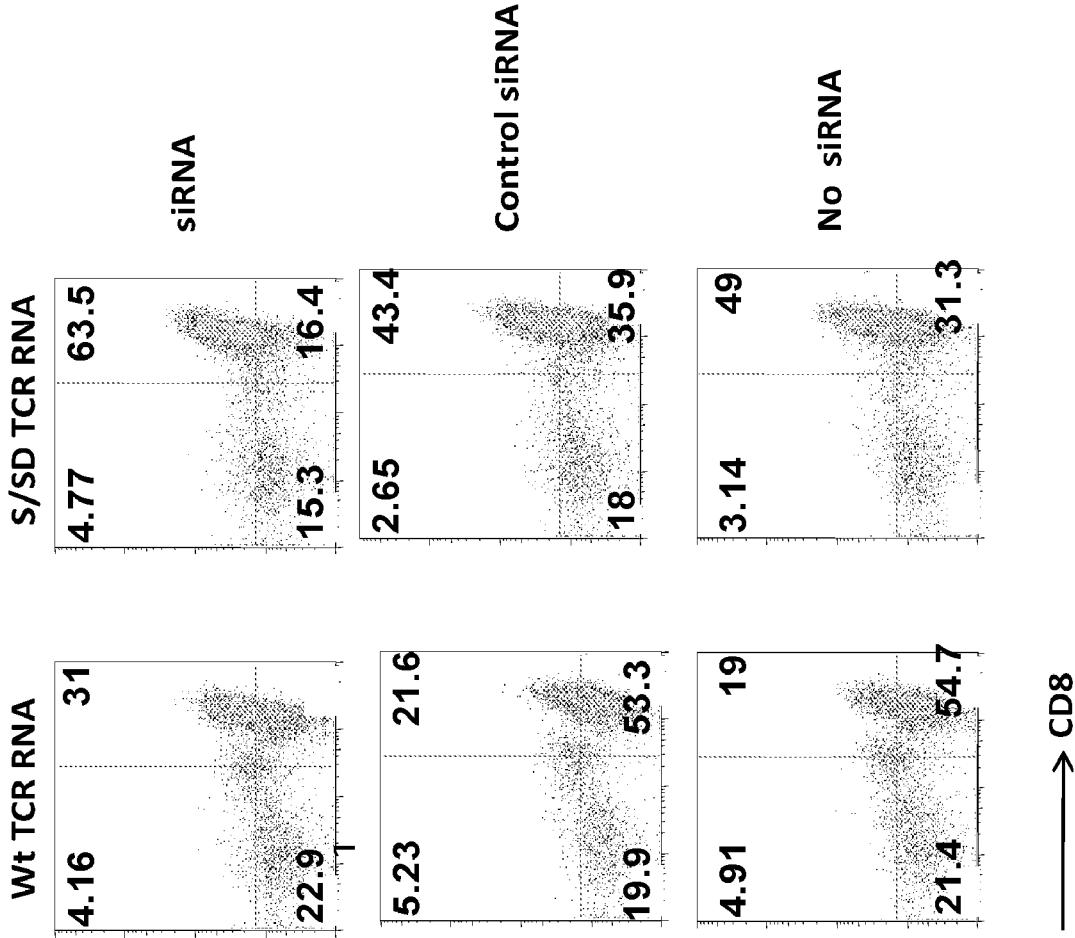


Figure 22



TCR alpha/beta

Figure 23A



→ CD8

Figure 23C

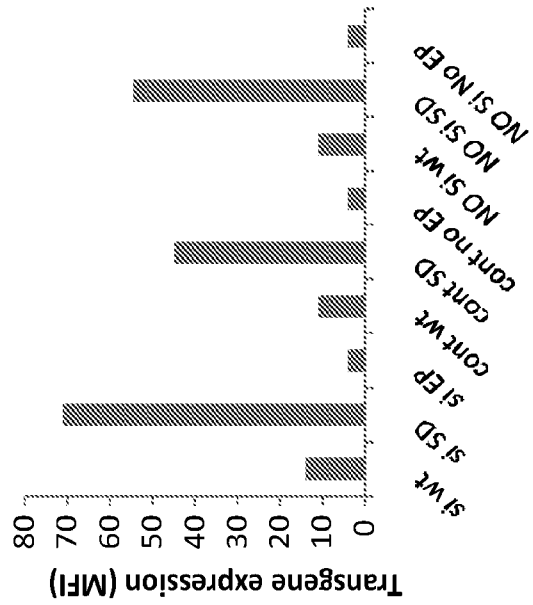


Figure 23B

2024203330 20 May 2024

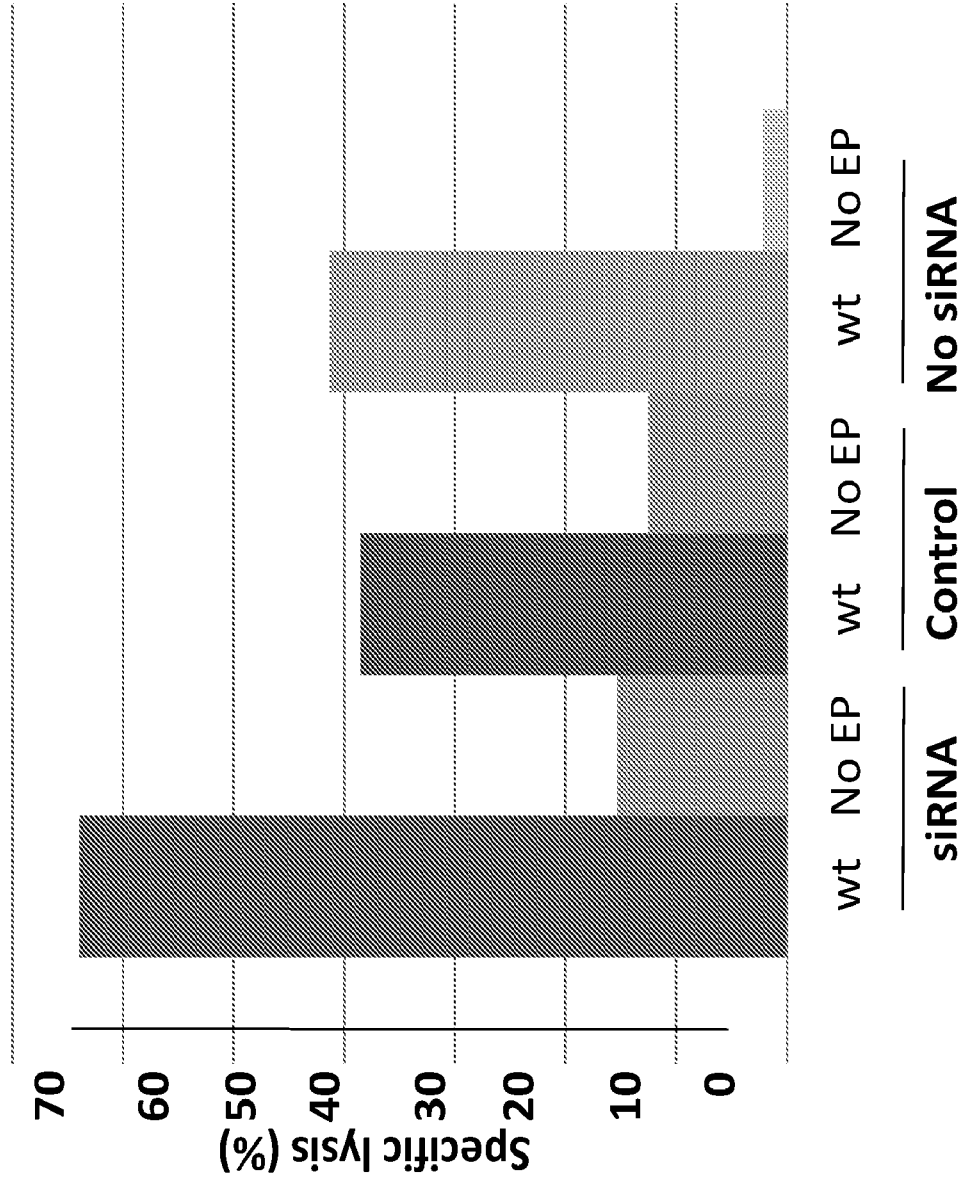


Figure 23D

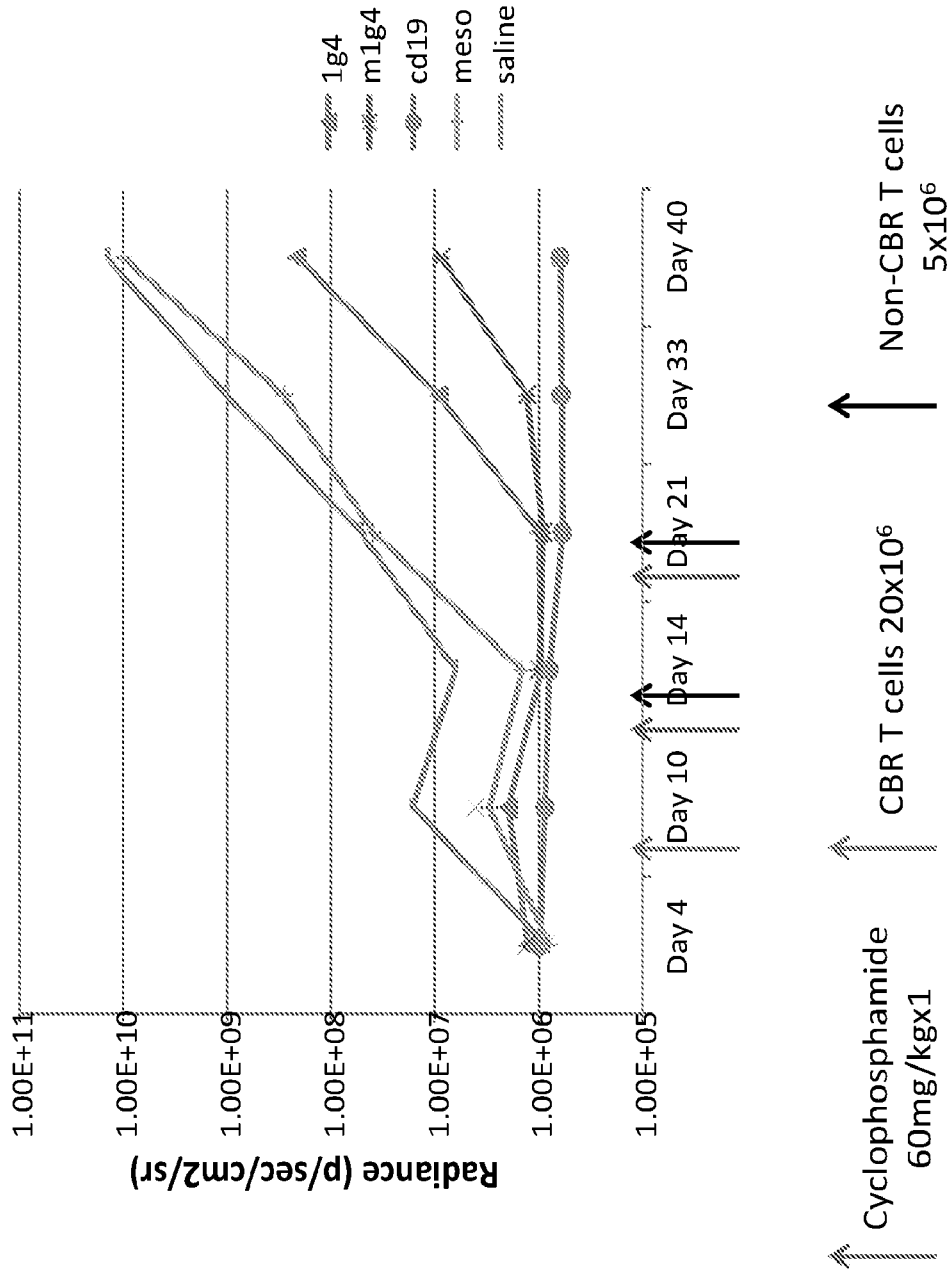


Figure 24

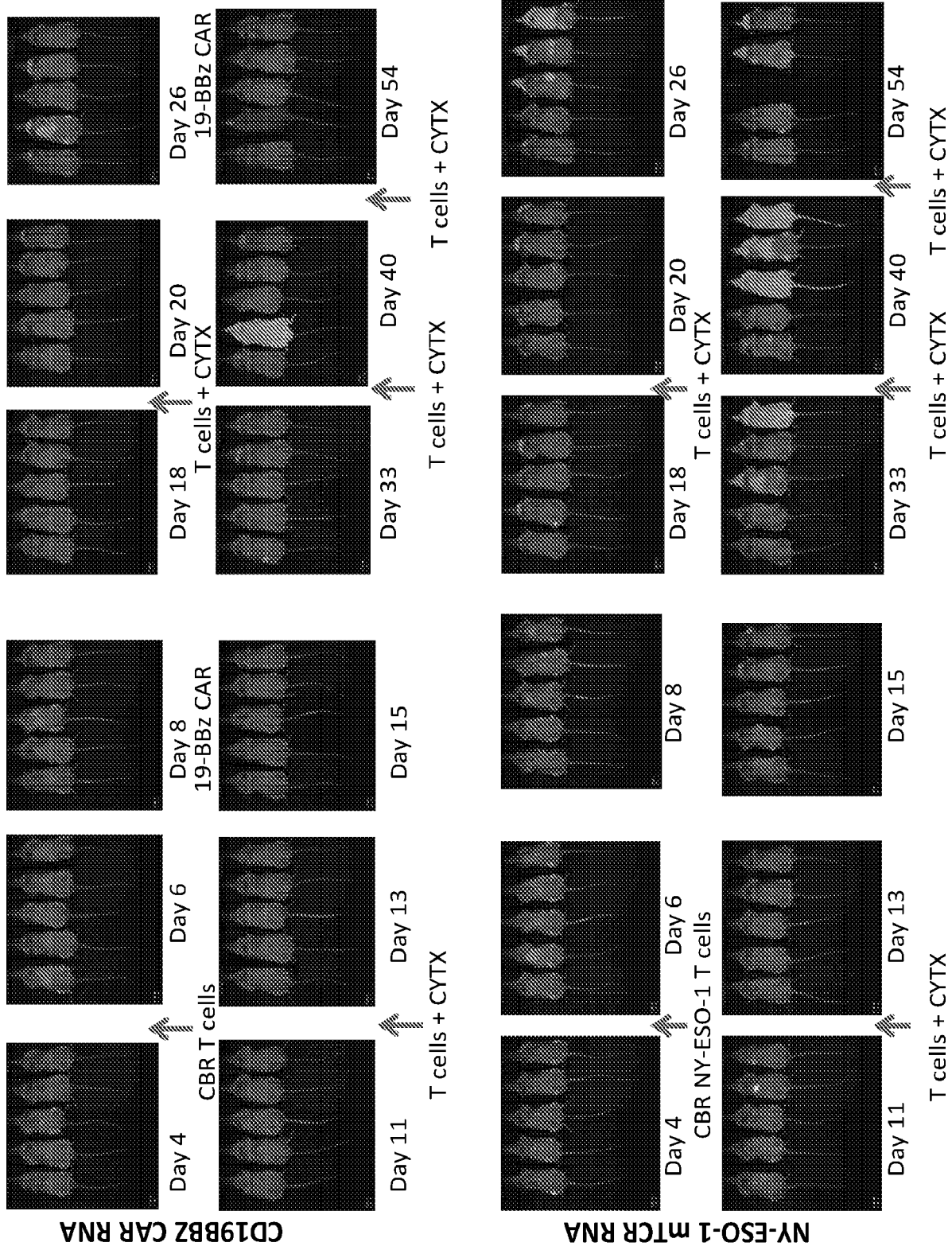


Figure 25

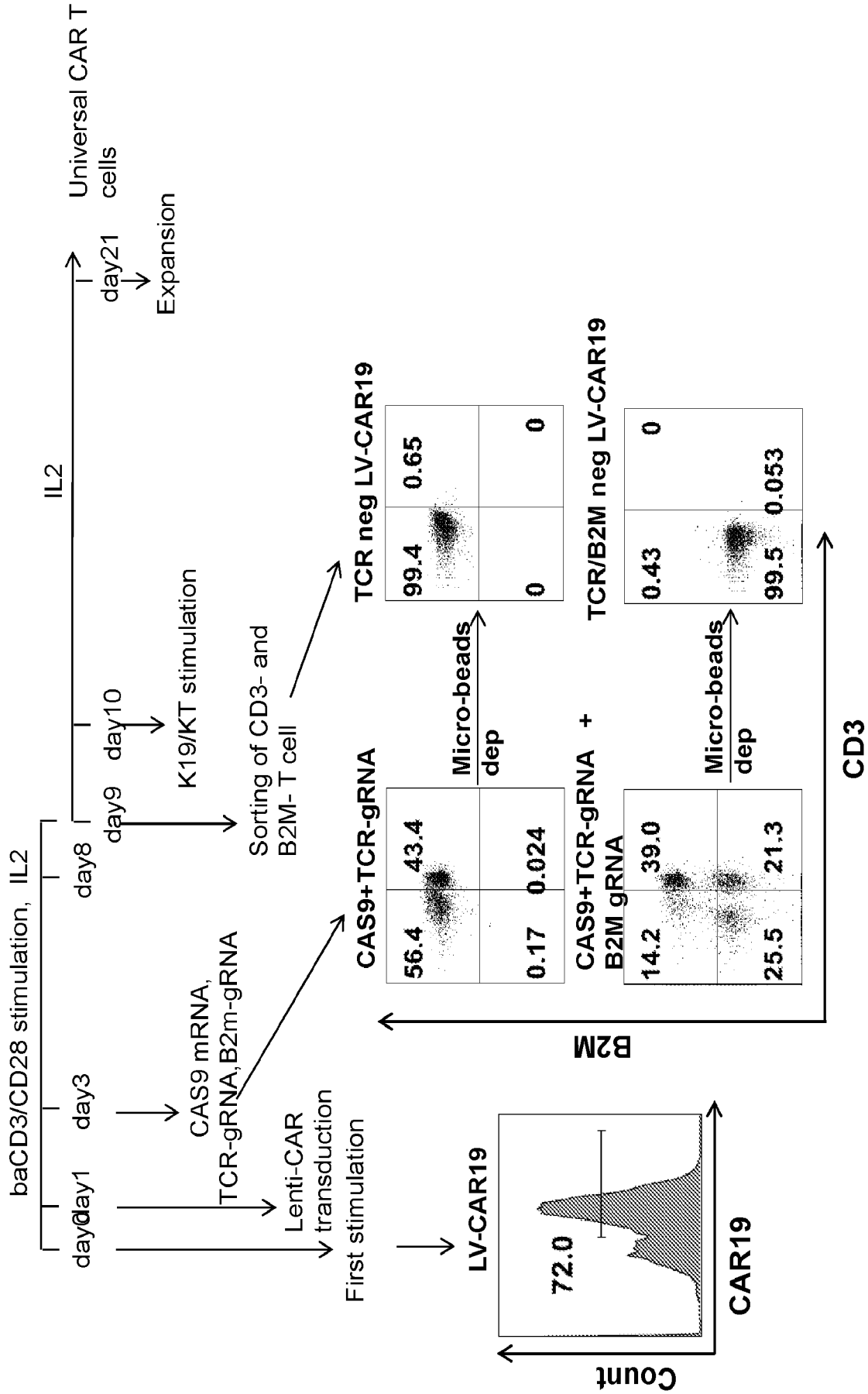


Figure 26

Fold increase	Day0	Day3	Day5	Day7	Day9	Day11	Final NO.
NT	1	2	6	17	38	64	320
LV-GFP	1	3	6	16	38	66	330
LV-CAR19	1	3	8	23	45	113	1700
TCR neg LV-CAR19	1	4	6	16	38	77	2300
TCR/B2M LV-CAR19	1	3	6	19	39	99	1680

NT, LV-GFP, LV-CAR19,
TCR neg LV-CAR19, TCR/B2M neg LV-CAR19

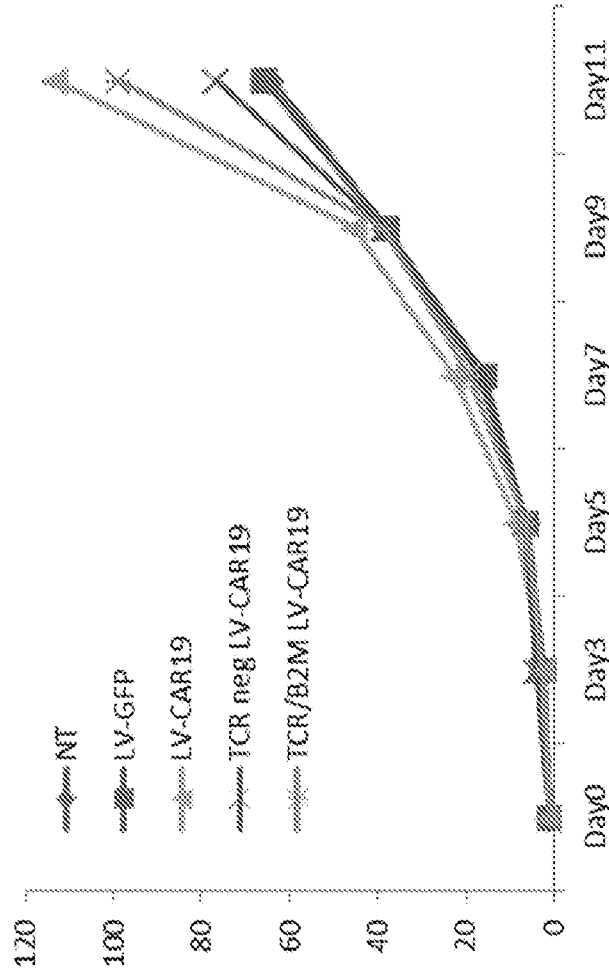
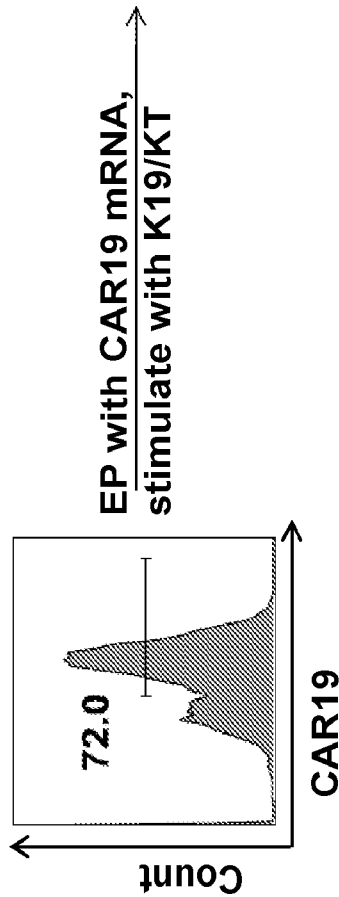


Figure 27

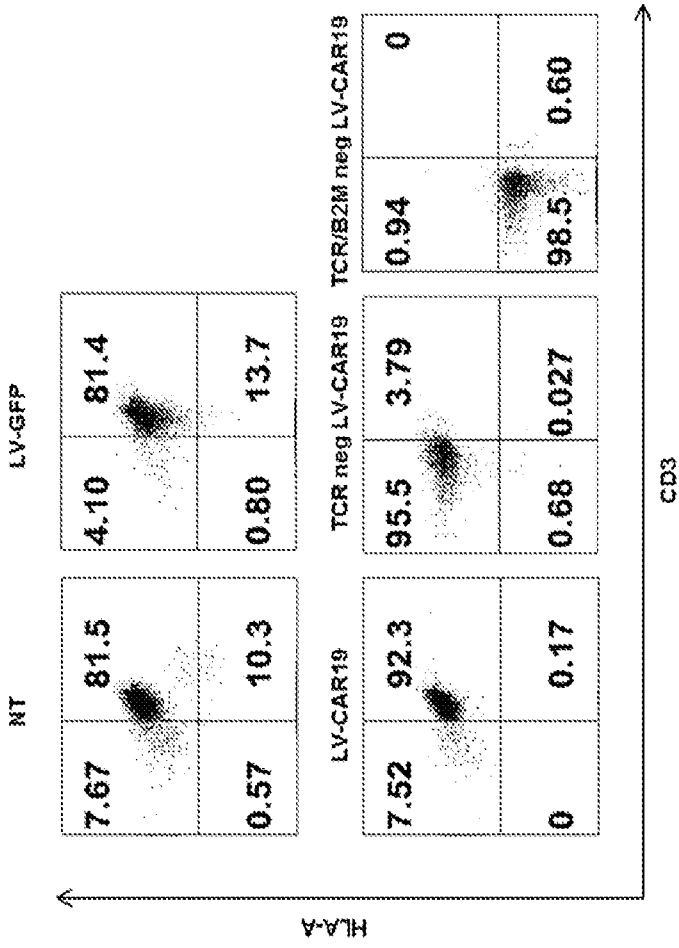


Figure 28A

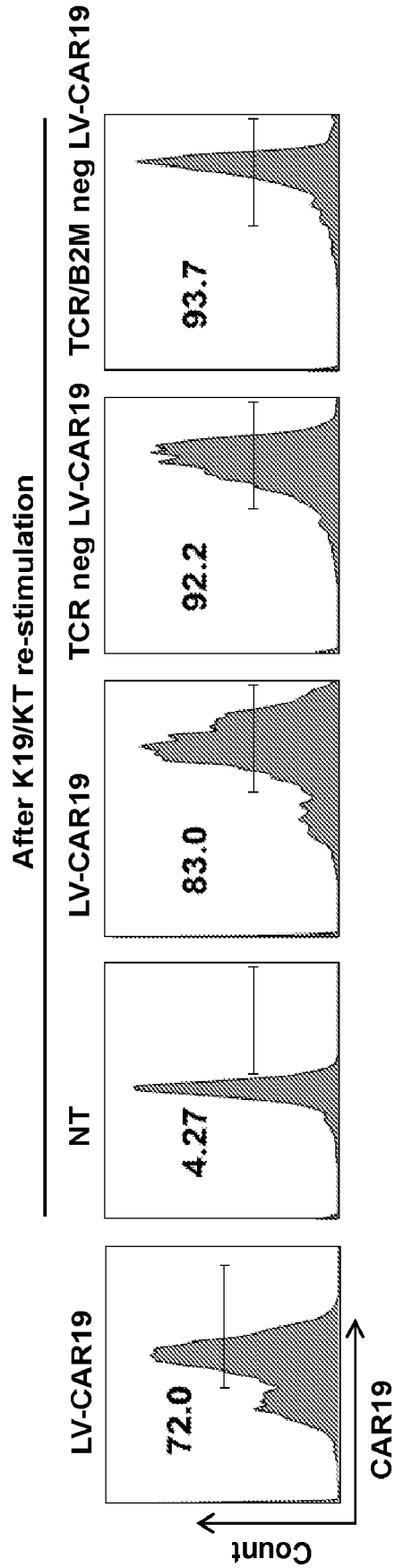


Figure 28B

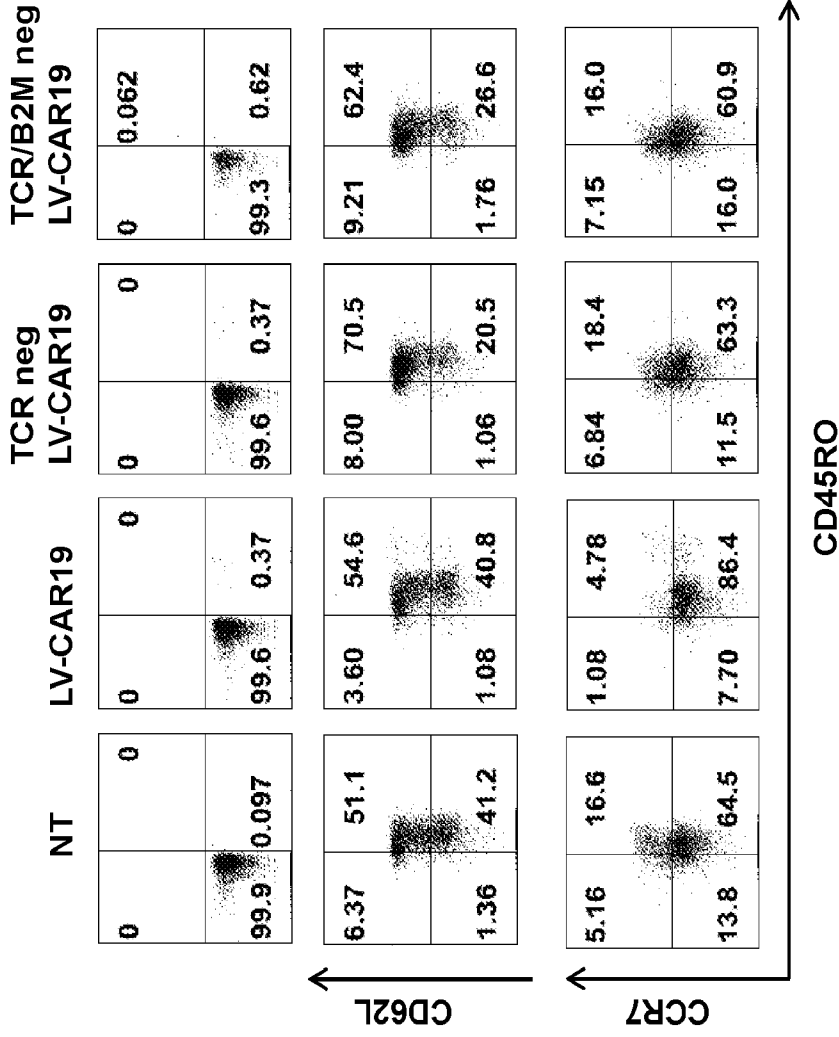


Figure 29B

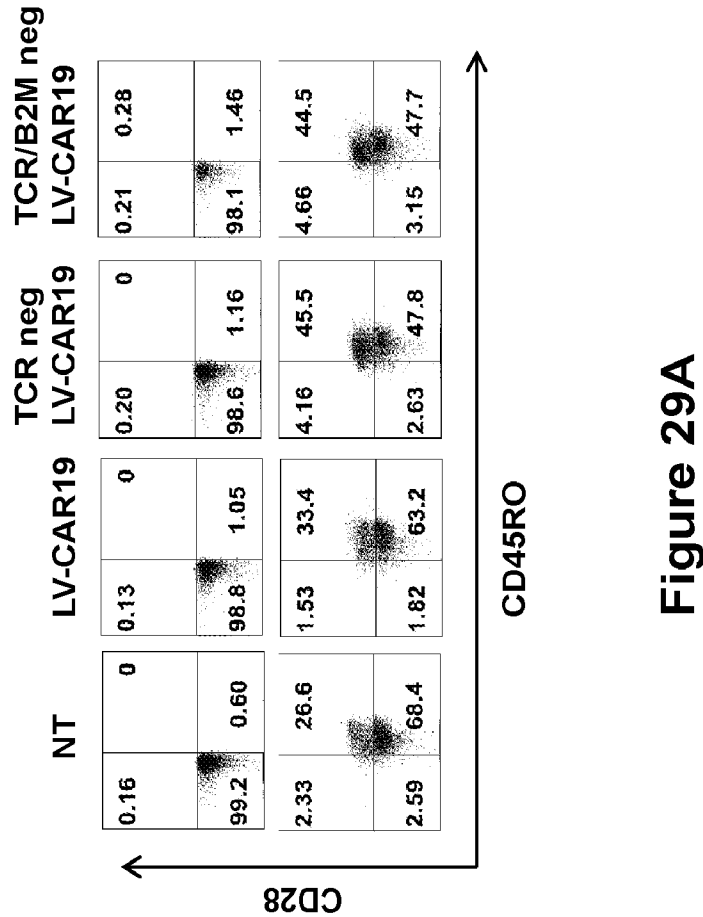
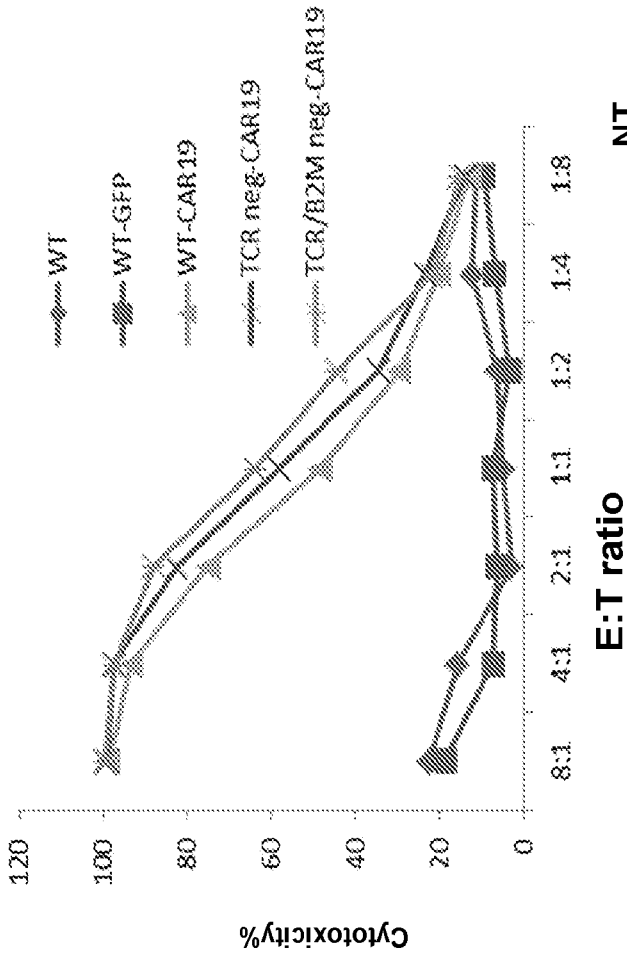
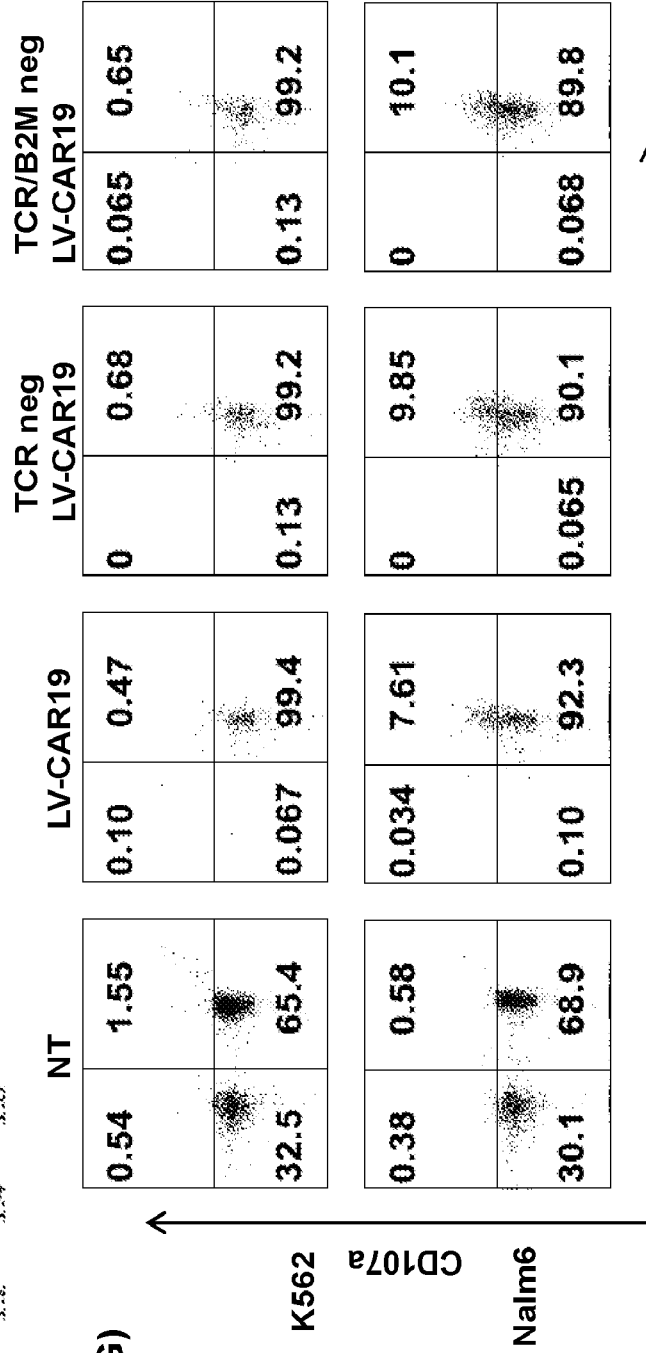


Figure 29A



(Nalm6-ESO-CBG)

Figure 30A



CD8 (gated on CD3 or CAR19 positive cells)

Figure 30B

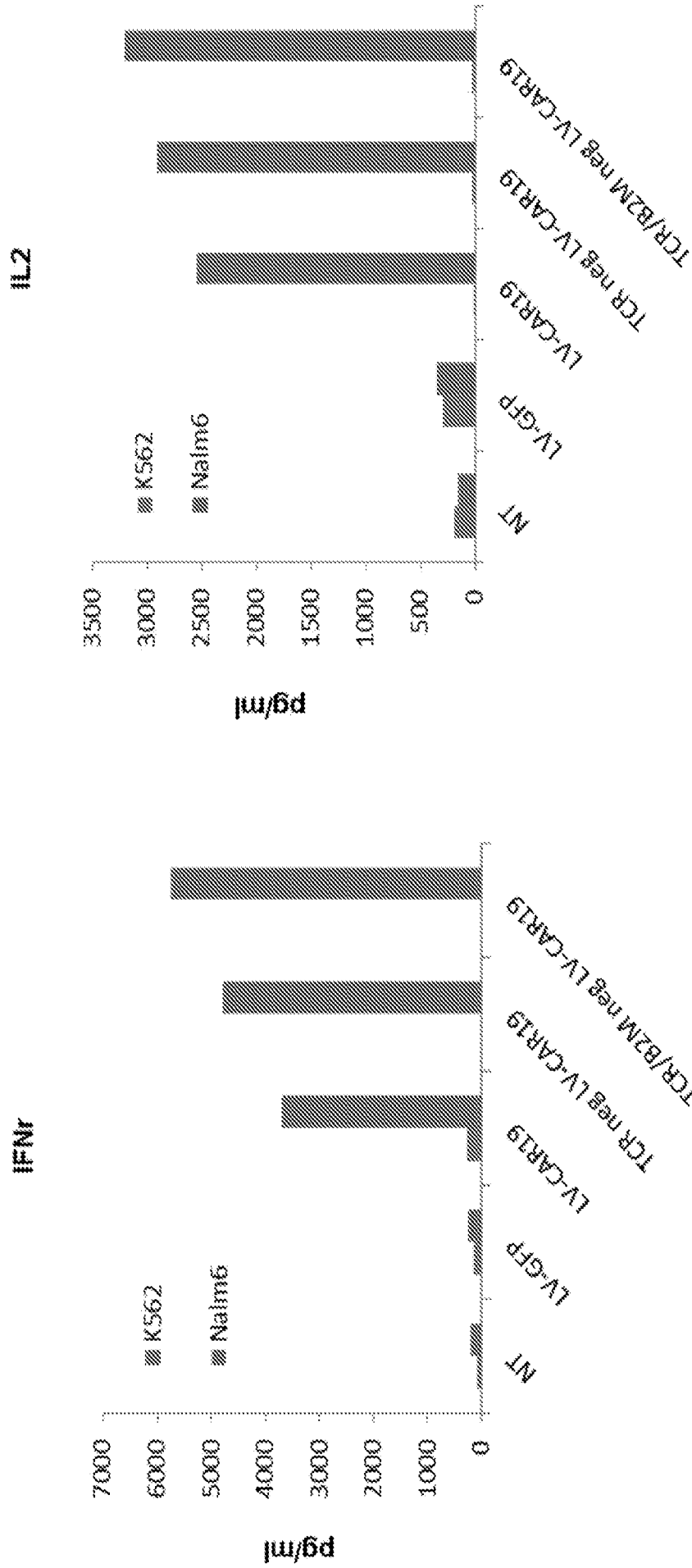
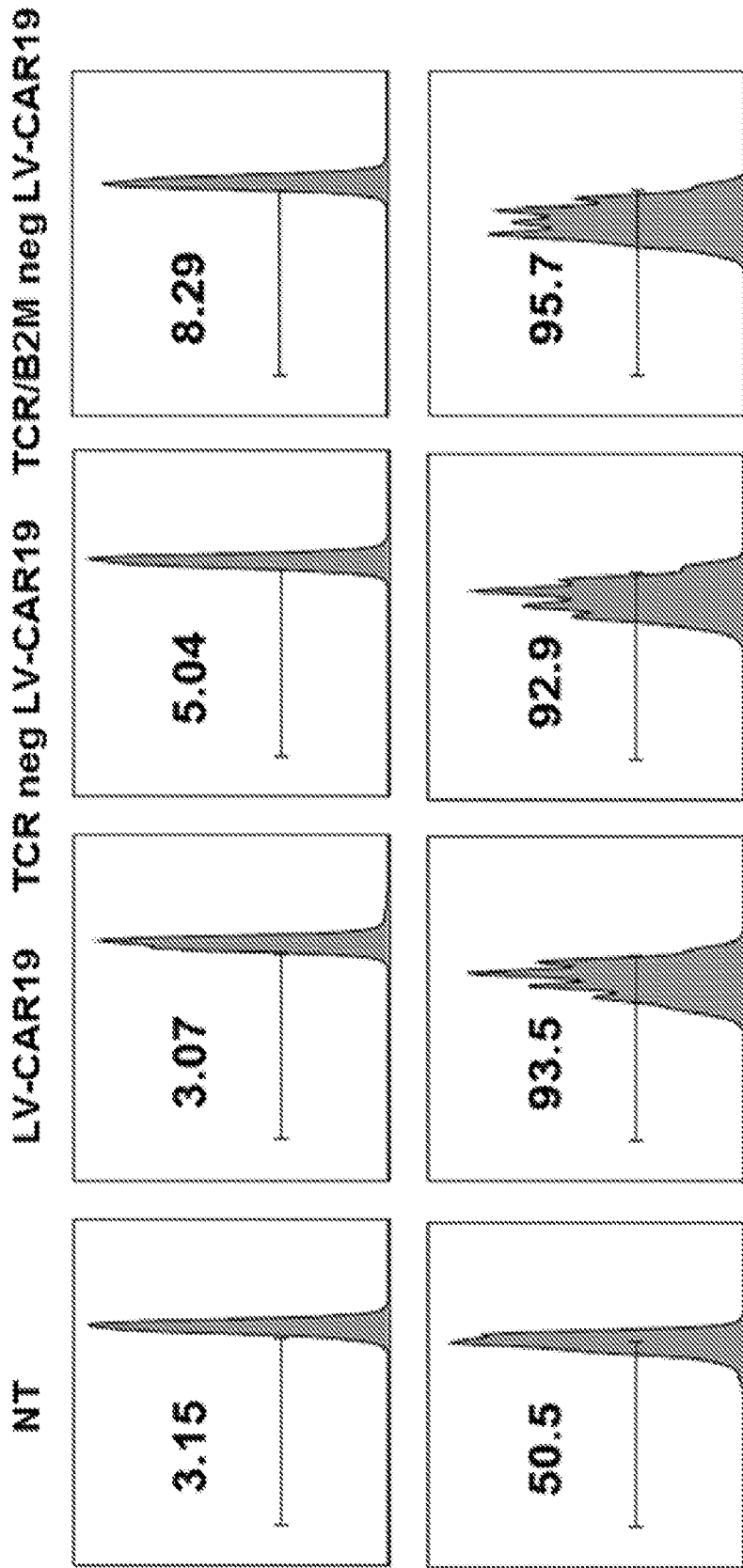


Figure 30C



E:T ratio is 1: 2

Figure 30D

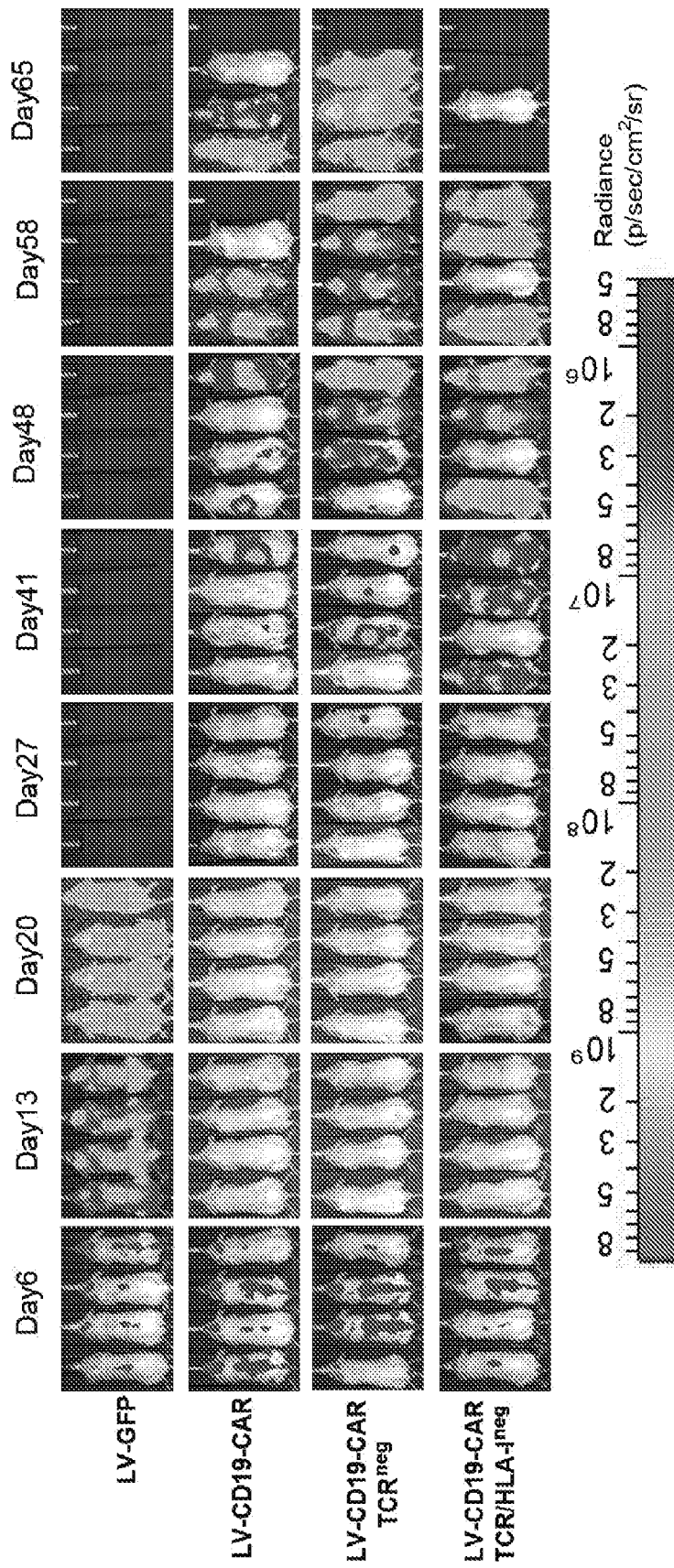


Figure 31

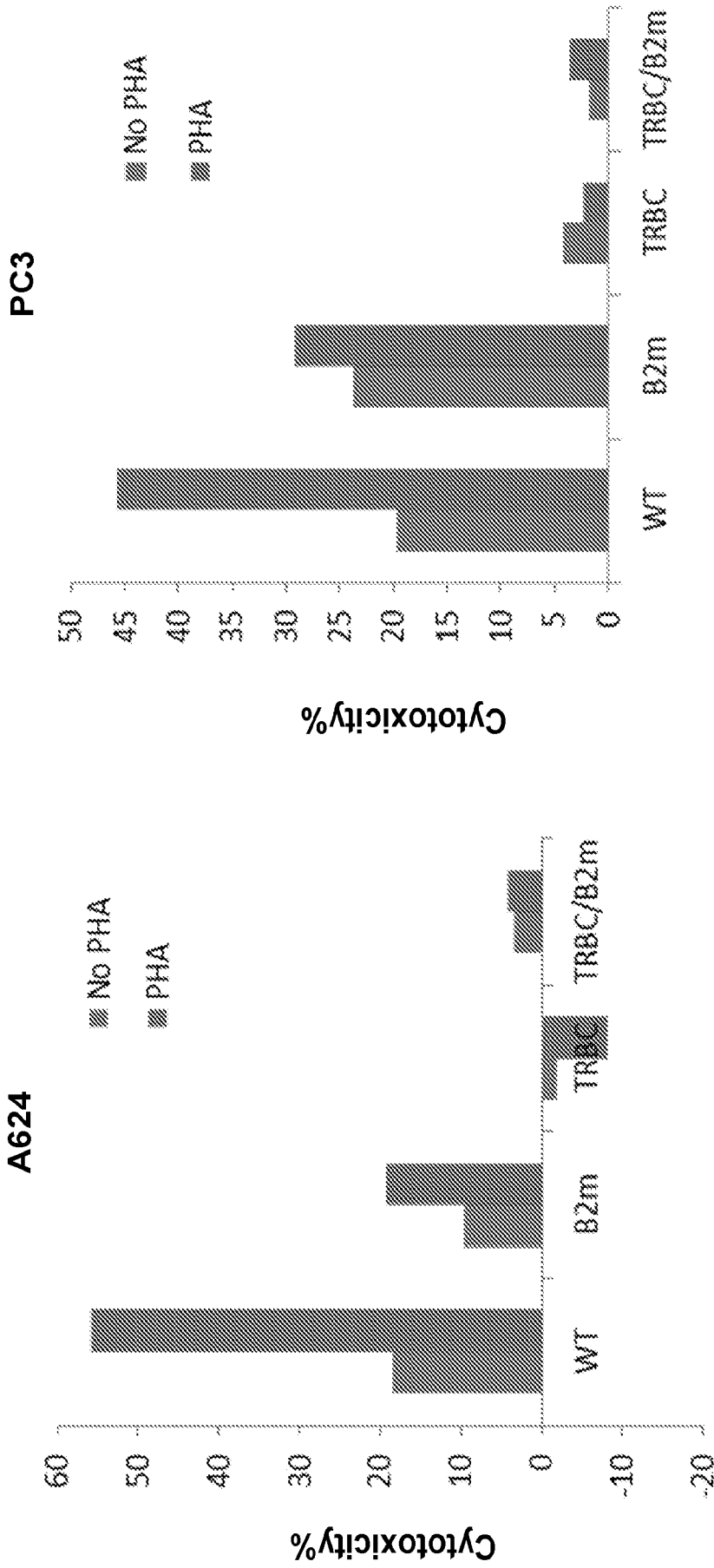


Figure 32A

CD65+ CD3- NK cell

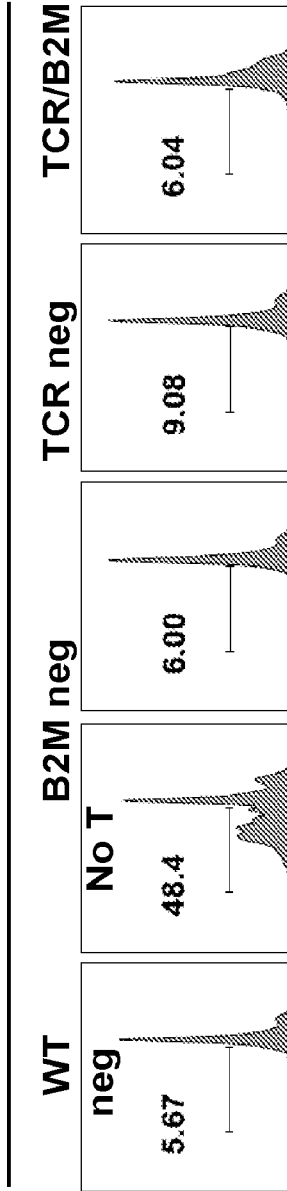


Figure 32B

Target: allogeneic PBMC

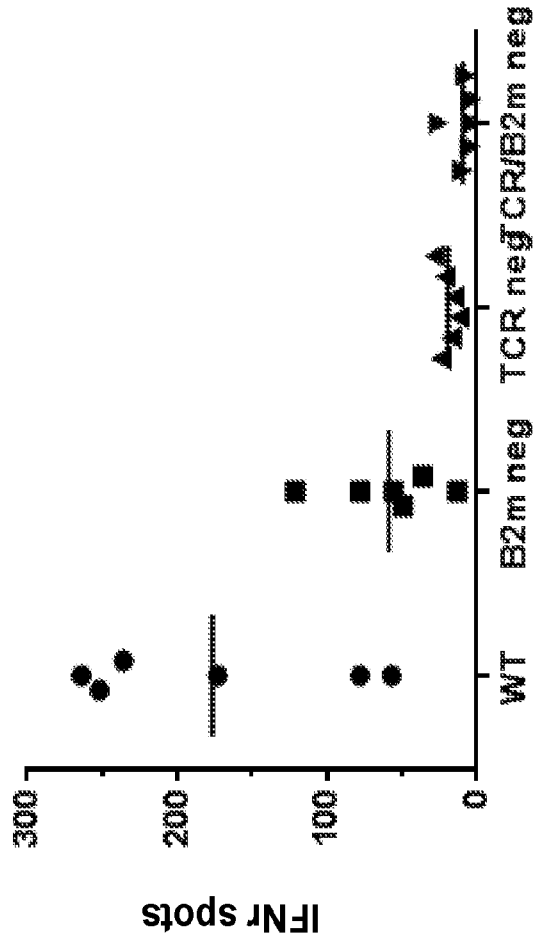


Figure 32C

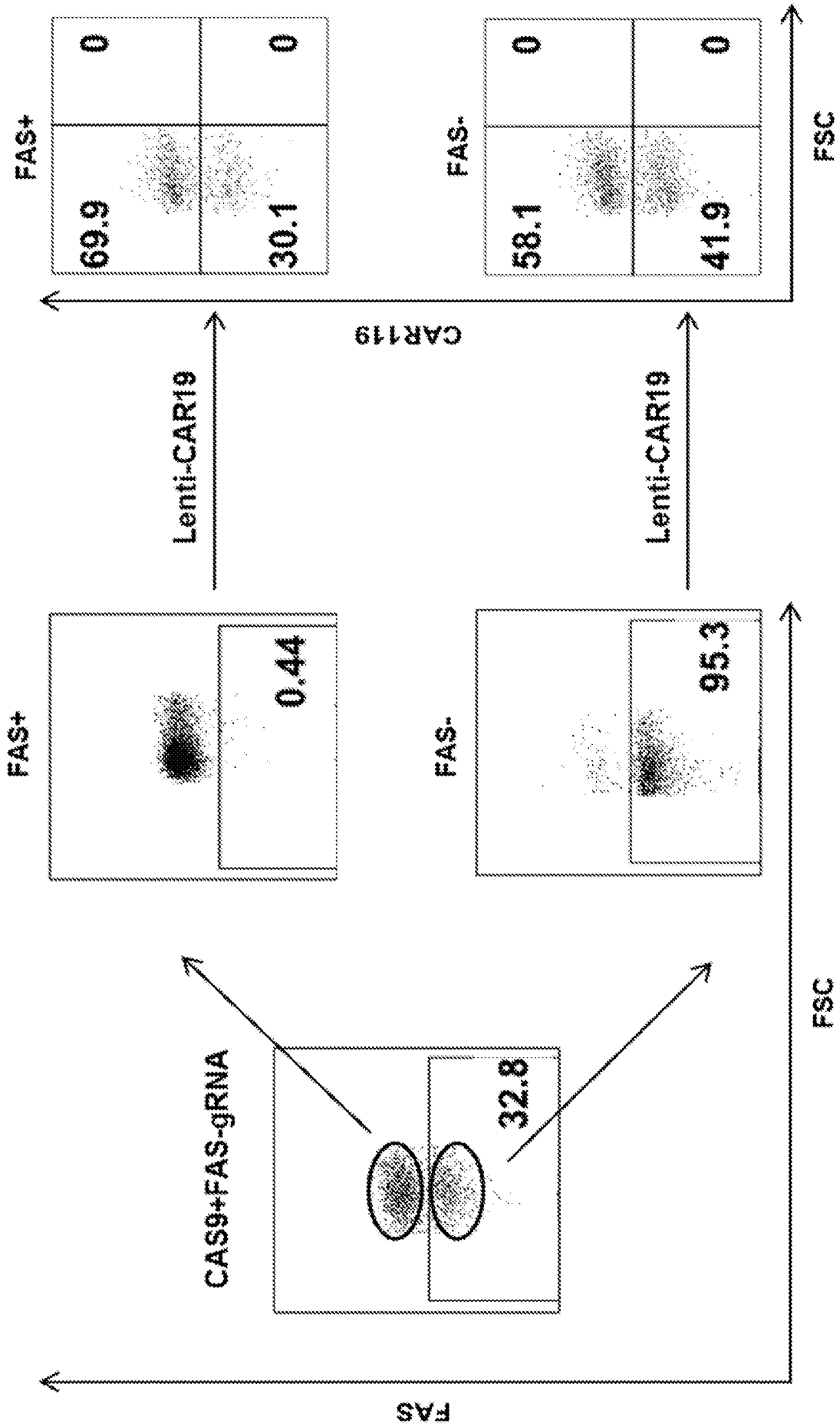


Figure 33

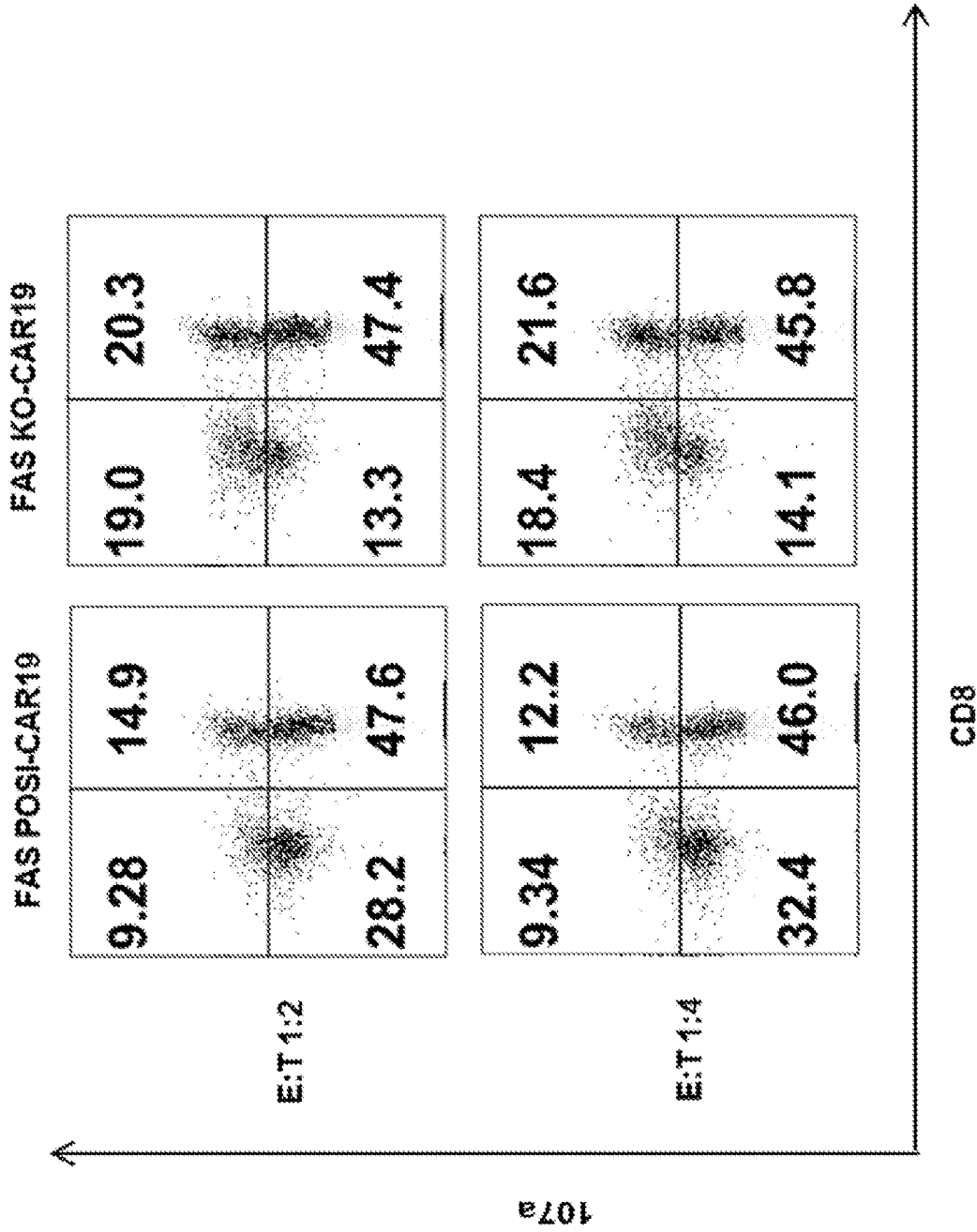


Figure 33 con't

2024203330 20 May 2024

Co-culture with K562-CD19

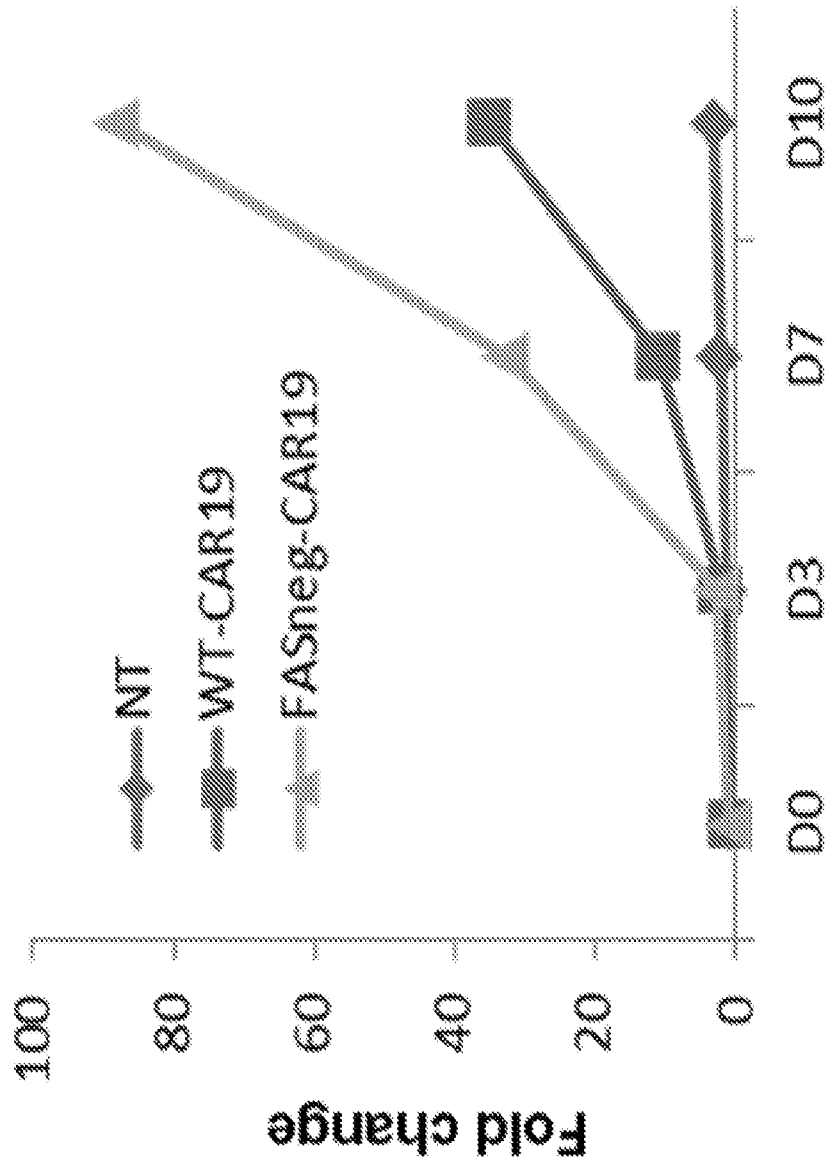


Figure 34A

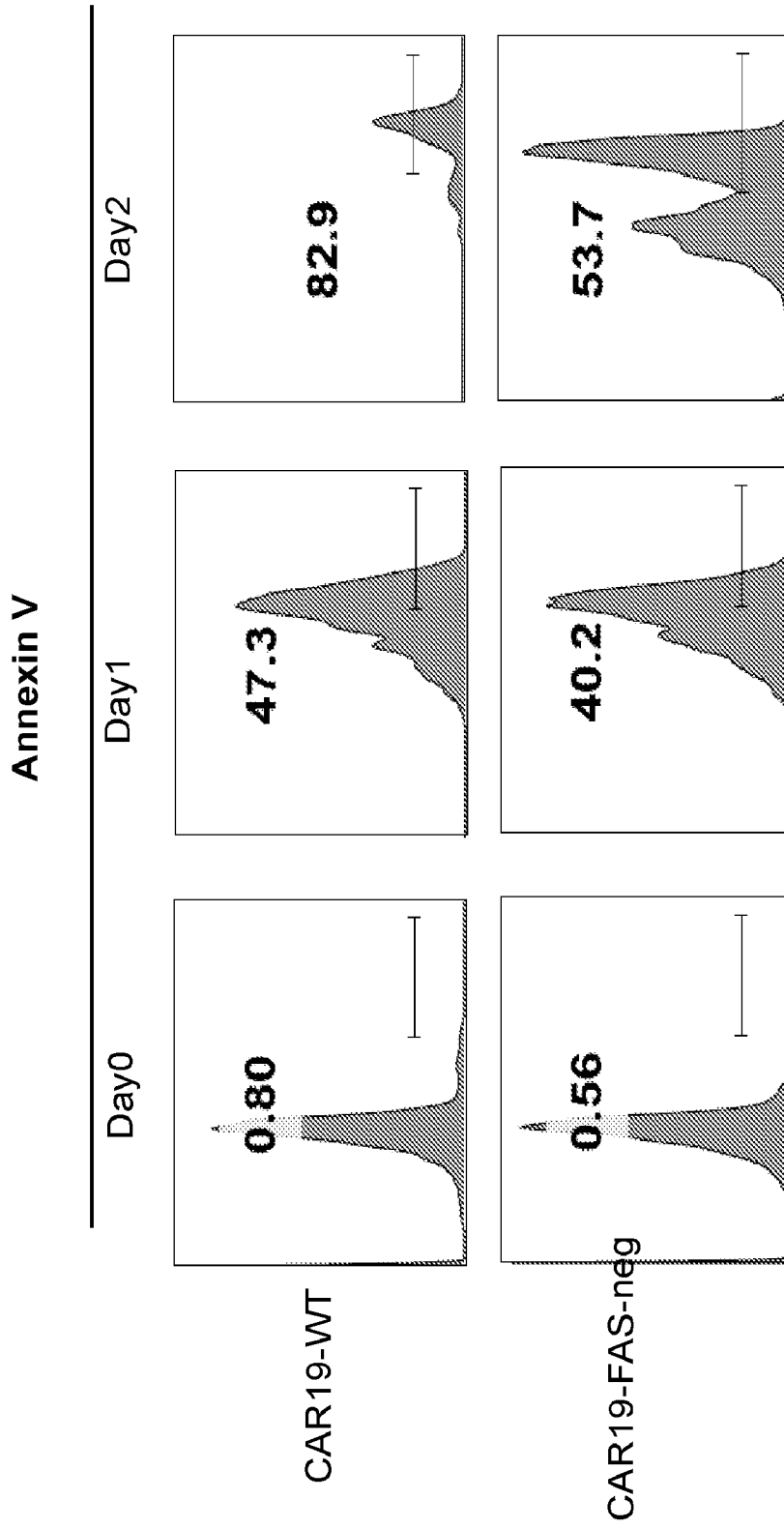


Figure 34B

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CD3 True Count assay

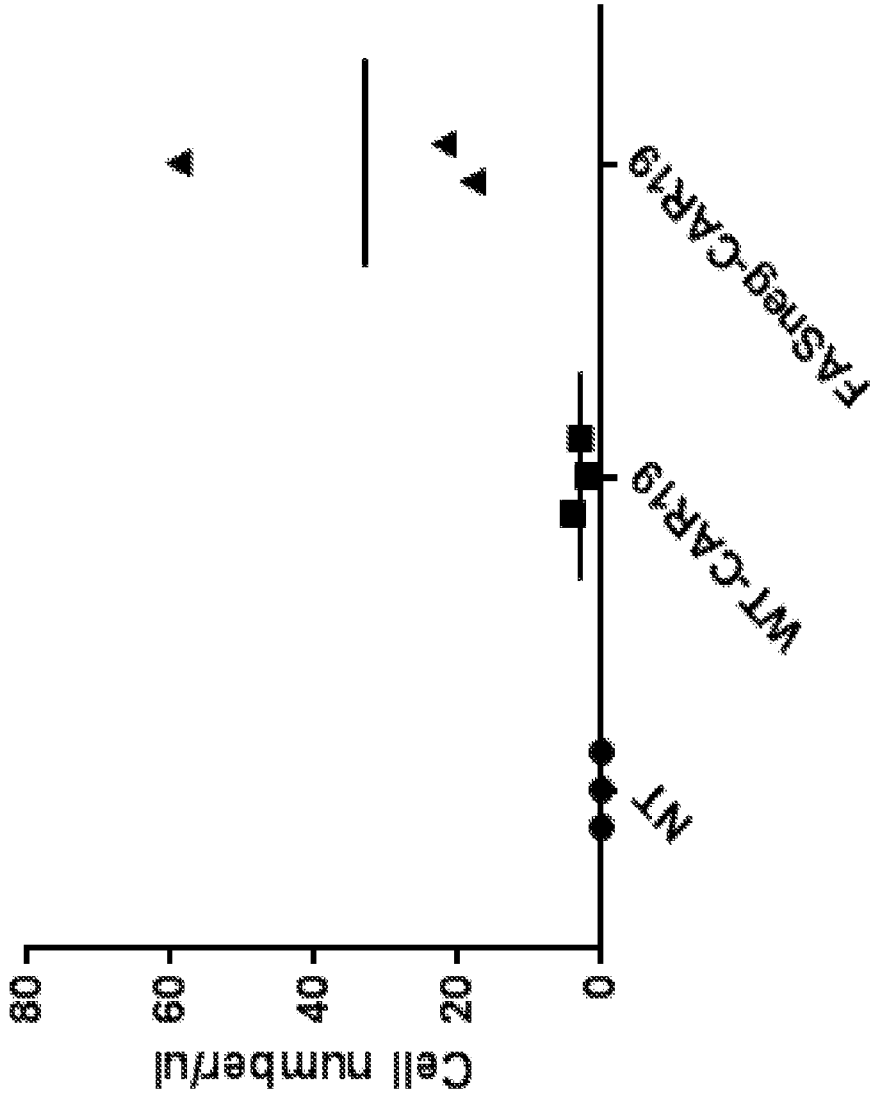


Figure 35A

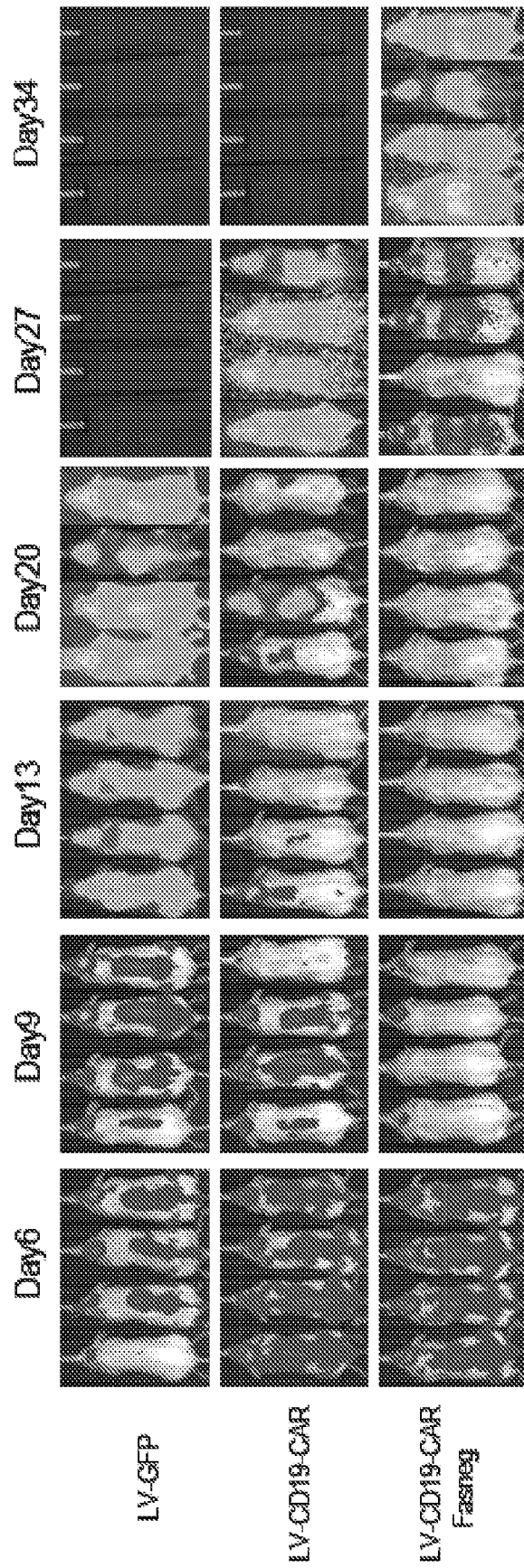


Figure 35B

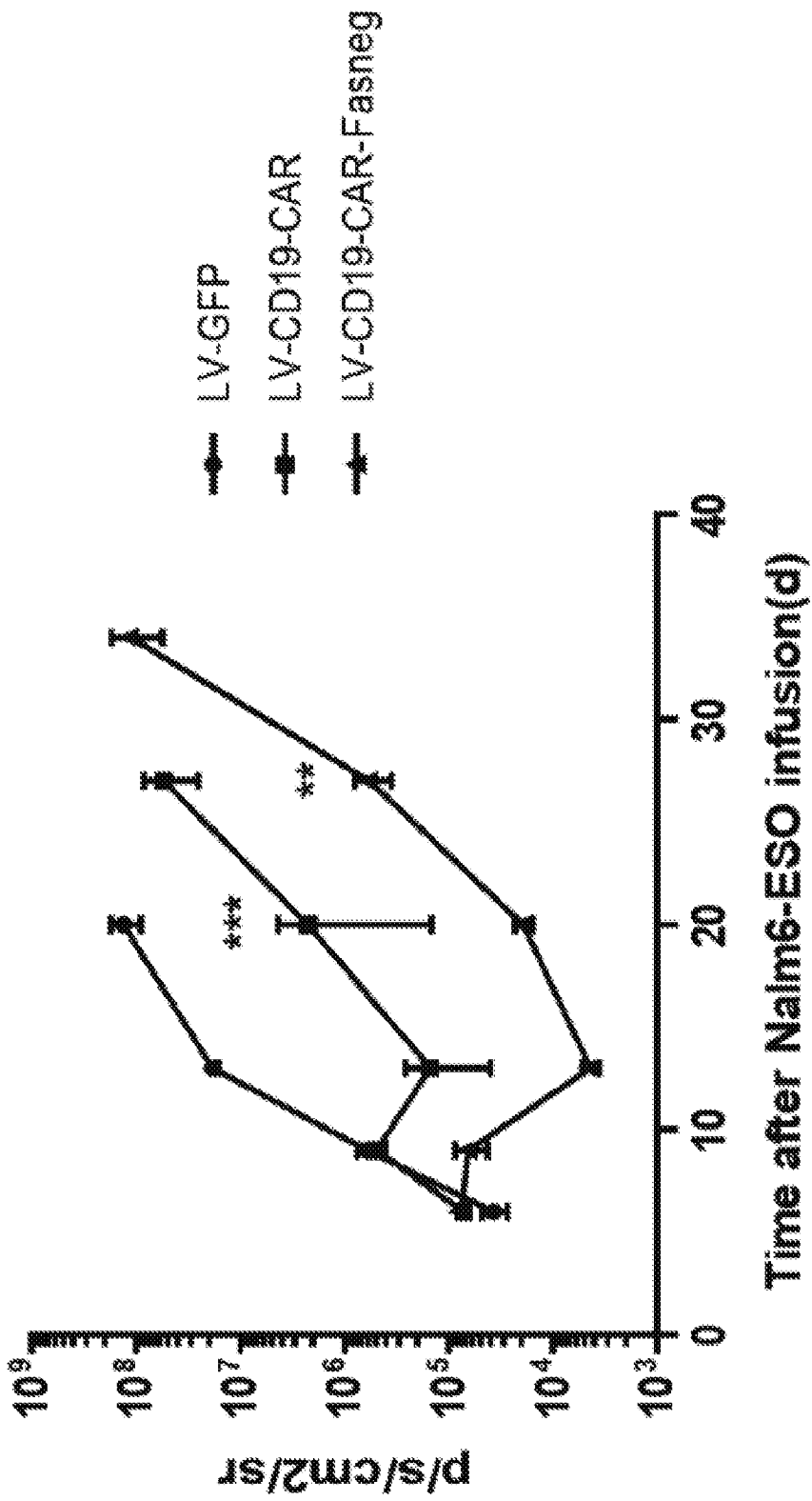


Figure 35C

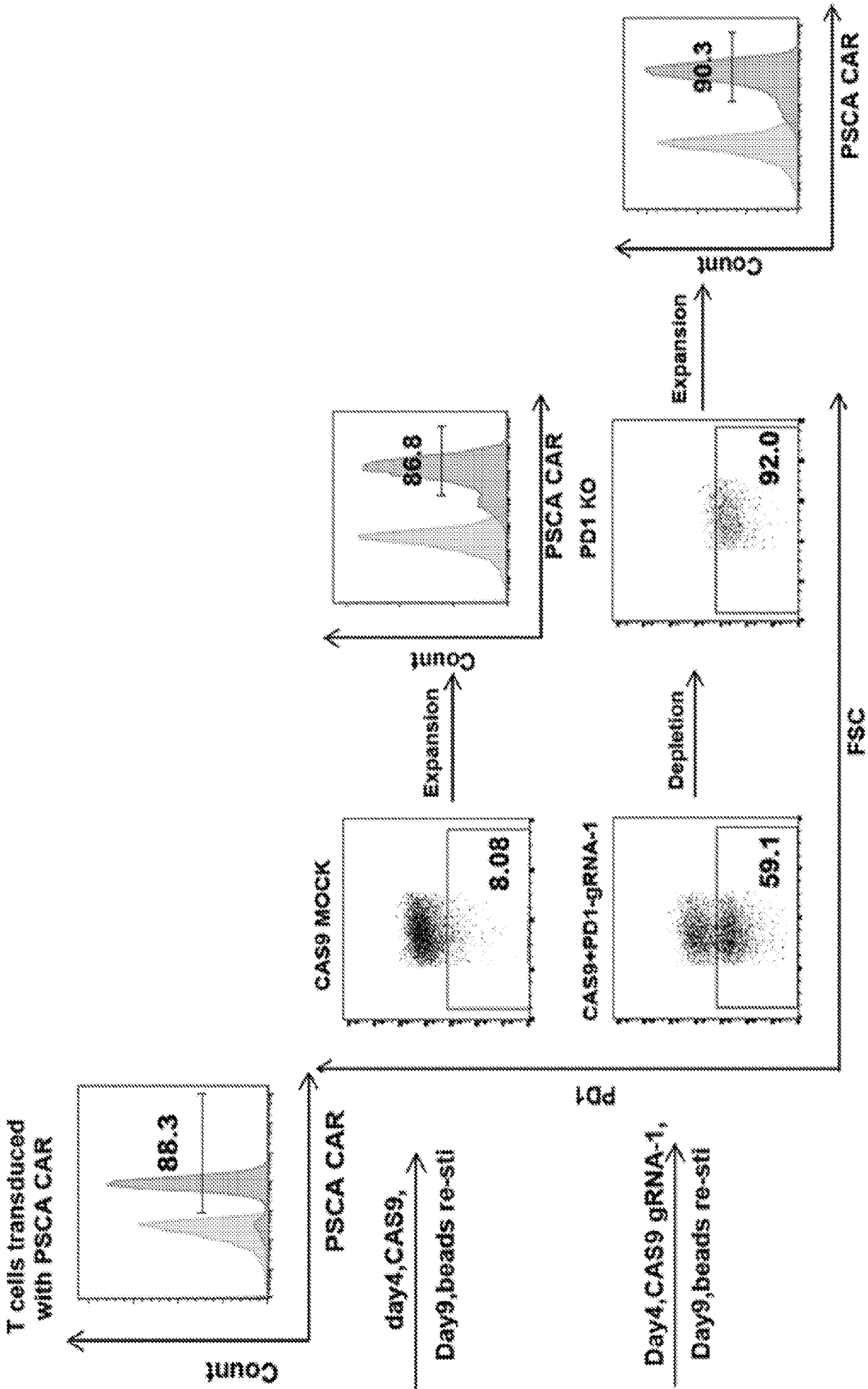


Figure 36

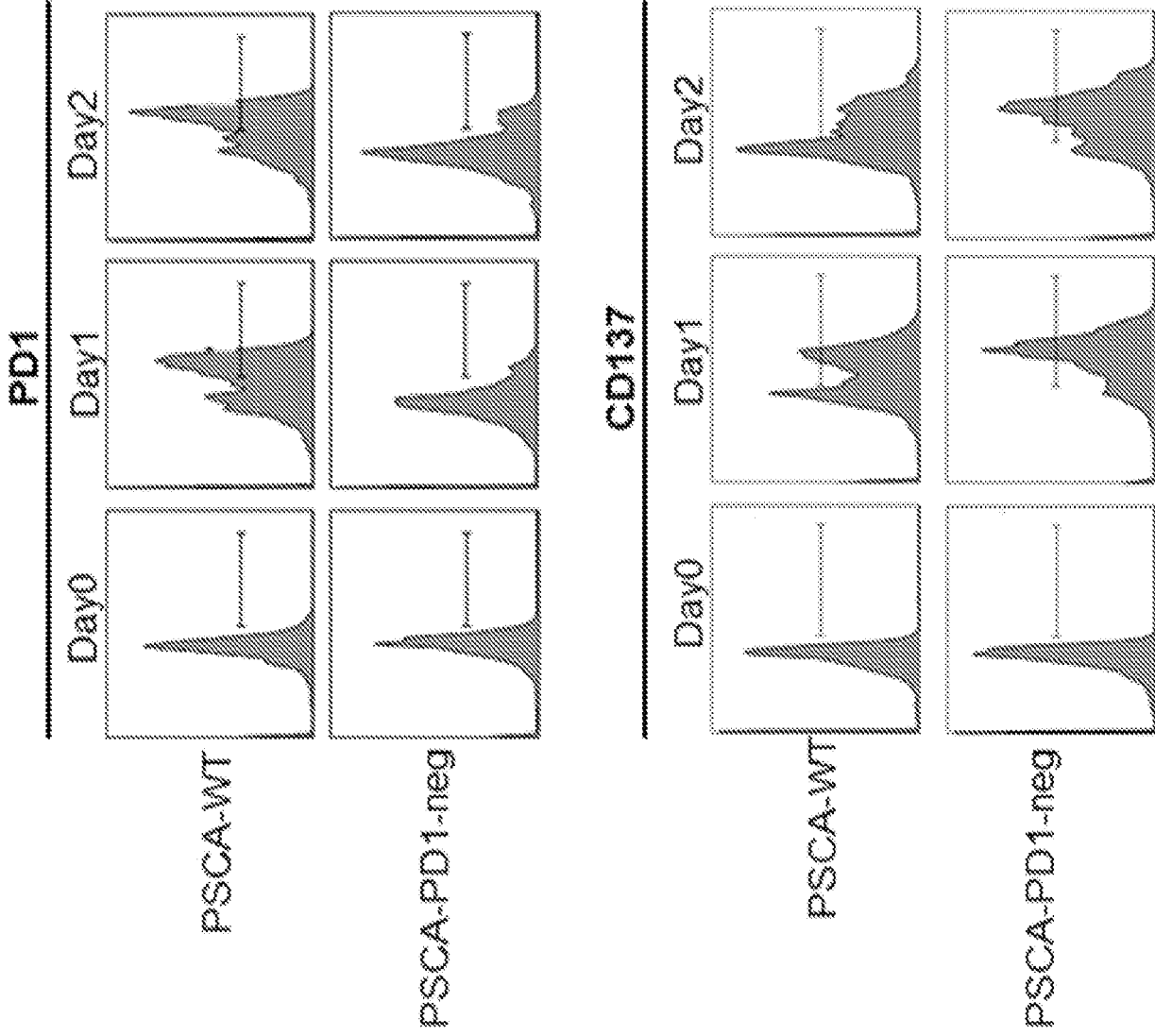


Figure 37

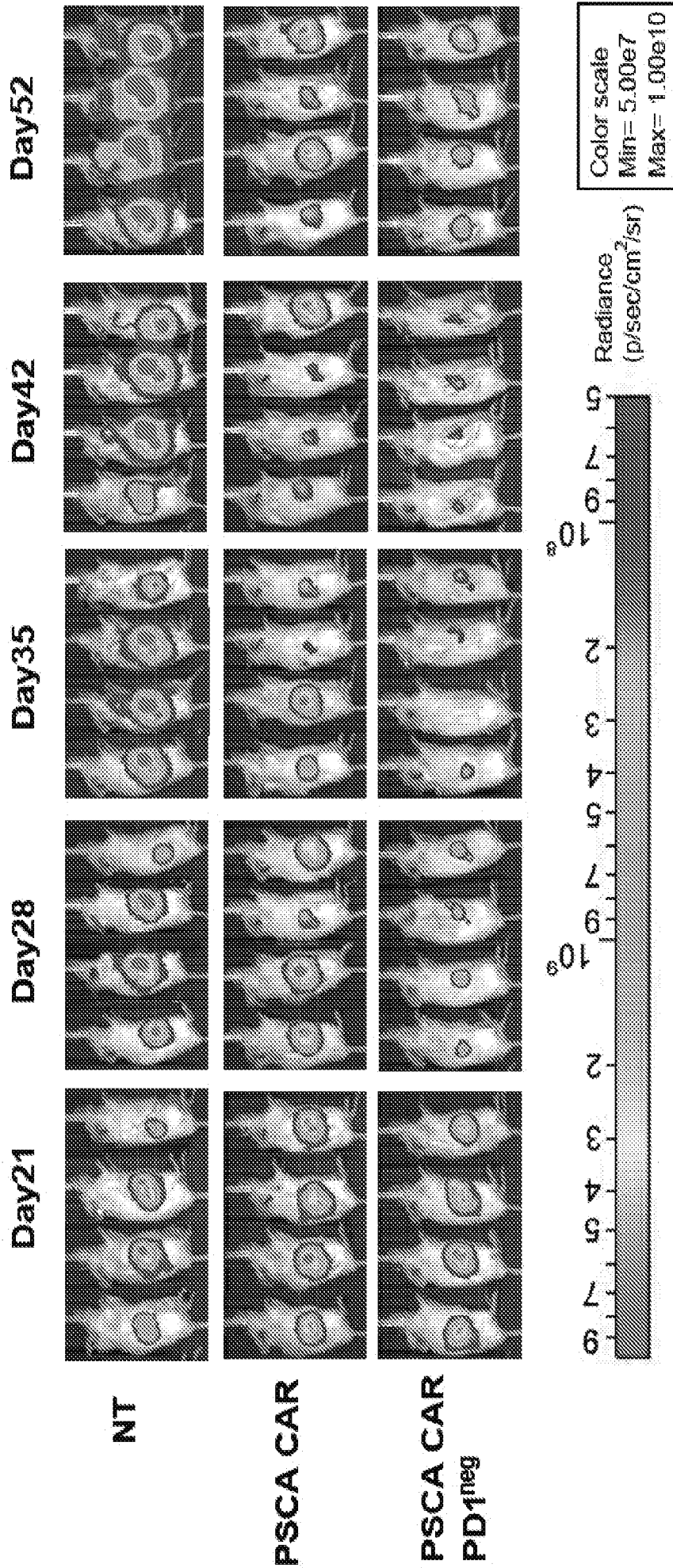


Figure 38A

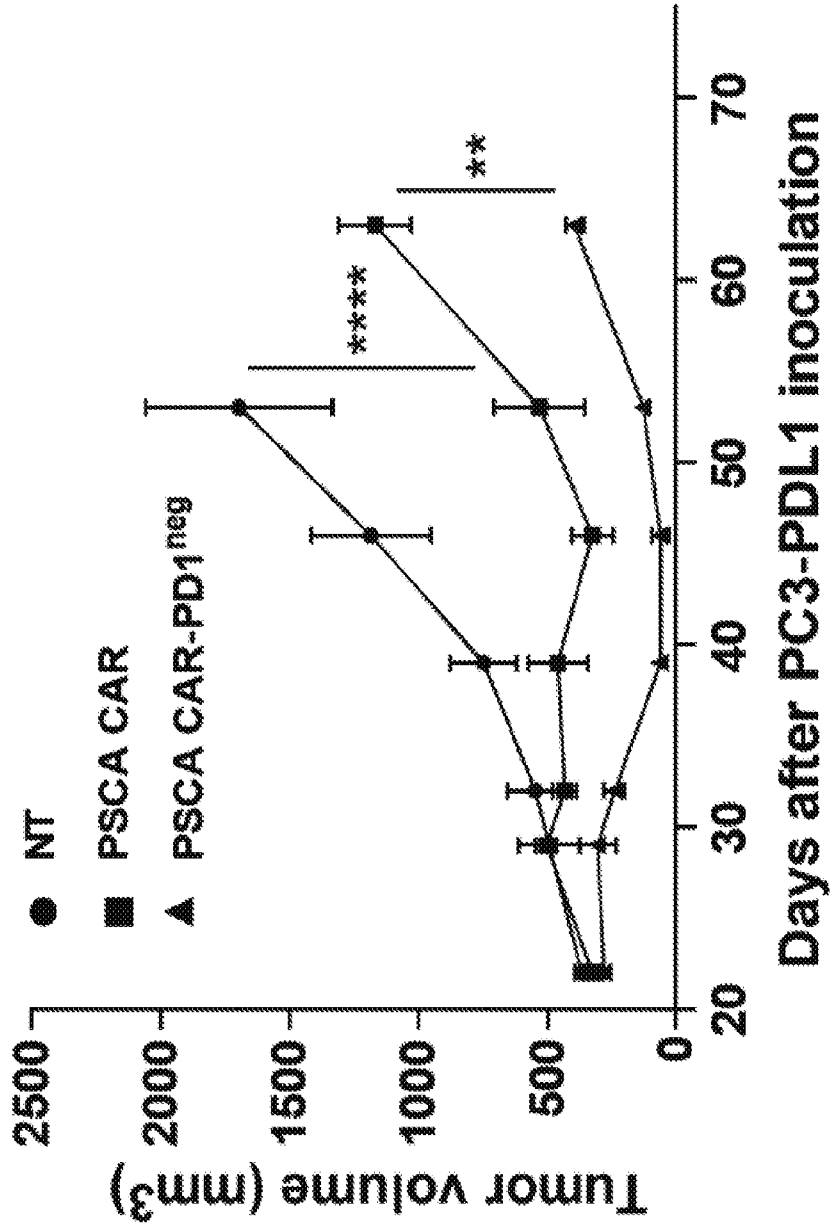


Figure 38B

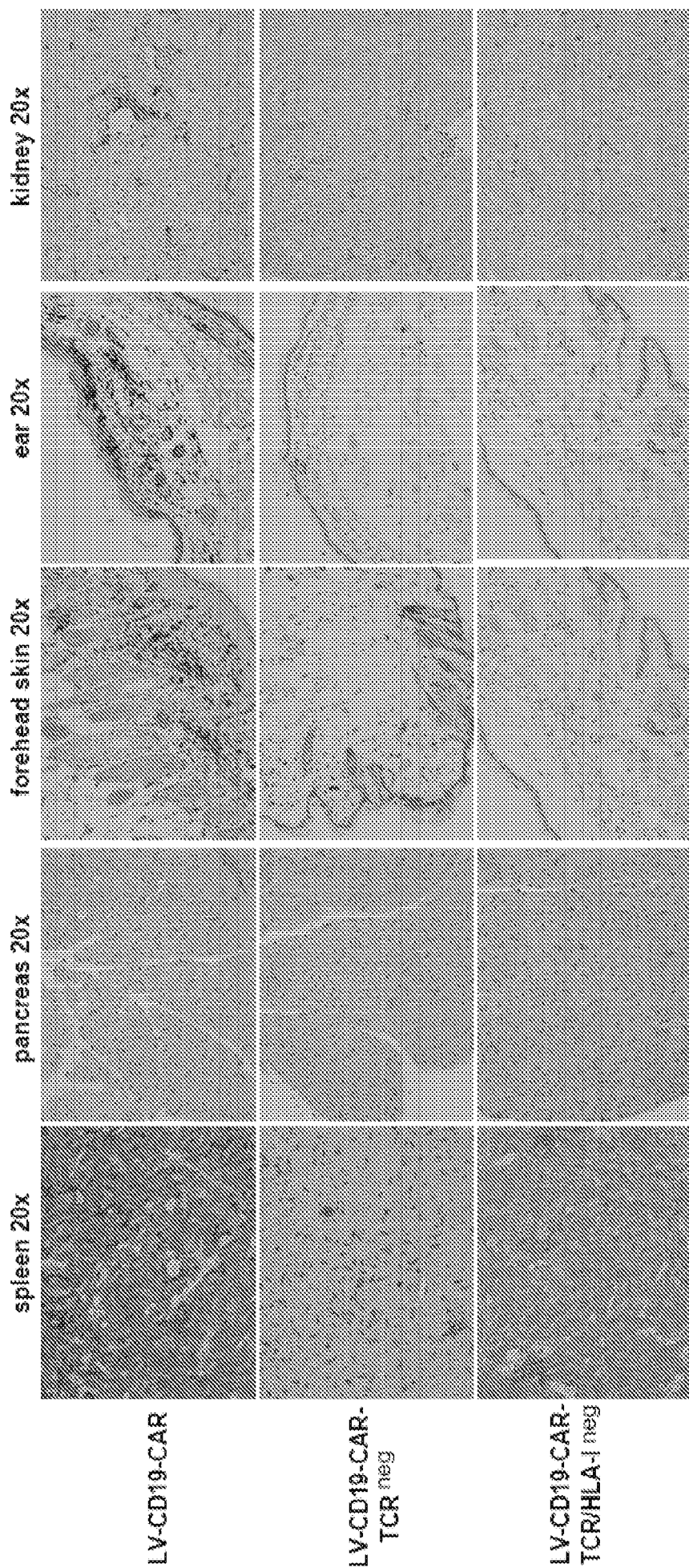


Figure 39

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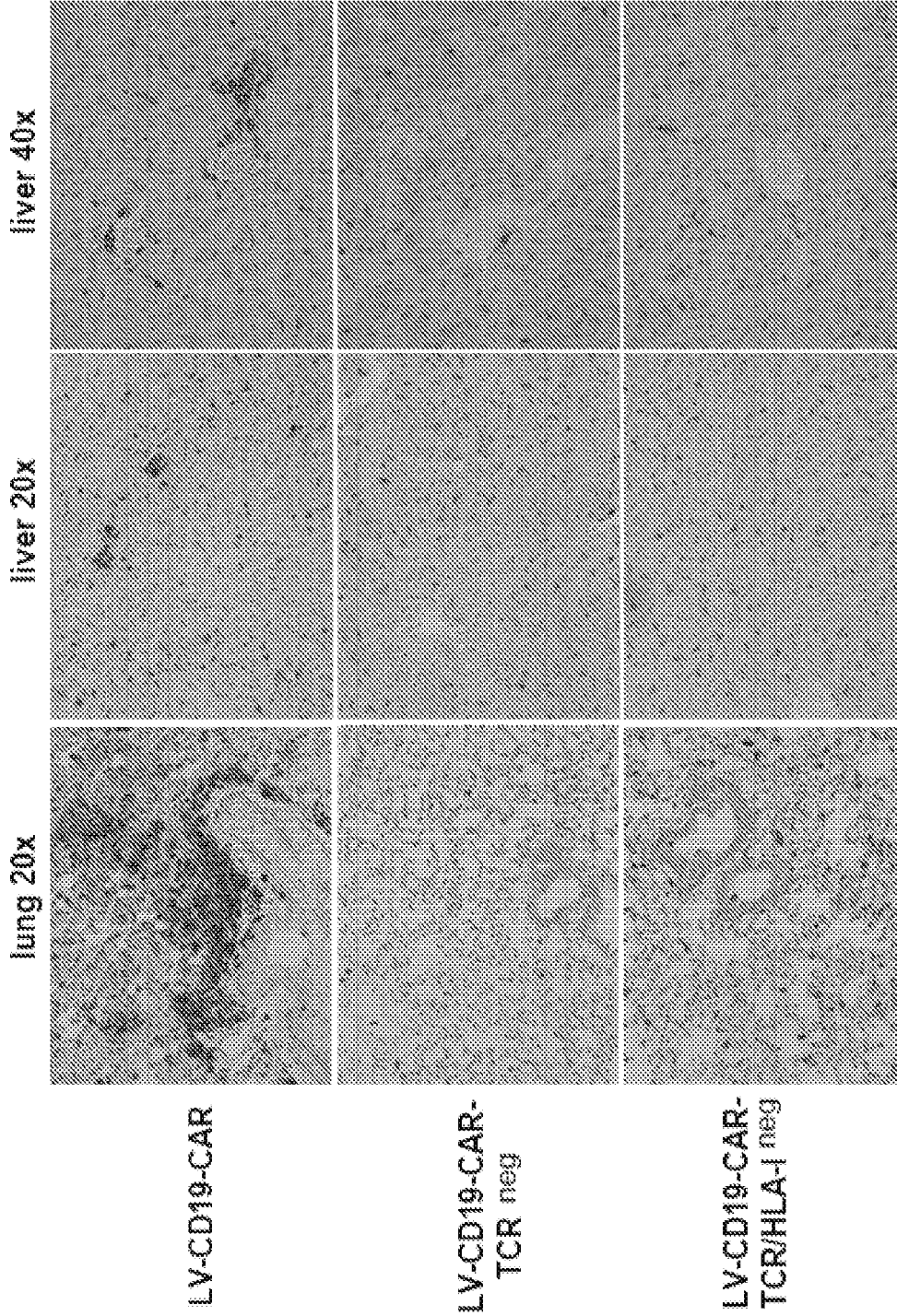


Figure 39 con't

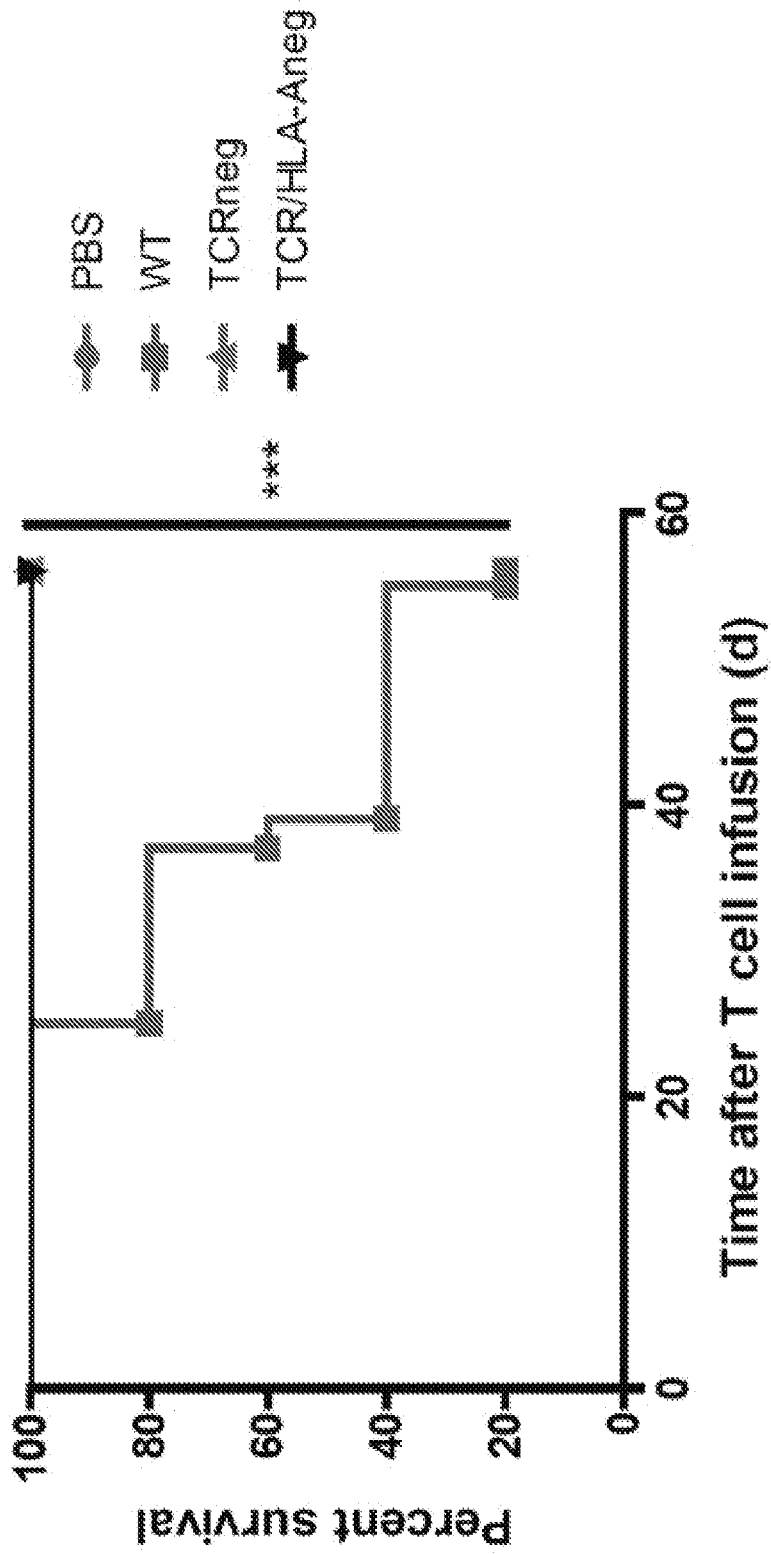


Figure 40A

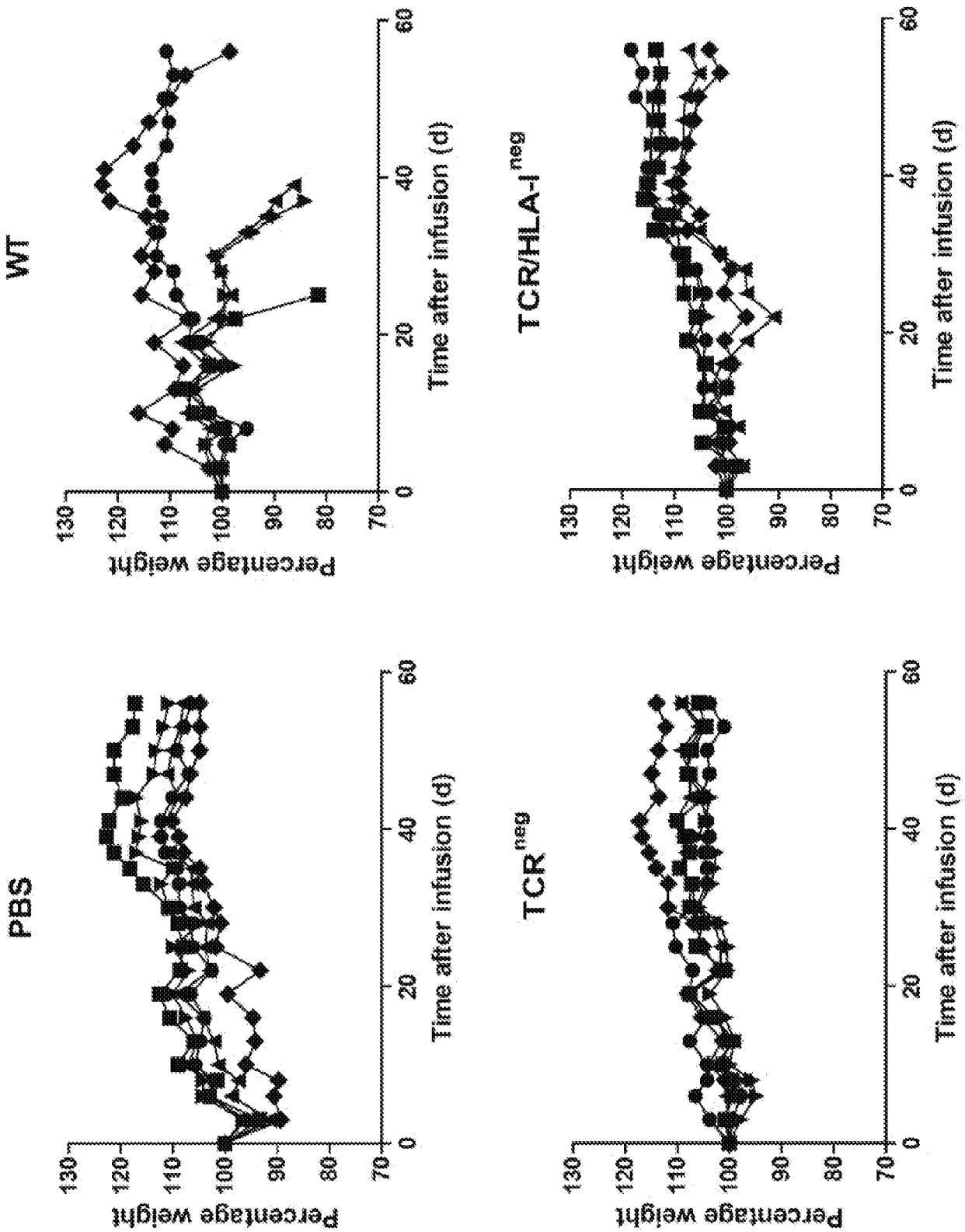


Figure 40B

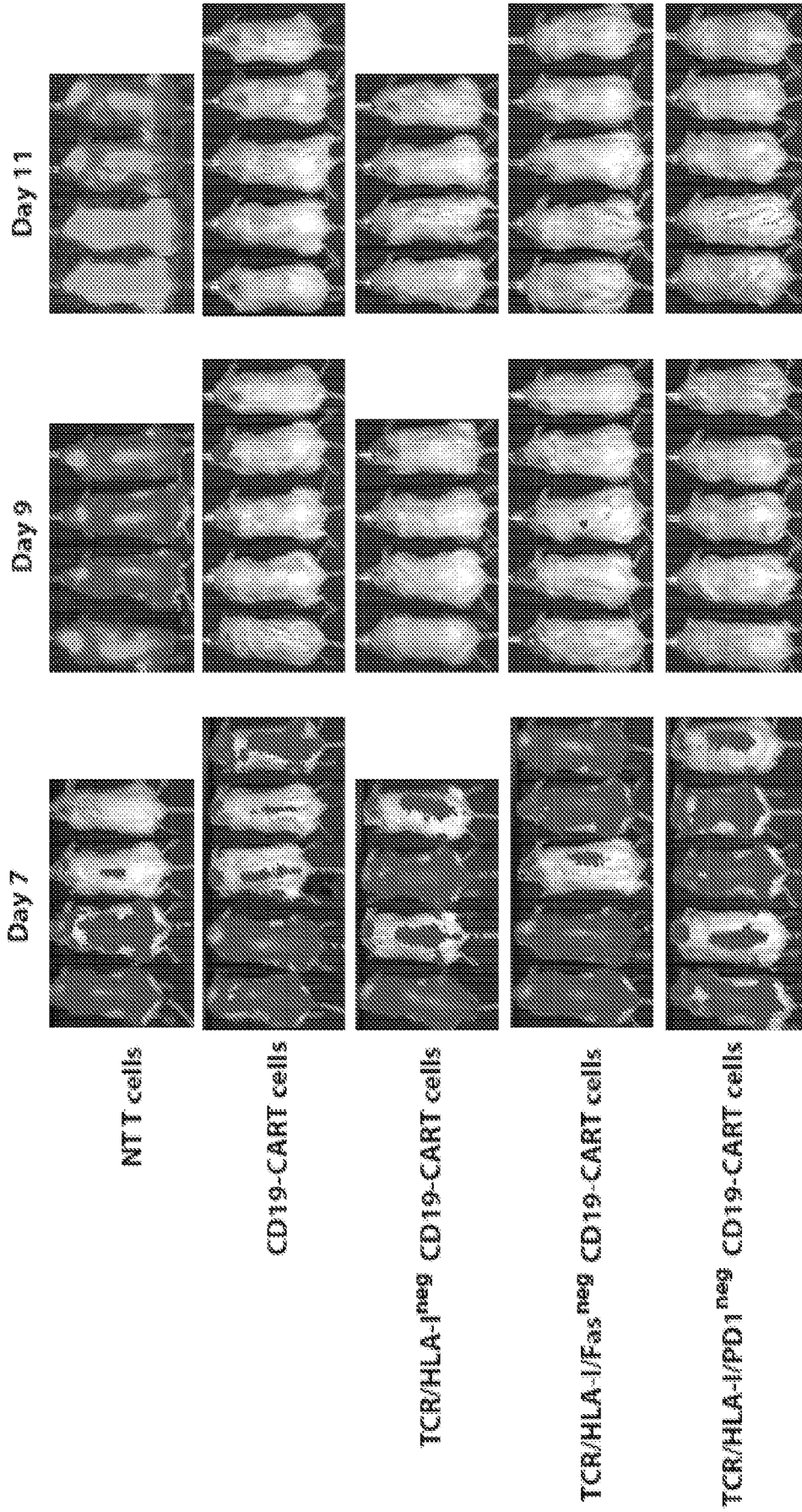


Figure 41A

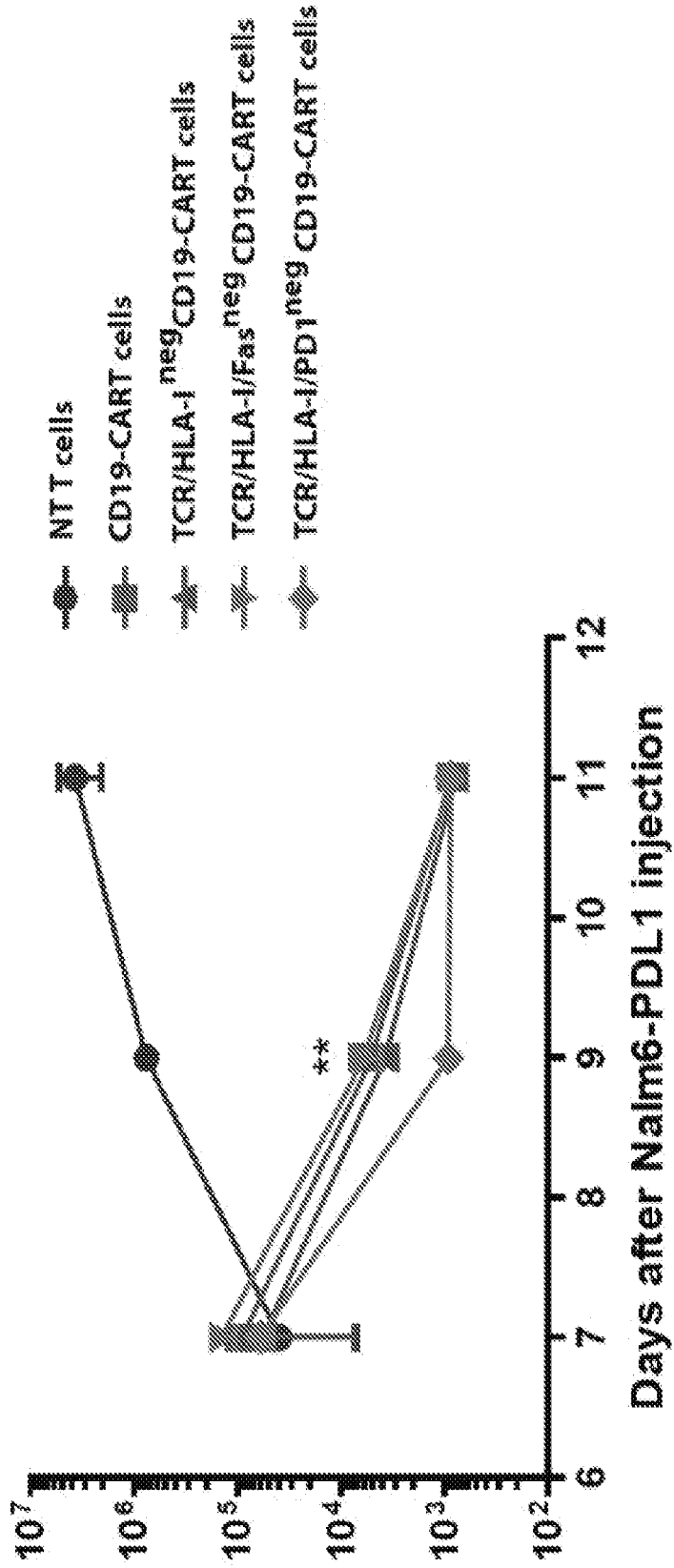


Figure 41B

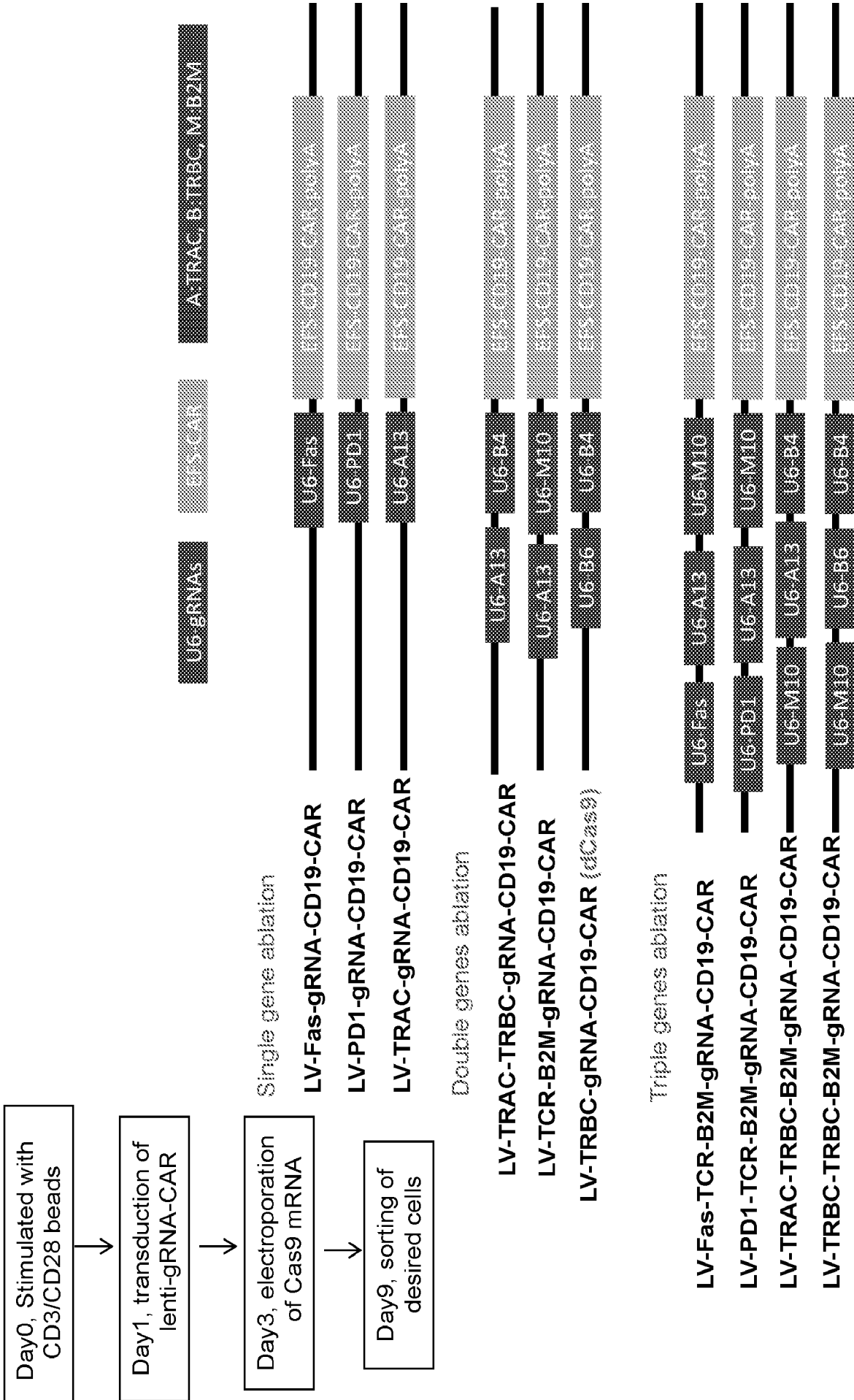


Figure 42

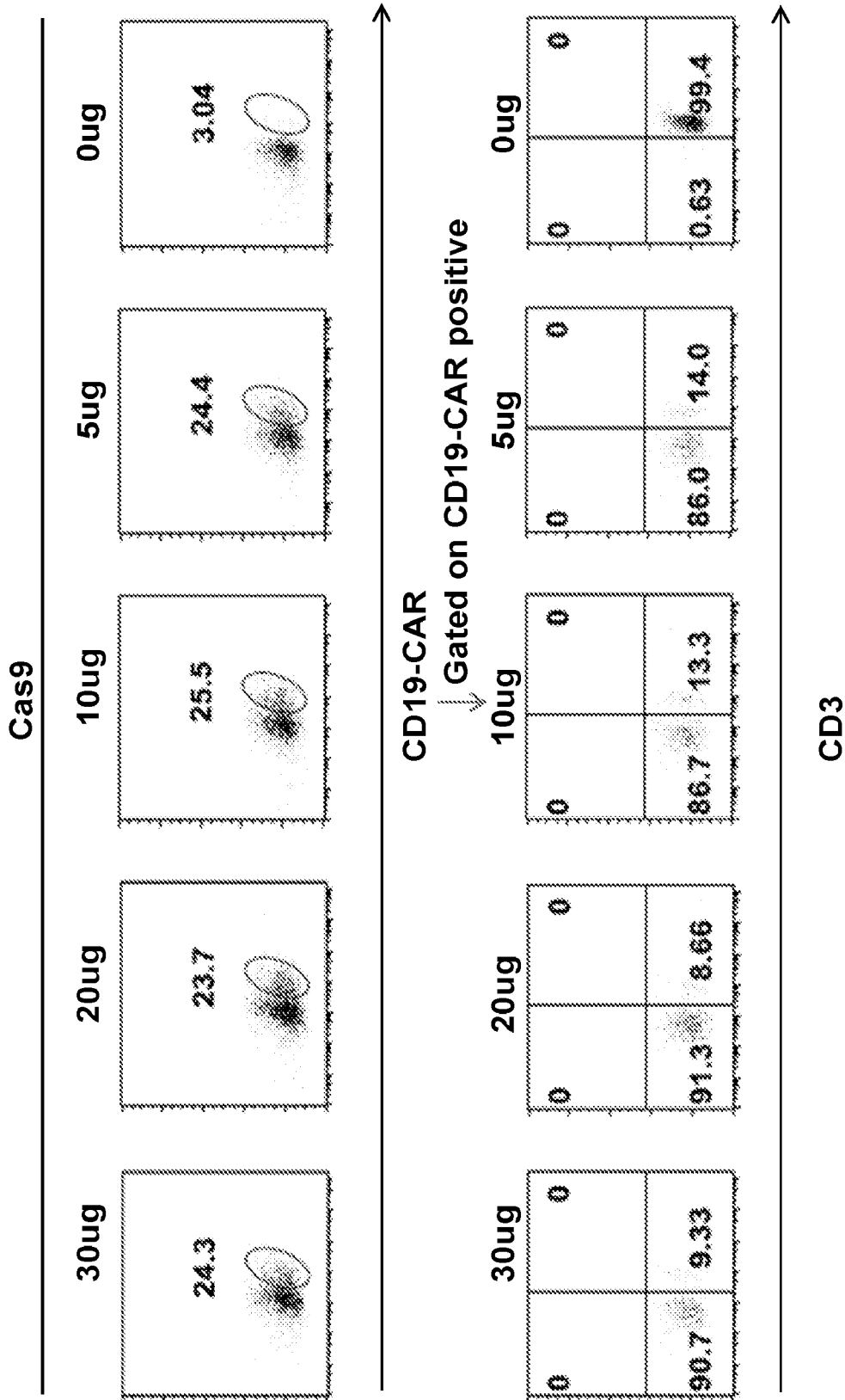


Figure 43

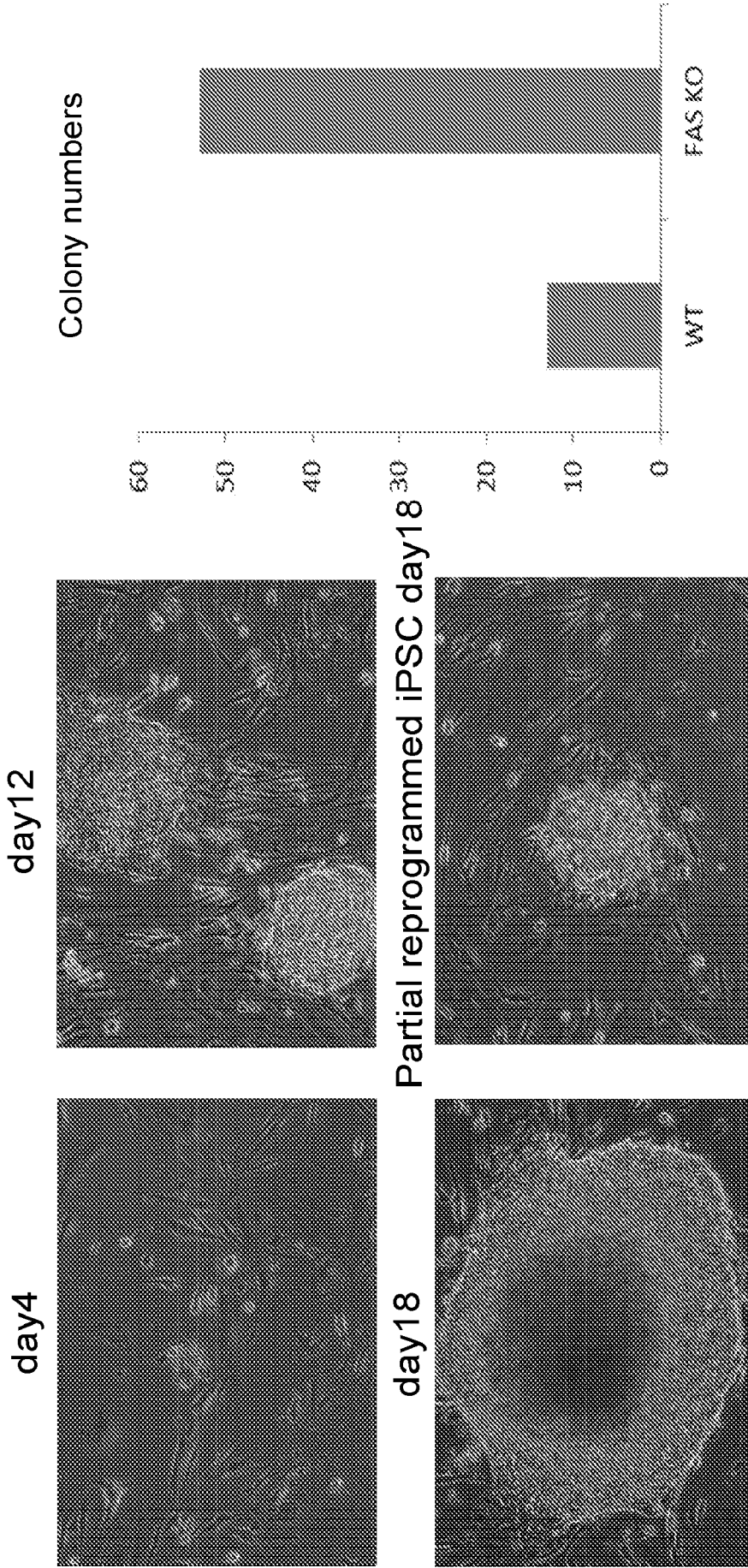


Figure 44B

Passage 0

Figure 44A

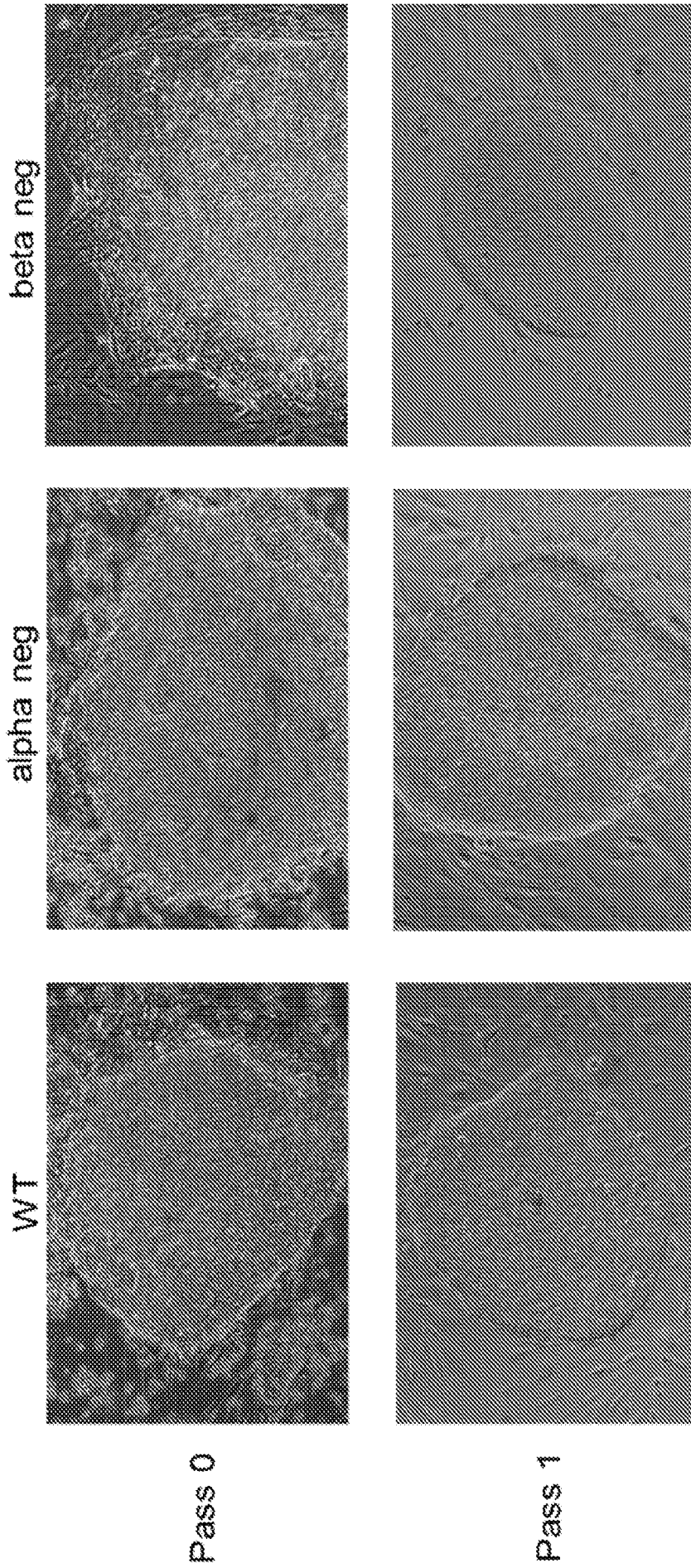


Figure 45A

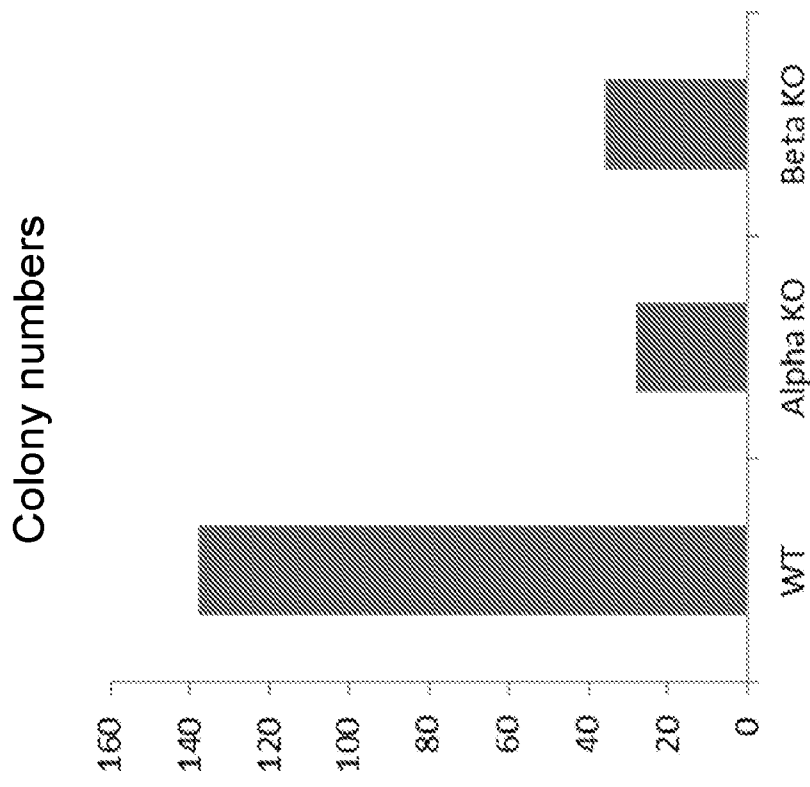


Figure 45B

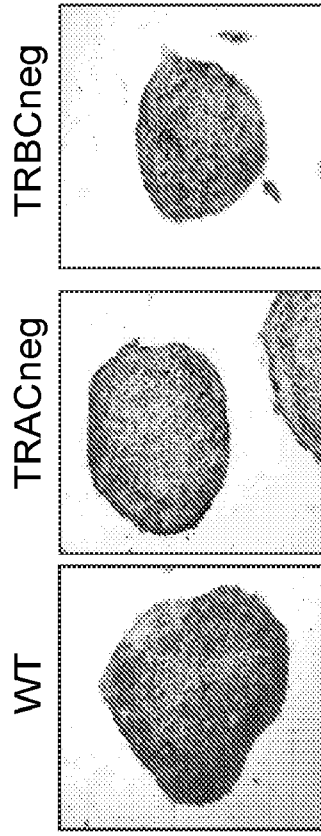


Figure 45C

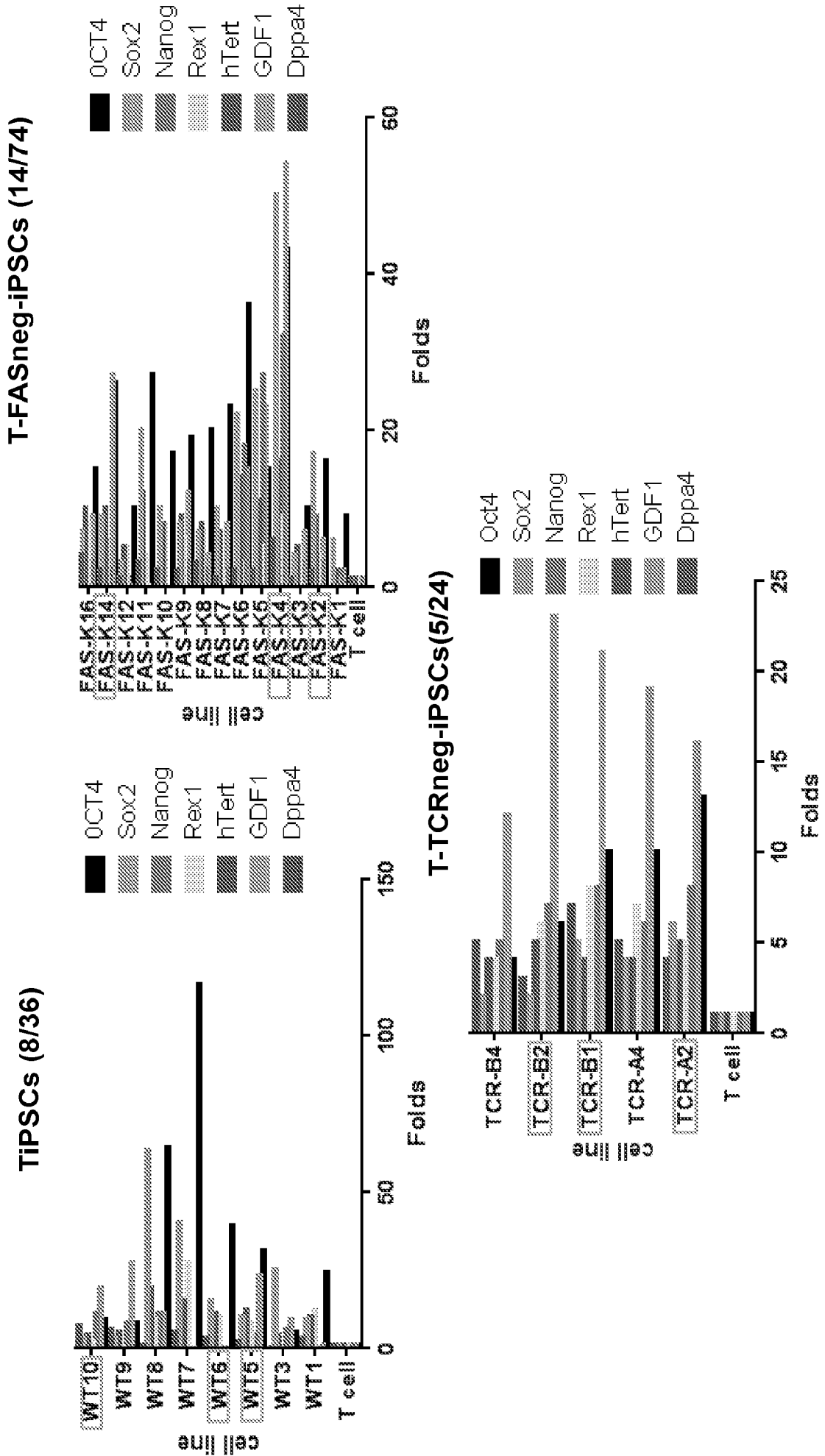
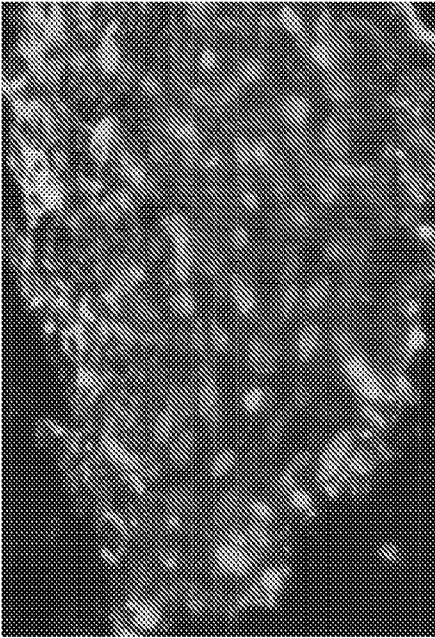


Figure 46

Tra-1-60



SSEA4

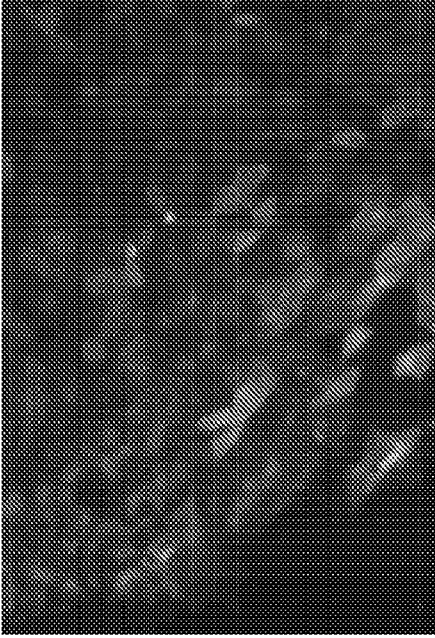


Figure 47A

T-FasKO-iPSC

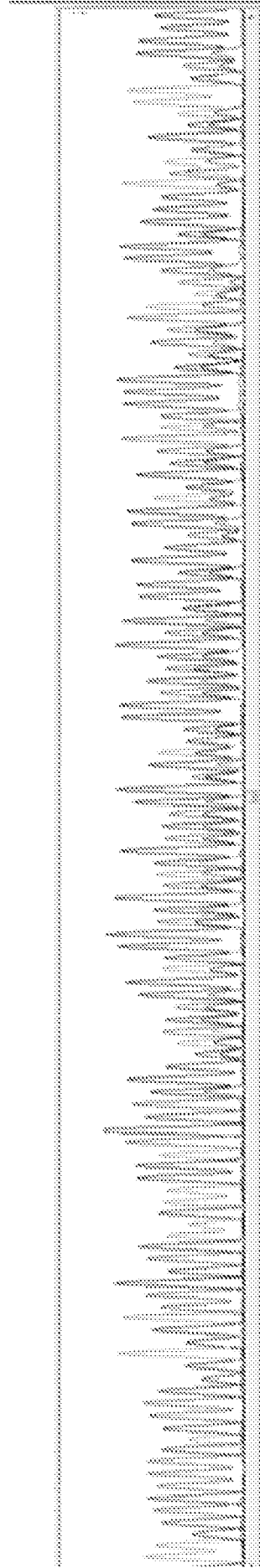


Figure 47B

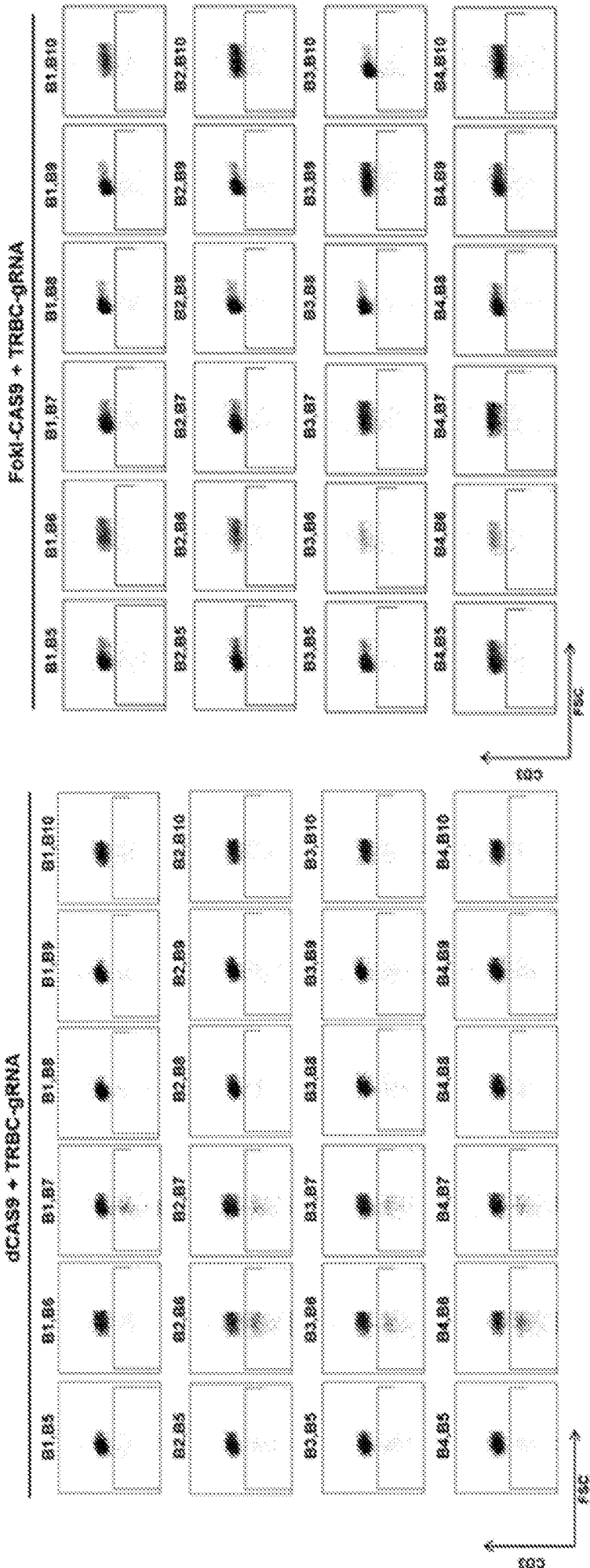


Figure 48A

D10A-CAS9 + TRBC-grNA

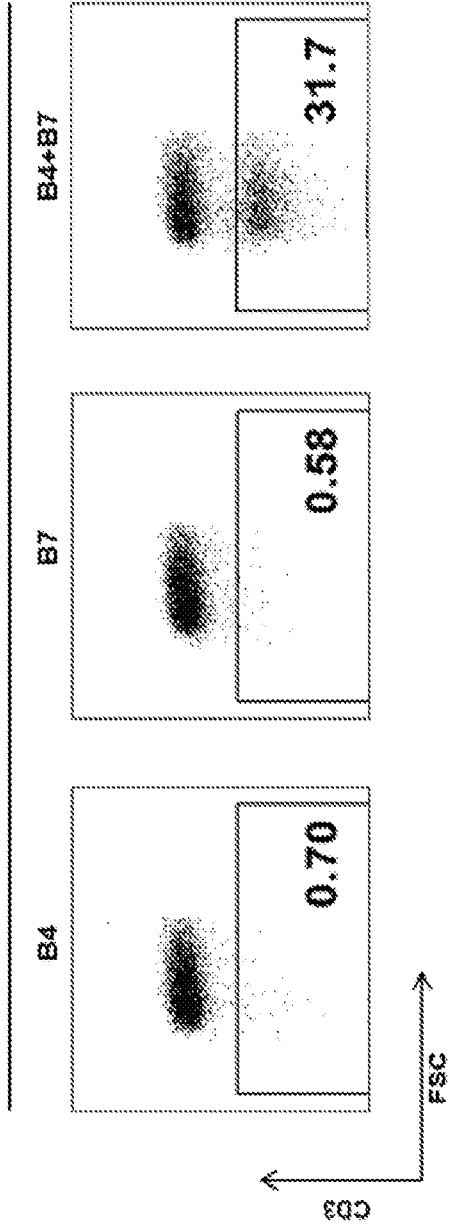


Figure 48B

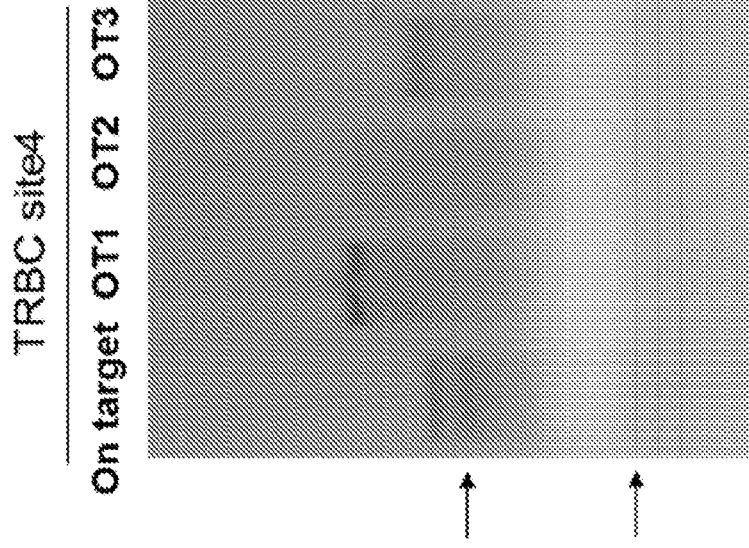
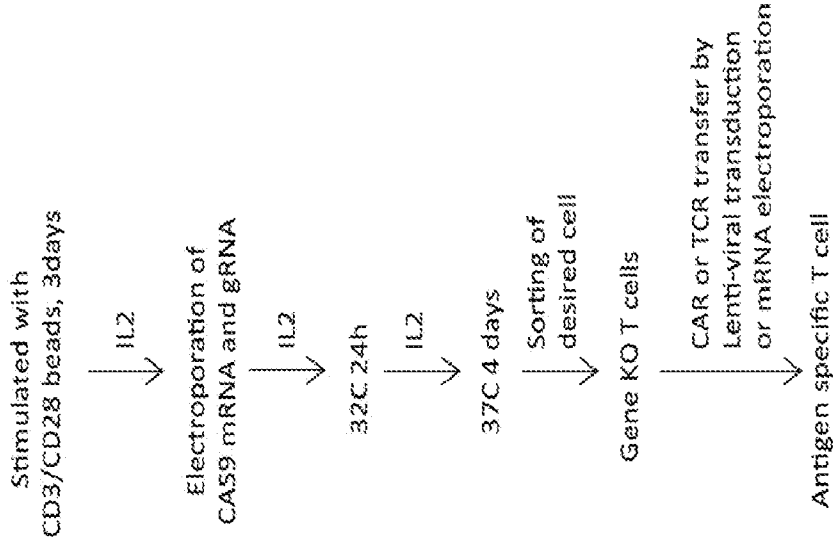


Figure 48C



T7 promoter + target RNA + guide RNA scaffold

TAATACGACTCACTATAGNNNNNNNNNNNNNNNNNNNNNN

NNNNNNNGTTTITAGAGCTAGAAATAGCAAGTTA

AAATAAGGCTAGTCGGTTATCAACTTGAAAAGT

GGCACCGAGTCGGTGGCTTTTIT

Figure 49

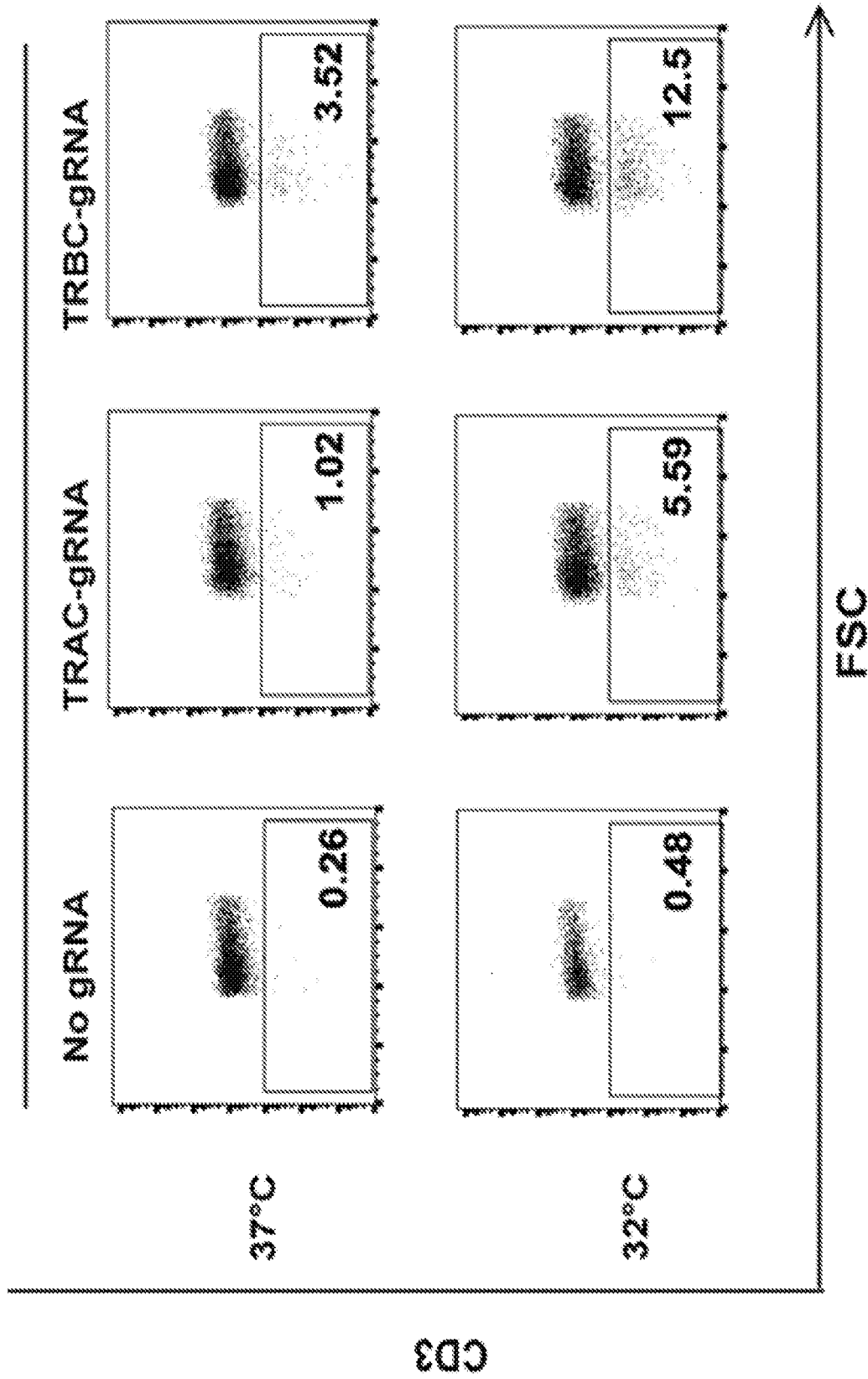


Figure 50A

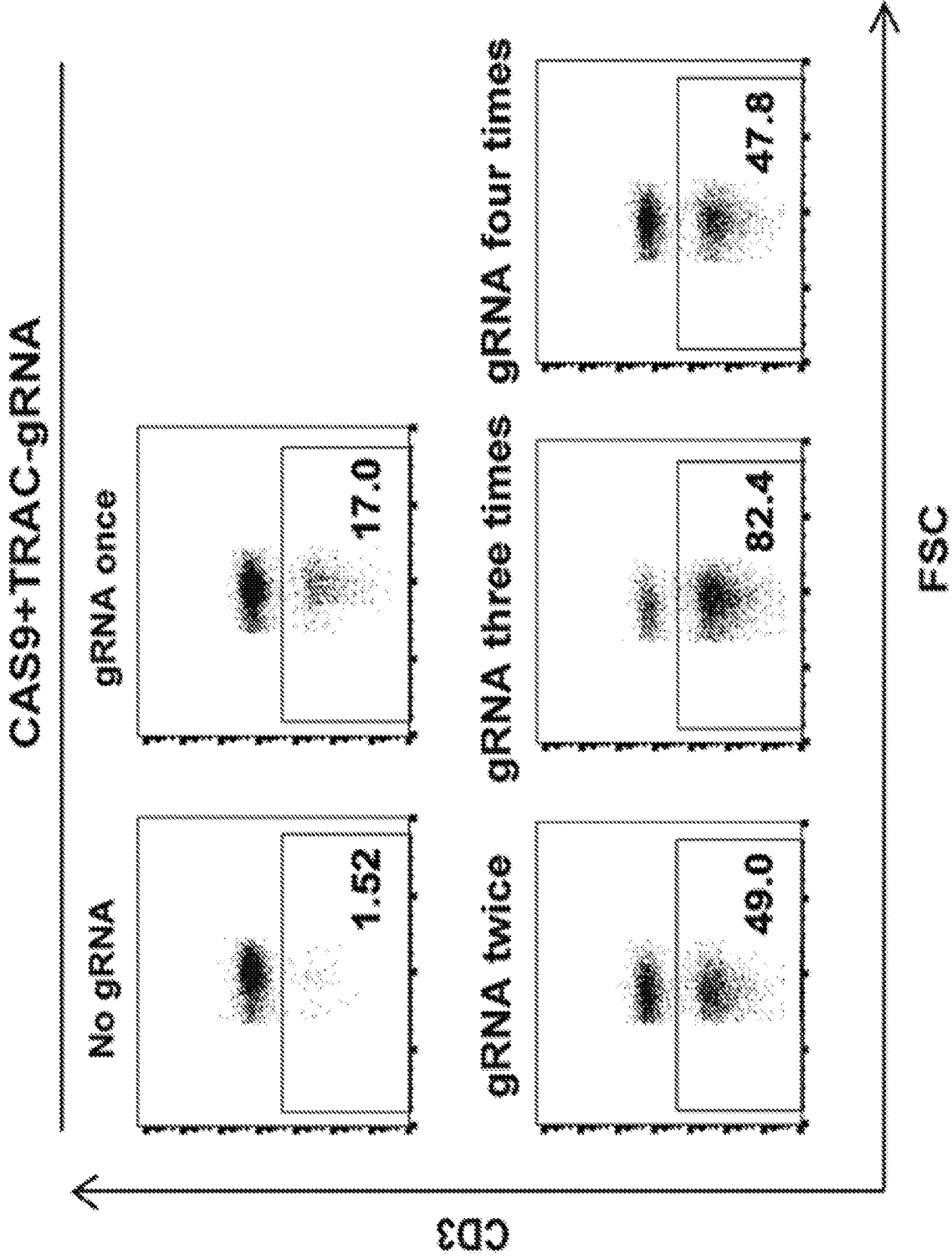


Figure 50B

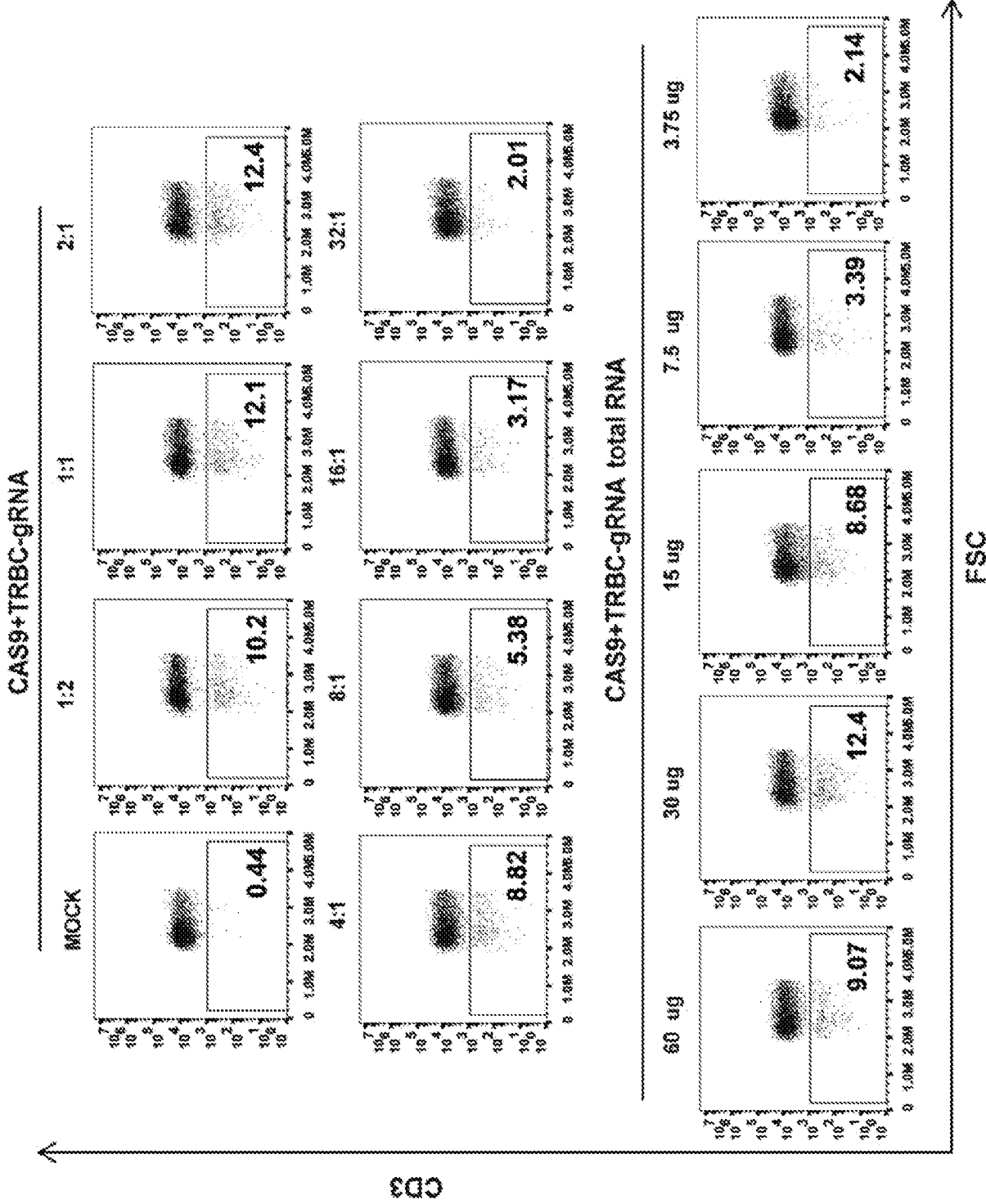


Figure 51A

2024203330 20 May 2024

CAS9/ times	gRNA/ times	Protein %	Genome %
1	0	0	0
1	1	17	27.7 (5/18)
1	2	49	60 (12/20)
1	3	82.4	89.4(17/19)
1	4	47.8	55 (11/20)

Figure 51B

2024203330 20 May 2024

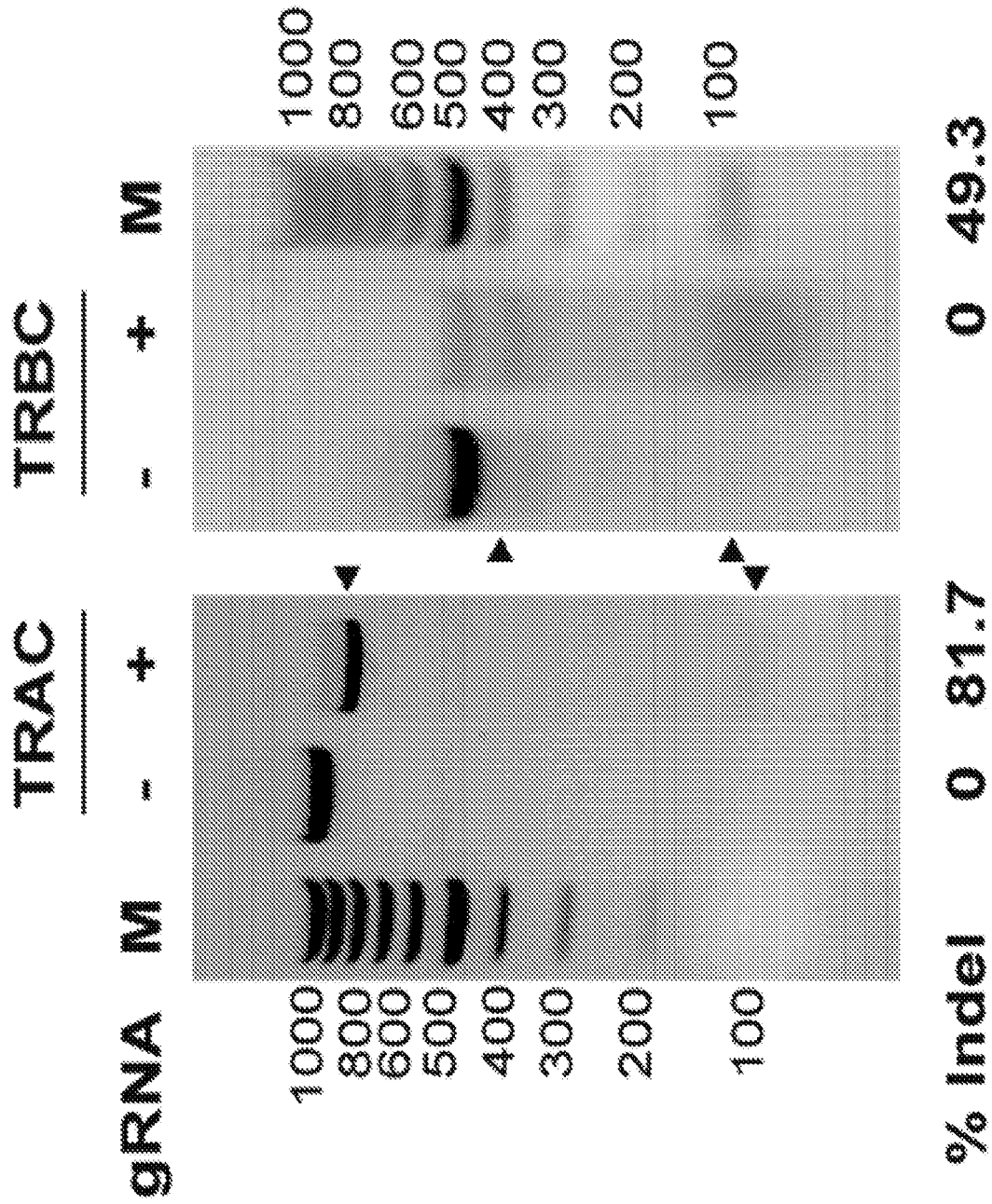


Figure 52

```

TRAC WT  TATATCACAGACAAAACCTGTGCTAGACATGAGGGICIAIGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X4)  TATATCACAGACAAAACCTGTGCTAGACATGAG- TCTAIGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X3)  TATATCACAGACAAAACCTGTGCTAGACATG- GCTAIGSSACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAGACATGA- GCTAIGSSACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAGACATG- - TCTAIGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAGAC- TGAGSSTCTATGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAG- - - TGAGSSTCTATGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCT- - - - TGAGSSTCTATGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAGACATGAGGTTCTAIGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAGACATGAGSSTCTAIGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X1)  TATATCACAGACAAAACCTGTGCTAGACATGAGGTTCTAIGGACTTCAAGAGCAACAGTGTCTGIGGGCT
(X2)  AGCAGCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X2)  AGCAGCCCGCCCTC- ATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X2)  AGCAGCCCGCC- TCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X1)  AGCAGCCCGCCCTCAATGACTC- AGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X1)  AGCAGCCCGCCCTCAATGACT- - AGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X1)  AGCAGCCCGCC- - - - GACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X1)  AGCAGCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCT
(X1)  AGCAGCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCT
(X1)  AGCAGCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCT
(X3)  AGCAGCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X2)  AGCAGCCCGCCCTCAATGACTC- AGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X1)  AGCAGCCCGCCCTCA- - - - - ACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCTG
(X1)  AGCAGCCCGCC- TCA- TGACT- CAGATACTG- CTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCT
(X1)  AGCAGCC- - - - - ACTG- - - - - C- G- - - - - TGAGCAGCCGCTGAGGGTCTCGGCCACCTTCT
(X1)  AGCAGCCCGCCCTCAATGACTCCAGATACTGCTGAGCAGCCGCTGAGGGTCTCGGCCACCTTCT

```

Figure 53A

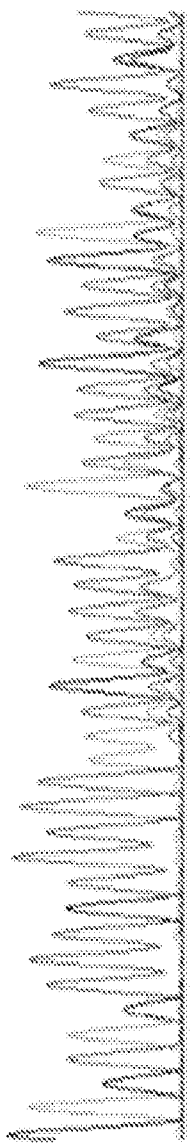
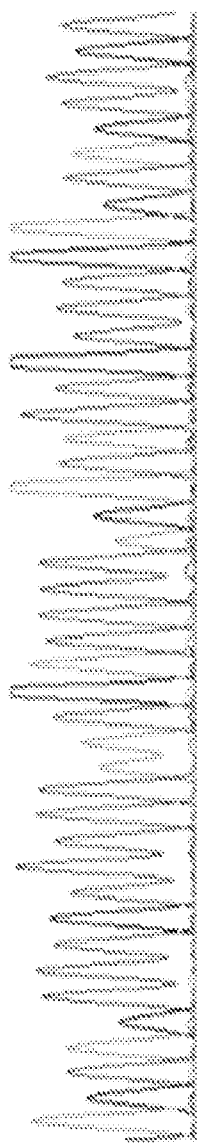
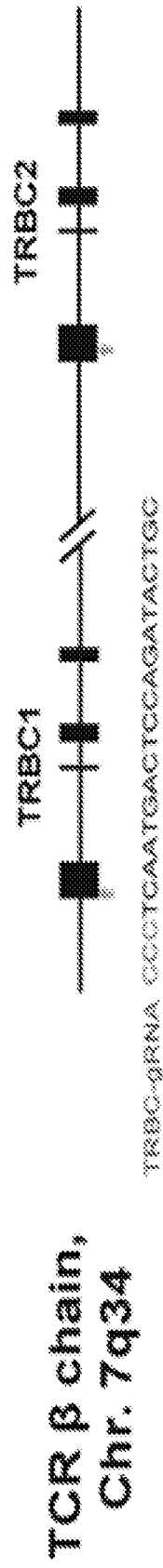
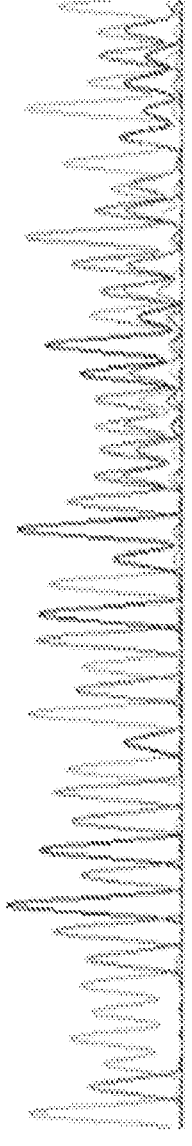
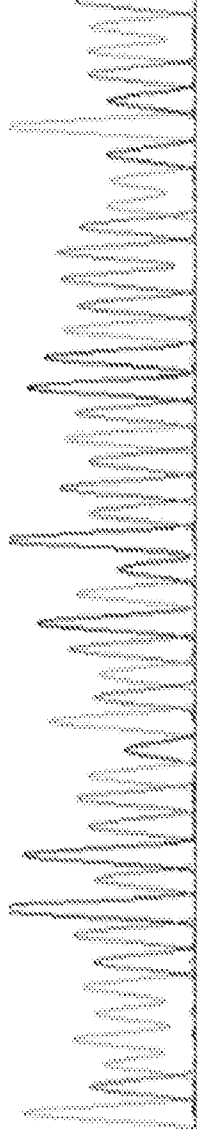
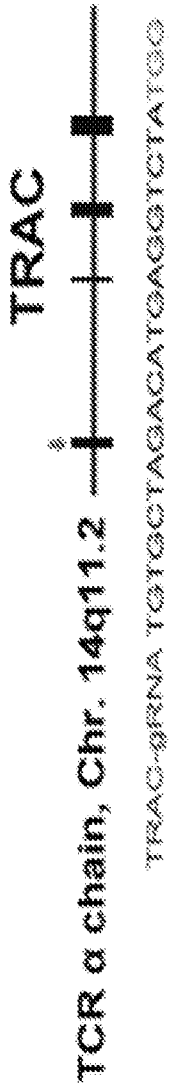


Figure 53B

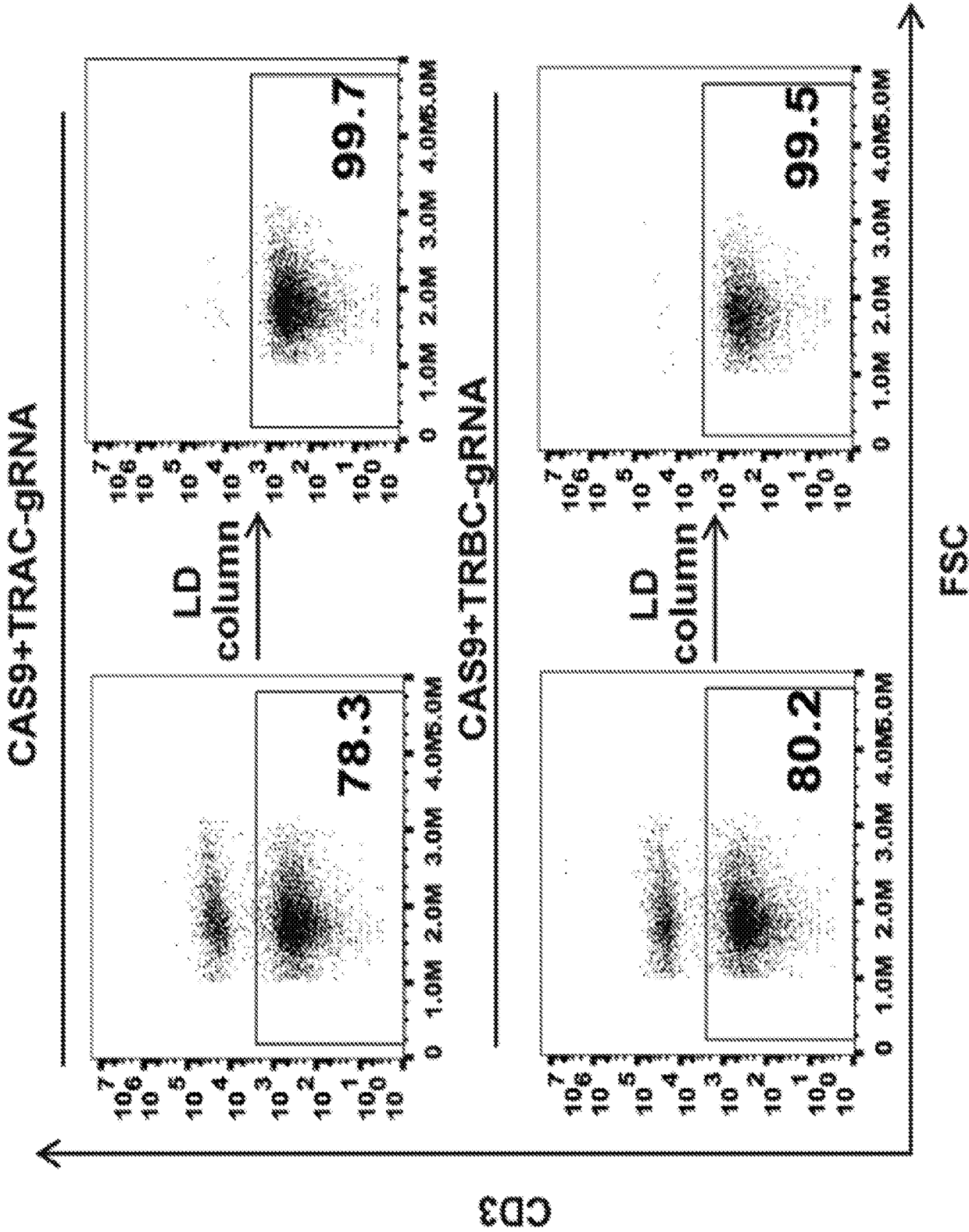


Figure 54

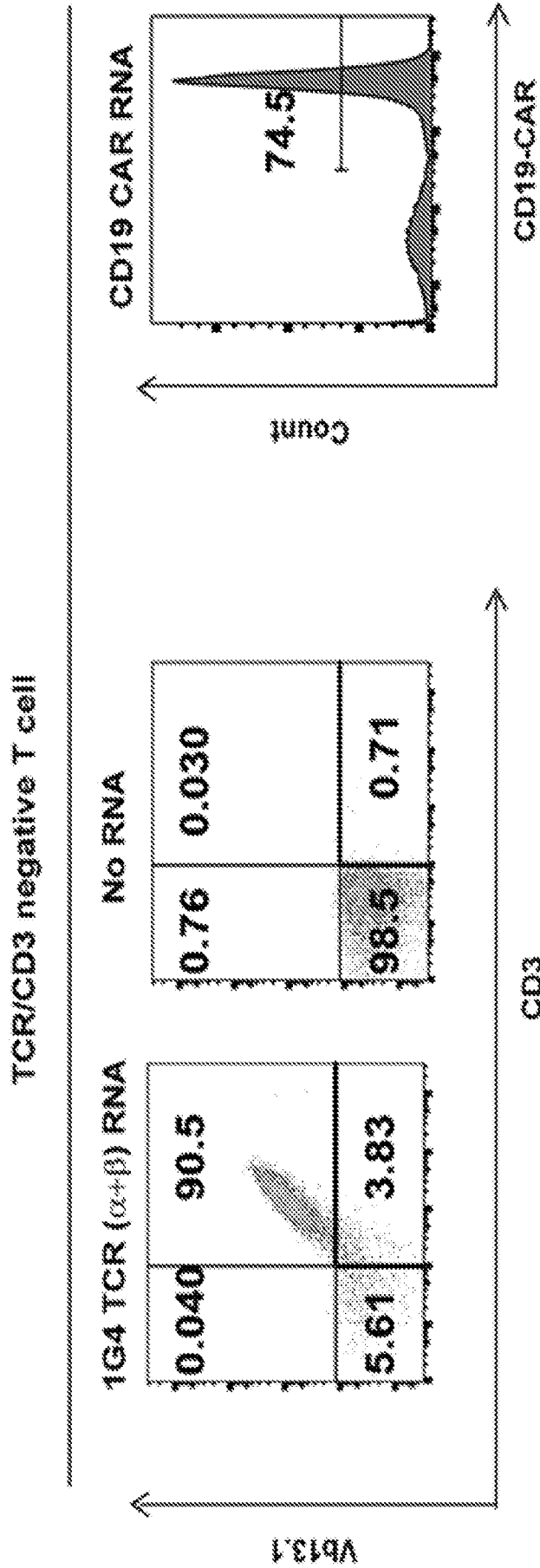


Figure 55

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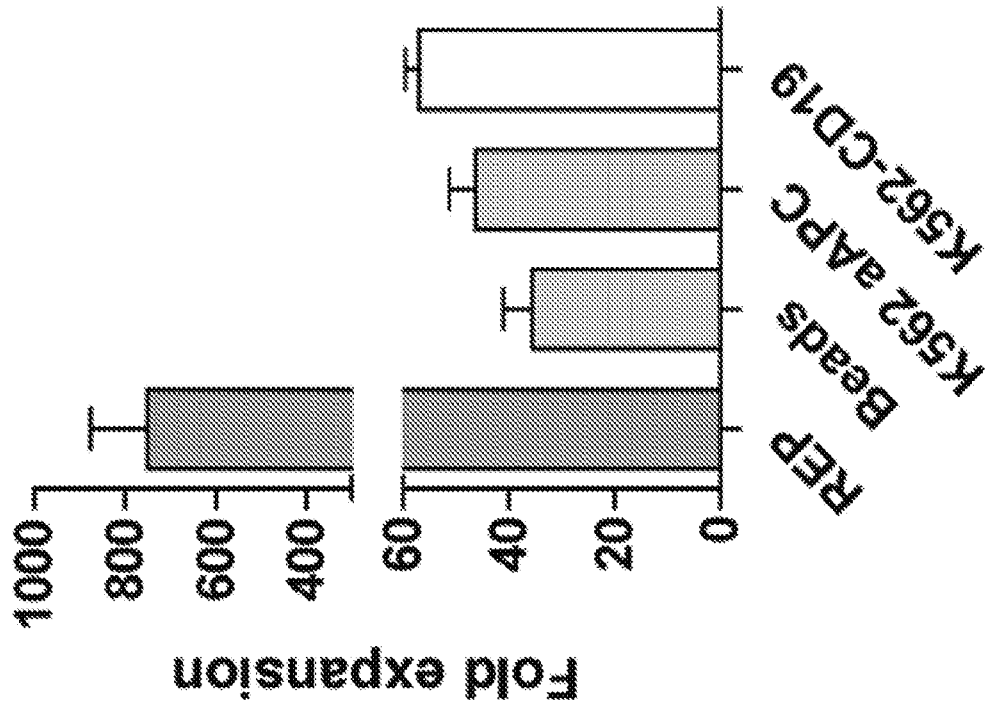


Figure 56

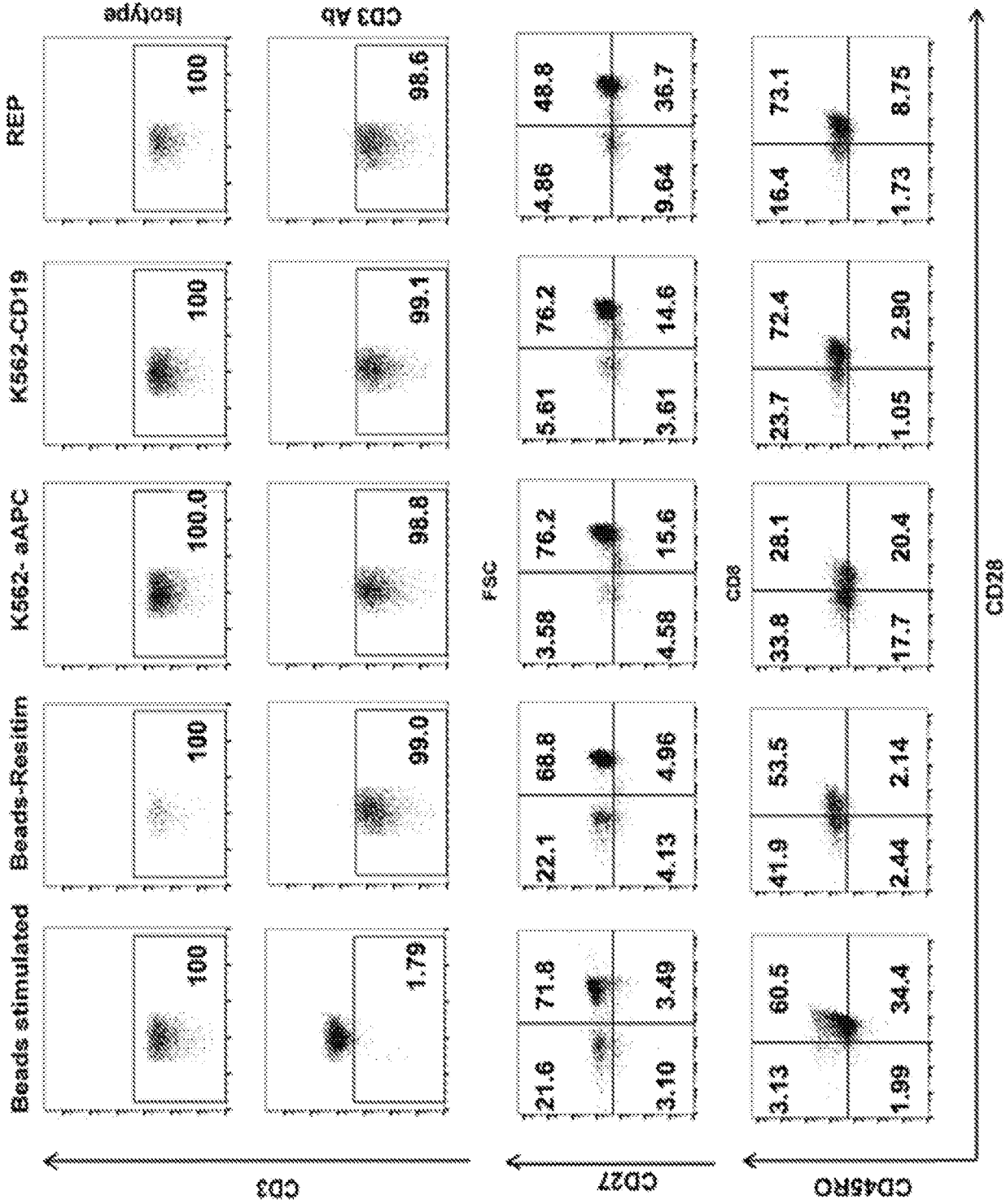


Figure 57

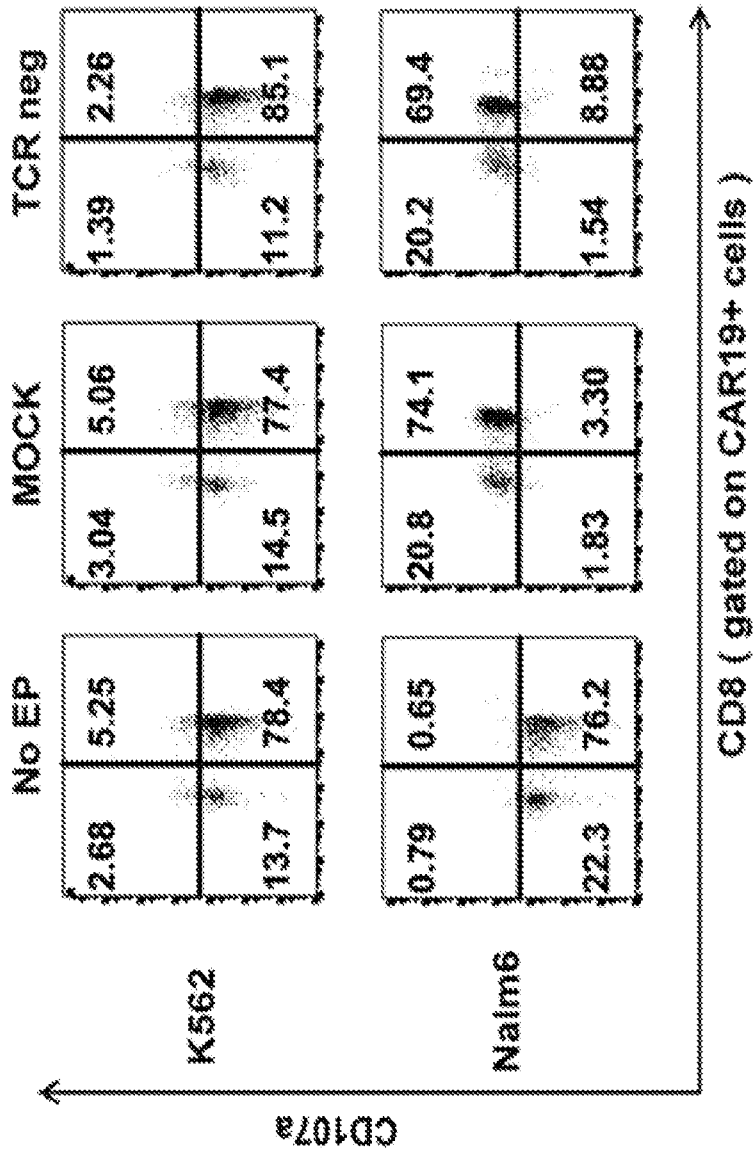
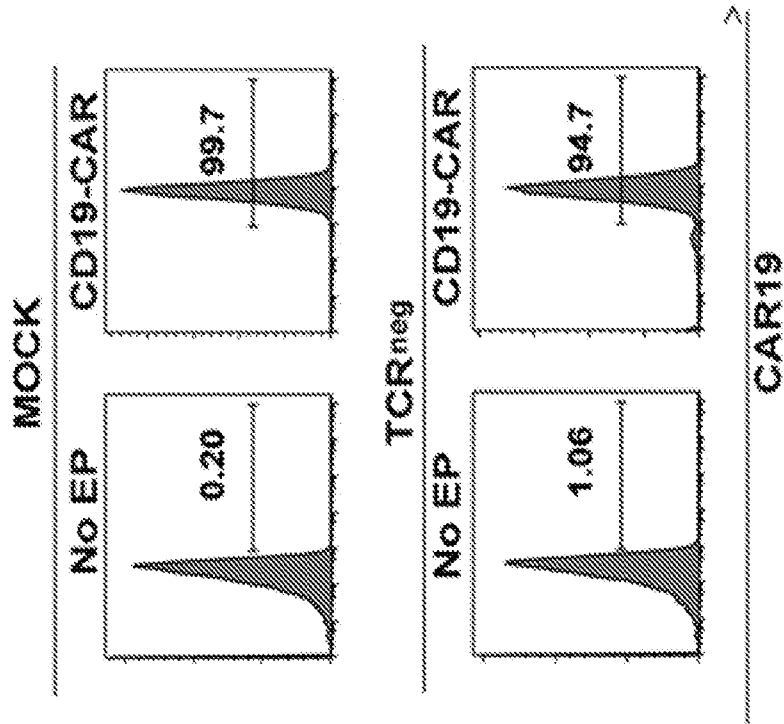


Figure 58

Figure 59A

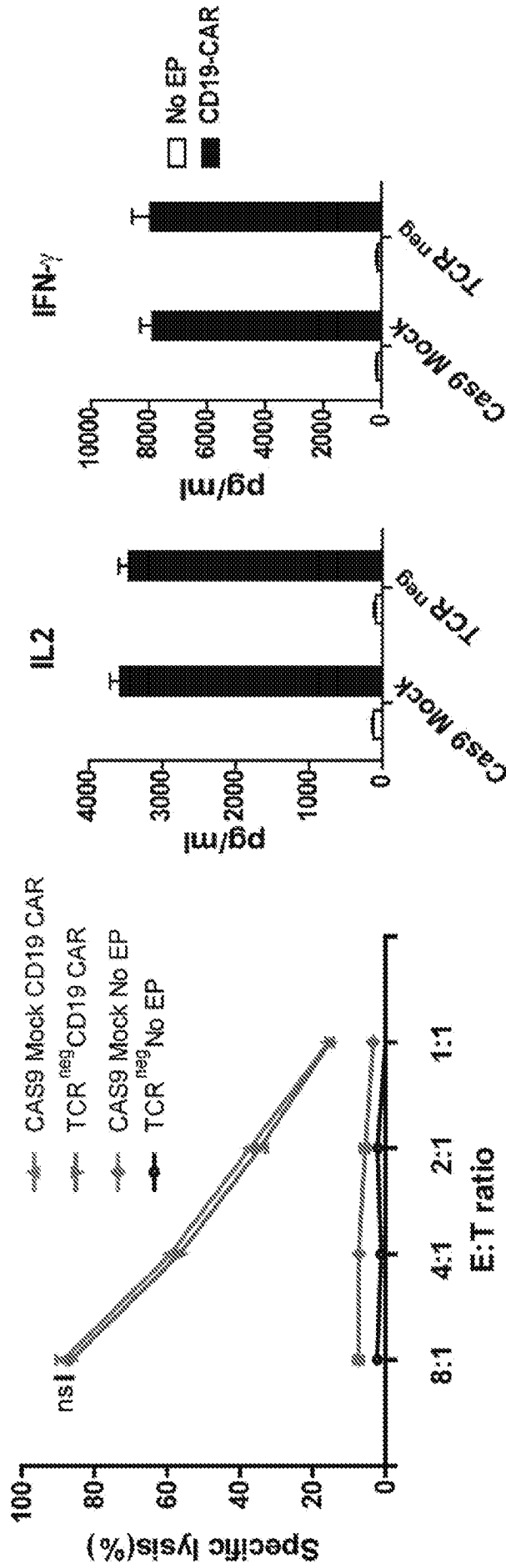


Figure 59B

Figure 59C

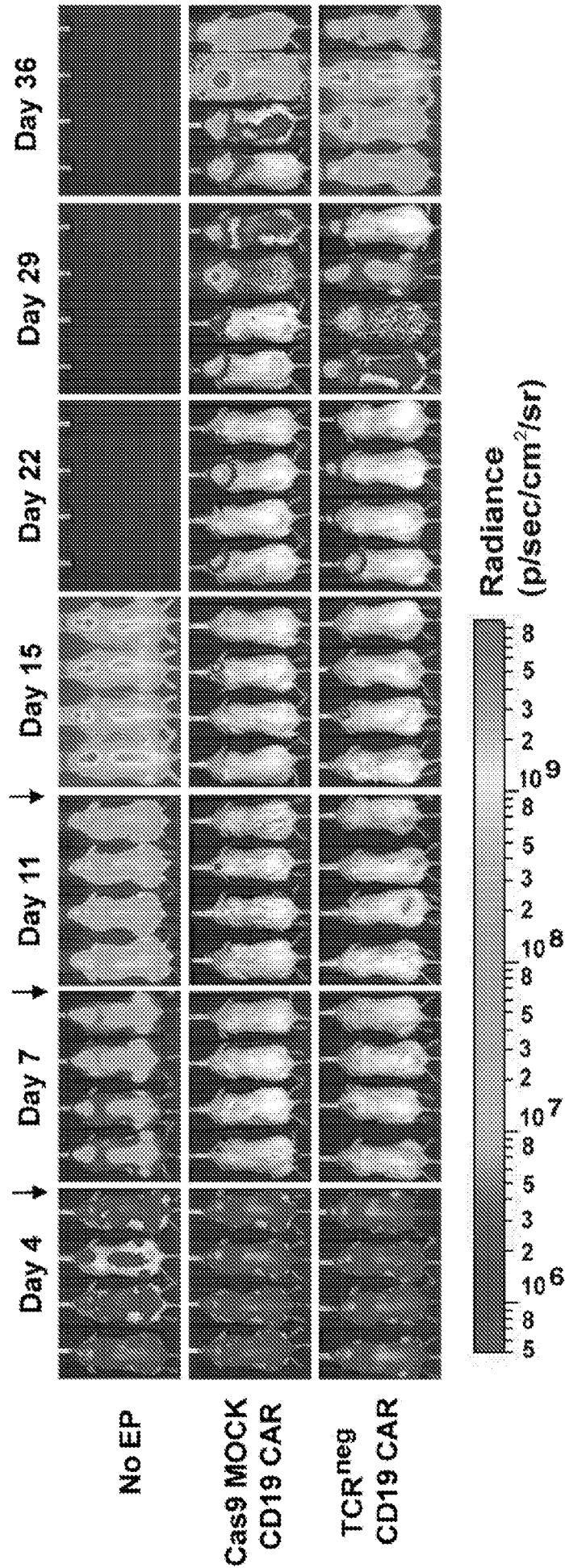


Figure 59D

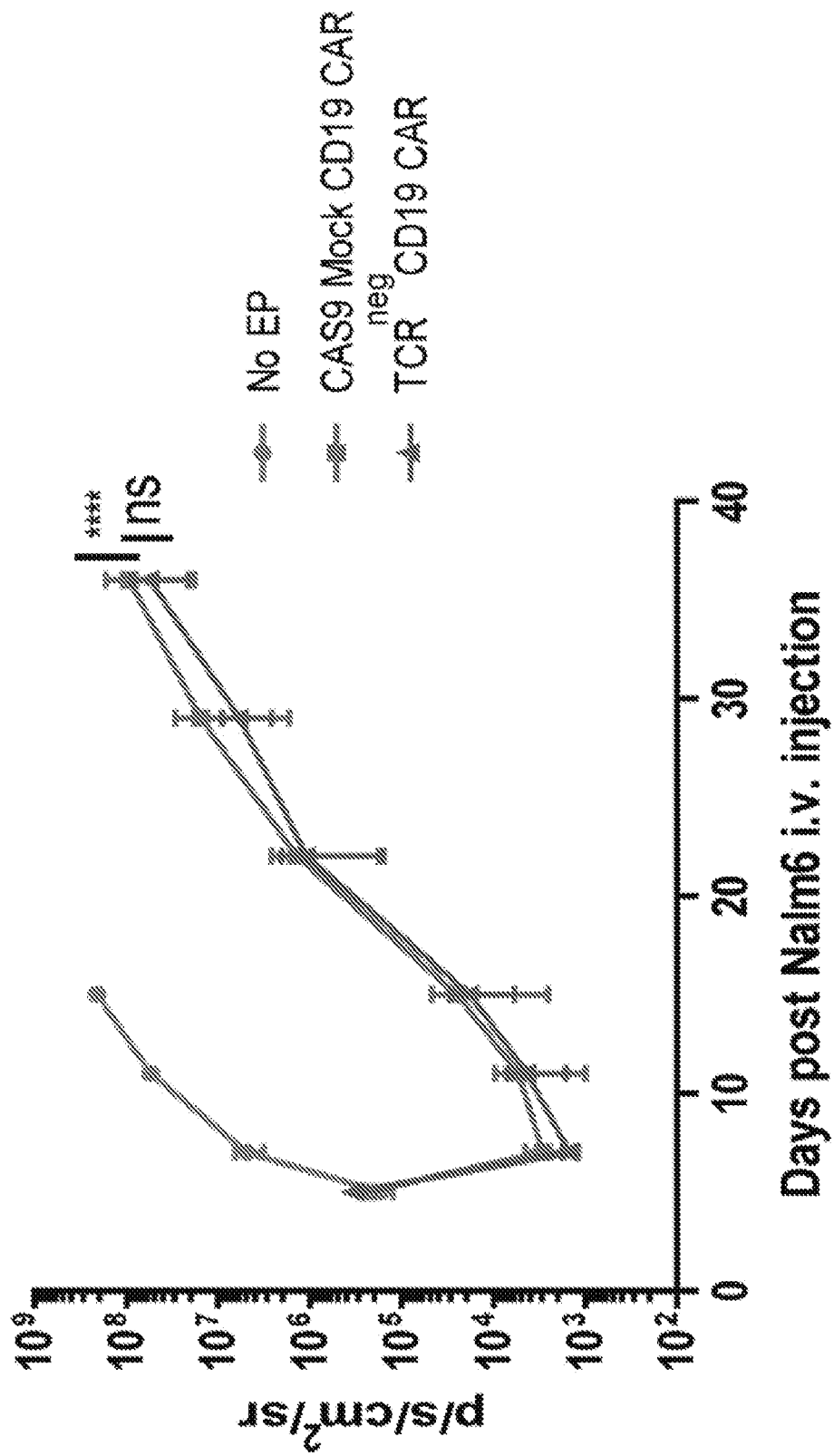


Figure 59E

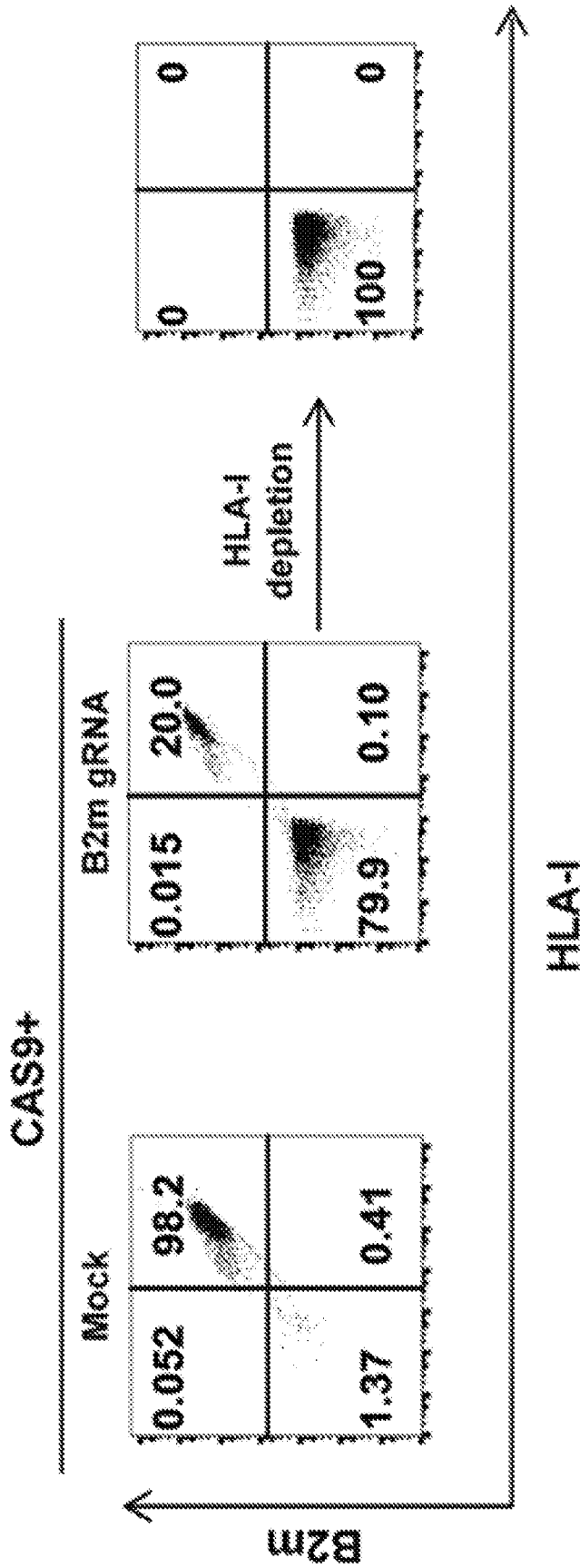


Figure 60

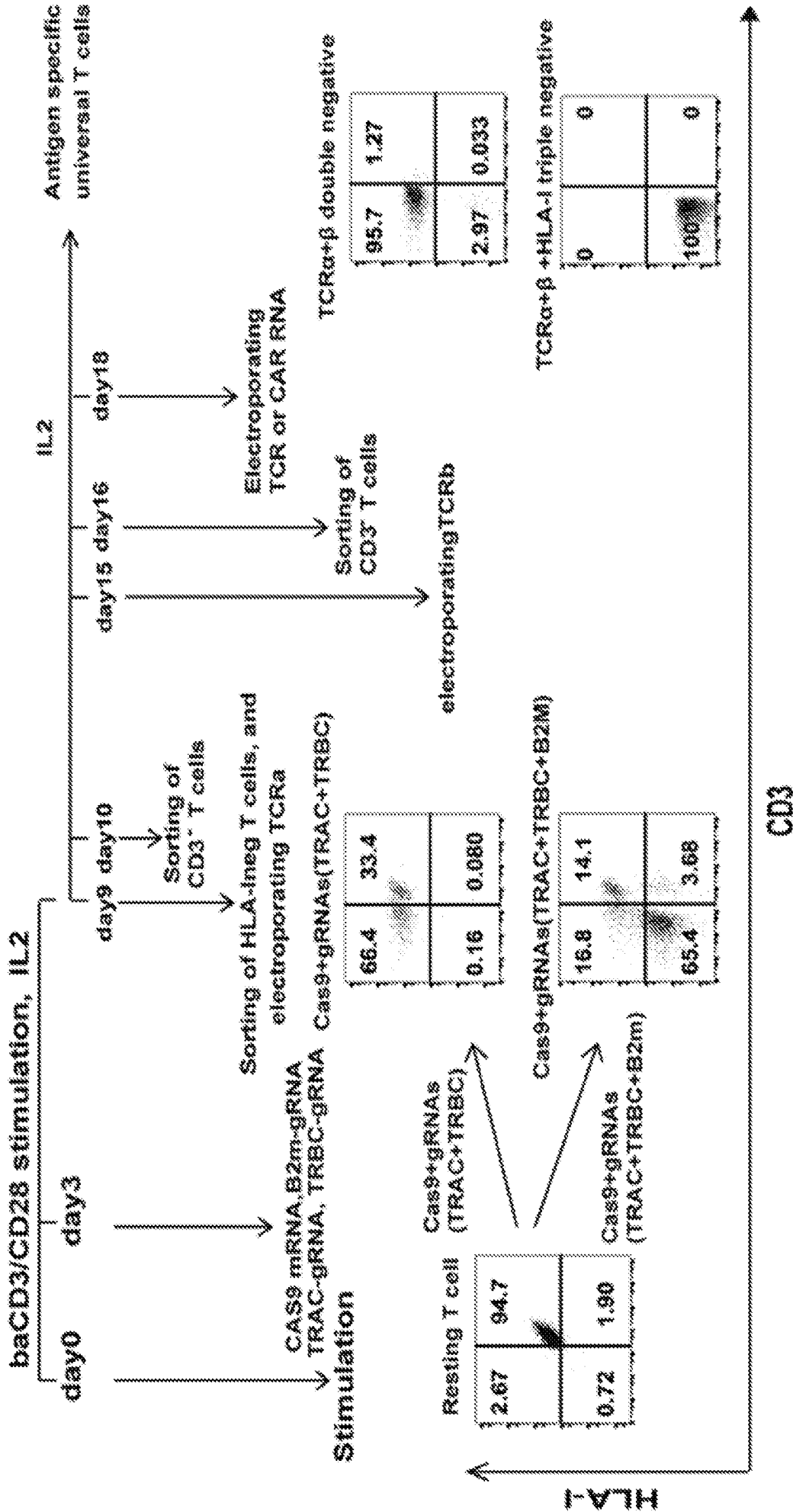


Figure 61

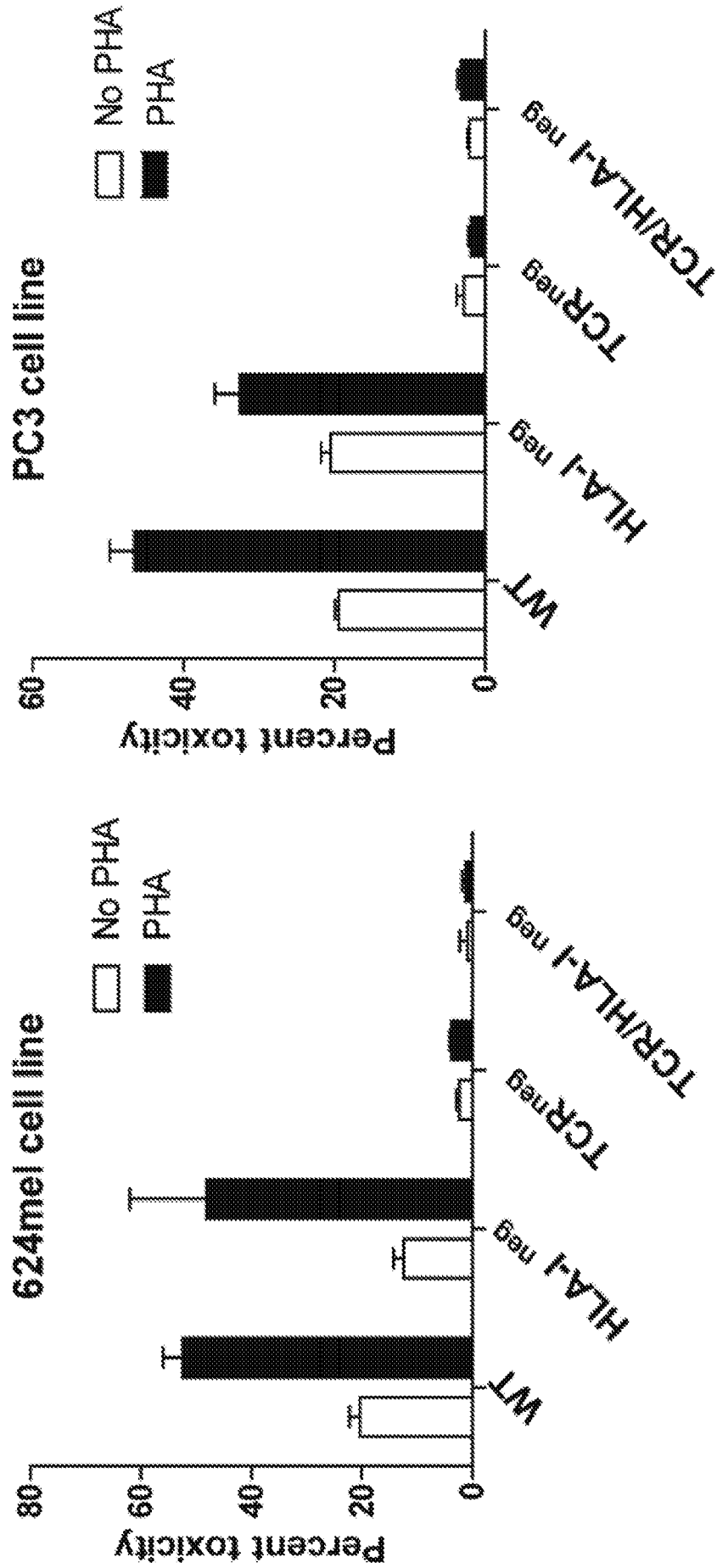


Figure 62

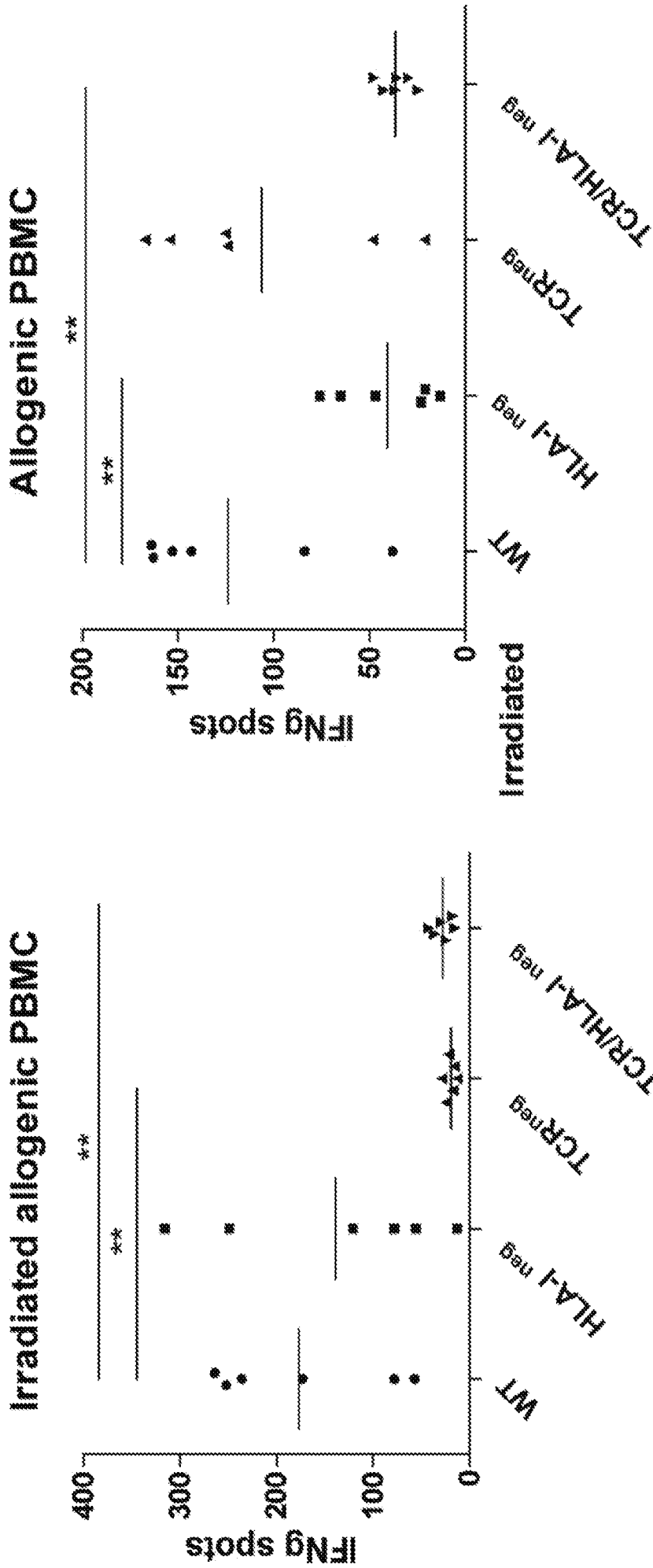


Figure 63

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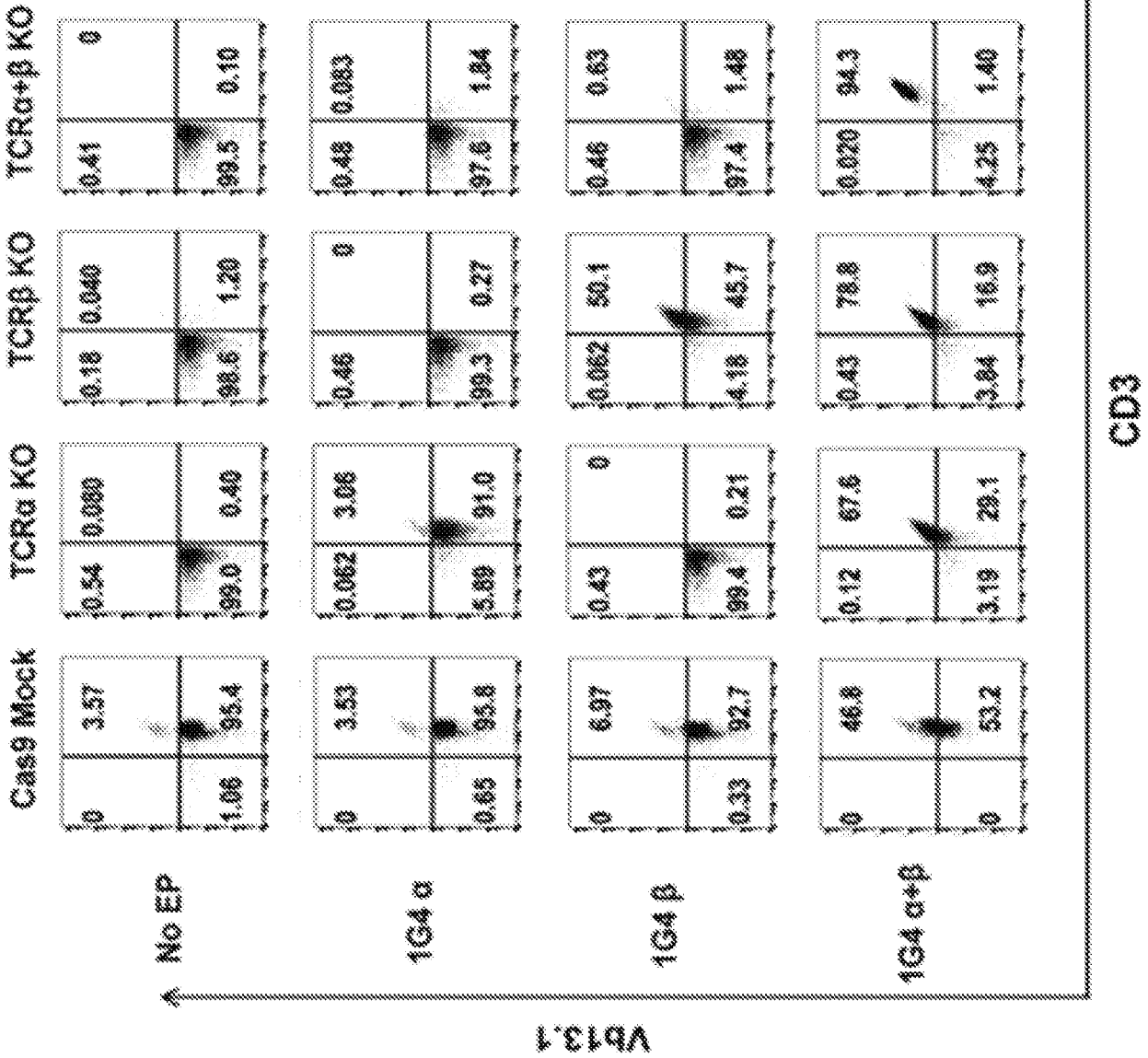
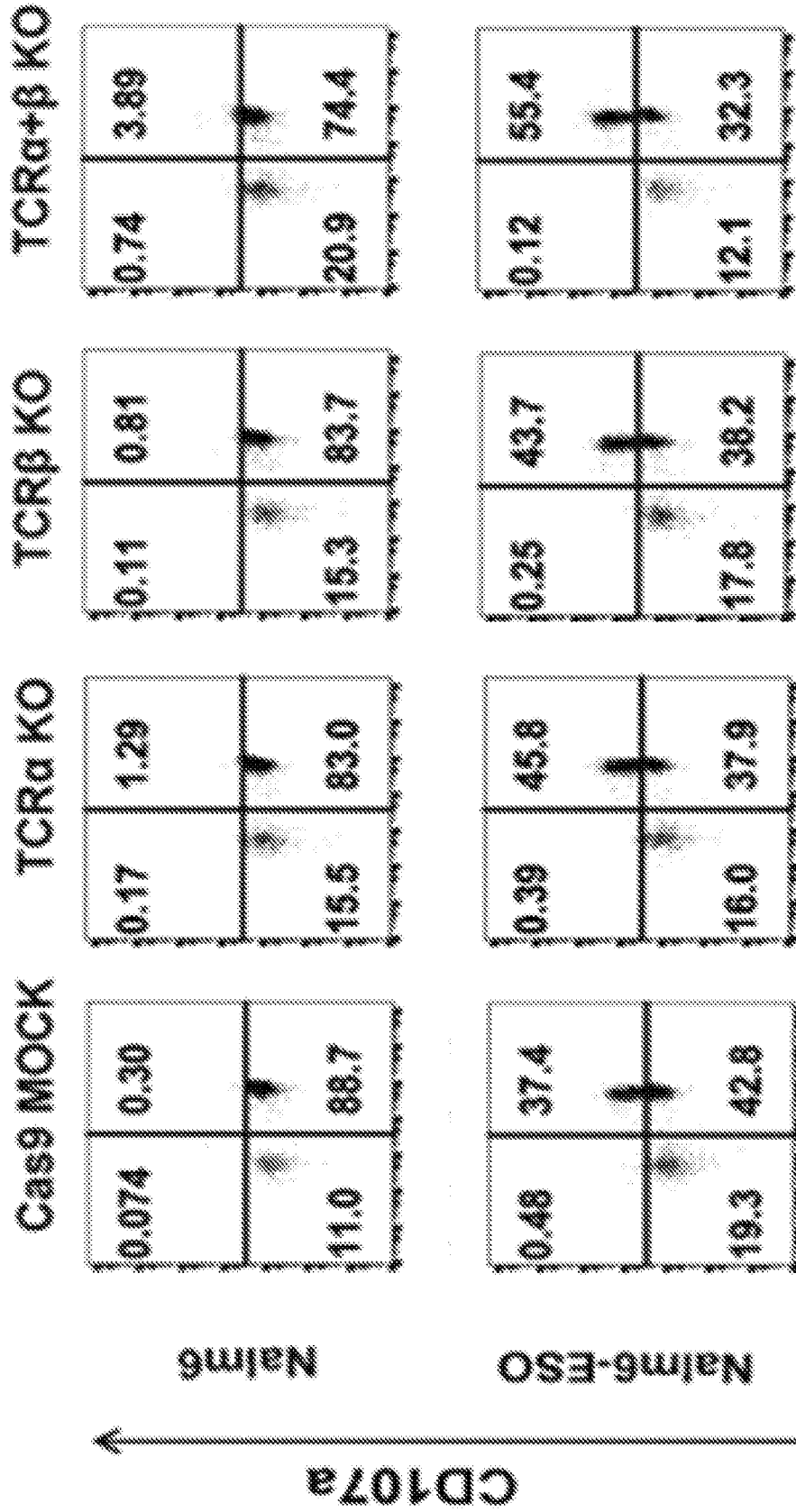


Figure 64

1G4TCR re-direction



CD8
Figure 65A

2024203330 20 May 2024

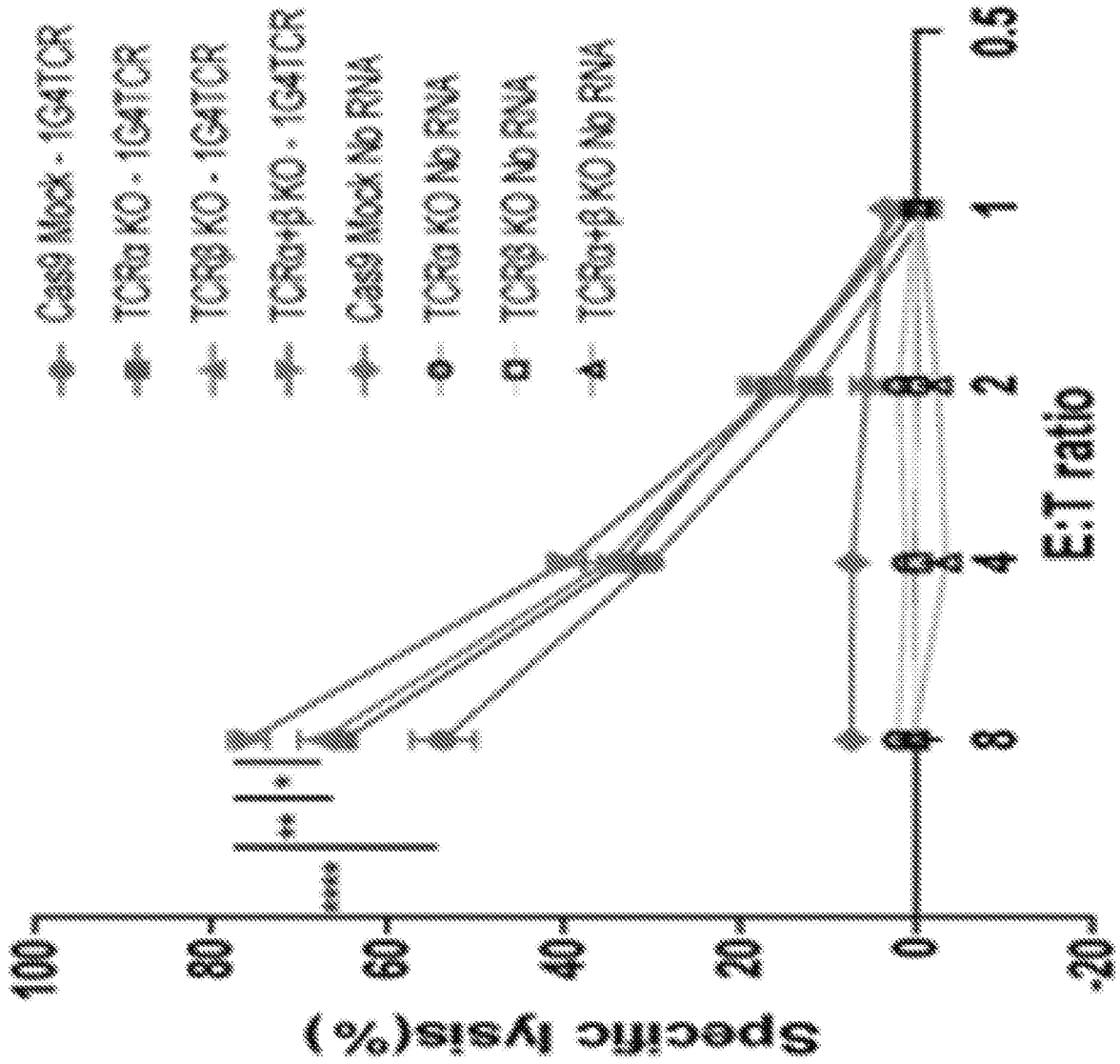


Figure 65B

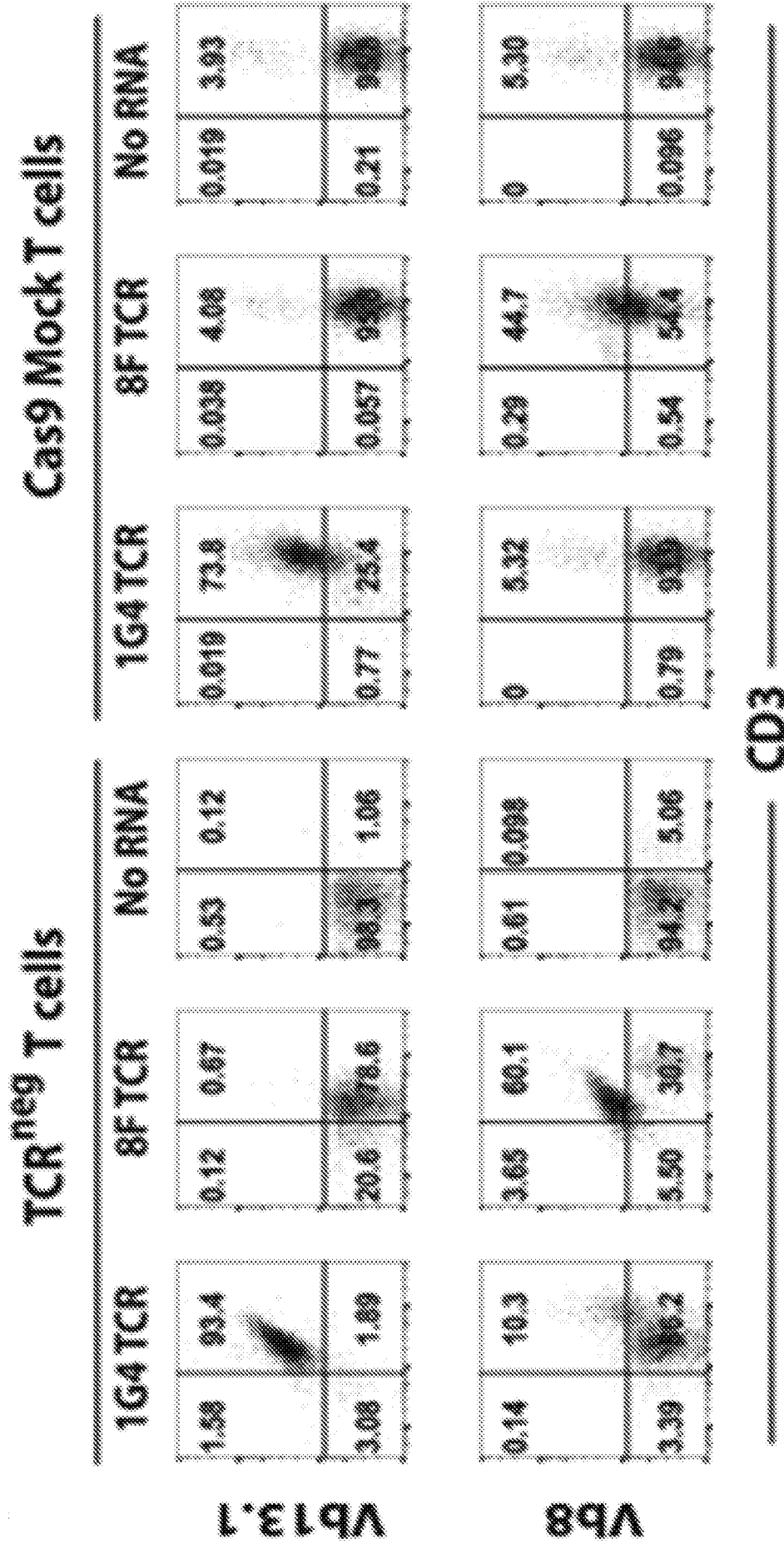


Figure 66

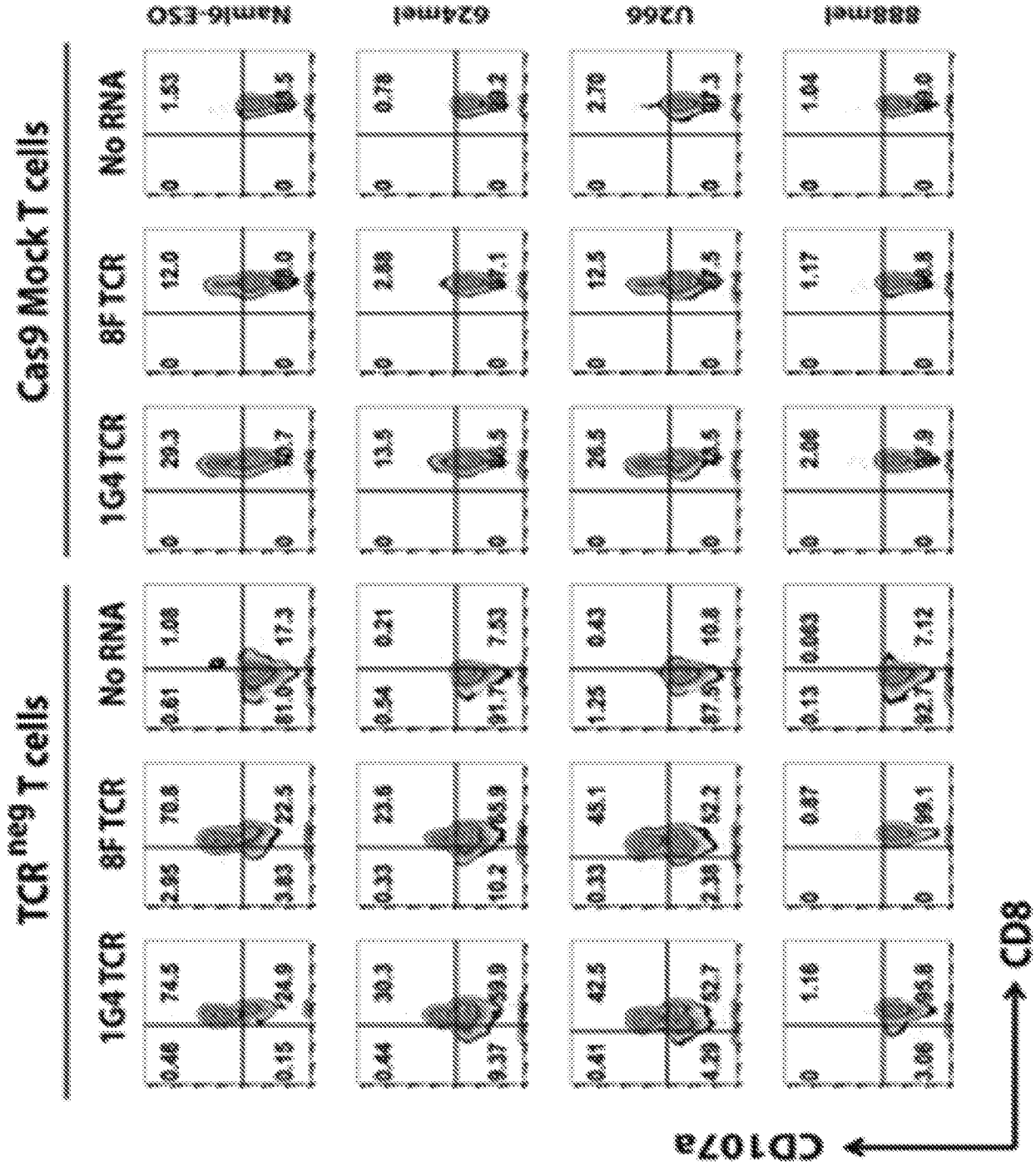


Figure 67A

2024203330 20 May 2024

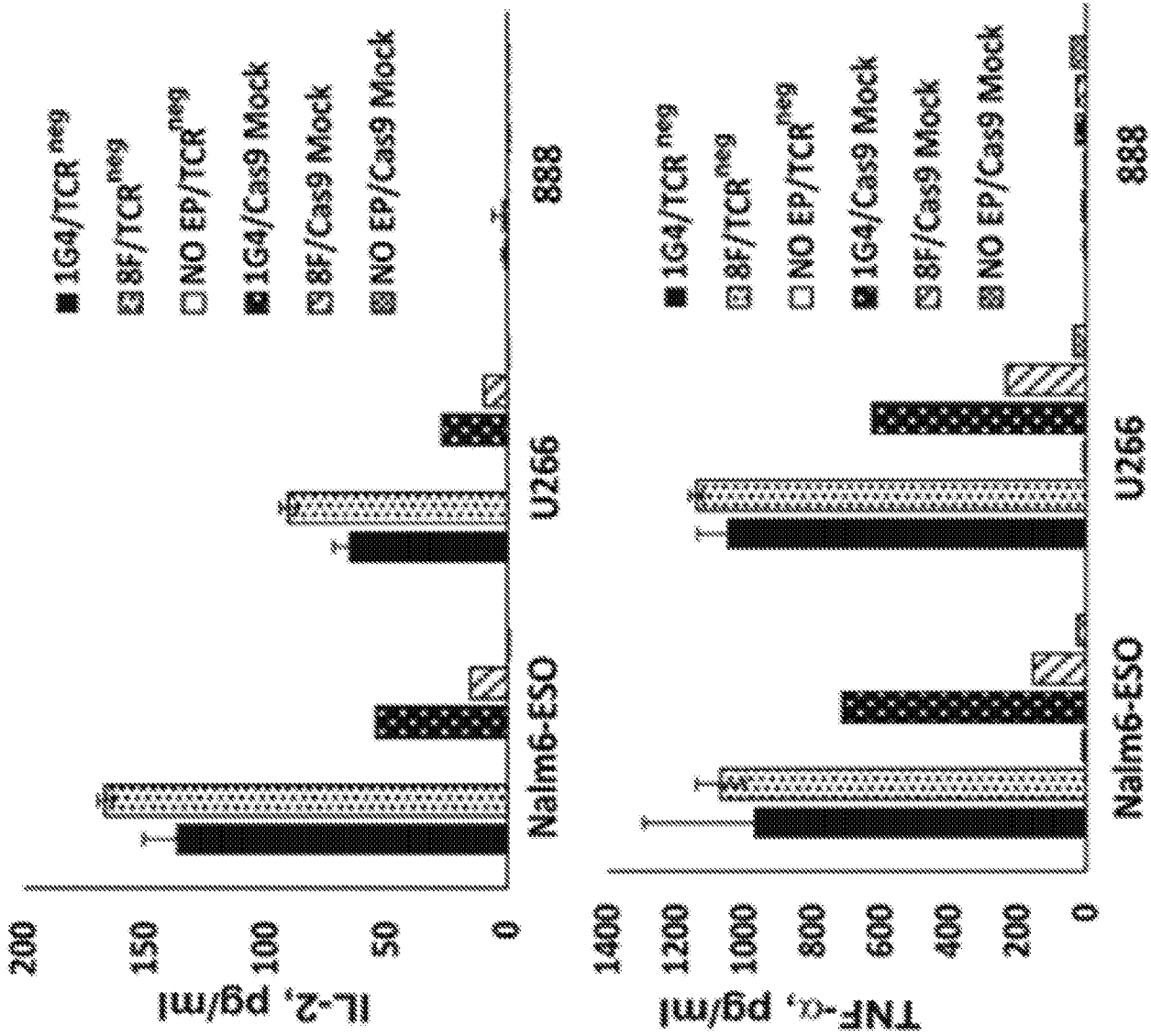


Figure 67B

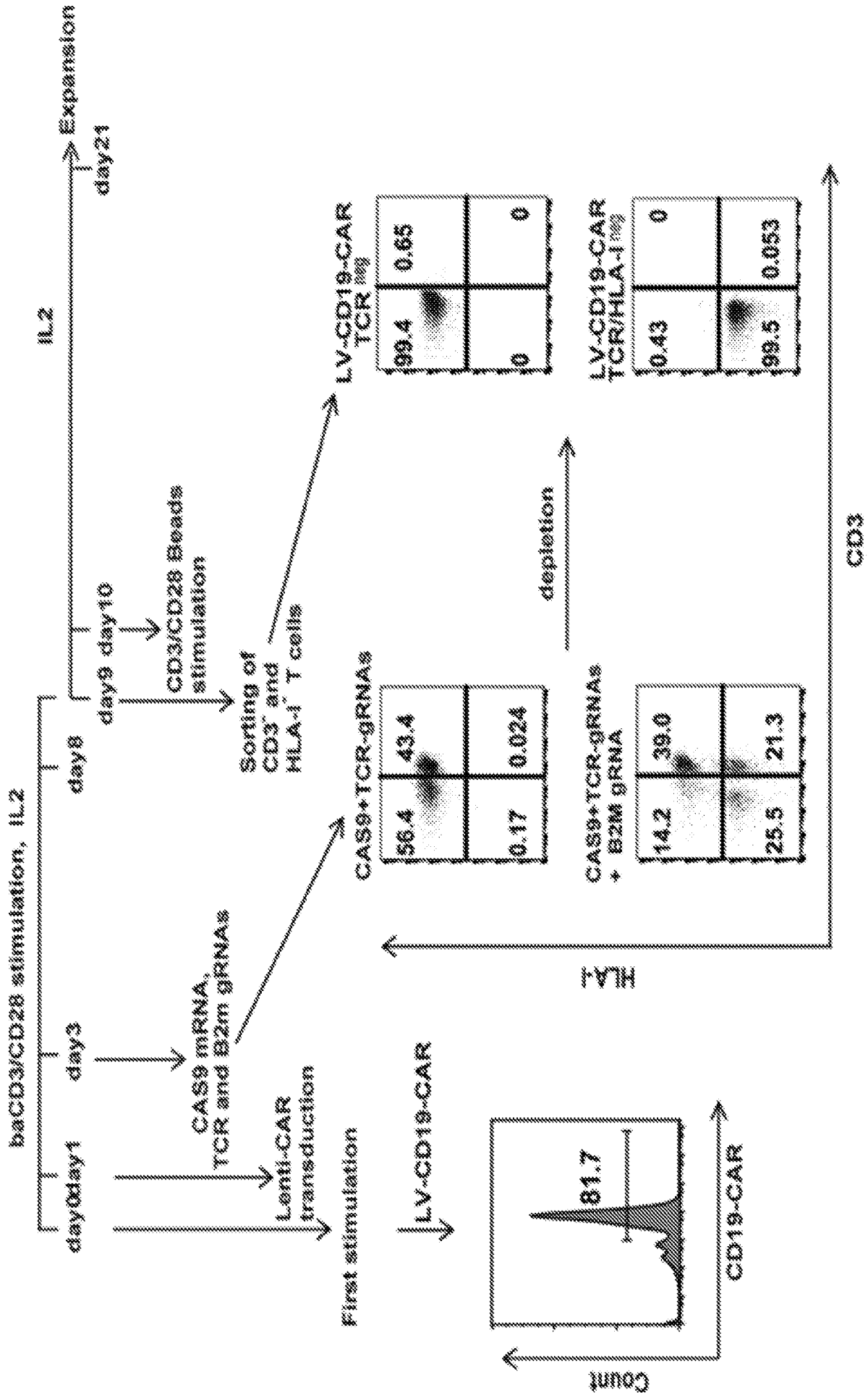


Figure 68

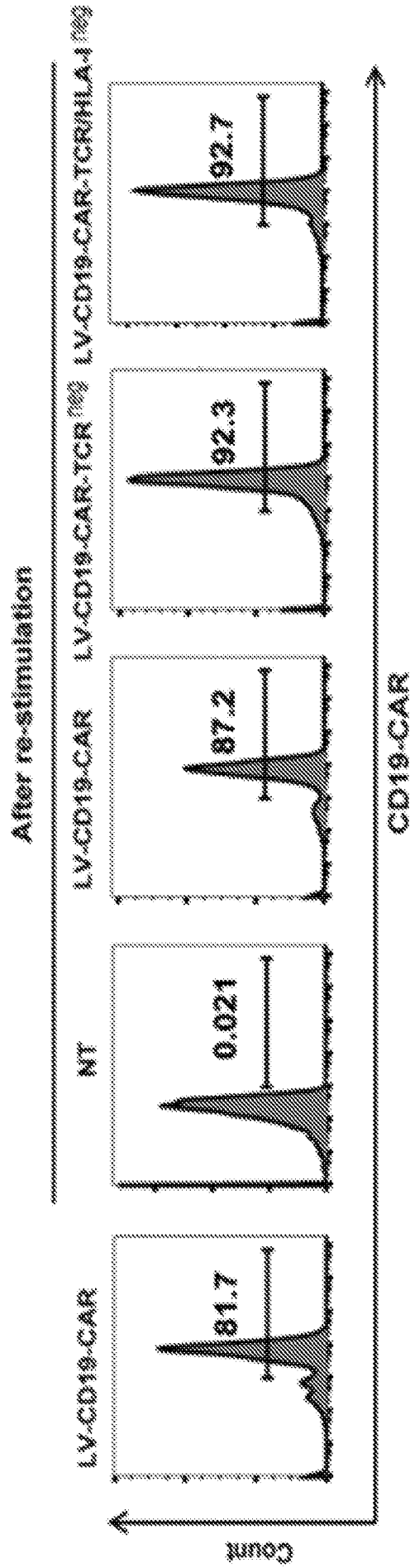
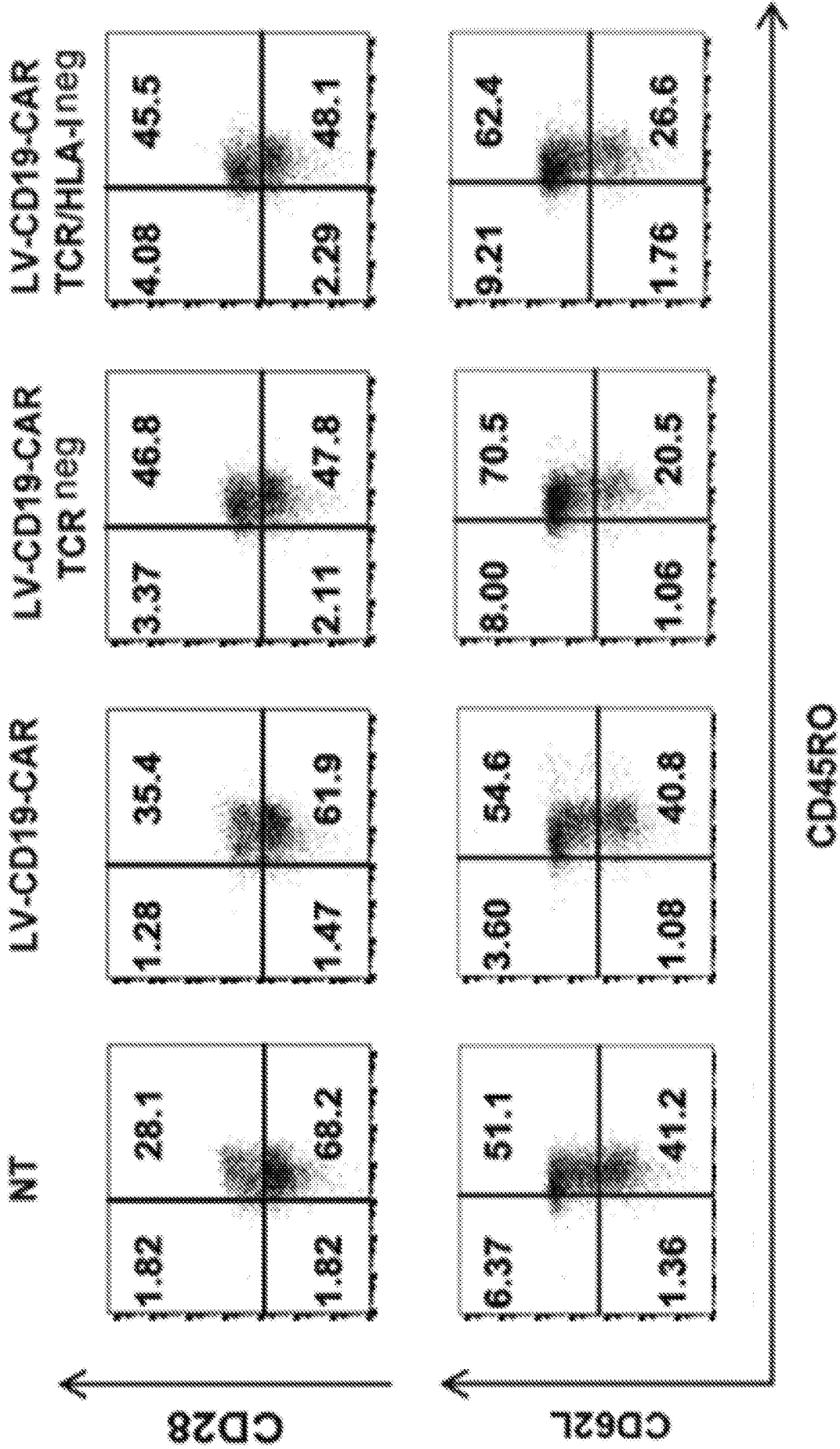


Figure 69



CD45RO
Figure 70

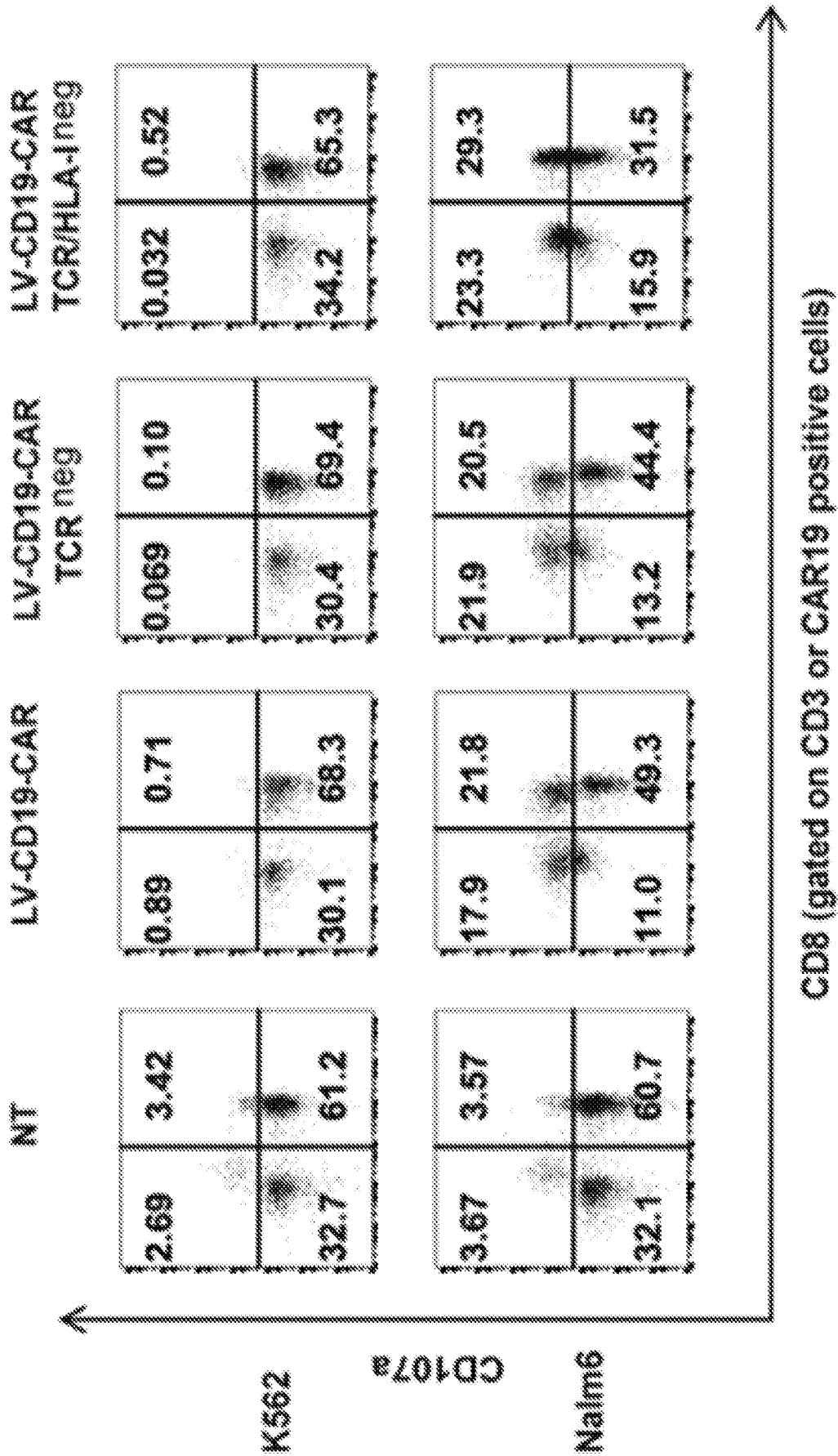


Figure 71

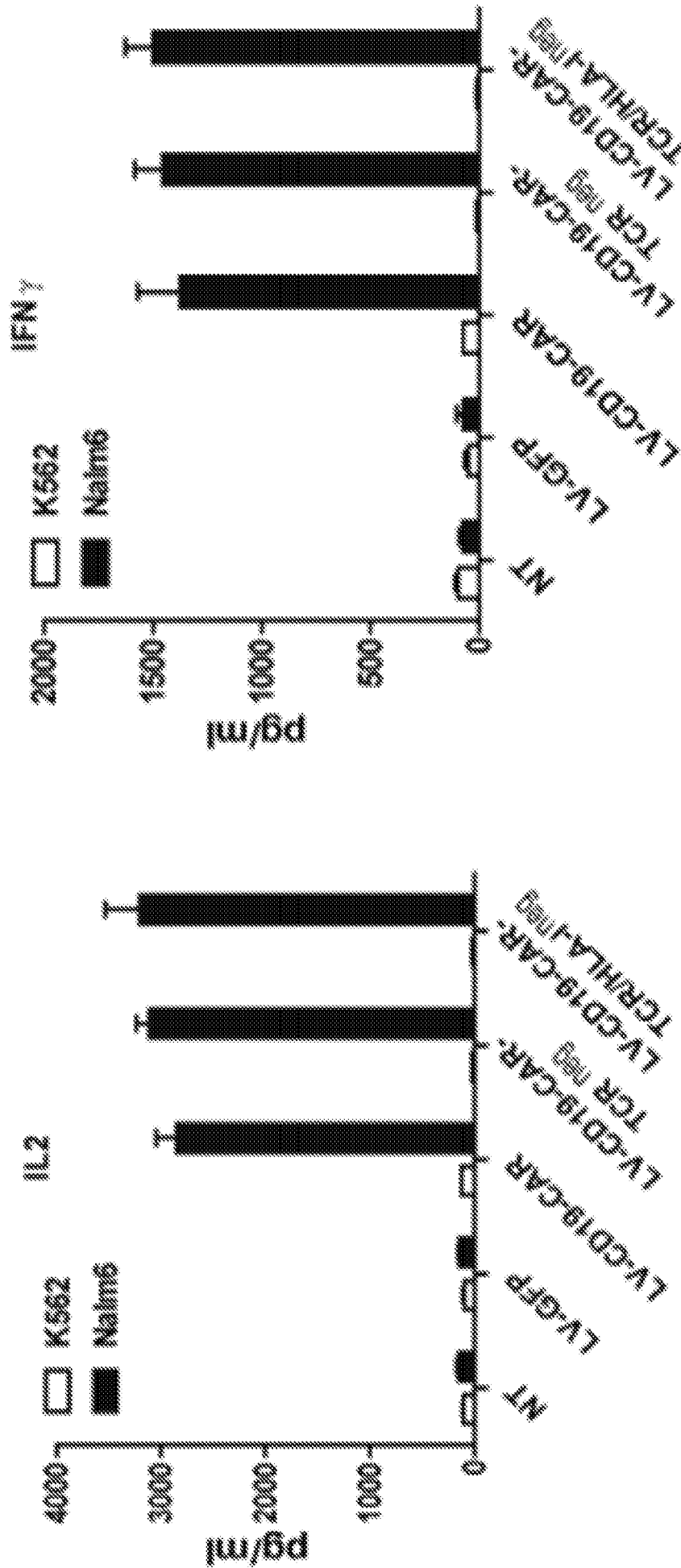


Figure 72

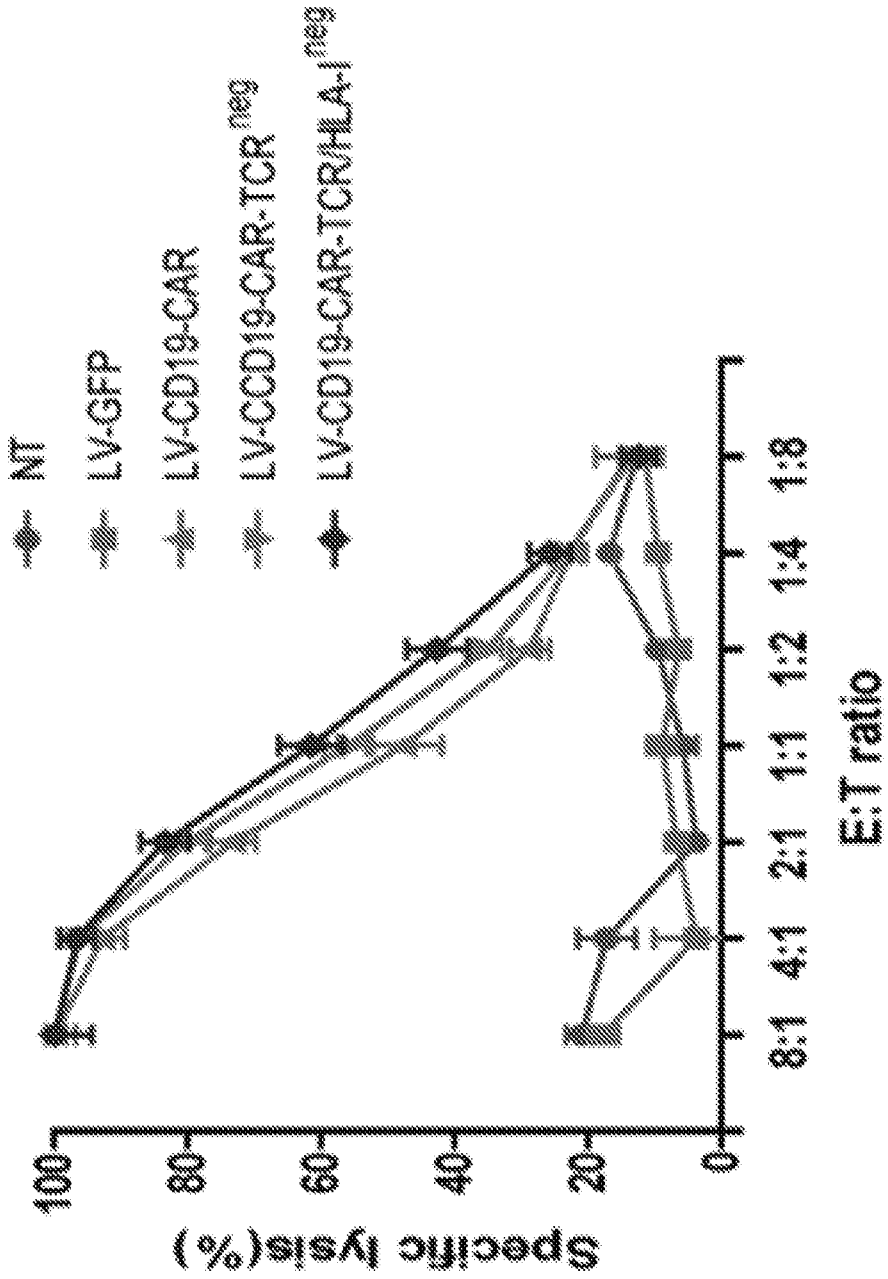


Figure 73

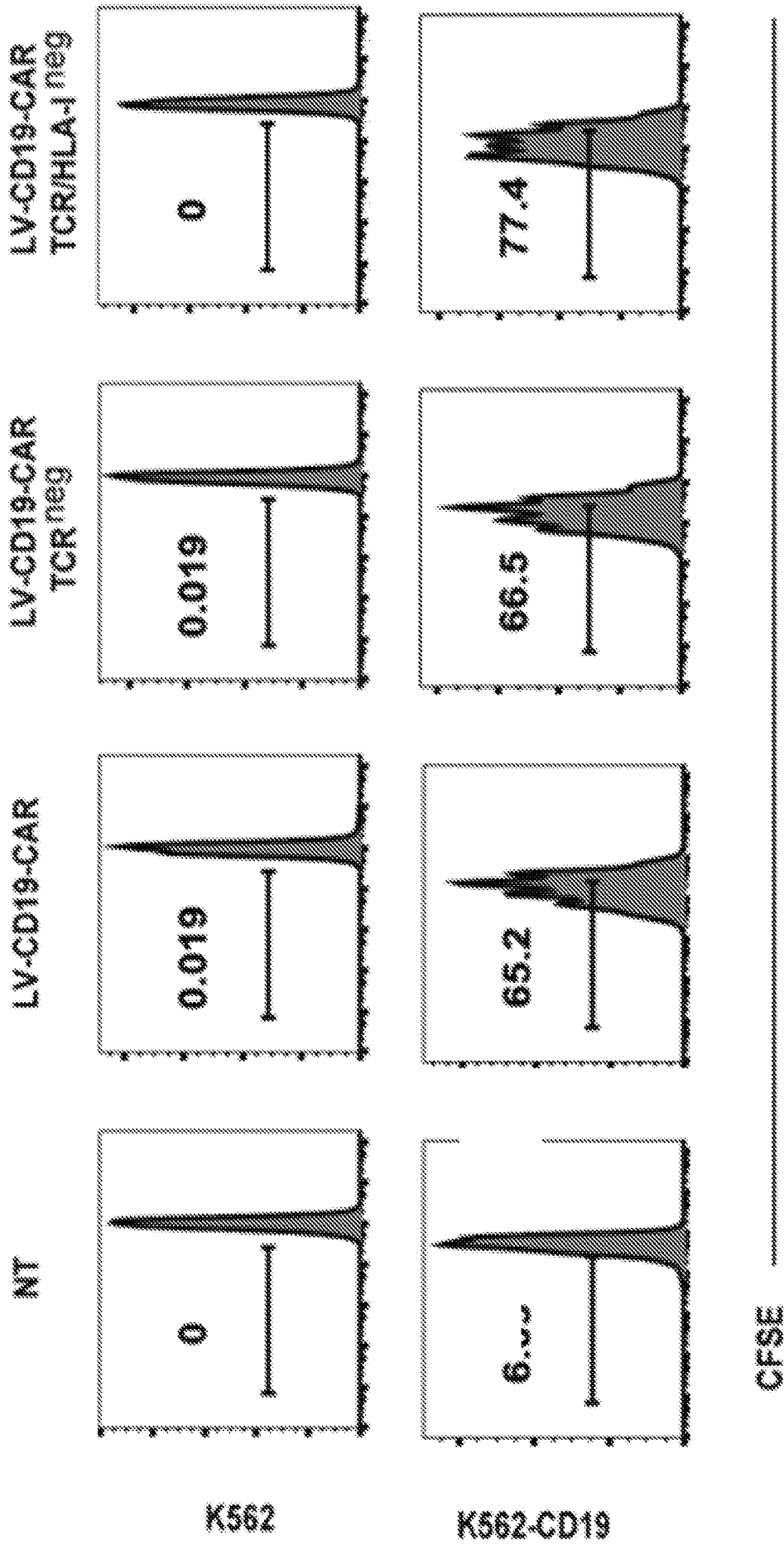


Figure 74

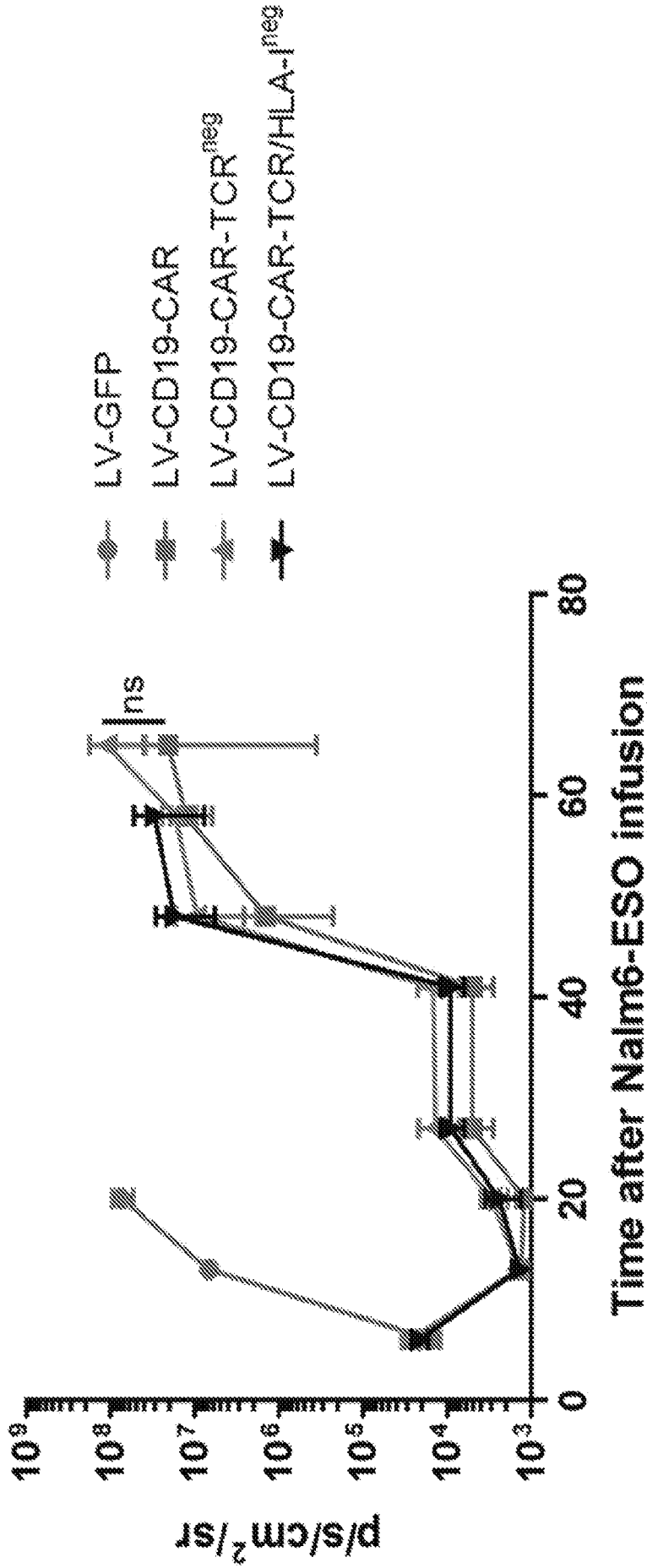


Figure 75A

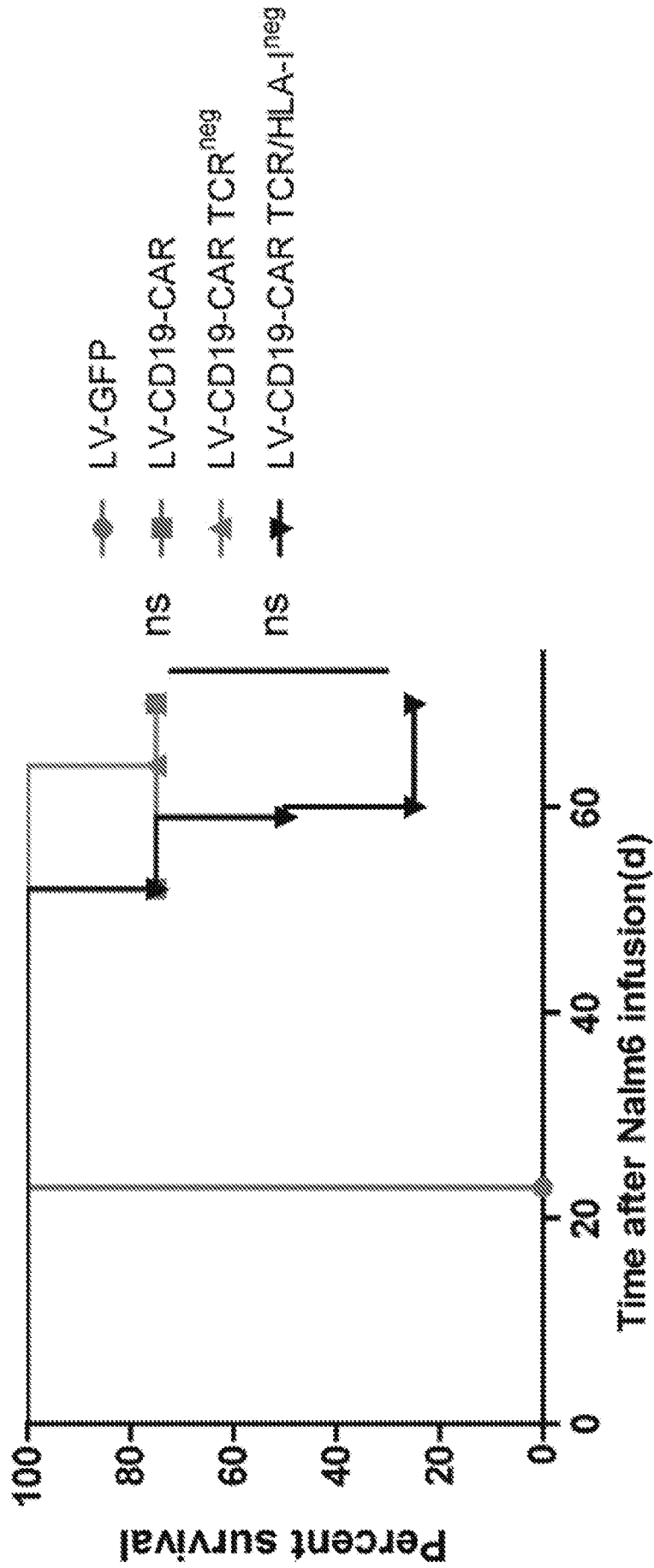


Figure 75B

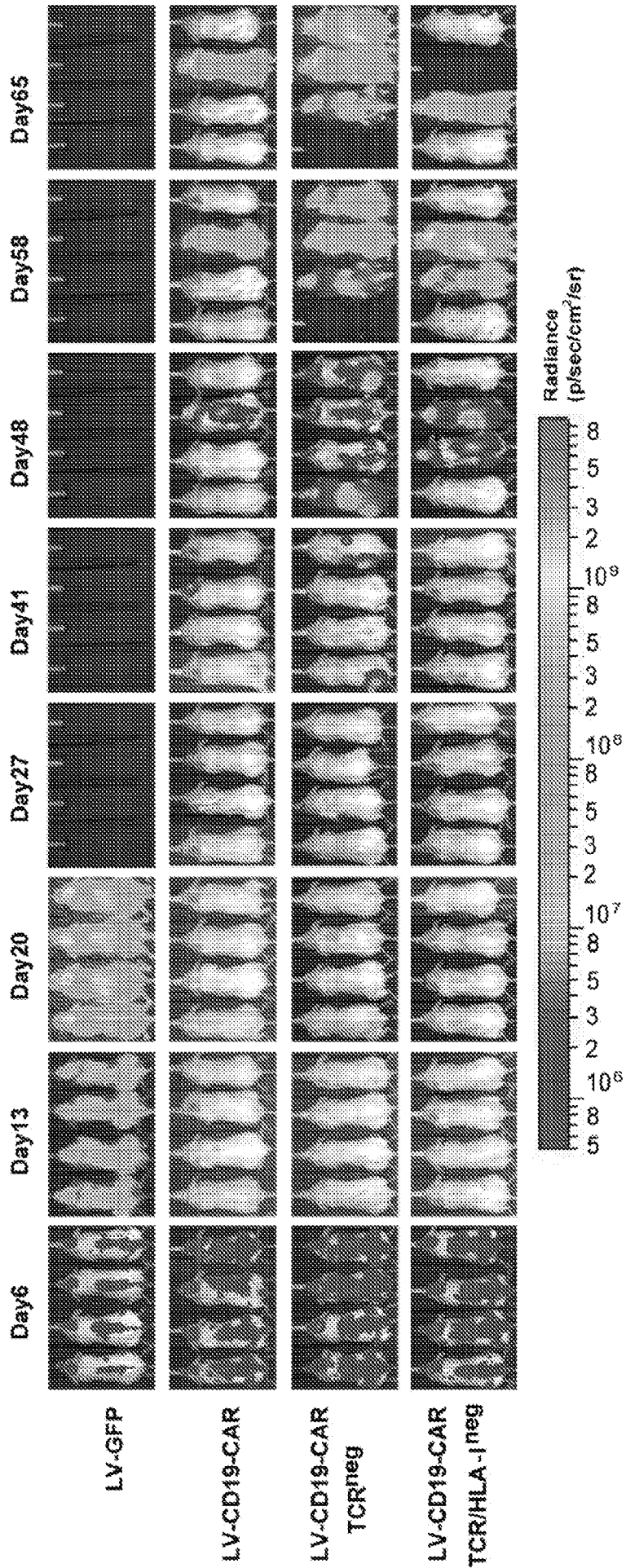


Figure 76

2024203330 20 May 2024

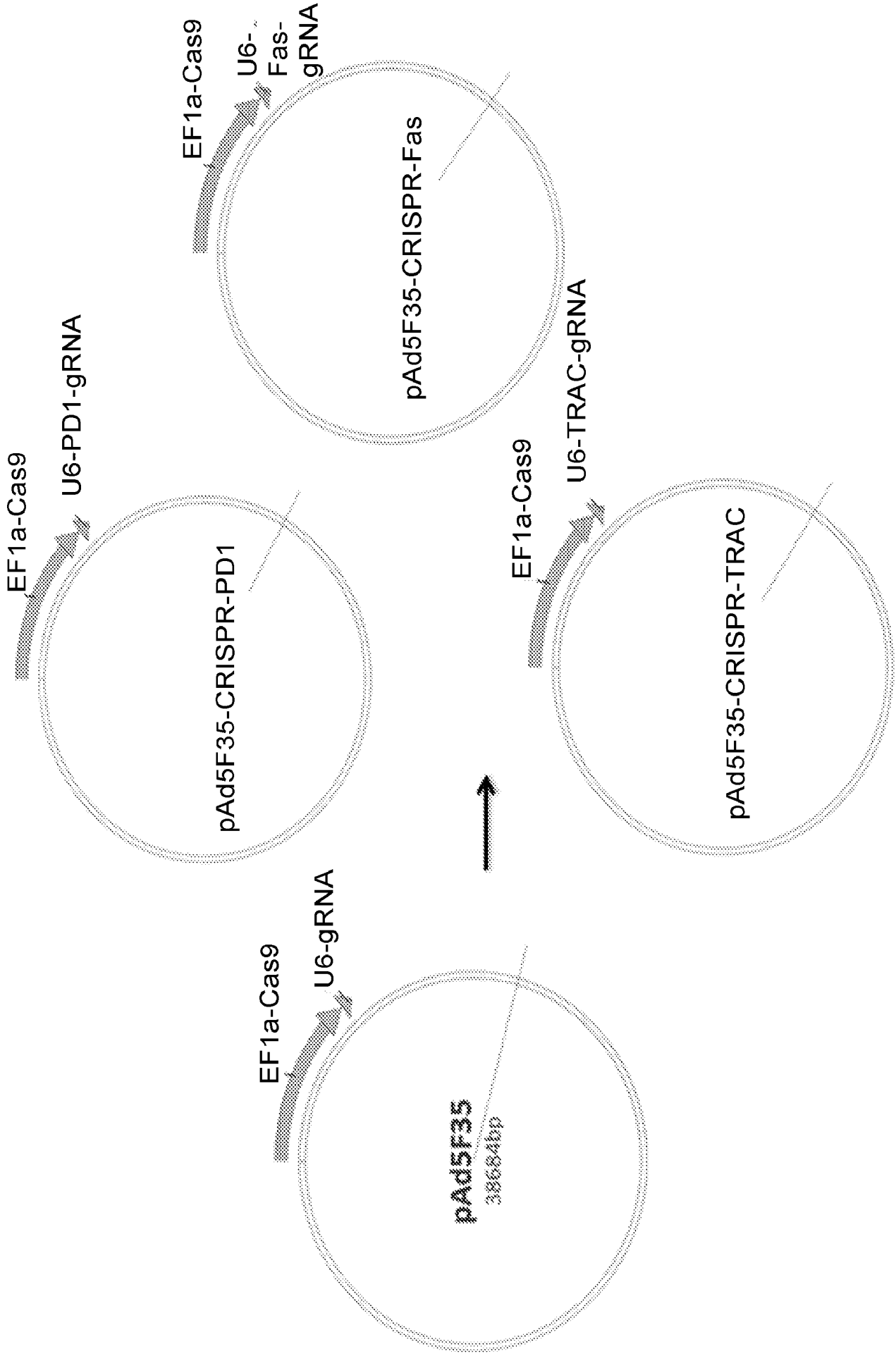


Figure 77

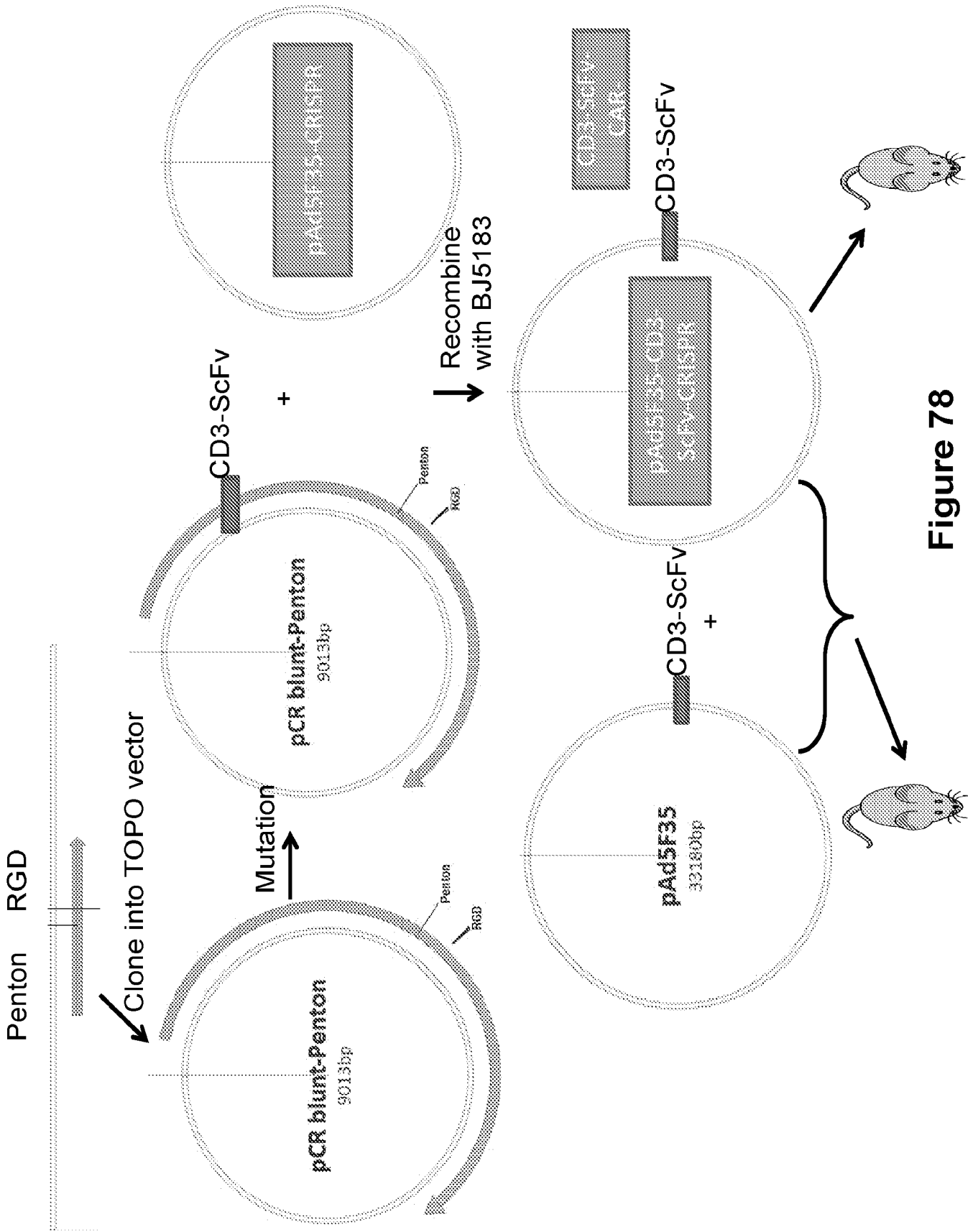


Figure 78

PD1-gRNA: GGCCAGGATGGTTCCTTAGGT

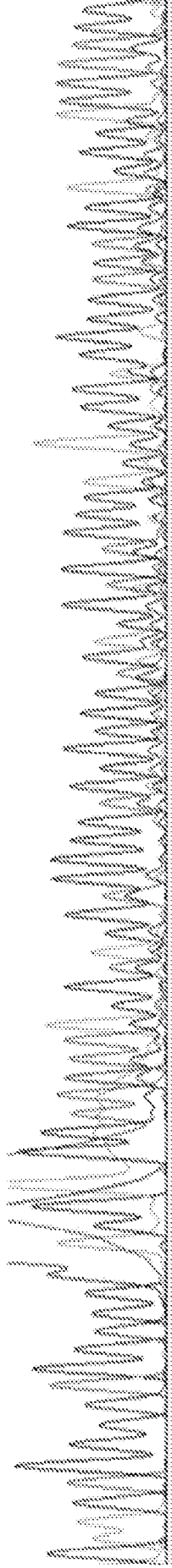


Figure 79A

WT: TGGGCTGGCGCC CAGGATGGTTCCTTAGGTAGGTGGGGT
 1: TGGGCTGGCGCC CAGGATGGTTCCTTAGGTAGGTGGGGT
 6: TGGGCTGGCGCC CAGGATGG ---TAGGTAGGTGGGGT
 7: TGGGCTGGCGCC CAGGATGGTT-TTAGGTAGGTGGGGT
 8: TGGGCTGGCGCC CAGGATGG ---TAGGTAGGTGGGGT
 13: TGGGCTGGCGCC CAGGATGGTTCCTTAGGTAGGTGG
 16: TGGGCTGGCGCC CAGGATGGTTCCTTAGGTAGGTGGGGT
 19: TGGGCTGGCGCC CAGGATGGTTCCTTAGGGCTAGGTGGG

Figure 79B

EP #	EP1 condition		EP2 condition		EP1 RNA (ug)		EP2 RNA (ug)		Cuvette	volume (ul)	CD3- %	B2M - %	Fold Exp.
	EP1 condition	EP2 condition	CAS9	gRNA	CAS9	gRNA	gRNA	gRNA					
1	360v	1ms	360v	1ms	40	20	10	10	2mm	200	81.8	88.6	2.7
2	360v	1ms	360v	1ms	40	10	5	5	2mm	200	71.6	77.5	4.7
3	360v	1ms	360v	1ms	40	7	5	5	2mm	200	66.2	59.5	7.3
4	360v	1ms	360v	1ms	40	0	10	10	2mm	200	76.9	75.4	8.5
5	360v	1ms	360v	1ms	20	10	5	5	2mm	200	68.2	56.2	5.5
6	360v	1ms	360v	1ms	40	20	10	10	4mm	200	35.6	17.8	16.8
7	360v	1ms	360v	1ms	20	10	5	5	4mm	200	15.8	3.8	19.1
8	360v	1ms	360v	1ms	80	40	20	20	4mm	400	38	33.8	9.7
9	500v	500us	500v	500us	40	20	10	10	4mm	200	37.4	17.8	17.2
10	500v	1ms	500v	500us	20	10	5	5	4mm	200	22.8	9.3	18.5
11	500v	700us	500v	500us	20	10	5	5	4mm	200	18.3	5	16.7
12	No EP	Only wash								200	1.8	1.5	18.8

Figure 80

EP #	EPI condition	EP2 condition	CAS9 gRNA	EP2 RNA (ug)	ERNA	Cuvette	EP volume (ul)	CD3-	B2M-	D7 Expansion
1	500v 1ms	500v 1ms	80	20	4mm	400	78.8	80.8	3.4	
2	500v 1ms	500v 1ms	80	20	4mm	400	58.4	69.9	5.6	
3	500v 1ms	500v 1ms	80	10	4mm	400	37.6	47.6	7.0	
4	500v 2ms	500v 2ms	80	20	4mm	400	74.2	83.7	2.9	
5	500v 2ms	500v 2ms	80	10	4mm	400	80.5	83.4	3.0	
6	400v 2ms	500v 1ms	80	10	4mm	400	40.5	51.0	6.7	
7	400v 2ms	500v 1ms	80	10	4mm	400	55.7	64.1	5.7	
8	400v 2ms	500v /000s	80	20	4mm	400	50.0	64.0	6.5	
9	400v 3ms	500v 1ms	80	20	4mm	400	61.8	74.1	6.1	
10	360v 1ms	360v 1ms	40	10	2mm	200	82.8	85.5	1.1	
11	360v 1ms	360v 1ms	40	10	2mm	200	84.8	82.4	0.8	
12	360v 1ms	360v 1ms	40	10	2mm	200	85.9	85.3	0.8	
13	360v 1ms	360v 1ms	40	10	2mm	200	67.8	79.9	1.0	
14	500v 1ms	500v 800us	80	20	4mm	400	51.3	62.8	6.9	
15	No EP					200	4.1	6.3	13.0	

Figure 81

	EP1 condition	EP2 condition	CAS9	gRNA	CD3-	B2M-	D7 Expansion
1	400v	3ms	80	20	62.2	84.4	3.2
2	400v	3ms	120	20	59.6	76.5	3.7
3	400v	3ms	120	30	72.6	85.1	3.4
4	400v	2ms	120	20	63.5	84.8	5.2
5	400v	2ms	120	30	67.3	86.7	3.6
6	400v	2ms	120	20	54.8	79.3	3.9
7	400v	2ms	120	30	69.7	87.1	3.6
8	500v	2ms	80	20	63.6	85.2	3.3
9	500v	2ms	120	20	64.2	83.0	2.9
10	500v	2ms	120	30	76.5	84.1	2.1
11	500v	2ms	120	30	75.9	87.6	2.0
12	500v	2ms	120	30	81.4	89.8	2.0
13	450v	2ms	80	20	66.3	88.1	3.1
14	450v	2ms	120	20	71.0	88.2	2.7
15	450v	3ms	120	20	74.8	88.2	1.8
16	400v	3ms	80	20	60.6	72.0	2.2
17	NO EP				0.5	0.7	9.9

Figure 82

EP #	I condition	EP2 condition	CAS9	gRNA	CD3-	B2m-	Day8 Exp.
1	400v	2ms 500v	120	20	68.8	78.9	7.8
2	400v	2ms 500v	120	10	51.5	60.3	9.7
3	500v	1ms 500v	120	20	70.6	79	8.3
4	500v	1ms 500v	120	10	52.1	62.9	10.1
5	500v	1ms 500v	120	5	30.6	31.4	11.4
6	500v	1ms 500v	120	1	8.91	5.51	12.4
7	500v	1ms 400v	120	20	55.9	67	10.3
8	400v	2ms 500v	120	20	61.6	73.4	8.6
9	400v	2ms 500v	120	10	42.9	49.9	10.4
10	400v	2ms 500v	80	10	41.8	49.3	10.0
11	400v	2ms 500v	120	20	59.1	68.1	6.8
12	NO EP				3.73	2.79	17.0

Figure 83

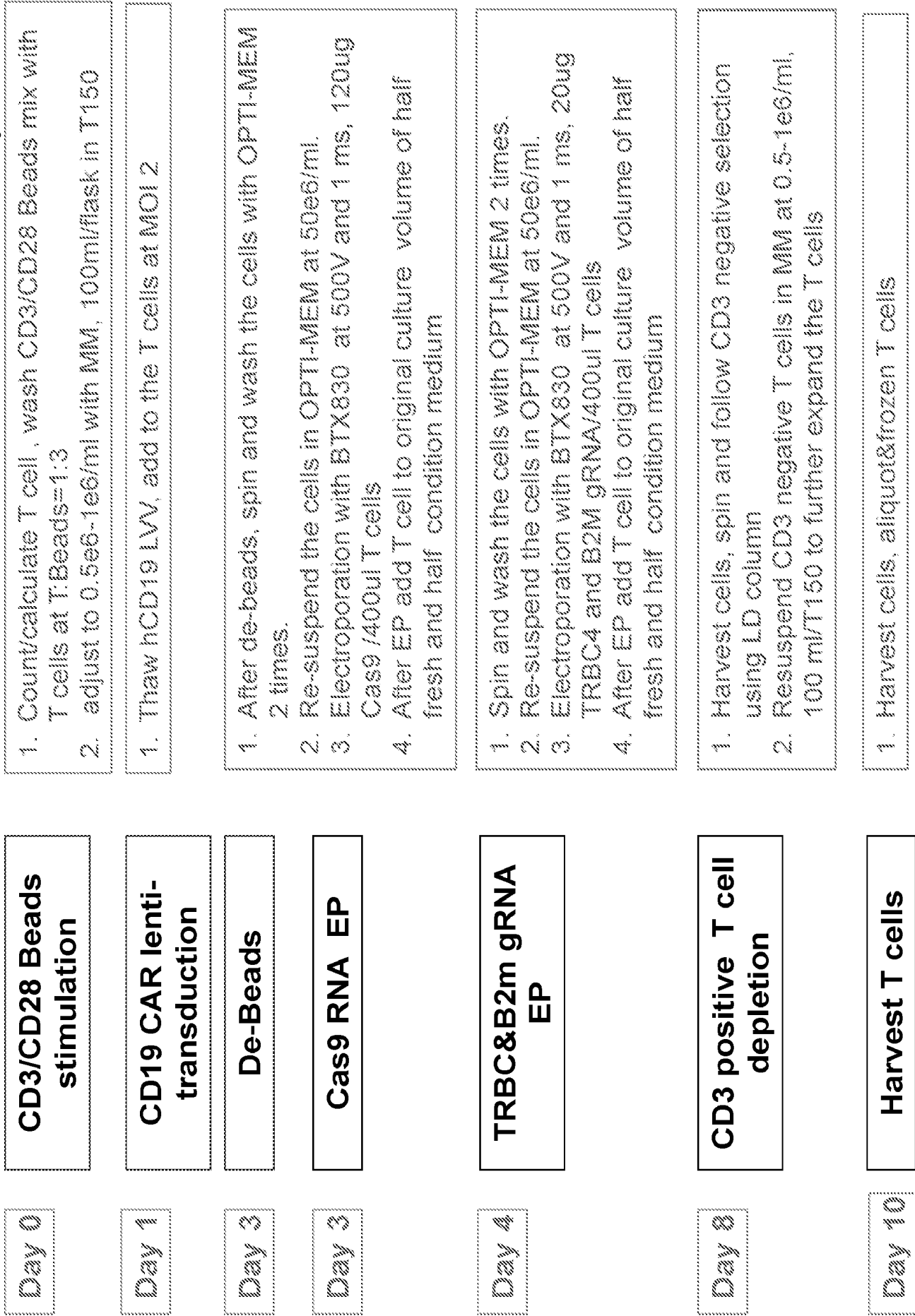


Figure 84

Cell#	Group # Donor	T cells	Day 0	Day 3	Day 5	Day 6	Day 7	Day 8	Day 10
	1	ND391 CART/MOCK EP	10.0	19.8	63.4	127.9	310.2	556.8	1090.8
	2	ND391 CART/CRISPR	100.0	178.2	175.4	350.4	571.6	1036.8	2985.6
	3	ND463 CART/MOCK EP	10.0	17.1	57.0	115.1	301.9	648.6	1282.5
	4	ND463 CART/CRISPR	90.0	153.5	150.8	309.6	522.8	861.6	2839.2
	5	ND463 UNMOD	30.0	43.5	173.8	305.2	733.2	1503.2	2935.2
	6	ND469 CART/MOCK EP	10.0	12.0	66.6	99.3	301.9	577.2	1017.9
	7	ND469 CART/CRISPR	60.0	108.2	74.1	147.0	252.0	521.6	1190.4
<i>Expansion</i>									
Cell#	Group # Donor	T cells	Day 0	Day 3	Day 5	Day 6	Day 7	Day 8	Day 10
	1	ND391 CART/MOCK EP	1	2	6	13	31	56	109
	2	ND391 CART/CRISPR	1	2	2	4	6	10	30
	3	ND463 CART/MOCK EP	1	2	6	12	30	65	128
	4	ND463 CART/CRISPR	1	2	2	3	6	10	32
	5	ND463 UNMOD	1	1	6	10	24	50	98
	6	ND469 CART/MOCK EP	1	1	7	10	30	58	102
	7	ND469 CART/CRISPR	1	2	1	2	4	9	20

Figure 85

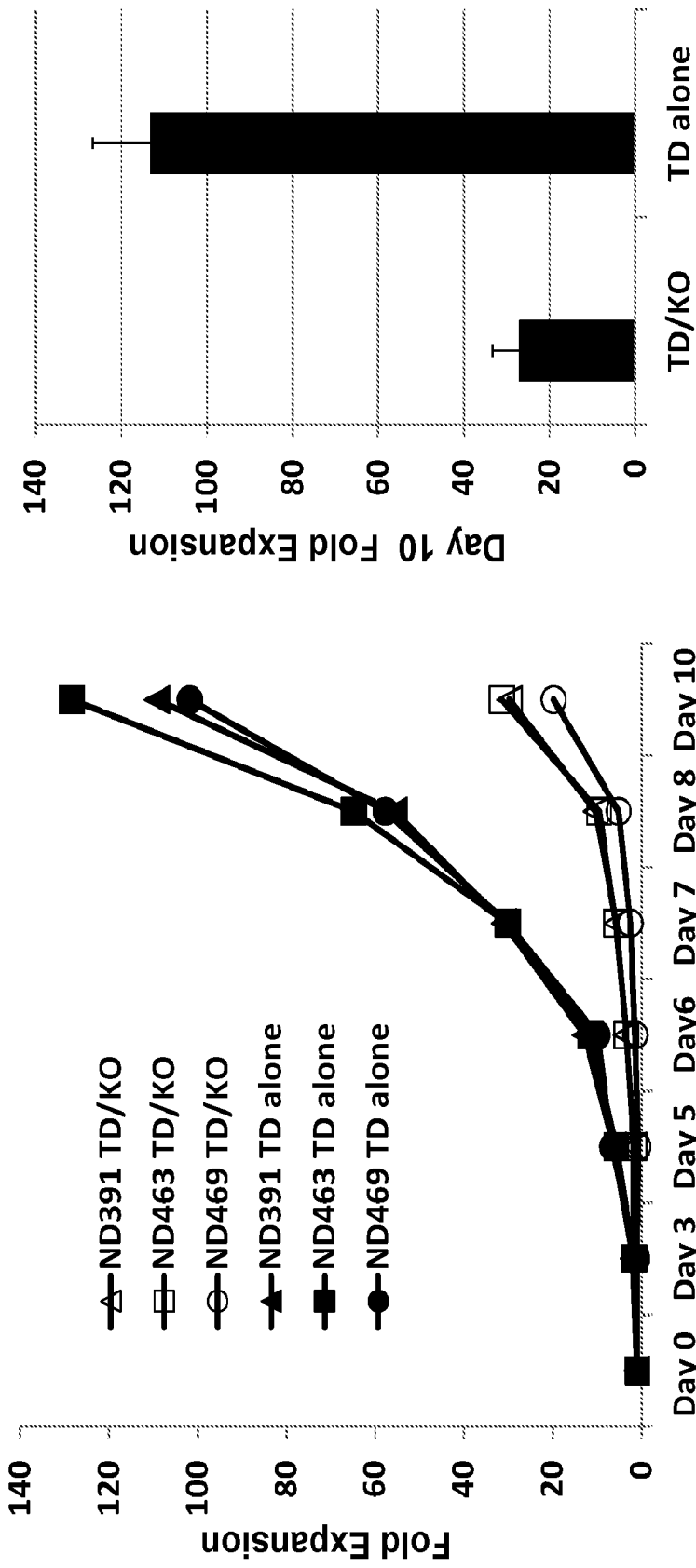
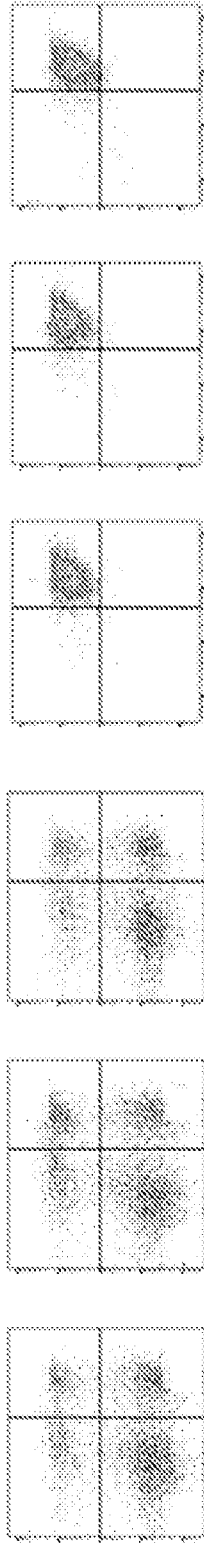


Figure 86

CD19 CAR TD/CRISPR

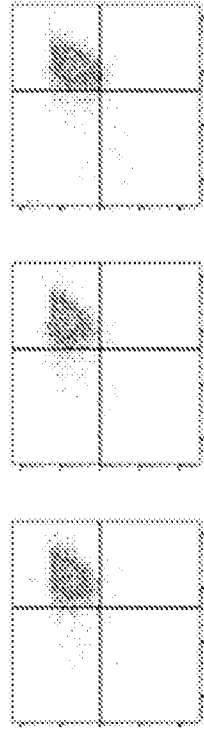
ND391 ND463 ND469 ND391 ND463 ND469



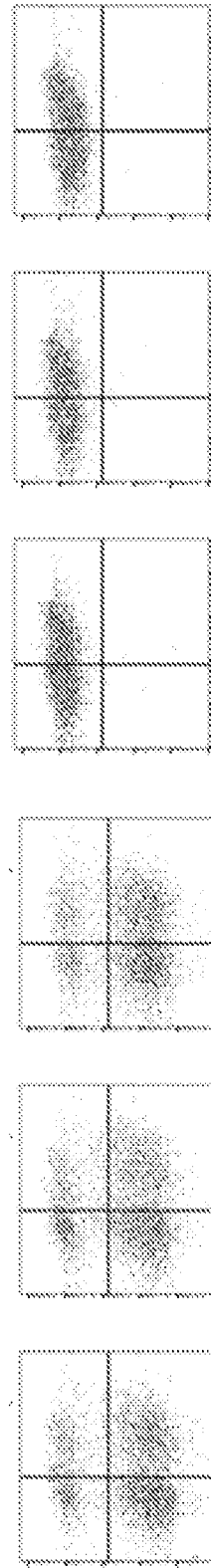
CD3
B2M

CD19 CAR TD

ND391 ND463 ND469



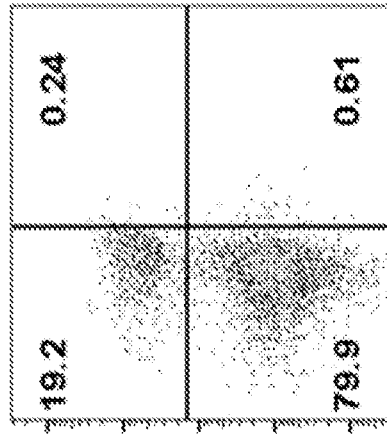
CD3
CAR



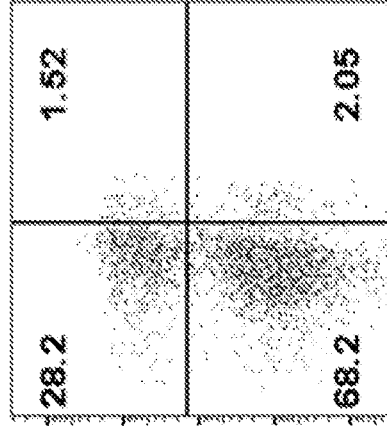
B2M
CAR

Figure 87

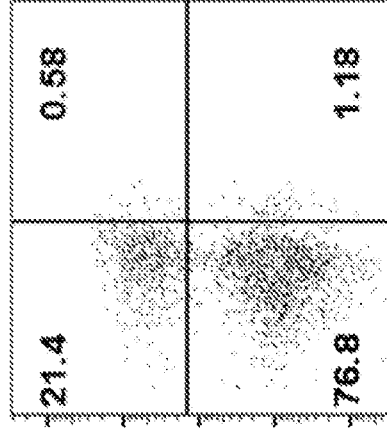
ND391



ND463



ND469



B2M
CD3

Day 8 T cells post CD3 depletion

Figure 88

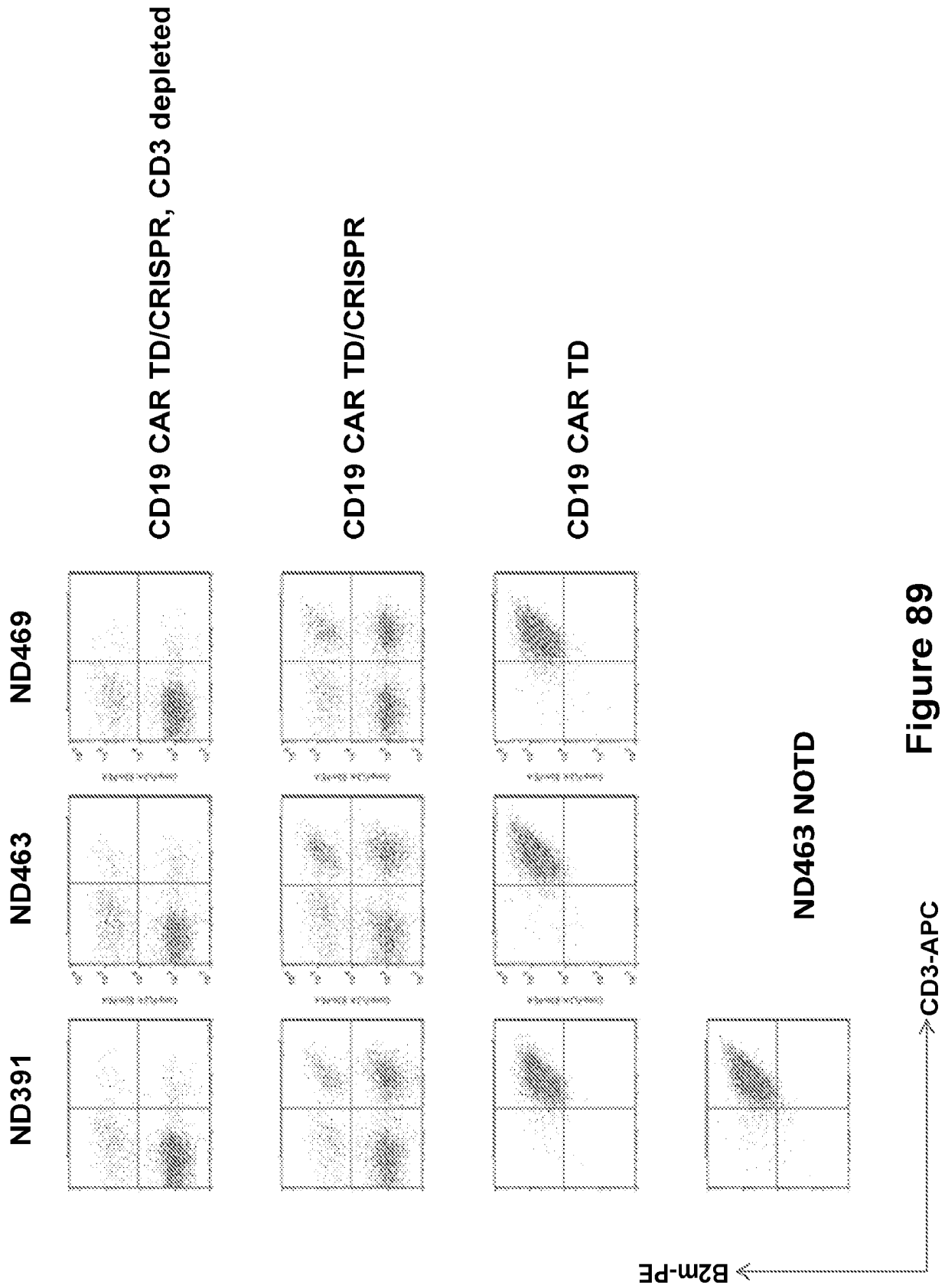


Figure 89

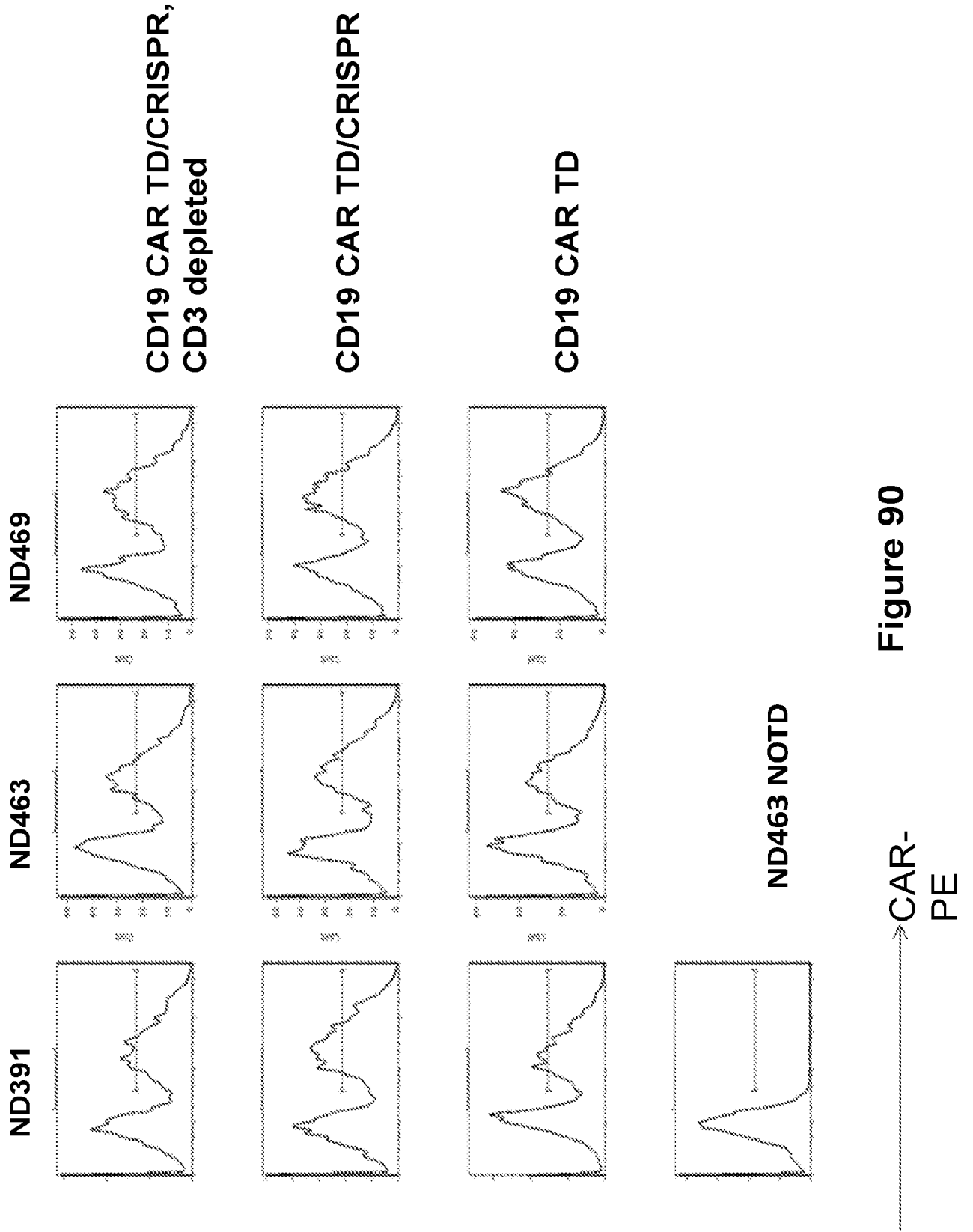
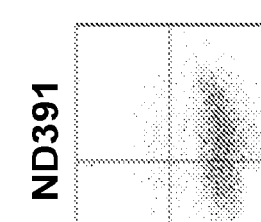
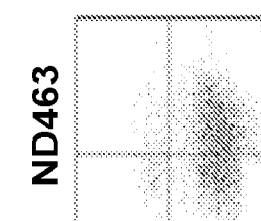
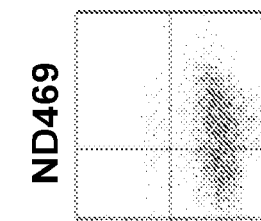
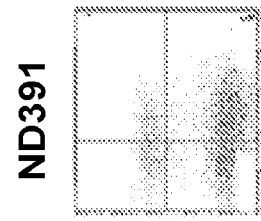
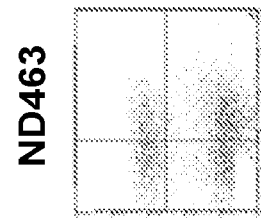
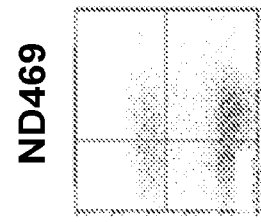


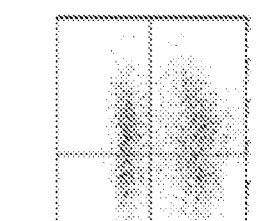
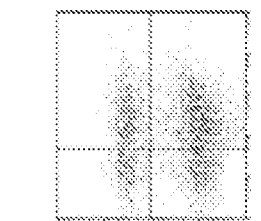
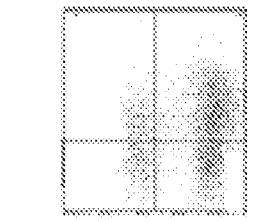
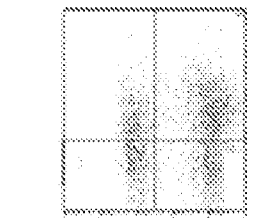
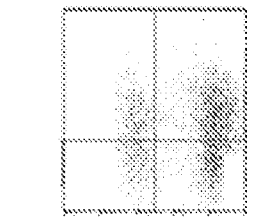
Figure 90

CD3 depleted
CRISPR,

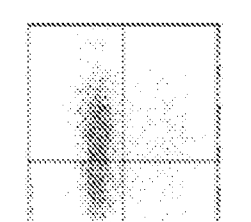
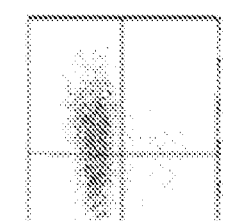
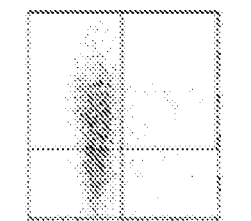
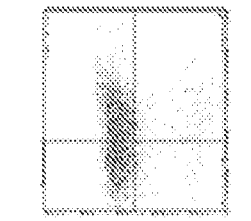
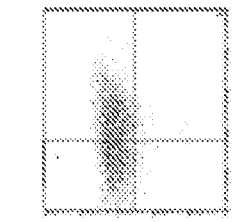
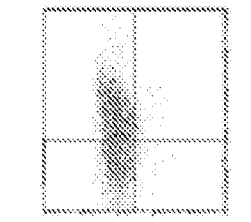
CD19 CAR TD/
CRISPR



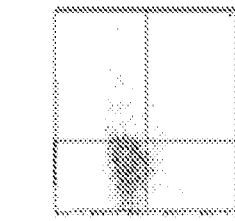
CD19 CAR TD/
CRISPR



CD19 CAR TD



ND463 NOTD



B2M-PE

CAR-APC

CD3-FITC

CAR-APC

CAR-APC

Figure 91

Donor cell ID	%CAR19+	%CAR19+ CD3- B2M-	% CAR19+ CD3- B2M+	%CAR19 +CD3+ B2M-	%CAR19 +CD3+ B2M+
ND391	51.4	85.2	12.8	1.6	0.4
ND463	48.5	73.4	22.9	1.8	1.9
ND469	55.8	82.1	16.5	0.8	0.6

Figure 92

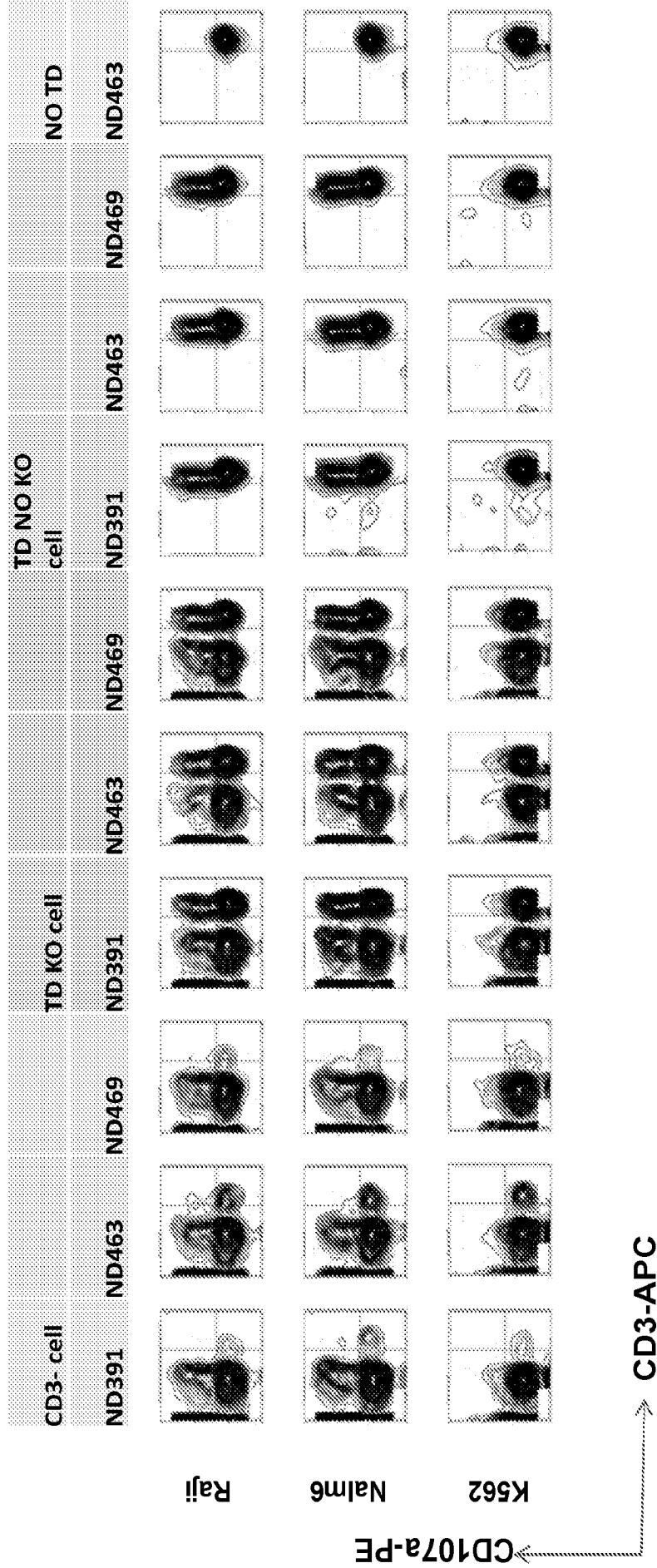


Figure 93

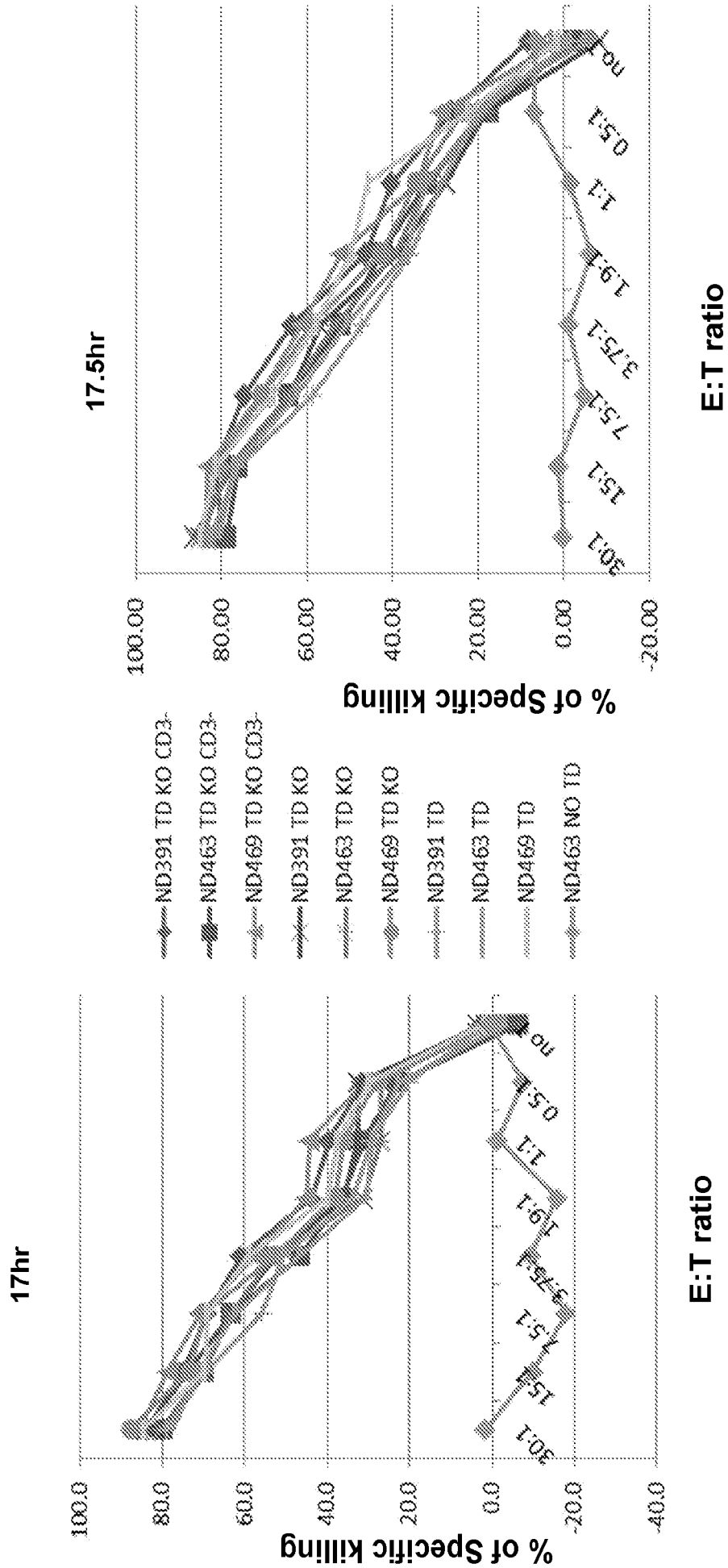


Figure 94

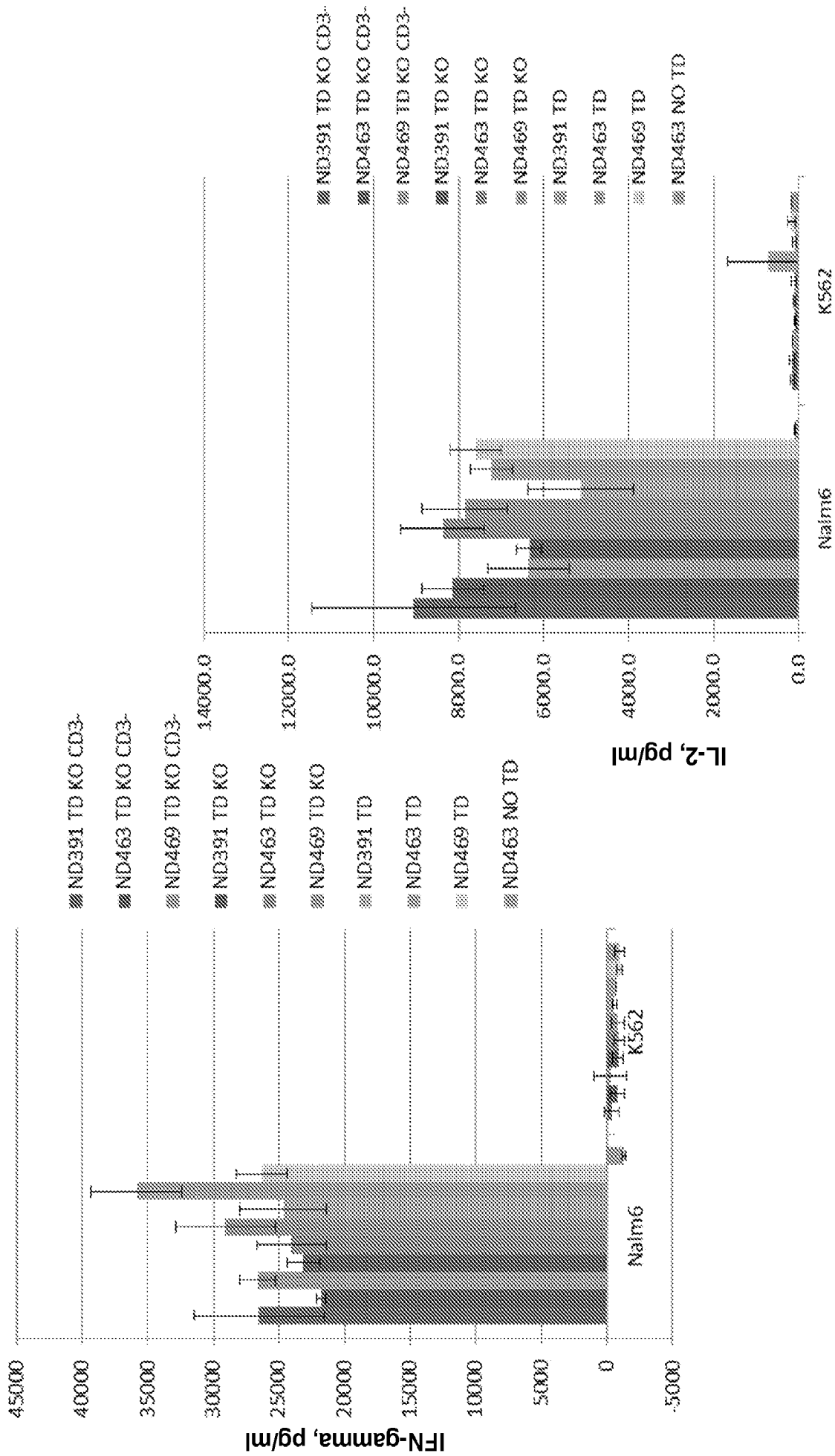


Figure 95

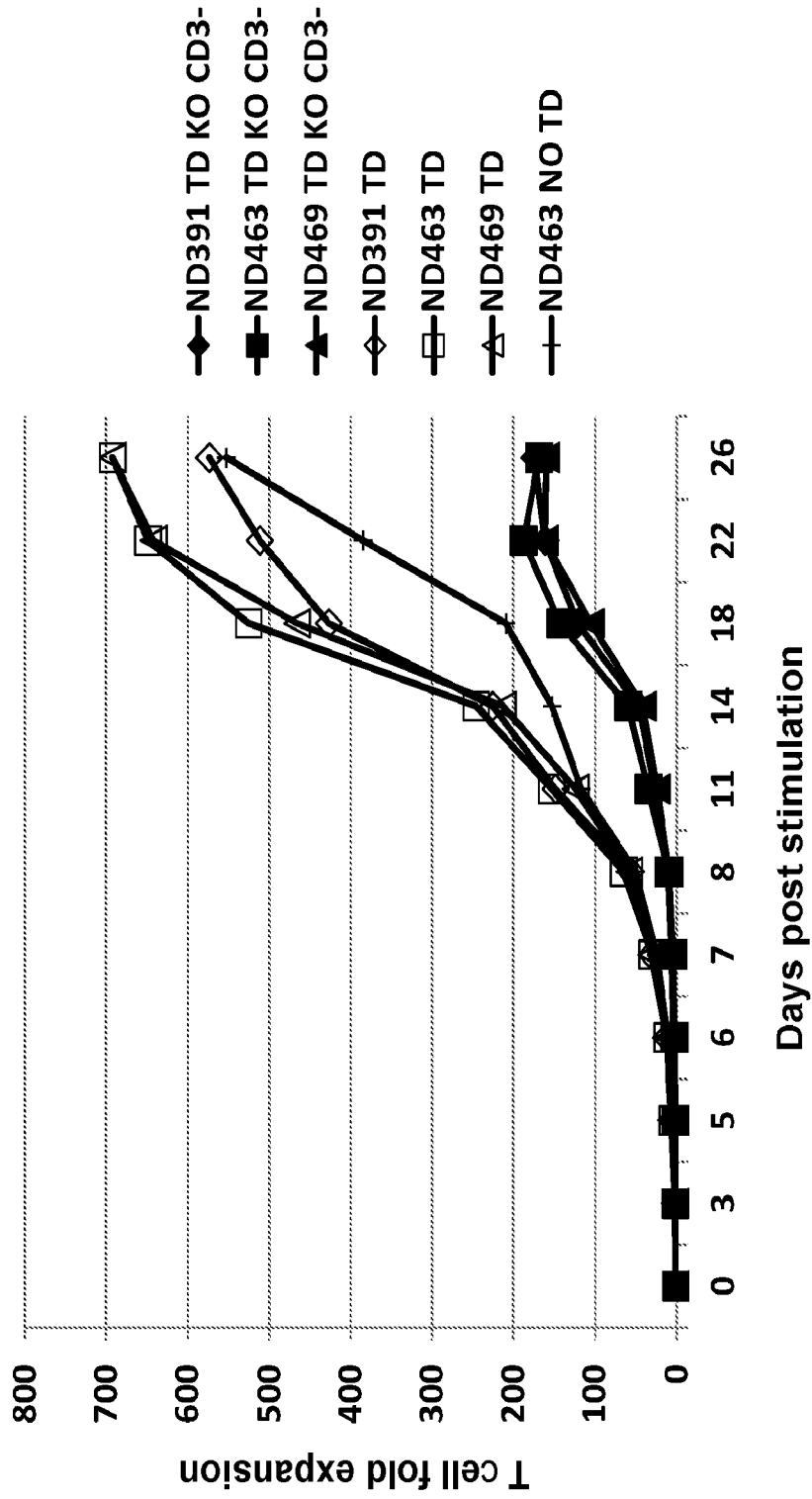


Figure 96

Sequence Listing

2024203330 18 Jul 2024

1	Sequence Listing Information	
1-1	File Name	046483-7100EP2.xml
1-2	DTD Version	V1_3
1-3	Software Name	WIPO Sequence
1-4	Software Version	2.3.0
1-5	Production Date	2024-07-09
1-6	Original free text language code	
1-7	Non English free text language code	
2	General Information	
2-1	Current application: IP Office	EP
2-2	Current application: Application number	24 171 941.8
2-3	Current application: Filing date	2024-04-23
2-4	Current application: Applicant file reference	
2-5	Earliest priority application: IP Office	US
2-6	Earliest priority application: Application number	62/073,651
2-7	Earliest priority application: Filing date	2014-10-31
2-8en	Applicant name	THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA
2-8	Applicant name: Name Latin	
2-9en	Inventor name	
2-9	Inventor name: Name Latin	
2-10en	Invention title	ALTERING GENE EXPRESSION IN CART CELLS AND USES THEREOF
2-11	Sequence Total Quantity	97

3-1	Sequences		
3-1-1	Sequence Number [ID]	1	
3-1-2	Molecule Type	DNA	
3-1-3	Length	20	
3-1-4	Features	misc_feature 1..20	
	Location/Qualifiers	note=Synthetic Sequence source 1..20 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-1-5	Residues	tgtgctagac atgaggtcta	20
3-2	Sequences		
3-2-1	Sequence Number [ID]	2	
3-2-2	Molecule Type	DNA	
3-2-3	Length	20	
3-2-4	Features	misc_feature 1..20	
	Location/Qualifiers	note=Synthetic Sequence source 1..20 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-2-5	Residues	gcagtatctg gagtcattga	20
3-3	Sequences		
3-3-1	Sequence Number [ID]	3	
3-3-2	Molecule Type	DNA	
3-3-3	Length	20	
3-3-4	Features	misc_feature 1..20	
	Location/Qualifiers	note=Synthetic Sequence source 1..20 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-3-5	Residues	cgcgagcaca gctaaggcca	20
3-4	Sequences		
3-4-1	Sequence Number [ID]	4	
3-4-2	Molecule Type	DNA	
3-4-3	Length	20	
3-4-4	Features	misc_feature 1..20	
	Location/Qualifiers	note=Synthetic Sequence source 1..20 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-4-5	Residues	ggcgccctgg ccagtcgtct	20
3-5	Sequences		
3-5-1	Sequence Number [ID]	5	
3-5-2	Molecule Type	DNA	
3-5-3	Length	20	
3-5-4	Features	misc_feature 1..20	
	Location/Qualifiers	note=Synthetic Sequence source 1..20 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-5-5	Residues	gaggggccag atgccagca	20
3-6	Sequences		
3-6-1	Sequence Number [ID]	6	
3-6-2	Molecule Type	DNA	
3-6-3	Length	24	
3-6-4	Features	misc_feature 1..24	
	Location/Qualifiers	note=Synthetic Sequence source 1..24 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-6-5	Residues	tcatgtccta accctgatcc tctt	24
3-7	Sequences		
3-7-1	Sequence Number [ID]	7	

3-7-2	Molecule Type	DNA	
3-7-3	Length	23	
3-7-4	Features	misc_feature 1..23	
	Location/Qualifiers	note=Synthetic Sequence source 1..23 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-7-5	Residues	ttggactttt cccagctgac aga	23
3-8	Sequences		
3-8-1	Sequence Number [ID]	8	
3-8-2	Molecule Type	DNA	
3-8-3	Length	24	
3-8-4	Features	misc_feature 1..24	
	Location/Qualifiers	note=Synthetic Sequence source 1..24 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-8-5	Residues	taccaggacc agacagctct taga	24
3-9	Sequences		
3-9-1	Sequence Number [ID]	9	
3-9-2	Molecule Type	DNA	
3-9-3	Length	24	
3-9-4	Features	misc_feature 1..24	
	Location/Qualifiers	note=Synthetic Sequence source 1..24 mol_type=other DNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-9-5	Residues	tctcacctaa tctcctccag gcat	24
3-10	Sequences		
3-10-1	Sequence Number [ID]	10	
3-10-2	Molecule Type	RNA	
3-10-3	Length	27	
3-10-4	Features	misc_feature 1..27	
	Location/Qualifiers	note=Synthetic Sequence source 1..27 mol_type=other RNA organism=synthetic construct	
	NonEnglishQualifier Value		
3-10-5	Residues	aggaggattc ggaaccaat cactgac	27
3-11	Sequences		
3-11-1	Sequence Number [ID]	11	
3-11-2	Molecule Type	DNA	
3-11-3	Length	25	
3-11-4	Features	source 1..25	
	Location/Qualifiers	mol_type=other DNA organism=synthetic construct misc_feature 1..23 note=Segment is RNA. misc_feature 24..25 note=Segment is DNA. misc_RNA 1..23 misc_feature 1..25 note=Synthetic Sequence	
	NonEnglishQualifier Value		
3-11-5	Residues	cagtgattgg gttccgaatc ctctct	25
3-12	Sequences		
3-12-1	Sequence Number [ID]	12	
3-12-2	Molecule Type	RNA	
3-12-3	Length	27	
3-12-4	Features	misc_feature 1..27	
	Location/Qualifiers	note=Synthetic Sequence source 1..27 mol_type=other RNA organism=synthetic construct	
	NonEnglishQualifier Value		

3-12-5	Residues	acctccttcc cattcaccca ccagctc	27
3-13	Sequences		
3-13-1	Sequence Number [ID]	13	
3-13-2	Molecule Type	DNA	
3-13-3	Length	25	
3-13-4	Features	misc_feature 1..25	
	Location/Qualifiers	note=Synthetic Sequence	
		misc_RNA 1..23	
		source 1..25	
		mol_type=other DNA	
		organism=synthetic construct	
		misc_feature 1..23	
		note=Segment is RNA.	
		misc_feature 24..25	
		note=Segment is DNA.	
	NonEnglishQualifier Value		
3-13-5	Residues	gctggtgggt gaatgggaag gaggt	25
3-14	Sequences		
3-14-1	Sequence Number [ID]	14	
3-14-2	Molecule Type	DNA	
3-14-3	Length	122	
3-14-4	Features	misc_feature 1..122	
	Location/Qualifiers	note=Synthetic Sequence; Figure 1B	
		source 1..122	
		mol_type=other DNA	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-14-5	Residues	ttaatacgac tcactatagg caccaaagct gcccttaccg ttttagagct agaaatagca 60 agttaaata aggctagtcc gttatcaact tgaaaaagtg gcaccgagtc ggtgcttttt 120 tt 122	
3-15	Sequences		
3-15-1	Sequence Number [ID]	15	
3-15-2	Molecule Type	DNA	
3-15-3	Length	23	
3-15-4	Features	misc_feature 1..23	
	Location/Qualifiers	note=Synthetic Sequence; Figure 1C	
		source 1..23	
		mol_type=other DNA	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-15-5	Residues	tgtgctagac atgaggtcta tgg	23
3-16	Sequences		
3-16-1	Sequence Number [ID]	16	
3-16-2	Molecule Type	DNA	
3-16-3	Length	23	
3-16-4	Features	misc_feature 1..23	
	Location/Qualifiers	note=Synthetic Sequence; Figure 1C	
		source 1..23	
		mol_type=other DNA	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-16-5	Residues	ccctcaatga ctccagatac tgc	23
3-17	Sequences		
3-17-1	Sequence Number [ID]	17	
3-17-2	Molecule Type	DNA	
3-17-3	Length	38	
3-17-4	Features	misc_feature 1..38	
	Location/Qualifiers	note=Synthetic Sequence; Figure 79B	
		source 1..38	
		mol_type=other DNA	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-17-5	Residues	tgggctggcg gccaggatgg ttcttggtag gtaggtgg	38
3-18	Sequences		
3-18-1	Sequence Number [ID]	18	
3-18-2	Molecule Type	DNA	
3-18-3	Length	38	
3-18-4	Features	misc_feature 1..38	

	Location/Qualifiers	note=Synthetic Sequence; Figure 79B source 1..38 mol_type=other DNA organism=synthetic construct	
3-18-5	NonEnglishQualifier Value Residues	tgggctggcg gccaggatgg ttcttagggg taggtggg	38
3-19	Sequences		
3-19-1	Sequence Number [ID]	19	
3-19-2	Molecule Type	DNA	
3-19-3	Length	67	
3-19-4	Features	misc_feature 1..67	
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct	
3-19-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg aggtctatgg acttcaagag caacagtgct gtggcct	60 67
3-20	Sequences		
3-20-1	Sequence Number [ID]	20	
3-20-2	Molecule Type	DNA	
3-20-3	Length	66	
3-20-4	Features	misc_feature 1..66	
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct	
3-20-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg agtctatgga cttcaagagc aacagtgctg tggcct	60 66
3-21	Sequences		
3-21-1	Sequence Number [ID]	21	
3-21-2	Molecule Type	DNA	
3-21-3	Length	65	
3-21-4	Features	misc_feature 1..65	
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..65 mol_type=other DNA organism=synthetic construct	
3-21-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg gtctatggac ttcaagagca acagtgctgt ggcct	60 65
3-22	Sequences		
3-22-1	Sequence Number [ID]	22	
3-22-2	Molecule Type	DNA	
3-22-3	Length	66	
3-22-4	Features	misc_feature 1..66	
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct	
3-22-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg agtctatgga cttcaagagc aacagtgctg tggcct	60 66
3-23	Sequences		
3-23-1	Sequence Number [ID]	23	
3-23-2	Molecule Type	DNA	
3-23-3	Length	64	
3-23-4	Features	misc_feature 1..64	
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..64 mol_type=other DNA organism=synthetic construct	
3-23-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg tctatggact tcaagagcaa cagtgctgtg gcct	60 64
3-24	Sequences		
3-24-1	Sequence Number [ID]	24	
3-24-2	Molecule Type	DNA	

3-24-3	Length	66
3-24-4	Features Location/Qualifiers	misc_feature 1..66 note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct
3-24-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagactga ggtctatgga cttcaagagc aacagtgctg 60 tggcct 66
3-25	Sequences	
3-25-1	Sequence Number [ID]	25
3-25-2	Molecule Type	DNA
3-25-3	Length	64
3-25-4	Features Location/Qualifiers	misc_feature 1..64 note=Synthetic Sequence; Figure 3D source 1..64 mol_type=other DNA organism=synthetic construct
3-25-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagtggagg tctatggact tcaagagcaa cagtgctgtg 60 gcct 64
3-26	Sequences	
3-26-1	Sequence Number [ID]	26
3-26-2	Molecule Type	DNA
3-26-3	Length	62
3-26-4	Features Location/Qualifiers	misc_feature 1..62 note=Synthetic Sequence; Figure 3D source 1..62 mol_type=other DNA organism=synthetic construct
3-26-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gcttgaggtc tatggacttc aagagcaaca gtgctgtggc 60 ct 62
3-27	Sequences	
3-27-1	Sequence Number [ID]	27
3-27-2	Molecule Type	DNA
3-27-3	Length	67
3-27-4	Features Location/Qualifiers	misc_feature 1..67 note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct
3-27-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg aggttctatg gacttcaaga gcaacagtgc 60 tgtggcc 67
3-28	Sequences	
3-28-1	Sequence Number [ID]	28
3-28-2	Molecule Type	DNA
3-28-3	Length	67
3-28-4	Features Location/Qualifiers	misc_feature 1..67 note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct
3-28-5	NonEnglishQualifier Value Residues	tatatcacag acaaaactgt gctagacatg agggctctatg gacttcaaga gcaacagtgc 60 tgtggcc 67
3-29	Sequences	
3-29-1	Sequence Number [ID]	29
3-29-2	Molecule Type	DNA
3-29-3	Length	67
3-29-4	Features Location/Qualifiers	misc_feature 1..67 note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct
3-29-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tccagatact gctgagcag ccgcctgagg gtctcggcca 60 cctctg 67

3-30	Sequences	
3-30-1	Sequence Number [ID]	30
3-30-2	Molecule Type	DNA
3-30-3	Length	66
3-30-4	Features	misc_feature 1..66
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct
3-30-5	NonEnglishQualifier Value Residues	agcagcccgc cctcatgact ccagatactg cctgagcagc gcctgaggg tctcgccac 60 cttctg 66
3-31	Sequences	
3-31-1	Sequence Number [ID]	31
3-31-2	Molecule Type	DNA
3-31-3	Length	66
3-31-4	Features	misc_feature 1..66
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct
3-31-5	NonEnglishQualifier Value Residues	agcagcccgc ctcaatgact ccagatactg cctgagcagc gcctgaggg tctcgccac 60 cttctg 66
3-32	Sequences	
3-32-1	Sequence Number [ID]	32
3-32-2	Molecule Type	DNA
3-32-3	Length	66
3-32-4	Features	misc_feature 1..66
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct
3-32-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tcagatactg cctgagcagc gcctgaggg tctcgccac 60 cttctg 66
3-33	Sequences	
3-33-1	Sequence Number [ID]	33
3-33-2	Molecule Type	DNA
3-33-3	Length	65
3-33-4	Features	misc_feature 1..65
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..65 mol_type=other DNA organism=synthetic construct
3-33-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tagatactgc ctgagcagcc gcctgaggg tctcgccacc 60 ttctg 65
3-34	Sequences	
3-34-1	Sequence Number [ID]	34
3-34-2	Molecule Type	DNA
3-34-3	Length	62
3-34-4	Features	misc_feature 1..62
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..62 mol_type=other DNA organism=synthetic construct
3-34-5	NonEnglishQualifier Value Residues	agcagcccgc ccgactccag atactgcctg agcagccgcc tgagggcttc ggccaccttc 60 tg 62
3-35	Sequences	
3-35-1	Sequence Number [ID]	35
3-35-2	Molecule Type	DNA
3-35-3	Length	67
3-35-4	Features	misc_feature 1..67
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct

3-35-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaaatga ctccagatac tgcoctgagca gccgcctgag ggtctcggcc 60 accttct 67
3-36	Sequences	
3-36-1	Sequence Number [ID]	36
3-36-2	Molecule Type	DNA
3-36-3	Length	67
3-36-4	Features Location/Qualifiers	misc_feature 1..67 note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct
3-36-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tcccagatac tgcoctgagca gccgcctgag ggtctcggcc 60 accttct 67
3-37	Sequences	
3-37-1	Sequence Number [ID]	37
3-37-2	Molecule Type	DNA
3-37-3	Length	67
3-37-4	Features Location/Qualifiers	misc_feature 1..67 note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct
3-37-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tccagatact gcoctgagcag ccgcctgagg gtctcggcca 60 ccttctg 67
3-38	Sequences	
3-38-1	Sequence Number [ID]	38
3-38-2	Molecule Type	DNA
3-38-3	Length	64
3-38-4	Features Location/Qualifiers	misc_feature 1..64 note=Synthetic Sequence; Figure 3D source 1..64 mol_type=other DNA organism=synthetic construct
3-38-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac agatactgcc tgagcagccg cctgagggtc tcggccacct 60 tctg 64
3-39	Sequences	
3-39-1	Sequence Number [ID]	39
3-39-2	Molecule Type	DNA
3-39-3	Length	66
3-39-4	Features Location/Qualifiers	misc_feature 1..66 note=Synthetic Sequence; Figure 3D source 1..66 mol_type=other DNA organism=synthetic construct
3-39-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tcagatactg cctgagcagc cgcoctgaggg tctcggccac 60 cttctg 66
3-40	Sequences	
3-40-1	Sequence Number [ID]	40
3-40-2	Molecule Type	DNA
3-40-3	Length	55
3-40-4	Features Location/Qualifiers	misc_feature 1..55 note=Synthetic Sequence; Figure 3D source 1..55 mol_type=other DNA organism=synthetic construct
3-40-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaactgc ctgagcagcc gcoctgagggt ctcggccacc ttctg 55
3-41	Sequences	
3-41-1	Sequence Number [ID]	41
3-41-2	Molecule Type	DNA
3-41-3	Length	62
3-41-4	Features Location/Qualifiers	misc_feature 1..62 note=Synthetic Sequence; Figure 3D source 1..62

3-41-5	NonEnglishQualifier Value Residues	mol_type=other DNA organism=synthetic construct agcagcccgc ctcatgactc agatactgct gagcagccgc ctgagggtct cggccacctt 60 ct 62
3-42	Sequences	
3-42-1	Sequence Number [ID]	42
3-42-2	Molecule Type	DNA
3-42-3	Length	46
3-42-4	Features	misc_feature 1..46
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..46 mol_type=other DNA organism=synthetic construct
3-42-5	NonEnglishQualifier Value Residues	agcagccact gcgtgagcag ccgcgtgagg gtctcggcca ccttct 46
3-43	Sequences	
3-43-1	Sequence Number [ID]	43
3-43-2	Molecule Type	DNA
3-43-3	Length	67
3-43-4	Features	misc_feature 1..67
	Location/Qualifiers	note=Synthetic Sequence; Figure 3D source 1..67 mol_type=other DNA organism=synthetic construct
3-43-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tccgagatac tgcoctgagca gccgcctgag ggtctcggcc 60 accttct 67
3-44	Sequences	
3-44-1	Sequence Number [ID]	44
3-44-2	Molecule Type	DNA
3-44-3	Length	23
3-44-4	Features	misc_feature 1..23
	Location/Qualifiers	note=Synthetic Sequence; Figure 9A source 1..23 mol_type=other DNA organism=synthetic construct
3-44-5	NonEnglishQualifier Value Residues	ccgtggcctt agctgtgctc gcg 23
3-45	Sequences	
3-45-1	Sequence Number [ID]	45
3-45-2	Molecule Type	DNA
3-45-3	Length	23
3-45-4	Features	misc_feature 1..23
	Location/Qualifiers	note=Synthetic Sequence; Figure 12A source 1..23 mol_type=other DNA organism=synthetic construct
3-45-5	NonEnglishQualifier Value Residues	ccatgctggg catctggacc ctc 23
3-46	Sequences	
3-46-1	Sequence Number [ID]	46
3-46-2	Molecule Type	DNA
3-46-3	Length	23
3-46-4	Features	misc_feature 1..23
	Location/Qualifiers	note=Synthetic Sequence; Figure 14A source 1..23 mol_type=other DNA organism=synthetic construct
3-46-5	NonEnglishQualifier Value Residues	gggcgggtgct acaactgggc tgg 23
3-47	Sequences	
3-47-1	Sequence Number [ID]	47
3-47-2	Molecule Type	DNA
3-47-3	Length	23
3-47-4	Features	misc_feature 1..23
	Location/Qualifiers	note=Synthetic Sequence; Figure 15A source 1..23

3-47-5	NonEnglishQualifier Value Residues	mol_type=other DNA organism=synthetic construct cataaagcca tggcttgct tgg	23
3-48	Sequences		
3-48-1	Sequence Number [ID]	48	
3-48-2	Molecule Type	DNA	
3-48-3	Length	64	
3-48-4	Features Location/Qualifiers	misc_feature 1..64 note=Synthetic Sequence; Figure 15B source 1..64 mol_type=other DNA organism=synthetic construct	
3-48-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaagcc atggcttgcc ttggatttca gcggcacaag gctcagctga acct	60 64
3-49	Sequences		
3-49-1	Sequence Number [ID]	49	
3-49-2	Molecule Type	DNA	
3-49-3	Length	59	
3-49-4	Features Location/Qualifiers	misc_feature 1..59 note=Synthetic Sequence; Figure 15B source 1..59 mol_type=other DNA organism=synthetic construct	
3-49-5	NonEnglishQualifier Value Residues	aacaccgctc ccataatggc ttgccttggg tttcagcggc acaaggctca gctgaacct	59
3-50	Sequences		
3-50-1	Sequence Number [ID]	50	
3-50-2	Molecule Type	DNA	
3-50-3	Length	62	
3-50-4	Features Location/Qualifiers	misc_feature 1..62 note=Synthetic Sequence; Figure 15B source 1..62 mol_type=other DNA organism=synthetic construct	
3-50-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaccat ggcttgctt ggatttcagc ggcacaaggc tcagctgaac ct	60 62
3-51	Sequences		
3-51-1	Sequence Number [ID]	51	
3-51-2	Molecule Type	DNA	
3-51-3	Length	62	
3-51-4	Features Location/Qualifiers	misc_feature 1..62 note=Synthetic Sequence; Figure 15B source 1..62 mol_type=other DNA organism=synthetic construct	
3-51-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaccat ggcttgctt ggatttcagc ggcacaaggc tcagctgaac ct	60 62
3-52	Sequences		
3-52-1	Sequence Number [ID]	52	
3-52-2	Molecule Type	DNA	
3-52-3	Length	64	
3-52-4	Features Location/Qualifiers	misc_feature 1..64 note=Synthetic Sequence; Figure 15B source 1..64 mol_type=other DNA organism=synthetic construct	
3-52-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaagtc catggcttgc cttggatttc agcggcacia ggctcagctg aacc	60 64
3-53	Sequences		
3-53-1	Sequence Number [ID]	53	
3-53-2	Molecule Type	DNA	
3-53-3	Length	63	
3-53-4	Features Location/Qualifiers	misc_feature 1..63 note=Synthetic Sequence; Figure 15B	

3-53-5	NonEnglishQualifier Value Residues	source 1..63 mol_type=other DNA organism=synthetic construct aacaccgctc ccataaacca tggcttgctc tggatttcag cggcacaagg ctccagctgaa 60 cct 63
3-54	Sequences	
3-54-1	Sequence Number [ID]	54
3-54-2	Molecule Type	DNA
3-54-3	Length	50
3-54-4	Features Location/Qualifiers	misc_feature 1..50 note=Synthetic Sequence; Figure 15B source 1..50 mol_type=other DNA organism=synthetic construct
3-54-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaaggg atttcagcgg cacaaggctc agctgaacct 50
3-55	Sequences	
3-55-1	Sequence Number [ID]	55
3-55-2	Molecule Type	DNA
3-55-3	Length	59
3-55-4	Features Location/Qualifiers	misc_feature 1..59 note=Synthetic Sequence; Figure 15B source 1..59 mol_type=other DNA organism=synthetic construct
3-55-5	NonEnglishQualifier Value Residues	aacaccgctc ccataatggc ttgccttggg tttcagcggc acaaggctca gctgaacct 59
3-56	Sequences	
3-56-1	Sequence Number [ID]	56
3-56-2	Molecule Type	DNA
3-56-3	Length	59
3-56-4	Features Location/Qualifiers	misc_feature 1..59 note=Synthetic Sequence; Figure 15B source 1..59 mol_type=other DNA organism=synthetic construct
3-56-5	NonEnglishQualifier Value Residues	aacaccgctc ccataatggc ttgccttggg tttcagcggc acaaggctca gctgaacct 59
3-57	Sequences	
3-57-1	Sequence Number [ID]	57
3-57-2	Molecule Type	DNA
3-57-3	Length	63
3-57-4	Features Location/Qualifiers	misc_feature 1..63 note=Synthetic Sequence; Figure 15B source 1..63 mol_type=other DNA organism=synthetic construct
3-57-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaacca tggcttgctc tggatttcag cggcacaagg ctccagctgaa 60 cct 63
3-58	Sequences	
3-58-1	Sequence Number [ID]	58
3-58-2	Molecule Type	DNA
3-58-3	Length	61
3-58-4	Features Location/Qualifiers	misc_feature 1..61 note=Synthetic Sequence; Figure 15B source 1..61 mol_type=other DNA organism=synthetic construct
3-58-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaccatg gcttgcttg gatttcagcg gcacaaggct cagctgaacc 60 t 61
3-59	Sequences	
3-59-1	Sequence Number [ID]	59
3-59-2	Molecule Type	DNA
3-59-3	Length	59
3-59-4	Features Location/Qualifiers	misc_feature 1..59 note=Synthetic Sequence; Figure 15B

3-59-5	NonEnglishQualifier Value Residues	source 1..59 mol_type=other DNA organism=synthetic construct aacaccgctc ccataatggc ttgccttggg tttcagcggc acaaggctca gctgaacct 59
3-60	Sequences	
3-60-1	Sequence Number [ID]	60
3-60-2	Molecule Type	DNA
3-60-3	Length	64
3-60-4	Features Location/Qualifiers	misc_feature 1..64 note=Synthetic Sequence; Figure 15B source 1..64 mol_type=other DNA organism=synthetic construct
3-60-5	NonEnglishQualifier Value Residues	aacaccgctc ccataaagcc atggcttggc ttggatttca gcggcacaag gctcagctga 60 acct 64
3-61	Sequences	
3-61-1	Sequence Number [ID]	61
3-61-2	Molecule Type	DNA
3-61-3	Length	23
3-61-4	Features Location/Qualifiers	misc_feature 1..23 note=Synthetic Sequence; Figure 16 source 1..23 mol_type=other DNA organism=synthetic construct
3-61-5	NonEnglishQualifier Value Residues	ggacatcaag cctgctaaca tgg 23
3-62	Sequences	
3-62-1	Sequence Number [ID]	62
3-62-2	Molecule Type	DNA
3-62-3	Length	23
3-62-4	Features Location/Qualifiers	misc_feature 1..23 note=Synthetic Sequence; Figure 16 source 1..23 mol_type=other DNA organism=synthetic construct
3-62-5	NonEnglishQualifier Value Residues	catcaagcct gctaacatgg agg 23
3-63	Sequences	
3-63-1	Sequence Number [ID]	63
3-63-2	Molecule Type	DNA
3-63-3	Length	122
3-63-4	Features Location/Qualifiers	misc_feature 1..122 note=Synthetic Sequence; Figure 49 misc_feature 19..39 note=n is a, c, g, or t source 1..122 mol_type=other DNA organism=synthetic construct
3-63-5	NonEnglishQualifier Value Residues	taatacgact cactatagnn nnnnnnnnnn nnnnnnnnng ttttagagct agaaatagca 60 agttaaaata aggctagtcc gttatcaact tgaaaaagtgc gcaccgagtc ggtgcttttt 120 tt 122
3-64	Sequences	
3-64-1	Sequence Number [ID]	64
3-64-2	Molecule Type	DNA
3-64-3	Length	20
3-64-4	Features Location/Qualifiers	misc_feature 1..20 note=Synthetic Sequence; Figure 79A source 1..20 mol_type=other DNA organism=synthetic construct
3-64-5	NonEnglishQualifier Value Residues	ggccaggatg gttcttaggt 20
3-65	Sequences	
3-65-1	Sequence Number [ID]	65
3-65-2	Molecule Type	DNA

3-65-3	Length	38	
3-65-4	Features Location/Qualifiers	misc_feature 1..38 note=Synthetic Sequence; Figure 79B source 1..38 mol_type=other DNA organism=synthetic construct	
3-65-5	NonEnglishQualifier Value Residues	tgggctggcg gccaggatgg ttcttaggta ggtggggt	38
3-66	Sequences		
3-66-1	Sequence Number [ID]	66	
3-66-2	Molecule Type	DNA	
3-66-3	Length	34	
3-66-4	Features Location/Qualifiers	misc_feature 1..34 note=Synthetic Sequence; Figure 79B source 1..34 mol_type=other DNA organism=synthetic construct	
3-66-5	NonEnglishQualifier Value Residues	tgggctggcg gccaggatgg taggtaggtg gggt	34
3-67	Sequences		
3-67-1	Sequence Number [ID]	67	
3-67-2	Molecule Type	DNA	
3-67-3	Length	37	
3-67-4	Features Location/Qualifiers	misc_feature 1..37 note=Synthetic Sequence; Figure 79B source 1..37 mol_type=other DNA organism=synthetic construct	
3-67-5	NonEnglishQualifier Value Residues	tgggctggcg gccaggatgg ttttaggtag gtggggt	37
3-68	Sequences		
3-68-1	Sequence Number [ID]	68	
3-68-2	Molecule Type	DNA	
3-68-3	Length	34	
3-68-4	Features Location/Qualifiers	misc_feature 1..34 note=Synthetic Sequence; Figure 79B source 1..34 mol_type=other DNA organism=synthetic construct	
3-68-5	NonEnglishQualifier Value Residues	tgggctggcg gccaggatgg taggtaggtg gggt	34
3-69	Sequences		
3-69-1	Sequence Number [ID]	69	
3-69-2	Molecule Type	DNA	
3-69-3	Length	113	
3-69-4	Features Location/Qualifiers	misc_feature 1..113 note=Synthetic Sequence; Figure 1C source 1..113 mol_type=other DNA organism=synthetic construct	
3-69-5	NonEnglishQualifier Value Residues	ctgtgctaga catgaggtct atggacttca agagcaacag tgctgtggcc tggagcaaca 60 aatctgactt tgcatgtgca aacgccttca acaacagcat tattccagaa gac 113	
3-70	Sequences		
3-70-1	Sequence Number [ID]	70	
3-70-2	Molecule Type	DNA	
3-70-3	Length	98	
3-70-4	Features Location/Qualifiers	misc_feature 1..98 note=Synthetic Sequence; Figure 1C source 1..98 mol_type=other DNA organism=synthetic construct	
3-70-5	NonEnglishQualifier Value Residues	gatgtgtata tcacagacaa aactgtgcta gacatgaggt ctatggactt caagagcaac 60 agtgtgtgg cctggagcaa caaatctgac ttgcatg 98	
3-71	Sequences		
3-71-1	Sequence Number [ID]	71	
3-71-2	Molecule Type	DNA	

3-71-3	Length	108
3-71-4	Features Location/Qualifiers	misc_feature 1..108 note=Synthetic Sequence; Figure 1C source 1..108 mol_type=other DNA organism=synthetic construct
3-71-5	NonEnglishQualifier Value Residues	accctcttc ctttccaga ggacctgaac aagggtgtcc caccgaggt cgctgtgttt 60 gagccatcag aagcagagat ctcccacacc caaaaggcca cactgggtg 108
3-72	Sequences	
3-72-1	Sequence Number [ID]	72
3-72-2	Molecule Type	DNA
3-72-3	Length	111
3-72-4	Features Location/Qualifiers	misc_feature 1..111 note=Synthetic Sequence; Figure 1C source 1..111 mol_type=other DNA organism=synthetic construct
3-72-5	NonEnglishQualifier Value Residues	gaccgcgagc cctcaagga gcagcccgcc ctcaatgact ccagatactg cctgagcagc 60 cgctgaggg tctcgccac cttctggcag aacccccgca accacttccg c 111
3-73	Sequences	
3-73-1	Sequence Number [ID]	73
3-73-2	Molecule Type	DNA
3-73-3	Length	74
3-73-4	Features Location/Qualifiers	misc_feature 1..74 note=Synthetic Sequence; Figure 3B source 1..74 mol_type=other DNA organism=synthetic construct
3-73-5	NonEnglishQualifier Value Residues	gattctgatg tgtatatcac agacaaaact gtgctagaca tgaggcttat ggacttcaag 60 agcaacagtg ctgt 74
3-74	Sequences	
3-74-1	Sequence Number [ID]	74
3-74-2	Molecule Type	DNA
3-74-3	Length	74
3-74-4	Features Location/Qualifiers	misc_feature 1..74 note=Synthetic Sequence; Figure 3B misc_feature 47 note=y is c or t misc_feature 48 note=y is c or t misc_feature 49 note=w is a or t misc_feature 54 note=m is a or c misc_feature 57 note=y is c or t misc_feature 58 note=m is a or c misc_feature 60 note=r is a or g misc_feature 64 note=m is a or c misc_feature 67 note=m is a or c source 1..74 mol_type=other DNA organism=synthetic construct
3-74-5	NonEnglishQualifier Value Residues	gattctgatg tgtatatcac agacaaaact gtgctagaca tgaggtyywt ggamttymar 60 aacmacmgtg gtgg 74
3-75	Sequences	
3-75-1	Sequence Number [ID]	75
3-75-2	Molecule Type	DNA
3-75-3	Length	64
3-75-4	Features Location/Qualifiers	misc_feature 1..64 note=Synthetic Sequence; Figure 3B

3-75-5	NonEnglishQualifier Value Residues	source 1..64 mol_type=other DNA organism=synthetic construct ccctcaagga gcagcccgcc ctcaatgact ccagatactg cctgagcagc cgctgaggg 60 tctc 64
3-76 3-76-1 3-76-2 3-76-3 3-76-4	Sequences Sequence Number [ID] Molecule Type Length Features Location/Qualifiers	76 DNA 64 misc_feature 1..64 note=Synthetic Sequence; Figure 3B misc_feature 33 note=m is a or c misc_feature 34 note=r is a or g misc_feature 36 note=w is a or t misc_feature 41 note=s is c or g misc_feature 46 note=r is a or g misc_feature 49 note=r is a or g misc_feature 52 note=s is c or g misc_feature 58 note=r is a or g source 1..64 mol_type=other DNA organism=synthetic construct
3-76-5	NonEnglishQualifier Value Residues	ccctcaagga gcagcccgcc ctcaatgact ccmrawactg sctgarcarc cscctgargg 60 tctc 64
3-77 3-77-1 3-77-2 3-77-3 3-77-4	Sequences Sequence Number [ID] Molecule Type Length Features Location/Qualifiers	77 DNA 82 misc_feature 1..82 note=Synthetic Sequence; Figure 9A source 1..82 mol_type=other DNA organism=synthetic construct
3-77-5	NonEnglishQualifier Value Residues	gagctgacag cattcgggcc gagatgtctc gctccgtggc cttagctgtg ctccgctac 60 tctctctttt ctggcctgga gg 82
3-78 3-78-1 3-78-2 3-78-3 3-78-4	Sequences Sequence Number [ID] Molecule Type Length Features Location/Qualifiers	78 DNA 120 misc_feature 1..120 note=Synthetic Sequence; Figure 9A misc_feature 35 note=y is c or t misc_feature 43 note=s is c or g misc_feature 45 note=s is c or g misc_feature 48 note=v is a, c, or g misc_feature 60 note=k is g or t misc_feature 108 note=y is c or t source 1..120 mol_type=other DNA organism=synthetic construct
3-78-5	NonEnglishQualifier Value Residues	attcgggccg agatgtctcg ctccgtggcc ttagygtgct tscscetvct ctctctttck 60

		ggcctggagg ctatccagcg tgagtctctc ctaccctccc gctctggycc ttcctctccc 120
3-79	Sequences	
3-79-1	Sequence Number [ID]	79
3-79-2	Molecule Type	DNA
3-79-3	Length	105
3-79-4	Features	misc_feature 1..105
	Location/Qualifiers	note=Synthetic Sequence; Figure 12A source 1..105 mol_type=other DNA organism=synthetic construct
3-79-5	NonEnglishQualifier Value Residues	gctcaacaac catgctgggc atctggacc tctacctct ggtgagccct ctccctgccc 60 ggtggaggct taccctgtct tagtcccggg gataggcaaa gtggg 105
3-80	Sequences	
3-80-1	Sequence Number [ID]	80
3-80-2	Molecule Type	DNA
3-80-3	Length	94
3-80-4	Features	misc_feature 1..94
	Location/Qualifiers	note=Synthetic Sequence; Figure 12A misc_feature 33 note=k is g or t misc_feature 36 note=s is c or g misc_feature 37 note=m is a or c misc_feature 38 note=w is a or t misc_feature 39 note=y is c or t misc_feature 40 note=y is c or t misc_feature 41 note=k is g or t misc_feature 43 note=r is a or g misc_feature 44 note=m is a or c misc_feature 47 note=y is c or t misc_feature 48 note=y is c or t misc_feature 50 note=y is c or t misc_feature 51 note=w is a or t misc_feature 52 note=m is a or c misc_feature 54 note=y is c or t misc_feature 55 note=y is c or t misc_feature 56 note=y is c or t misc_feature 57 note=k is g or t misc_feature 60 note=k is g or t misc_feature 63 note=s is c or g misc_feature 79 note=k is g or t misc_feature 94 note=k is g or t source 1..94 mol_type=other DNA organism=synthetic construct
3-80-5	NonEnglishQualifier Value Residues	ttcacttcgg aggattgctc aacaaccatg ctkggsmwyy kgrmccyy cy wmcyykkggk 60 gascctctc cggcccggkg gaaggcttac cck 94

3-81	Sequences	
3-81-1	Sequence Number [ID]	81
3-81-2	Molecule Type	DNA
3-81-3	Length	85
3-81-4	Features	misc_feature 1..85
	Location/Qualifiers	note=Synthetic Sequence; Figure 14A source 1..85 mol_type=other DNA organism=synthetic construct
3-81-5	NonEnglishQualifier Value Residues	gccagtcgtc tgggcggtgc tacaactggg ctggcggcca ggatggttct taggtagggtg 60 gggtcggcgg tcagggtgcc cagag 85
3-82	Sequences	
3-82-1	Sequence Number [ID]	82
3-82-2	Molecule Type	DNA
3-82-3	Length	91
3-82-4	Features	misc_feature 1..91
	Location/Qualifiers	note=Synthetic Sequence; Figure 14A misc_feature 35 note=y is c or t misc_feature 36 note=k is g or t misc_feature 53 note=y is c or t misc_feature 63 note=k is g or t misc_feature 87 note=r is a or g source 1..91 mol_type=other DNA organism=synthetic construct
3-82-5	NonEnglishQualifier Value Residues	cctggccagt cgtctgggcg gtgctacaac tgggykggcg gccaggatgg ttyttaggta 60 ggkggggctcg gcggtcaggt gtcccaragc c 91
3-83	Sequences	
3-83-1	Sequence Number [ID]	83
3-83-2	Molecule Type	DNA
3-83-3	Length	90
3-83-4	Features	misc_feature 1..90
	Location/Qualifiers	note=Synthetic Sequence; Figure 15A source 1..90 mol_type=other DNA organism=synthetic construct
3-83-5	NonEnglishQualifier Value Residues	ggttttgctc tacttcctga agacctgaac accgctccca taaagccatg gcttgccctg 60 gatttcagcg gcacaaggct cagctgaacc 90
3-84	Sequences	
3-84-1	Sequence Number [ID]	84
3-84-2	Molecule Type	DNA
3-84-3	Length	114
3-84-4	Features	misc_feature 1..114
	Location/Qualifiers	note=Synthetic Sequence; Figure 15A misc_feature 32 note=y is c or t misc_feature 34 note=r is a or g misc_feature 37 note=k is g or t misc_feature 56 note=r is a or g misc_feature 62 note=m is a or c source 1..114 mol_type=other DNA organism=synthetic construct
3-84-5	NonEnglishQualifier Value Residues	cttcctgaag acctgaacac cgctcccata ayrgtgkgtc gtgtagtatt tcggcrrcac 60 gmtgatctga tcatgctgac tacgacgtgc tgctcctgctc ttctgctggt tctt 114
3-85	Sequences	

3-85-1	Sequence Number [ID]	85
3-85-2	Molecule Type	DNA
3-85-3	Length	80
3-85-4	Features	misc_feature 1..80
	Location/Qualifiers	note=Synthetic Sequence; Figure 16 source 1..80 mol_type=other DNA organism=synthetic construct
3-85-5	NonEnglishQualifier Value Residues	cgagttccac cgcaccagt gcaacgtggt cgtctacagc agtagcaaag ggaccatccg 60 cctgtgtgac atgcgctcct 80
3-86	Sequences	
3-86-1	Sequence Number [ID]	86
3-86-2	Molecule Type	DNA
3-86-3	Length	93
3-86-4	Features	misc_feature 1..93
	Location/Qualifiers	note=Synthetic Sequence; Figure 16 misc_feature 43 note=m is a or c misc_feature 44 note=w is a or t misc_feature 45 note=k is g or t misc_feature 62 note=k is g or t misc_feature 73 note=m is a or c misc_feature 74 note=s is c or g source 1..93 mol_type=other DNA organism=synthetic construct
3-86-5	NonEnglishQualifier Value Residues	taaccatgca ctcccagaca tcgtggacat caagcctgct aamwkggagg aactaaccaa 60 aktcatcctt gmsccaatt tcaccccca cca 93
3-87	Sequences	
3-87-1	Sequence Number [ID]	87
3-87-2	Molecule Type	DNA
3-87-3	Length	93
3-87-4	Features	misc_feature 1..93
	Location/Qualifiers	note=Synthetic Sequence; Figure 16 misc_feature 43 note=r is a or g misc_feature 46 note=r is a or g misc_feature 51 note=m is a or c misc_feature 56 note=r is a or g misc_feature 59 note=m is a or c misc_feature 62 note=m is a or c misc_feature 66 note=s is c or g misc_feature 67 note=m is a or c misc_feature 68 note=r is a or g misc_feature 71 note=s is c or g misc_feature 73 note=r is a or g source 1..93 mol_type=other DNA organism=synthetic construct
3-87-5	NonEnglishQualifier Value Residues	tgcactccca gacatcgtgg acatcaagcc tgctaacatg gargarctga mcgaartcmt 60 cmctgsmrcc sarttccacc cgcaccagtg caa 93

3-88	Sequences	
3-88-1	Sequence Number [ID]	88
3-88-2	Molecule Type	DNA
3-88-3	Length	92
3-88-4	Features	misc_feature 1..92
	Location/Qualifiers	note=Synthetic Sequence; Figure 21 source 1..92 mol_type=other DNA organism=synthetic construct
	NonEnglishQualifier Value	
3-88-5	Residues	gattctgatg tgtatatcac agacaaaact gtgctagaca tgagggtctat ggacttcaag 60 agcaacagtg ctgtggcctg gagcaacaaa tc 92
3-89	Sequences	
3-89-1	Sequence Number [ID]	89
3-89-2	Molecule Type	DNA
3-89-3	Length	92
3-89-4	Features	misc_feature 1..92
	Location/Qualifiers	note=Synthetic Sequence; Figure 21 misc_feature 47 note=y is c or t misc_feature 48 note=y is c or t misc_feature 49 note=w is a or t misc_feature 54 note=m is a or c misc_feature 57 note=y is c or t misc_feature 58 note=m is a or c misc_feature 60 note=r is a or g misc_feature 64 note=m is a or c misc_feature 67 note=m is a or c source 1..92 mol_type=other DNA organism=synthetic construct
	NonEnglishQualifier Value	
3-89-5	Residues	gattctgatg tgtatatcac agacaaaact gtgctagaca tgagggttywt ggamttymar 60 aacmacmgtg gtggggcctg gaacaacaaa tc 92
3-90	Sequences	
3-90-1	Sequence Number [ID]	90
3-90-2	Molecule Type	DNA
3-90-3	Length	79
3-90-4	Features	misc_feature 1..79
	Location/Qualifiers	note=Synthetic Sequence; Figure 21 source 1..79 mol_type=other DNA organism=synthetic construct
	NonEnglishQualifier Value	
3-90-5	Residues	ccctcaagga gcagcccgcc ctcaatgact ccagatactg cctgagcagc cgcctgaggg 60 tctcgccac cttctggca 79
3-91	Sequences	
3-91-1	Sequence Number [ID]	91
3-91-2	Molecule Type	DNA
3-91-3	Length	79
3-91-4	Features	misc_feature 1..79
	Location/Qualifiers	note=Synthetic Sequence; Figure 21 misc_feature 33 note=m is a or c misc_feature 34 note=r is a or g misc_feature 36 note=w is a or t misc_feature 41 note=s is c or g misc_feature 46

3-91-5	NonEnglishQualifier Value Residues	<p>note=r is a or g misc_feature 49 note=r is a or g misc_feature 52 note=s is c or g misc_feature 58 note=r is a or g misc_feature 69 note=m is a or c misc_feature 74 note=y is c or t source 1..79 mol_type=other DNA organism=synthetic construct</p> <p>ccctcaagga gcagcccgcc ctcaatgact ccmrawactg sctgarcarc cscctgargg 60 tctcgccmc cttytggca 79</p>
<p>3-92 3-92-1 3-92-2 3-92-3 3-92-4</p>	<p>Sequences Sequence Number [ID] Molecule Type Length Features Location/Qualifiers</p>	<p>92 DNA 129 misc_feature 1..129 note=Synthetic Sequence; Figure 47B misc_feature 40 note=k is g or t misc_feature 43 note=s is c or g misc_feature 46 note=y is c or t misc_feature 47 note=y is c or t misc_feature 66 note=k is g or t misc_feature 69 note=r is a or g misc_feature 90 note=r is a or g misc_feature 101 note=k is g or t misc_feature 106 note=r is a or g misc_feature 118 note=r is a or g misc_feature 120 note=s is c or g misc_feature 124 note=r is a or g source 1..129 mol_type=other DNA organism=synthetic construct</p>
3-92-5	NonEnglishQualifier Value Residues	<p>aagctctttc acttcggagg attgctcaac aaccatgctk ggsatyggga ccctcctacc 60 tctggkgarc cctctcctgg ceggggtggar gcttaccocg kottartccc ggggatargs 120 aaartgggg 129</p>
<p>3-93 3-93-1 3-93-2 3-93-3 3-93-4</p>	<p>Sequences Sequence Number [ID] Molecule Type Length Features Location/Qualifiers</p>	<p>93 DNA 44 misc_feature 1..44 note=Synthetic Sequence; Figure 53B source 1..44 mol_type=other DNA organism=synthetic construct</p>
3-93-5	NonEnglishQualifier Value Residues	<p>acaaaactgt gctagacatg aggtctatgg acttcaagag caac 44</p>
<p>3-94 3-94-1 3-94-2 3-94-3</p>	<p>Sequences Sequence Number [ID] Molecule Type Length</p>	<p>94 DNA 43</p>

3-94-4	Features Location/Qualifiers	misc_feature 1..43 note=Synthetic Sequence; Figure 53B misc_feature 25 note=y is c or t misc_feature 26 note=y is c or t misc_feature 27 note=v is a, c, or g misc_feature 32 note=m is a or c misc_feature 35 note=y is c or t misc_feature 36 note=m is a or c misc_feature 38 note=r is a or g misc_feature 42 note=m is a or c source 1..43 mol_type=other DNA organism=synthetic construct	
3-94-5	NonEnglishQualifier Value Residues	acaaaactgt gctagacatg aggtyyvtgg amttymaraa cma	43
3-95	Sequences		
3-95-1	Sequence Number [ID]	95	
3-95-2	Molecule Type	DNA	
3-95-3	Length	44	
3-95-4	Features Location/Qualifiers	misc_feature 1..44 note=Synthetic Sequence; Figure 53B source 1..44 mol_type=other DNA organism=synthetic construct	
3-95-5	NonEnglishQualifier Value Residues	agcagcccgc cctcaatgac tccagatact gctgagcag ccgc	44
3-96	Sequences		
3-96-1	Sequence Number [ID]	96	
3-96-2	Molecule Type	DNA	
3-96-3	Length	45	
3-96-4	Features Location/Qualifiers	misc_feature 1..45 note=Synthetic Sequence; Figure 53B misc_feature 25 note=m is a or c misc_feature 26 note=r is a or g misc_feature 28 note=w is a or t misc_feature 33 note=s is c or g misc_feature 38 note=r is a or g misc_feature 41 note=r is a or g misc_feature 44 note=s is c or g source 1..45 mol_type=other DNA organism=synthetic construct	
3-96-5	NonEnglishQualifier Value Residues	gagcagcccg cctcaatga ctccmrawac tgsctgarca rccsc	45
3-97	Sequences		
3-97-1	Sequence Number [ID]	97	
3-97-2	Molecule Type	DNA	
3-97-3	Length	86	
3-97-4	Features Location/Qualifiers	misc_feature 1..86 note=Synthetic Sequence; Figure 79A misc_feature 66 note=r is a or g source 1..86	

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3-97-5	NonEnglishQualifier Value Residues	mol_type=other DNA organism=synthetic construct caactgggct ggcggccagg atggttctta ggtaggtggg gtcggcggtc aggtgtccca 60 gagccrgggg tctggagga ccttcc 86
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