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(54) Title: FOXP3 ENGINEERED CD4+ T CELLS FOR USE IN TREG-BASED IMMUNOTHERAPY

(57) Abstract: Engineered Treg-like cells, $CD4^{LVFOXP3}$ T cells, and their use in cellular therapy to promote immune tolerance are disclosed. In particular, $CD4^{LVFOXP3}$ T cells are produced by transduction of $CD4^{+}$ T cells with a lentiviral vector expressing FOXP3 under the control of a constitutive promoter. Transduced cells express FOXP3 at high and persistent levels and acquire immune suppressive characteristics resembling naturally occurring Treg cells.

FOXP3 ENGINEERED CD4+ T CELLS FOR USE IN TREG-BASED IMMUNOTHERAPY

Cross Reference

[0001] This application claims benefit of U.S. Provisional Patent Application No. 62/858,828, filed June 7, 2019, U.S. Provisional Patent Application No. 62/976,233, filed February 13, 2020, and U.S. Provisional Patent Application No. 62/994,454, filed March 25, 2020, which applications are incorporated herein by reference in their entirety.

Background

[0002] Immune regulation, including suppression of autoreactivity, is a fundamental function of a healthy immune system. Regulatory T (Treg) cells actively engage in the maintenance of immunological self-tolerance and immune homeostasis. The transcription factor, Forkhead Box P3 (*FOXP3*), is the key transcription factor for the function of Treg cells, which are of thymic origin, highly express CD25 and downregulate CD127. Thymic derived Treg cells are strictly dependent on persistent, high FOXP3 expression to exert their suppressive function and modulate immune responses, maintain tolerance to self-antigens and prevent autoimmunity. The occurrence of severe autoimmunity, allergy and immunopathology in humans and rodents with mutated FOXP3 genes demonstrates that deficiency or dysfunction of thymic derived $CD4^+CD25^+Foxp3^+$ Treg cells alone is sufficient to break self-tolerance in an otherwise normal immune system (reviewed in Bacchetta R. et al., Ann N Y Acad Sci., 2016).

[0003] The key role of Treg cells in maintaining tolerance is exemplified by loss-of-function *FOXP3* mutations resulting in primary Treg cell dysfunction and the monogenic autoimmune immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Barzaghi F, et al. Front Immunol., 2012). IPEX syndrome is a deadly X-linked immunodeficiency with severe multiple autoimmune manifestations, most notably in the skin, gastrointestinal tract, and endocrine organs, leading to acute life-threatening or chronic inflammation, lymphoproliferation and autoantibody-mediated pathologies. Without treatment, IPEX is rapidly fatal.

[0004] A worldwide survey of the IPEX disease course and the treatment outcomes by collection of data on 96 IPEX patients from multiple centers was performed, including those that are part of the Primary Immune Deficiency Treatment Consortium (PIDTC) and the European Society of Immune Deficiencies Inborn Errors Working Party (ESID-IEWP) (Barzaghi F JACI, 2018). Results from this survey, the largest available for IPEX patients, revealed that 50% of the patients have onset of their disease in the first month of life, while 45% have onset during the first year of life. Only 5% of patients have onset of their disease after the first year, and these cases are usually less severe or atypical. The typical onset is characterized by refractory diarrhea, less often by neonatal type 1 diabetes (T1D) as the first

symptom, both associated with severe eczema. However, with disease progression, one out of two patients develop T1D. Usually, IPEX has an acute and devastating onset, which is poorly controlled by pharmacological immunosuppression (IS). During this initial phase of the disease which is the most critical for survival, severe and irreversible organ damage may occur, negatively impacting disease prognosis and treatment outcome. Although rare, the disease has wide geographic distribution and its diagnosis has progressively increased in the past decade, as knowledge of the disease has been disseminated. Although the number of diagnosed patients has increased, advances in therapies for IPEX have not followed.

[0005] IPEX patients need prompt therapeutic intervention at onset. The treatment of IPEX syndrome currently relies on nutritional support, replacement therapy for endocrine organ failure, multiple IS drugs, and hematopoietic stem cell transplantation (HSCT). Allogenic HSCT has proven curative, however, it is not available to every patient. Therefore, limited donor availability and the toxicities of allogenic HSCT make this approach less than ideal, especially for patients under 1-year of age. Data from our survey showed that overall survival of IPEX patients after HSCT is 73% with the greatest number of deaths occurring early after transplantation (Barzaghi JACI, 2018). In this same study, the degree of organ impairment at the time of HSCT was the most important variable impacting outcome, independent of age, donor source or conditioning regimen. This strongly supports the importance of stabilizing the patient's clinical condition before HSCT.

[0006] Patients not eligible for HSCT, who survive the acute onset period, may stabilize and become more responsive to IS, while this possibility might depend on type of mutation and relative residual protein function. In such cases, patients may reach young adulthood, despite the persistence of disease recurrence, significant organ damage, and new autoimmune manifestations through the years. Indeed, our survey showed that, although the overall survival of patients on chronic IS is not statistically different from that of patients who received HSCT, there was a progressive deterioration of the clinical status of patients receiving IS.

[0007] Overall, there is substantial evidence to demonstrate that available treatments are only partially beneficial. Therefore, a therapy aimed specifically at restoring Treg-functions by replacing the dysfunctional Treg cell subsets, targeting the main defect in this rare disease caused by *FOXP3* mutations, would address a significant unmet medical need.

[0008] IPEX syndrome clinical manifestations recapitulate those occurring in many other autoimmune diseases more commonly observed in the general population. These autoimmune diseases are not due to monogenic defects but rather results from genetic predisposition and environmental co-morbidity factors. Nevertheless, abnormal function or number of Treg cells are involved in their pathogenesis and Treg cell immunotherapy is envisaged as innovative treatment for these diseases (Bluestone JA, Science 2018).

SUMMARY

[0009] Engineered $CD4^{LVFOXP3}$ T cells and their use in cellular therapy to promote immune tolerance are provided. In particular, $CD4^{LVFOXP3}$ T cells are produced by transfection of $CD4^+$ T cells with a lentiviral vector expressing FOXP3 under the control of a ubiquitous constitutive promoter, including without limitation the human EF1α promoter. Transduced cells persistently express FOXP3 at high levels and acquire immune suppressive characteristics resembling naturally occurring Treg cells.

[0010] In an embodiment, a method of producing $CD4^{LVFOXP3}$ T cells is provided, the method comprising: a) collecting a sample comprising $CD4^+$ T lymphocytes from an individual; b) activating $CD4^+$ T cells, e.g. by polyclonal activation through binding the T cell receptor; c) transducing the $CD4^+$ T lymphocytes with the recombinant lentiviral vector under conditions wherein FOXP3 is constitutively expressed, resulting in conversion of the $CD4^+$ T lymphocytes into regulatory T cell (Treg)-like cells, i.e. $CD4^{LVFOXP3}$ T cells. The cells thus generated find use, for example, in screening assays, therapeutic methods, and the like. Figure 1 provides an exemplary flow chart.

[0011] In another embodiment, a method of adoptive cellular immunotherapy for treating an inflammatory condition is provided, the method comprising: a) collecting a sample comprising $CD4^+$ T lymphocytes from a subject; b) activating $CD4^+$ T cells, e.g. by polyclonal activation through binding the T cell receptor; c) transducing the $CD4^+$ T lymphocytes with the recombinant lentiviral vector under conditions wherein the FOXP3 is constitutively expressed resulting in conversion of the $CD4^+$ T lymphocytes into regulatory T cell (Treg)-like cells, i.e. $CD4^{LVFOXP3}$ T cells; d) purifying by positive isolation the transduced $CD4^{LVFOXP3}$ T cells; e) activating the isolated transduced $CD4^{LVFOXP3}$ T cells e.g. by polyclonal activation through binding the T cell receptor for expansion; and f) administering a therapeutically effective amount of the expanded $CD4^{LVFOXP3}$ T cells to the subject. The biological sample can be any sample comprising $CD4^+$ T lymphocytes, including, for example, without limitation, blood or tissue where $CD4^+$ T lymphocytes are present. In some embodiments, the method further comprises isolating $CD4^+$ T lymphocytes from the biological sample.

[0012] The biological sample can be any sample comprising $CD4^+$ T lymphocytes, including, for example, without limitation, blood or tissue where $CD4^+$ T lymphocytes are present. $CD4^+$ T lymphocyte populations may comprise naïve T cells, memory T cells, total T cells; etc. In some embodiments, the method further comprises isolating $CD4^+$ T lymphocytes from the biological sample.

[0013] In certain embodiments, the method further comprises substantially purifying the $CD4^{LVFOXP3}$ T cells. In some embodiments, the $CD4^{LVFOXP3}$ T cells are substantially purified by

positive selection for the cell surface marker encoded by the recombinant lentiviral vector. For example, if the cell surface marker is a truncated nerve growth factor receptor (tNGFR), the $CD4^{LVFOXP3}$ T cells can be substantially purified by positive selection for the tNGFR cell surface marker using for example, without limitation, immunomagnetic separation or flow cytometry. Alternatively, to enrich for transduced cells the culture is maintained in the presence of a concentration of rapamycin effective to reduce the number of non-transduced cells.

[0014] In certain embodiments, the method further comprises culturing the $CD4^+$ T lymphocytes during and after transduction. In some embodiments, the method of claim further comprises adding IL-2 and IL-7 to a culture of $CD4^+$ T lymphocytes to expand the number of $CD4^+$ T lymphocytes in the culture.

[0015] In certain embodiments, the method further comprises culturing the $CD4^{LVFOXP3}$ T cells. In some embodiments, the method further comprises adding IL-2 and IL-15 to a culture of $CD4^{LVFOXP3}$ T cells to expand the number of $CD4^{LVFOXP3}$ T cells in the culture.

[0016] In certain embodiments, the method further comprises inducing or isolating a subpopulation of T lymphocytes comprising a T cell receptor specific for an antigen of interest such as, an alloantigen, an autoantigen, or an allergen, and transducing as described herein to generate a population of antigen-specific $CD4^{LVFOXP3}$ T cells.

[0017] In one aspect, a bidirectional lentiviral vector of not more than about 11,000 bp, usually not more than about 10,500 bp and may not more than about 10,100 bp, comprising full-length *FOXP3* cDNA expressed under an EF1α promoter and *NGFR* expressed under the minimal CMV promoter from the opposite strand is utilized. The vector may comprise each of: a) a polynucleotide encoding forkhead box protein 3 (FOXP3), conferring to the CD4+ T lymphocytes specific regulatory function; b) an elongation factor 1a (EF1α) promoter, wherein the EF1α promoter is operably linked to the polynucleotide encoding FOXP3; c) a polynucleotide encoding a cell surface marker for tracking and selection of cells transfected with the vector; d) a mutated Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) modified to remove a WHx start codon; e) human immunodeficiency virus (HIV)-derived elements comprising a 5' long terminal repeat (5' LTR), a Ψ packaging signal, a truncated Gag sequence, a rev response element (RRE) sequence, a central polypurine tract (cPPT), a central termination sequence (CTS), a truncated negative regulatory factor (NEF) sequence, and a partially deleted and self-inactivated (SIN) 3' long terminal repeat (3' LTR); f) a polyadenylation sequence; g) an SV40 origin of replication; h) a bacterial high copy origin of replication (Ori) and (i) kanamycin resistance gene. In one embodiment, the cell surface marker is a truncated nerve growth factor receptor (tNGFR). An exemplary lentiviral expression vector comprises the components arranged as depicted in the vector map shown in FIG. 2, or as set out in Table 2. In one embodiment, the recombinant

lentiviral vector comprises the nucleotide sequence of SEQ ID NO:2 or a sequence having at least about 80-100% sequence identity thereto, including any percent identity within this range, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity thereto, wherein the recombinant lentiviral vector is capable of generating a $CD4^{LVFOXP3}$ T cells by transduction of a $CD4^+$ T lymphocyte. The $CD4^{LVFOXP3}$ T cells produced by this method are Treg-like cells called $CD4^{LVFOXP3}$ T cells.

[0018] In certain embodiments, the method further comprises transducing the $CD4^+$ T lymphocytes with a recombinant polynucleotide encoding an exogenous T cell receptor or a chimeric antigen receptor (CAR). In other embodiments, the method further comprises culturing the transduced Treg-like lymphocytes to increase expression of homing receptors, e.g. increasing expression of receptor that provide for enhanced migration to the intestinal mucosa, which requires the expression of very specific homing receptors on T cells, integrin α4β7 and chemokine receptor CCR9.

[0019] In another aspect, $CD4^{LVFOXP3}$ T cells produced by the methods described herein are provided. In another aspect, a composition comprising $CD4^{LVFOXP3}$ T cells for use in treatment of an inflammatory condition is provided. In some embodiments, a composition of $CD4^{LVFOXP3}$ T cells is substantially purified free of other cells. In some embodiments, the composition further comprises a pharmaceutically acceptable excipient. In another embodiment, a composition comprising a $CD4^{LVFOXP3}$ T cell for use in treatment of IPEX syndrome is provided.

[0020] In another aspect, a method of treating an inflammatory, e.g. an autoimmune, condition in a subject is provided, the method comprising administering a therapeutically effective amount of a composition comprising $CD4^{LVFOXP3}$ T cells to the subject. The composition is generally administered in an amount sufficient to reduce inflammation in the subject. In certain embodiments, multiple cycles of treatment are administered to the subject.

[0021] In certain embodiments, the $CD4^+$ T lymphocytes, from which the $CD4^{LVFOXP3}$ T cells are derived (i.e., by transduction with a lentiviral vector expressing FOXP3 as described herein), are autologous or allogeneic.

[0022] In certain embodiments, the method further comprises activating the $CD4^+$ T lymphocytes before transduction. In some embodiments, the method further comprises adding IL-2 and IL-15 to a culture of $CD4^+$ T lymphocytes to expand the number of $CD4^+$ T lymphocytes in the culture. Culturing may be performed *in vitro* or *ex vivo.*

[0023] In certain embodiments, the method further comprises culturing the $CD4^{LVFOXP3}$ T cells before administration to a subject. In some embodiments, the method further comprises adding IL-2 and IL-15 to a culture of $CD4^{LVFOXP3}$ T cells to expand the number of $CD4^{LVFOXP3}$ T cells in the culture. Culturing may be performed *in vitro* or *ex vivo.*

[0024] The methods described herein can be used to treat inflammatory conditions, including for example, without limitation, Treg deficiency, autoimmune disorders, allergies, acute inflammatory syndromes, graft-versus-host disease, and transplant rejection. In one embodiment, the Treg deficiency/autoimmune disorder is IPEX syndrome. In another embodiment, the method is performed prior to performance of a tissue or organ transplant, in conjunction with a tissue or organ transplant, or after a tissue or organ transplant.

[0025] In another embodiment, a method of treating immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome is provided, the method comprising administering a therapeutically effective amount of a composition comprising $CD4^{LVFOXP3}$ T cells to the subject, as described herein.

[0026] $CD4^{LVFOXP3}$ T cells may be administered by any suitable mode of administration. In some embodiments, the $CD4^{LVFOXP3}$ T cells are administered intravenously or intra-arterially. In another embodiment, the $CD4^{LVFOXP3}$ T cells are administered locally at a site of inflammation. In another embodiment, the $CD4^{LVFOXP3}$ T cells are administered locally at a site of a tissue or organ transplant.

[0027] In one embodiment, an animal model for FOXP3 deficiency is provided. In such an animal model, FOXP3 deficient $CD34^{+}$ hematopoietic stem and progenitor cells (HSPCs) from a human donor are transplanted intrahepatically in neonatal pups, e.g. of about 3-5 days old, of immune deficient mice. Suitable mouse strains include, for example, NSG mice, SGM3_NSG mice, etc. that lack mature T cells, B cells, and functional NK cells, and are deficient in mouse cytokine signaling. The FOXP3 deficient $CD34^{+}$ HSPCs can be obtained from a human donor that is deficient in FOXP3, e.g. an IPEX patient; or alternatively obtained from an individual with normal FOXP3 expression, where the HSPCs are genetically manipulated *in vitro* to reduce FOXP3 expression, e.g. by targeting FOXP3 with a CRISPR-Cas9 RNP construct. The resulting animal model has human immune system cells deficient in FOXP3 expression.

[0028] The efficacy of engineered $CD4^{LVFOXP3}$ T cells in preventing and controlling lymphoproliferation of FOXP3 deficient CD4+T cells can be determined by administering such engineered cells to the mouse model described above and determining the effect on lymphoproliferation.

[0029] The safety and/or efficacy of engineered $CD4^{LVFOXP3}$ T cells in completion and expansion of an immune response to pathogens, including but not limited to fungi, e.g. candida albicans; virus. e.g. adenovirus; protozoan pathogens, bacterial pathogens, etc. can be determined by administering such engineered cells to the immune deficient mouse model mentioned above in combination with human T cells primed with a pathogen, and determining the effect of the engineered cells on the immune response.

[0030] The safety and/or efficacy of engineered $CD4^{LVFOXP3}$ T cells in immune surveillance and tumor clearance can be determined by administering such engineered cells to the immune deficient mouse model mentioned above in combination with a tumor xenograft, e.g. skin sarcoma, and determining the effect of the engineered cells on the ability of human peripheral blood mononuclear cells (PBMC) to develop an immune response and clear the tumor.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0032] FIG. 1 shows a process flow chart for $CD4^{LVFOXP3}$ production.

[0033] FIG. 2. A. shows the LVFOXP3 Transfer Plasmid Map. The map shows all components of the 10,036 bp transfer plasmid pLVFOXP3 used for vector production by transient transfection of HEK293T cells. B. LVFOXP3 Transfer Plasmid Component Descriptions.

[0034] FIGS. 3A-3E show $CD4^{LVFOXP3}$ obtained upon optimization protocol from total $CD4^+$ T cells from healthy donors (n = 8). (A) Transduction efficiency, measured as percentage of $NGFR^+$ cells, at day 6 post-transduction; purity of transduced cells measured at day 10 post-transduction as percentage of $NGFR^+$ cells and vector copy number measured by PCR at day 24 (end of culture). Results of $CD4^{LVFOXP3}$ (CD4FOXP3) and of $CD4^{LVNGFR}$ (CD4NGFR) control cells are shown. (B) Percentage of positive cells for FOXP3, NGFR, CD25 and CD127 at day 24. (C) Cytokine production by $CD4^{LVFOXP3}$ (CD4FOXP3) and of $CD4^{LVNGFR}$ (CD4NGFR) control cells. All cytokines are measured by ELISA. IL-2 at 24 hours and the other cytokines at 72 hours after anti-CD3/CD28 activation (1:25=bead: cell). (D) Expression of other Tregs-related molecules. CTLA4, PD-1, ICOS, HELIOS, GITR, IL-1R1, IL-6R, TIGIT, CD226, TIM3 and LAG3 were measured by FACS. (n=6, MEAN+SEM) or (E) mRNA (FOXP3, NGFR, EOS, LGMN, IL-12A and EBI3) were measured by real time PCR.

[0035] FIGS. 4A-4B show $CD4^{LVFOXP3}$ cells obtained upon optimization protocol from total $CD4^+$ T cells from healthy donors (n=8) and two IPEX patients. (A) Percentage of suppression (median and SEM) from 8 different donors obtained either with the previously described methods (APC/Feeder) or with the recently optimized method more GMP compliant (TransAct optimized cGMP). Proliferation of responder T cells was determined by CFSE staining and measured by division index after 4 days of activation. $CD4^{LVFOXP3}$ were added at different responder: suppressor cells ratios as indicated. (B) Percentage of suppression by $CD4^{LVFOXP3}$

obtained from IPEX #37, with c.1150 G>A and preserved endogenous mutFOXP3 and IPEX #64, c.1270_1272delinsC which abrogates endogenous FOXP3 expression.

[0036] FIG. 5 shows an assessment of FOXP3 expression from fresh or frozen $CD4^{LVFOXP3}$. $CD4^{LVFOXP3}$ were either tested at the end of the culture (fresh) or were frozen and thawed (Frozen). The result of this experiment performed with cells from three different subjects is shown.

[0037] FIG. 6 shows the gene expression profile of the $CD4^{LVFOXP3}$ in comparison to freshly isolated Tregs or non-Tregs or controls transduced cells with NGFR only (no FOXP3). (a) Principle component analysis (PCA) (b) Hierarchical cluster analysis (HCA) (c) Shared DEGs (up-regulated or downregulated) between FOXP3 Tregs and $CD4^{LVFOXP3}$ and the 7 genes shared between FOXP3 Tregs and $CD4^{LVFOXP3}$.

[0038] FIG.7 shows that the TCR repertoire of $CD4^{LVFOXP3}$ remains polyclonal and similar to that of the starting population of CD4+ T cells.

[0039] FIG. 8 shows that both $CD4^{LVFOXP3}$ autologous or allogeneic to the responder T cells can efficiently prevent xenoGvHD. The experimental design (a) and results from one representative experiment are shown (b). Autologous and allogeneic $CD4^{LVFOXP3}$ generated in parallel were co-injected with effector CD4+ T cells. $CD4^{LVFOXP3}$autologous or allogeneic showed similar engraftment and *in vivo* survival. Percentage engraftment of total CD45+ human cells (c) and of NGFR+ transduced cells (d) is higher when $CD4^{LVFOXP3}$ are autologous to the effector cells.

[0040] FIG. 9 shows the phenotype of human T cells obtained from the spleen of xenoGVHD-hu-mice treated or not with $CD4^{LVFOXP3}$. Results (dot plot) obtained from one mouse (above) and cumulative data from multiple mice (below) are shown, comparing mice injected with $CD4^+$ T cells alone (Teff) or co-injected with allogeneic or autologous $CD4^{LVFOXP3}$ (LVFOXP3 Allo and LVFOXP3 Auto, respectively) or control $CD4^{LVNGFR}$ (LVNGFR). CD45 expression indicates the human cell engraftment. CD25, CD69, CD71 and HLA-DR indicate activation markers. Despite comparable level of engraftment, T cells isolated from spleen of mice treated with $CD4^{LVFOXP3}$ expressed significantly lower levels of activation markers.

[0041] FIG. 10 shows rechallenge with $CD4^+$ Teff cells in a xenoGVHD hu-mouse model. Overall survival (a), weight (b), human CD45+ cell engraftment (c) and NGFR+ transduced cell engraftment (d) are shown. Sublethally irradiated mice were injected with human $CD4^+$ Teff cells alone (black line) or with $CD4^{LVFOXP3}$ (blue line) or control $CD4^{LVNGFR}$ (grey line). After one week the mice injected with Teff alone or with control $CD4^{LVNGFR}$ started to lose weight and by week 3 had to be sacrificed because of the xenoreaction, whereas the mice coinjected with $CD4^{LVFOXP3}$ continued to be healthy. At about week 2, these mice were re-injected with Teff cells at the same initial dose ($2x10^6$/mouse). Despite re-challenge the mice that were

previously exposed to $CD4^{LVFOXP3}$ did not develop xenoGVHD. As an additional control, some of the mice were injected with Teff alone for the first time (Teff-late, dashed line) in parallel to the rechallenged mice. Also, these mice died earlier than the $CD4^{LVFOXP3}$ and rechallenged mice.

[0042] FIG. 11 Diagram of the FOXP3-deficient hu-mouse model (a) in which FOXP3 is KO using CRISPR/Cas9 RNP in human CD34+ HSPCs (HSC) and the resulting cells engraft and reconstitute an immunodeficient mouse (NSG or SGM3-NSG), leading to increased CD4+ T cell lymphoproliferation (b) and reduced FOXP3+/CD25+/CD127neg Treg cells (c).

[0043] FIG. 12 shows the results obtained using the FOXP3-deficient hu-mouse model, after injecting these mice with $CD4^{LVFOXP3}$. Survival of the mice who received at week 12 the $CD4^{LVFOXP3}$ was better as compared to those reconstituted with FOXP3 -deficient HSC (a). Despite the transduced T cells slowly decreased in the peripheral blood of the mice (b), the animal that received $CD4^{LVFOXP3}$, normalized their engraftment (c), CD4 and CD8 percentages and absolute numbers (d and e).

[0044] FIG 13. Shows the experimental protocol (a) and the results of the experiments assessing the impact of $CD4^{LVFOXP3}$ injection on the expansion of an immune response to pathogens such as candida albicans (b and c) or adenovirus (d and e), using PBMC+ mature dendritic cells (mDC) pulsed with the antigen. Mice injected with $CD4^{LVFOXP3}$ preserved an immune response that was comparable to that of control mice uninjected or injected with NGFR transduced cells. Results are expressed as IFNγ+ cells determined by Elispot.

[0045] FIG 14 shows the experimental design (a) and the results of the experiment assessing the impact of $CD4^{LVFOXP3}$ on tumor clearance by normal PBMC (b-e). Clearly, the tumor size increased only in the control mice that were not injected with PBMC, while it was reduced in the mice injected with PBMC as well as in the mice injected with PBMC+$CD4^{LVFOXP3}$ indicating that the transduced cells did not impair tumor clearance.

[0046] FIG 15 shows the experimental design (a) and results of *in vivo* reduction of the $CD4^{LVFOXP3}$ upon *in vivo* exposure to Basiliximab, indicating that this drug could be used *in vivo* to eliminate the $CD4^{LVFOXP3}$ in case of undesired toxicity.

[0047] FIG 16 shows in vitro phenotypic stability and survival of the $CD4^{LVFOXP3}$ in the presence of rapamycin, indicating that $CD4^{LVFOXP3}$ could be administered together with rapamycin without this drug negatively affecting the $CD4^{LVFOXP3}$.

Detailed Description of Embodiments

[0048] Engineered $CD4^{LVFOXP3}$ T cells and their use in cellular therapy to promote immune tolerance are disclosed. In particular, $CD4^{LVFOXP3}$ T cells are produced by transduction of $CD4^+$ T cells with a lentiviral vector expressing FOXP3 under the control of a constitutive promoter.

Transduced cells express FOXP3 at high and persistent levels and acquire immune phenotypic and immune suppressive characteristics resembling naturally occurring Treg cells. Methods of preclinical assessments of efficacy and safety of the $CD4^{LVFOXP3}$ T cells and methods of using such $CD4^{LVFOXP3}$ T cells in cellular therapy for treating various inflammatory conditions benefitting from increased immune tolerance, such as IPEX syndrome, as well as other conditions associated with Treg deficiency of different origin, inflammation, and/or immune dysregulation such as autoimmune diseases, graft-versus-host disease, transplant rejection, and allergies are also provided.

[0049] Before the engineered $CD4^{LVFOXP3}$ T cells and their use in treatment of inflammatory conditions are further described, it is to be understood that this invention is not limited to a particular method or composition described, as such may, of course, vary. 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, since the scope of the present invention will be limited only by the appended claims.

[0050] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0051] 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 this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0052] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention.

Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0053] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the regulatory T cell-like cells" includes reference to one or more regulatory T cell-like cells and equivalents thereof, e.g. $CD4^{LVFOXP3}$ cells, Treg-like cells, or engineered Tregs, known to those skilled in the art, and so forth.

[0054] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0055] "Immune tolerance", or "tolerogenic" refers to cells capable of suppressing or down-modulating an adaptive or innate immunological response.

[0056] The term "biological sample" encompasses a clinical sample. The types of "biological samples" include, but are not limited to: tissue obtained by surgical resection, tissue obtained by biopsy, cells in culture, cell supernatants, cell lysates, tissue samples, organs, bone marrow, blood, plasma, serum, fine needle aspirate, lymph node aspirate, cystic aspirate, a paracentesis sample, a thoracentesis sample, and the like.

[0057] The terms "obtained" or "obtaining" as used herein can also include the physical extraction or isolation of a biological sample (e.g., comprising $CD4^+$ T lymphocytes) from a subject. Accordingly, a biological sample comprising $CD4^+$ T lymphocytes can be isolated from a subject (and thus "obtained") by the same person or same entity that subsequently isolates $CD4^+$ T lymphocytes from the sample and produces $CD4^{LVFOXP3}$ T cells (transduced with a lentiviral vector expressing FOXP3) from the original unmodified $CD4^+$ T lymphocytes in the sample. When a biological sample is "extracted" or "isolated" from a first party or entity and then transferred (e.g., delivered, mailed, etc.) to a second party, the sample was "obtained" by the first party (and also "isolated" by the first party), and then subsequently "obtained" (but not "isolated") by the second party. Accordingly, in some embodiments, the step of obtaining does not comprise the step of isolating a biological sample.

[0058] In some embodiments, the step of obtaining comprises the step of isolating a biological sample (e.g., a pre-treatment biological sample, a post-treatment biological sample, etc.). Methods and protocols for isolating various biological samples (e.g., a blood sample, a biopsy sample, an aspirate, etc.) will be known to one of ordinary skill in the art and any convenient method may be used to isolate a biological sample.

[0059] "Substantially purified" generally refers to isolation of a component of a sample (e.g., cell or substance), such that the component comprises the majority percent of the sample in which it resides. Typically, in a sample, a substantially purified component comprises at least 70%, preferably at least 80%-85%, more preferably at least 90-99% of the sample.

[0060] The terms "treatment", "treating", "treat" and the like are used herein to generally refer to obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disease or symptom(s) thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. The term “treatment" encompasses any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease and/or symptom(s) from occurring in a subject who may be predisposed to the disease or symptom but has not yet been diagnosed as having it; (b) inhibiting the disease and/or symptom(s), i.e., arresting their development; or (c) relieving the disease symptom(s), i.e., causing regression of the disease and/or symptom(s). In terms of clinical trials for evaluation of safety, efficacy may not be a primary endpoint, but rather lack of toxicity may be considered treatment. Those in need of treatment include those already inflicted as well as those in which prevention is desired (e.g., those with increased susceptibility to an autoimmune disease, etc.)

[0061] A therapeutic treatment includes one in which the subject is inflicted prior to administration and a prophylactic treatment is one in which the subject is not inflicted prior to administration. In some embodiments, the subject has an increased likelihood of becoming inflicted or is suspected of being inflicted prior to treatment. In some embodiments, the subject is suspected of having an increased likelihood of becoming inflicted.

[0062] "Pharmaceutically acceptable excipient or carrier" refers to an excipient that may optionally be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient.

[0063] "Pharmaceutically acceptable salt" includes, but is not limited to, amino acid salts, salts prepared with inorganic acids, such as chloride, sulfate, phosphate, diphosphate, bromide, and nitrate salts, or salts prepared from the corresponding inorganic acid form of any of the preceding, e.g., hydrochloride, etc., or salts prepared with an organic acid, such as malate, maleate, fumarate, tartrate, succinate, ethylsuccinate, citrate, acetate, lactate, methanesulfonate, benzoate, ascorbate, para-toluenesulfonate, palmoate, salicylate and stearate, as well as estolate, gluceptate and lactobionate salts. Similarly salts containing pharmaceutically acceptable cations include, but are not limited to, sodium, potassium, calcium, aluminum, lithium, and ammonium (including substituted ammonium).

[0064] An "effective amount" of a composition comprising $CD4^{LVFOXP3}$ T cells (i.e., $CD4^{+}$ T lymphocytes transfected with a lentiviral vector expressing FOXP3) is an amount sufficient to

safely effect beneficial or desired results, such as an amount that suppresses activation and proliferation of effector T cells and increases immune tolerance. An effective amount can be administered in one or more administrations, applications, or dosages.

[0065] By "therapeutically effective dose or amount" of a composition comprising $CD4^{LVFOXP3}$ T cells (i.e., $CD4^+$ T lymphocytes transfected with a lentiviral vector expressing FOXP3) is intended an amount that, when administered as described herein, brings about a positive therapeutic response, such as improved recovery from an inflammatory condition such as, but not limited to, an autoimmune manifestation, an allergy, an acute inflammatory response, graft-versus-host disease, and transplant rejection. Improved recovery may include a reduction in inflammation, pain, or autoimmune-induced tissue damage, or better graft tolerance and prolonged survival of transplanted cells, tissue or organs. Improved recovery may include an improvement towards normalization of parameters such as ALC or specific cell subsets, eosinophils, CRP, cytokines in plasma, markers of specific organ function, Ab levels, and the like. Additionally, a therapeutically effective dose or amount may compensate for functional (e.g., IPEX syndrome) or quantitative Treg-deficiency and reduce the need for immunosuppressive or anti-inflammatory drugs. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, the particular drug or drugs employed, mode of administration, and the like. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation, based upon the information provided herein. For example, an effective unit dose may be 10^6 $CD4^{LVFOXP3}$ T cells /kg, 3×10^6 $CD4^{LVFOXP3}$ T cells /kg, 10^7 $CD4^{LVFOXP3}$ T cells /kg, 10^8 $CD4^{LVFOXP3}$ T cells /kg, 10^9 $CD4^{LVFOXP3}$ T cells /kg, or more.

[0066] By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macro molecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome. The term "isolated" when referring to a cell, is a cell that is separate and discrete from the whole organism with which the cell is found in nature.

[0067] "Substantially purified" generally refers to isolation of a substance (compound, drug, polynucleotide, protein, polypeptide) such that the substance comprises the majority percent of the sample in which it resides. Typically in a sample, a substantially purified component comprises 50%, preferably 80%-85%, more preferably 90-95% of the sample. Techniques for

purifying substances of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density.

[0068] The terms “recipient”, “individual”, “subject”, “host”, and “patient”, are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, sheep, goats, pigs, etc. Preferably, the mammal is human.

[0069] The term “unit dosage form,” as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the agents calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the unit dosage forms for use in the present invention depend on the particular compound employed and the effect to be achieved, the pharmacodynamics associated with each compound in the host, and the like.

[0070] "Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, viral, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation, is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

[0071] The term "transformation" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion. For example, direct uptake, transduction or f-mating are included. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. Transduction, where an exogenous polynucleotide is integrated into the host genome, i.e. the T lymphocyte genome, is preferred for the methods described herein.

[0072] "Recombinant host cells", "host cells," "cells", "cell lines," "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transferred DNA, and include the original progeny of the original cell which has been transfected.

[0073] "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter

operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper enzymes are present. Expression is meant to include the transcription of mRNA from a DNA or RNA template and can further include translation of a protein from an mRNA template. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

[0074] A "vector" is capable of transferring nucleic acid sequences to target cells (e.g., viral vectors, non-viral vectors, particulate carriers, and liposomes). Typically, "vector construct," "expression vector, " and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a nucleic acid of interest and which can transfer nucleic acid sequences to target cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

[0075] The terms "variant" refers to biologically active derivatives of the reference molecule that retain desired activity. In general, the term "variant" refers to molecules having a native sequence and structure with one or more additions, substitutions (generally conservative in nature) and/or deletions, relative to the native molecule, so long as the modifications do not destroy biological activity, and which are "substantially homologous" to the reference molecule. In general, the sequences of such variants will have a high degree of sequence homology to the reference sequence, e.g., sequence homology of more than 50%, generally more than 60%-70%, even more particularly 80%-85% or more, such as at least 90%-95% or more, when the two sequences are aligned.

[0076] "Gene transfer" or "gene delivery" refers to methods or systems for reliably inserting DNA or RNA of interest into a host cell. Such methods can result in transient expression of non-integrated transferred DNA, extrachromosomal replication and expression of transferred replicons (e.g., episomes), or integration of transferred genetic material into the genomic DNA of host cells.

[0077] The term "derived from" is used herein to identify the original source of a molecule but is not meant to limit the method by which the molecule is made which can be, for example, by chemical synthesis or recombinant means.

[0078] A polynucleotide "derived from" a designated sequence refers to a polynucleotide sequence which comprises a contiguous sequence of approximately at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, and even more preferably at least about 15-20 nucleotides corresponding, i.e., identical or complementary to, a region of the designated nucleotide sequence. The derived

polynucleotide will not necessarily be derived physically from the nucleotide sequence of interest, but may be generated in any manner, including, but not limited to, chemical synthesis, replication, reverse transcription or transcription, which is based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. As such, it may represent either a sense or an antisense orientation of the original polynucleotide.

Engineered Treg-Like Cells, $CD4^{LVFOXP3}$ T cells.

[0079] Compositions, methods, and kits are provided for producing and using engineered $CD4^{LVFOXP3}$ T cells expressing FOXP3. FOXP3 is a transcription factor essential for the function of natural Tregs in maintenance of immune tolerance. Lentivirus-mediated expression of FOXP3 in $CD4^+$ T lymphocytes endows cells with Treg-like characteristics, including the ability to suppress immune responses of effector T cells and other immune cells. $CD4^{LVFOXP3}$ T cells are useful for increasing immune tolerance to antigens in a subject such as alloantigens, autoantigens, and allergens. Accordingly, pharmaceutical compositions comprising such engineered $CD4^{LVFOXP3}$ T cells are useful for treating inflammatory and immune dysregulatory conditions benefitting from increased immunological tolerance, including, but not limited to, Treg deficiency, autoimmune diseases, allergies, graft-versus-host disease, and transplant rejection.

[0080] $CD4^{LVFOXP3}$ can be obtained from total $CD4^+$ T cells, requiring only limited $CD4^{LVFOXP3}$ *in vitro* expansion with cytokines, for example up to 5 to 10-fold expansion in a 2-3-week-culture.

[0081] In the process of generating clinically acceptable $CD4^{LVFOXP3}$, patient CD4+ T cells are isolated from a biological sample, e.g. from non-mobilized apheresis product. The CD4+ T cells are genetically modified in vitro by lentiviral-mediated gene transfer with the recombinant lentiviral vector LVFOXP3.

[0082] In some embodiments, $CD4^+$ T cells are isolated from non-mobilized apheresis by immunomagnetic separation using cGMP compliant reagents and device. The isolated $CD4^+$ T cells are cultured for up to 24 hours in medium comprising IL-2 at a concentration of from about 10 to about 1000 U/ml, and may be from about 50 to about 500 U/ml, e.g. from about 75 to about 150 U/ml; and IL-7 at a concentration of from about 0.5 to about 500 ng/ml, and may be from about 1 to about 100 ng/ml, e.g. from about 5 to about 50 ng/ml. Following transduction on Day 1, the transduced cells are cultured in medium comprising IL-2 at a concentration of from about 10 to about 1000 U/ml, and may be from about 50 to about 500 U/ml, e.g. from about 75 to about 150 U/ml; and IL-15 at a concentration of from about 0.5 to about 500 ng/ml, and may be from about 1 to about 100 ng/ml, e.g. from about 5 to about 50 ng/ml for a period of from 5 to 15 days, e.g. from 6 to 12 days, for example from 6 to 8 days.

The transduced $CD4^+$ T cells are then selected for expression of a marker from the vector, including without limitation NGFR. Selection may utilize magnetic microbead. Post-selection, cells are cultured in in the IL-2/IL-15 medium as described above. The transduced selected $CD4^+$ cells may be re-stimulated by the addition of an agent that binds to and activates polycloncal T cell receptor, e.g. anti-CD3; MACS® GMP T Cell TransAct™; etc. The expanded cells are harvested between about day 16 to 21.

[0083] $CD4^{LVFOXP3}$ T cells express high and stable levels of CD25 on the membrane that is directly regulated by FOXP3. As a safety measure, these cells can be eliminated in vivo by administration of an anti-CD25 mAb, including without limitation the commercially available antibody Basiliximab, which is approved for in use in pediatric and adult patients undergoing transplantation.

Conversion of $CD4^+$ T lymphocytes into $CD4^{LVFOXP3}$ T cells

[0084] A recombinant lentiviral vector comprising a *FOXP3* gene under the control of a constitutive promoter is used to convert $CD4^+$ T lymphocytes into $CD4^{LVFOXP3}$ T cells. For example, nucleic acids encoding the forkhead box protein 3 (FOXP3) transcription factor can be inserted into the lentivirus vector to create an expression cassette capable of expressing the FOXP3 in a suitable host $CD4^+$ T lymphocyte.

[0085] In some embodiments, the recombinant lentiviral vector comprises: a) a polynucleotide encoding forkhead box protein 3 (FOXP3); b) an elongation factor 1a (EF1α) promoter, wherein the EF1α promoter is operably linked to the polynucleotide encoding FOXP3; c) a polynucleotide encoding a cell surface marker for *in vitro* selection and *in vivo* tracking of cells transduced with the vector; d) a modified Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) modified to remove a WHx start codon; e) human immunodeficiency virus (HIV)-derived elements comprising a 5' long terminal repeat (5' LTR), a Ψ packaging signal, a truncated Gag sequence, a rev response element (RRE) sequence, a central polypurine tract (cPPT), a central termination sequence (CTS), a truncated negative regulatory factor (NEF) sequence, and a 3' long terminal repeat (5' LTR); f) a polyadenylation sequence; g) an SV40 origin of replication; and h) a bacterial high copy origin of replication (Ori).

[0086] In one embodiment, the cell surface marker is a truncated nerve growth factor receptor (NGFR). In another embodiment, the recombinant lentiviral vector further comprises a CMV promoter operably linked to the polynucleotide encoding the NEF. An exemplary lentiviral expression vector comprises the components arranged as depicted in the vector map shown in FIG.1. In one embodiment, the recombinant lentiviral vector comprises the nucleotide sequence of SEQ ID NO:2 or a sequence having at least about 80-100% sequence identity

thereto, including any percent identity within this range, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity thereto, wherein the recombinant lentiviral vector is capable of generating a Treg-like cell by transfection of a $CD4^+$ T lymphocyte.

[0087] The ability of constructs to produce FOXP3 can be empirically determined, for example, by using a real-time RT-PCR assay of FOXP3 mRNA levels or a Western Blot assay of FOXP3 protein levels. Additionally, the ability of the lentiviral vector to confer Treg-like characteristics on $CD4^+$ T lymphocytes can be evaluated with a suppression assay *in vitro* (see Examples).

[0088] FOXP3 nucleic acid and protein sequences may be derived from any source. A number of FOXP3 nucleic acid and protein sequences are known. A representative example of a human *FOXP3* gene sequence is presented in SEQ ID NO:1 and additional representative sequences including various isoforms of the FOXP3 transcription factor are listed in the National Center for Biotechnology Information (NCBI) database. See, for example, NCBI entries: Accession Nos. NM_001114377, NM_014009, NG_007392, XM_006724533, XM_017029567, NP_001107849, NP_054728, XP_016885056, and XP_006724596; all of which sequences (as entered by the date of filing of this application) are herein incorporated by reference. Any of these sequences or a variant thereof comprising a sequence having at least about 80-100% sequence identity thereto, including any percent identity within this range, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity thereto, can be used to construct a lentiviral construct, wherein the expressed variant FOXP3 retains biological activity, including transcription factor activity and the ability to convert $CD4^+$ T lymphocytes into $CD4^{LVFOXP3}$ T cells.

[0089] The $CD4^+$ T lymphocytes can optionally be purified before or after transduction by any method known in the art, including, but not limited to, density gradient centrifugation (e.g., Ficoll Hypaque, percoll, iodoxanol and sodium metrizoate), immunoselection (positive selection or negative selection for surface markers) with immunomagnetic beads or immunoaffinity columns, or fluorescence-activated cell sorting (FACS). For example, $CD4^+$ T lymphocytes can be isolated from apheresis products by immunomagnetic $CD4^+$ cell selection, cultured in the presence of IL-2 and IL-7, then activated and transduced with the lentiviral vector, followed by immunoselection for the cell surface marker (e.g., truncated NGFR) expressed by the recombinant lentiviral vector to separate transduced $CD4^{LVFOXP3}$ T cells from non-transduced T cells (see, Examples). The resulting cells may be restimulated and cultured in the presence of IL-2 and IL-15.

[0090] The ability of the engineered $CD4^{LVFOXP3}$ T cells to suppress proliferation and activation of effector T cells and other immune cells can be assayed by methods well known in the art

including, for example, without limitation, performing an *in vitro* suppression assay or ^{3}H-thymidine assay that measures suppression of T cell proliferation by $CD4^{LVFOXP3}$ T cells, or a flow cytometry-based suppression assay that measures suppression of proliferation and cytokine production in subpopulations of T cells and other immune cells (see, e.g., Thornton et al. (1998) J. Exp. Med. 1998. 188:287-296, Schneider et al. (2011) Methods Mol. Biol. 707:233-241, Baecher-Allan et al. (2005) Clin. Immunol. 115:10-18, McMurchy et al. (2012) Eur. J. Immunol. 42:27-34; herein incorporated by reference.

Antigen specificity

[0091] In some embodiments, antigen-specific Treg therapy is performed with $CD4^{LVFOXP3}$ T cells expressing a T cell receptor specific for a particular antigen of interest, wherein the Treg induces immunological tolerance selectively to the antigen of interest. The use of antigen-specific Tregs avoids the risk of general immunosuppression. In certain embodiments, the antigen specificity of a Treg is controlled by introducing an exogenous T cell receptor having specificity for an antigen of interest into a Treg. For example, Tregs can be transduced with a viral vector encoding a natural T cell receptor or an artificial T cell receptor such as a chimeric antigen receptor (CAR) having the desired specificity for an antigen of interest. CARs are artificial T cell receptors that typically comprise a single-chain variable fragment (scFv), which provides antigen specificity, fused to a T cell co-stimulatory domain and an activation domain. CARs can recognize epitopes of a target antigen of interest expressed on a cell surface (e.g., B cells or antigen presenting cells), but are not MHC-restricted. In $CD4^{LVFOXP3}$ T cells, designed with exogenous T cell receptors (e.g., natural or artificial) specific for an antigen of interest, the gene for the endogenous T cell receptor may be inactivated or deleted. For a review of engineering antigen-specific Tregs with exogenous T cell receptors and CAR receptors, see, e.g., Dawson et al. (2017) Transl. Res. 187:53-58, Boardman et al. (2016) Biochem. Soc. Trans. 44(2):342-8, Adair et al. (2017) Front. Immunol. 8:1117, Zhang et al. (2018) Front. Immunol. 2018; 9: 2359, Tsang et al. (2008) J. Clin. Invest. 118(11):3619-28, Kim et al. (2015) Blood 125(7):1107-15, Brusko et al. (2010) PLoS One 5(7):e11726, Wright et al. (2009) Proc. Natl. Acad. Sci. U.S.A. 106(45):19078-83, Schubert et al. (2018) Int. J. Cancer. 142(9):1738-1747; herein incorporated by reference.

[0092] $CD4^{+}$ T lymphocytes may be obtained from a biological sample collected from either healthy individuals or patients with an inflammatory and immune dysregulatory condition (e.g., Treg deficiency/autoimmune disease such as IPEX syndrome). The biological sample may be any sample containing $CD4^{+}$ T lymphocytes, such as a blood sample, a sample of peripheral blood mononuclear cells (PBMCs), or inflamed tissue in which the $CD4^{+}$ T lymphocytes have infiltrated. $CD4^{+}$ T lymphocytes can be isolated from a bodily fluid (e.g., blood) or tissue and

cultured prior to gene transfer with a recombinant lentiviral vector expressing FOXP3 to generate $CD4^{LVFOXP3}$ T cells. Culture media may be supplemented with interleukin 2 (IL-2) and IL-7 to expand the numbers of cells before and/or after transduction.

[0093] In some embodiments, antigen-specific $CD4^+$ T lymphocytes are obtained by eliciting an immune response to an antigen of interest. $CD4^+$ T lymphocytes may be contacted with an antigen of interest (in the presence of an antigen presenting cell) *in vivo*, *ex vivo*, or *in vitro*. For example, the antigen of interest can be administered to a subject to elicit a $CD4^+$ T cell response followed by collection of a biological sample from the subject comprising T cells recognizing the antigen of interest. The biological sample may be any sample containing $CD4^+$ T lymphocytes specific for the antigen of interest, such as a blood sample, a sample of peripheral blood mononuclear cells (PBMCs), or inflamed tissue in which antigen-specific $CD4^+$ T lymphocytes have infiltrated. Alternatively, a biological sample comprising $CD4^+$ T lymphocytes can be collected from a subject and treated with an antigen of interest in the presence of an antigen-presenting cell *ex vivo* or *in vitro*. Such induced antigen-specific $CD4^+$ T lymphocytes can be at the same time transduced with a recombinant lentiviral vector expressing FOXP3 and if desired a marker gene of interest, to generate antigen-specific $CD4^{LVFOXP3}$ T cells. Antigens of interest may include, for example, alloantigen or autoantigen specific for an autoimmune disease of interest.

[0094] Examples of suitable antigen presenting cells that can present an antigen of interest to $CD4^+$ T lymphocytes include dendritic cells, macrophages, and activated B cells. Alternately, artificial antigen presenting cells may be used, such as soluble major histocompatibility complex (MHC)-multimers or cellular or acellular artificial antigen presenting cells. MHC-multimers typically range in size from dimers to octamers (tetramers commonly used) and can be used to display class 1 or class 2 MHC (Hadrup et al. (2009) Nature Methods 6:520-526, Nepom et al. (2003) Antigen 106:1-4, Bakker et al. (2005) Current Opinion in Immunology 17:428-433). Cellular artificial antigen presenting cells may include cells that have been genetically modified to express T-cell co-stimulatory molecules, MHC alleles and /or cytokines. For example, artificial antigen presenting cells have been generated from fibroblasts modified to express HLA molecules, the co-stimulatory signal, B7.1, and the cell adhesion molecules, ICAM-1 and LFA-3 (Latouche et al. (2000) Nature Biotechnology. 18 (4):405–409). Acellular antigen presenting cells comprise biocompatible particles such as microparticles or nanoparticles that carry T cell activating proteins on their surface (Sunshine et al. (2014) Biomaterials. 35 (1): 269–277), Perica et al. (2014) Nanomedicine: Nanotechnology, Biology and Medicine. 10 (1):119–129). For a review of artificial antigen presenting cells, see, e.g., Oelke et al. (2004) Clin. Immunol. 110(3):243-251, Wang et al. (2017) Theranostics 7(14):3504-3516, Butler et al. (2014) Immunol Rev. 257(1):191-209,

Eggermont et al. (2014) Trends Biotechnol. 32(9):4564-4565, Sunshine et al. (2013) Nanomedicine (Lond) 8(7):1173-1189, and Rhodes et al. (2018) Mol. Immunol. 98:13-18; herein incorporated by reference.

[0095] Typically, the antigen of interest is at a concentration ranging from about 10 µg/ml to about 40 µg/ml in the biological sample. The antigen of interest may be pre-incubated with the antigen presenting cells for periods ranging from 1 to 18 hours prior to stimulation of the $CD4^+$ T lymphocytes. Culture media may be supplemented with interleukin 2 (IL-2) and interleukin 15 (IL-15) during intervals between stimulations to induce amino acid uptake and protein synthesis in antigen-activated T cells to promote growth and proliferation of antigen-specific $CD4^+$ T lymphocytes. The antigen-specific CD4+ T lymphocytes can subsequently be isolated from biological samples and transduced with a lentiviral vector expressing FOXP3, as described herein.

[0096] The cells may additionally be cultured with agents during Treg expansion that enhance expression of specific homing receptors. For example, culture in the presence of IFNγ and IL12 can enhance expression of CXCR3. Addition of retinoic acid (RA) during Treg expansion can induce expression of the gut-homing receptors α4β7-integrin and CCR9. See, for example, Hoeppli et al. (2019) Am J Transplant.19(1):62-76.

Applications

[0097] The methods described herein are useful for treating various immune conditions and disorders benefitting from increased immunological tolerance, such as inflammatory conditions including for example, without limitation, Treg deficiency, autoimmune disorders, allergies, acute inflammatory syndromes, graft-versus-host disease, and organ or tissue transplantation. In some embodiments, polyclonal $CD4^{LVFOXP3}$ T cells comprising a plurality of different T cell receptors are used for immunosuppression and promoting immune tolerance generally. In other embodiments, $CD4^{LVFOXP3}$ T cells comprising a T cell receptor specific for an antigen of interest are used to dampen adaptive antigen-specific immune responses to the antigen of interest selectively. The antigen specificity of a Treg-like cell can be controlled by further genetic modification of a $CD4^+$ T lymphocyte to include an exogenous T cell receptor or chimeric antigen receptor (CAR) having a desired antigen specificity. For known antigens, including without limitation autoantigens, T cells can be isolated by MHC-peptide tetramers and then transduced and expanded.

[0098] Treg deficiency and autoimmune and other inflammatory conditions that may be treated with engineered $CD4^{LVFOXP3}$ T cells by the methods described herein include, but are not limited to, immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome.

[0099] Other conditions associated with autoimmunity and undesirable inflammation include, for example, multiple sclerosis (MS), rheumatoid arthritis (RA), reactive arthritis, psoriasis, pemphigus vulgaris, Sjogren's disease, autoimmune thyroid disease (AITD), Hashimoto's thyroiditis, myasthenia gravis, diabetes mellitus type 1, stomatitis, lupus erythematosus, acute disseminated encephalomyelitis (ADEM), Addison's disease, agammaglobulinemia, alopecia areata, amyotrophic lateral sclerosis, ankylosing spondylitis, antiphospholipid syndrome, antisynthetase syndrome, atopic dermatitis, autoimmune aplastic anemia, autoimmune enteropathy, autoimmune hemolytic anemia, autoimmune hemolytic anemia, autoimmune inner ear disease, autoimmune lymphoproliferative syndrome, autoimmune peripheral neuropathy, autoimmune pancreatitis, autoimmune polyendocrine syndrome, autoimmune progesterone dermatitis, autoimmune thrombocytopenic purpura, autoimmune urticaria, autoimmune uveitis, Balo disease/Balo concentric sclerosis, Behçet's disease, Berger's disease, Bickerstaff's encephalitis, Blau syndrome, Bullous pemphigoid, Castleman's disease, celiac disease, Chagas disease, chronic inflammatory demyelinating polyneuropathy, chronic recurrent multifocal osteomyelitis, chronic obstructive pulmonary disease, Churg-Strauss syndrome, cicatricial pemphigoid, Cogan syndrome, cold agglutinin disease, complement component 2 deficiency, contact dermatitis, cranial arteritis, CREST syndrome, Crohn's disease, Cushing's Syndrome, cutaneous leukocytoclastic angiitis, Dego's disease, Dercum's disease, dermatitis herpetiformis, dermatomyositis, diffuse cutaneous systemic sclerosis, Dressler's syndrome, eczema, endometriosis, enthesitis-related arthritis, eosinophilic fasciitis, eosinophilic gastroenteritis, eosinophilic pneumonia, epidermolysis bullosa acquisita, erythema nodosum, erythroblastosis fetalis, essential mixed cryoglobulinemia, Evan's syndrome, fibrodysplasia ossificans progressiva, fibrosing alveolitis (or idiopathic pulmonary fibrosis), gastritis, gastrointestinal pemphigoid, glomerulonephritis, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's encephalopathy, Henoch-Schonlein purpura, gestational pemphigoid, hidradenitis suppurativa, Hughes-Stovin syndrome, hypogammaglobulinemia, idiopathic inflammatory demyelinating diseases, idiopathic pulmonary fibrosis, IgA nephropathy, inclusion body myositis, chronic inflammatory demyelinating polyneuropathy, interstitial cystitis, juvenile idiopathic arthritis, Kawasaki's disease, Lambert-Eaton myasthenic syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, linear IgA disease (LAD), lupoid hepatitis, Majeed syndrome, Ménière's disease, microscopic polyangiitis, Miller-Fisher syndrome, mixed connective tissue disease, morphea, Mucha-Habermann disease, microscopic colitis, myositis, narcolepsy, neuromyelitis optica, neuromyotonia, ocular cicatricial pemphigoid, opsoclonus myoclonus syndrome, Ord's thyroiditis, palindromic rheumatism, PANDAS, paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonage-Turner

syndrome, Pars planitis, pemphigus vulgaris, pernicious anaemia, perivenous encephalomyelitis, POEMS syndrome, polyarteritis nodosa, polymyalgia rheumatica, polymyositis, primary biliary cirrhosis, primary sclerosing cholangitis, progressive inflammatory neuropathy, psoriatic arthritis, pyoderma gangrenosum, pure red cell aplasia, Rasmussen's encephalitis, Raynaud phenomenon, relapsing polychondritis, Reiter's syndrome, restless leg syndrome, retroperitoneal fibrosis, rheumatic fever, sarcoidosis, Schmidt syndrome, Schnitzler syndrome, scleritis, scleroderma, serum sickness, Sjögren's syndrome, spondyloarthropathy, Still's disease, Stiff person syndrome, Subacute bacterial endocarditis (SBE), Susac's syndrome, Sweet's syndrome, Sydenham chorea, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis, thrombocytopenia, Tolosa-Hunt syndrome, transverse myelitis, undifferentiated connective tissue disease, undifferentiated spondyloarthropathy, urticarial vasculitis, vasculitis, vitiligo, Wegener's granulomatosis, autoimmune cardiomyopathy, ischemic heart disease, atherosclerosis, cancer, fibrosis, inflammatory bowel disease, inflammatory myopathy, giant cell arteritis (GCA), asthma, allergy, Parkinson's disease, schizophrenia, Alzheimer's disease, and acute respiratory distress syndrome ARDS, e.g. resulting from coronavirus infection.

[00100] Treatment of primates, more particularly humans is of interest, but other mammals may also benefit from treatment, particularly domestic animals such as equine, bovine, ovine, feline, canine, murine, lagomorpha, and the like.

Pharmaceutical Compositions

[00101] Pharmaceutical compositions can be prepared by formulating the $CD4^{LVFOXP3}$ T cells (i.e., $CD4^+$ T lymphocytes transduced with a lentiviral vector expressing FOXP3) into dosage forms by known pharmaceutical methods. For example, a pharmaceutical composition comprising $CD4^{LVFOXP3}$ T cells can be formulated for parenteral administration, as liquids, suspensions, emulsions, and injections (such as venous injections, drip injections, and the like).

[00102] In formulation into these dosage forms, the $CD4^{LVFOXP3}$ T cells can be combined as appropriate, with pharmaceutically acceptable carriers or media, in particular, sterile water and physiological saline, vegetable oils, resolvents, bases, emulsifiers, suspending agents, surfactants, stabilizers, vehicles, antiseptics, binders, diluents, tonicity agents, soothing agents, bulking agents, disintegrants, buffering agents, coating agents, lubricants, coloring agents, solution adjuvants, or other additives. The $CD4^{LVFOXP3}$ T cells cells may be also used in combination with known pharmaceutical compositions, immunosuppressants, cytokines, or other therapeutic agents.

[00103] In some embodiments, the pharmaceutical composition comprising the $CD4^{LVFOXP3}$ T cells is a sustained-release formulation, or a formulation that is administered using a sustained-release device. Such devices are well known in the art, and include, for example, transdermal patches, and miniature implantable pumps that can provide for delivery of the $CD4^{LVFOXP3}$ T cells over time in a continuous, steady-state fashion at a variety of doses to achieve a sustained-release effect with a non-sustained-release pharmaceutical composition.

[00104] Usually, but not always, the subject who receives the $CD4^{LVFOXP3}$ T cells (i.e., the recipient) is also the subject from whom the original, unmodified $CD4^+$ T lymphocytes (from which the $CD4^{LVFOXP3}$ T cells are produced) are harvested or obtained, which provides the advantage that the donated cells are autologous. However, $CD4^+$ T lymphocytes can be obtained from another unrelated subject (i.e., donor), a culture of cells from a donor, or from established cell culture lines. $CD4^+$ T lymphocytes may be obtained from the same species than the subject to be treated, and more preferably of the same immunological profile as the subject. Such cells can be obtained, for example, from a biological sample comprising $CD4^+$ T lymphocytes from a close relative or matched donor, and the $CD4^{LVFOXP3}$ T cells that are produced (i.e., by transduction with a lentiviral vector expressing FOXP3) can be administered to a subject in need of treatment for an inflammatory condition.

[00105] In certain embodiments, the $CD4^{LVFOXP3}$ T cells that are administered to a subject are derived from autologous or allogeneic $CD4^+$ T lymphocytes. The patients or subjects who donate or receive the cells are typically mammalian, and usually human. However, this need not always be the case, as veterinary applications are also contemplated.

[00106] Additionally, $CD4^{LVFOXP3}$ T cells may be expanded in culture prior to administration to a subject. In some embodiments, IL-2 is added to a culture to help stabilize and expand the number of $CD4^{LVFOXP3}$ T cells. In addition, a heparan sulfate-containing proteoglycan or heparin may be added to the culture to improve utilization of IL-2 by the $CD4^{LVFOXP3}$ T cells. For a description of techniques for culturing T cells, see, e.g., *Regulatory T Cells: Methods and Protocols* (Methods in Molecular Biology, Vol. 707, G. Kassiotis and A. Liston eds., Humana Press, 2011), Raulf-Heimsoth "T Cell - Primary Culture from Peripheral Blood" *Allergy Methods and Protocols* pp 17-30 (Humana Press Inc., Totowa, NJ, 2008); herein incorporated by reference in their entireties.

Administration

[00107] At least one therapeutically effective cycle of treatment with $CD4^{LVFOXP3}$ T cells (i.e., $CD4^+$ T lymphocytes transduced with a lentiviral vector expressing FOXP3) will be administered to a subject for treatment of an inflammatory condition. By "therapeutically effective dose or amount" of a composition comprising $CD4^{LVFOXP3}$ T cells is intended an

amount that, when administered as described herein, brings about a positive therapeutic response, such as improved recovery from an inflammatory condition benefitting from increased immunological tolerance, such as an autoimmune disorder, an allergy, acute inflammatory syndromes, graft-versus-host disease, or a tissue transplant. The administration of cells has a clinically acceptable safety profile. Improved recovery may include a reduction in inflammation, pain, or autoimmune-induced tissue damage, decreased allergic response, or prolonged survival of transplanted tissue or organs. Additionally, a therapeutically effective dose or amount may compensate for Treg-deficiency (e.g., IPEX syndrome) and reduce the need for immunosuppressive or anti-inflammatory drugs.

[00108] In certain embodiments, multiple therapeutically effective doses of compositions comprising $CD4^{LVFOXP3}$ T cells and/or one or more other therapeutic agents, such as other drugs for treating immune diseases or conditions, or other medications will be administered. The compositions of the present invention are typically, although not necessarily, administered via injection (subcutaneously, intravenously, intra-arterially, or intramuscularly), by infusion, or locally. Additional modes of administration are also contemplated, such as intraperitoneal, intrathecal, intralymphatic, intravascular, intralesion, transdermal, intraarticular, and so forth. In some embodiments, the $CD4^{LVFOXP3}$ T cells are administered locally, for example, to the site of a tissue or organ transplant or an inflamed region needing treatment. The pharmaceutical compositions comprising $CD4^{LVFOXP3}$ T cells and other agents may be administered using the same or different routes of administration in accordance with any medically acceptable method known in the art.

[00109] In another embodiment, the pharmaceutical compositions comprising $CD4^{LVFOXP3}$ T cells are administered prophylactically, e.g., to prevent Treg deficiency, etc. Such prophylactic uses will be of particular value for subjects who have a disease or a genetic predisposition to developing an inflammatory condition, such as an autoimmune disease, inflammation, or allergy. For example, $CD4^{LVFOXP3}$ T cells may be administered prior to transplant to prolong graft survival or to a patient with an autoimmune disease to prevent a disease flare, or in IPEX patients with mixed donor chimerism and disease relapse.

[00110] Those of ordinary skill in the art will appreciate which conditions compositions comprising $CD4^{LVFOXP3}$ T cells can effectively treat. The actual dose and number of doses to be administered will vary depending upon the age, weight, and general condition of the subject as well as the severity of the condition being treated, the judgment of the health care professional, and conjugate being administered. Therapeutically effective amounts can be determined by those skilled in the art, and will be adjusted to the particular requirements of each particular case.

[00111] Compositions comprising $CD4^{LVFOXP3}$ T cells, prepared as described herein (again, preferably provided as part of a pharmaceutical preparation), can be administered alone or in combination with one or more other therapeutic agents for treating an immune disease or condition, such as, but not limited to very low doses of immunosuppressive agents, such as steroidal agents (e.g., prednisone) or non-steroidal agents (e.g., mycophenolate, sirolimus (Rapamune, Wyeth-Ayerst Canada), tacrolimus (Prograf, Fujisawa Canada), 15-deoxyspergualin, cyclosporin, methotrexate, rapamycin, Rapamune (sirolimus/rapamycin), FK506, and Lisofylline (LSF); small molecules such as Jak-inhibitors; CTLA-4-Ig (Abatacept); and anti-inflammatory/analgesic agents, including, but not limited to, non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, and naproxen, paracetamol, acetaminophen, COX-2 inhibitors, such as rofecoxib, celecoxib, and etoricoxib; opioids, such as morphine, codeine, oxycodone, hydrocodone, dihydromorphine, pethidine; and immune selective anti-inflammatory derivatives (ImSAIDs); or other medications used to treat a particular condition or disease according to a variety of dosing schedules depending on the judgment of the clinician, needs of the patient, and so forth. The specific dosing schedule will be known by those of ordinary skill in the art or can be determined experimentally using routine methods. Exemplary dosing schedules include, without limitation, administration five times a day, four times a day, three times a day, twice daily, once daily, three times weekly, twice weekly, once weekly, twice monthly, once monthly, and any combination thereof. Preferred compositions are those requiring dosing no more than once a day.

[00112] Compositions comprising $CD4^{LVFOXP3}$ T cells can be administered prior to, concurrent with, or subsequent to other agents. If provided at the same time as other agents, the $CD4^{LVFOXP3}$ T cells can be provided in the same or in a different composition. Thus, the $CD4^{LVFOXP3}$ T cells and one or more other agents can be presented to the individual by way of concurrent therapy. By "concurrent therapy" is intended administration to a subject such that the therapeutic effect of the combination of the substances is caused in the subject undergoing therapy. For example, concurrent therapy may be achieved by administering a dose of a pharmaceutical composition comprising $CD4^{LVFOXP3}$ T cells and a dose of a pharmaceutical composition comprising at least one other agent, such as a drug for treating an immune disease or condition, which in combination comprise a therapeutically effective dose, according to a particular dosing regimen. Similarly, the $CD4^{LVFOXP3}$ T cells and one or more other therapeutic agents can be administered in at least one therapeutic dose. Administration of the separate pharmaceutical compositions can be performed simultaneously or at different times (i.e., sequentially, in either order, on the same day, or on different days), as long as the therapeutic effect of the combination of these substances is caused in the subject undergoing therapy.

Kits

[00113] Any of the compositions described herein may be included in a kit. For example, $CD4^{LVFOXP3}$ T cells (i.e., $CD4^+$ T lymphocytes transduced with a lentiviral vector expressing FOXP3) may be included in a kit. Alternatively, a recombinant lentiviral vector, as described herein, for expression of FOXP3 in $CD4^+$ T lymphocytes to produce $CD4^{LVFOXP3}$ T cells may be included in the kit. In some embodiments, untransduced $CD4^+$ T lymphocytes are provided with the lentiviral vector separate. The kit may also comprise transfection agents, agents for purification of cells (e.g., microbeads for selection of transfected cells having the NGFR surface marker), agents for maintaining or culturing cells, such as media, and optionally one or more other factors, such as cytokines (e.g., IL-2), growth factors, antibiotics, and the like.

[00114] Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or solution. The kit may comprise one or more containers holding the $CD4^{LVFOXP3}$ T cells and/or lentiviral vector, and other agents. Suitable containers for the compositions include, for example, bottles, vials, syringes, and test tubes. Containers can be formed from a variety of materials, including glass or plastic. A container may have a sterile access port (for example, the container may be a vial having a stopper pierceable by a hypodermic injection needle).

[00115] The kit can further comprise a container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. It can also contain other materials useful to the end-user, including other pharmaceutically acceptable formulating solutions such as buffers, diluents, filters, needles, and syringes or other delivery devices. The delivery device may be pre-filled with the compositions.

[00116] The kit can also comprise a package insert containing written instructions for methods of treating inflammatory conditions with the $CD4^{LVFOXP3}$ T cells, as described herein. The package insert can be an unapproved draft package insert or can be a package insert approved by the Food and Drug Administration (FDA) or other regulatory body.

[00117] In one embodiment, the kit comprises a lentiviral expression vector comprising the components arranged as depicted in the vector map shown in FIG. 2. In another embodiment, the kit comprises a recombinant lentiviral vector comprising the nucleotide sequence of SEQ ID NO:2 or a sequence having at least about 80-100% sequence identity thereto, including any percent identity within this range, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity thereto, wherein the recombinant lentiviral vector is capable of generating a Treg-like cell by transfection of a $CD4^+$ T lymphocyte.

[00118] It will be apparent to one of ordinary skill in the art that various changes and modifications can be made without departing from the spirit or scope of the invention.

Experimental

[00119] 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 present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.* amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[00120] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[00121] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention.

Example 1

FOXP3 engineered CD4+ T cells for Treg-based immunotherapy of IPEX Syndrome

[00122] FOXP3 is a transcription factor essential for the function of T regulatory (Treg) cells, a key $CD4^+$ T cell subset for control of immune responses and maintenance of immune tolerance. Mutations of FOXP3 cause severe early onset autoimmunity in mice and man, due to loss of functional Treg cells. Typical clinical manifestations include severe refractory enteropathy, Type 1 Diabetes (T1D), eczema, cytopenia, hepatitis, nephritis and thyroiditis. The disease, Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) Syndrome, was clinically described in 1982 in a family with 19 males affected, and in 2000 the causative gene, FOXP3 was confirmed in 7 additional IPEX patients. The link between FOXP3 gene, its importance in the functioning of Treg cells and preventing autoimmune manifestations was first demonstrated in transgenic and knock-out murine models in early 2003 (Bacchetta ANN NYA Science, 2016). Bacchetta, R. et al. (2006) published the first data

demonstrating that IPEX patients' Treg cells have impaired regulatory function (Bacchetta JCI, 2006).

[00123] The genetic reprogramming of mammalian cells for clinical purposes has recently become an available option, with the completion of clinical trials for the treatment of genetic diseases and their translation in market-authorized therapies. To control the devastating autoimmunity resulting from mutations of *FOXP3* in IPEX syndrome, adoptive transfer of functional autologous Tregs generated *in vitro* by wildtype FOXP3 enforced expression using lentiviral-mediated gene transfer is performed.

[00124] Engineered murine FOXP3 over-expression as a method to generate Treg cells has been previously attempted in murine cells. In human cells, however, the expression pattern and function of FOXP3 are more complex, and retrovirus-mediated overexpression of FOXP3 does not consistently result in the generation of potent suppressive T cells *in vitro*, mainly due to unstable and activation dependent retroviral-mediated transgene expression (Allan SE., 2005). However, we found that lentivirus-mediated expression of FOXP3 under the control of the human elongation factor EF1α promoter can efficiently convert T effector into Treg cells (Allan SE, 2008).

[00125] Transduction of peripheral $CD4^+$ T lymphocytes with LVFOXP3 and *in vitro* expansion of transduced cells led to the generation of a homogeneous pool of T cells constitutively expressing FOXP3 at high intensity. The resulting $CD4^{LVFOXP3}$ behave as functional and stable $FOXP3^+$- $CD4^{LVFOXP3}$ T cells, with potent *in vitro* suppressive activity, reduced proliferative capacity, and decreased cytokine production (Allan et al., 2008; Passerini et al., 2013). $CD4^{LVFOXP3}$ stably express FOXP3 independent of activation, in steady-state, and inflammatory conditions, especially when generated from naïve T cells, and maintain inhibitory functions *in vivo* in a model of xenogeneic-GvHD (Passerini et al., 2013).

[00126] $CD4^{LVFOXP3}$ can be obtained from total $CD4^+$ T cells, rendering the manufacturing process easy and cost-effective because $CD4^+$ T cells are present at high frequency (about 40% of the lymphocytes) in the peripheral blood and there is an available standardized process to isolate $CD4^+$ T cells in high numbers from peripheral blood. $CD4^{LVFOXP3}$ require limited *in vitro* expansion with cytokines. The current pre-clinical small-scale method for the generation of $CD4^{LVFOXP3}$ resulted in up to 5 to 10-fold expansion in a 3-week-culture.

[00127] The preclinical studies described here assess the feasibility, safety and impact on disease manifestations of LVFOXP3 gene transfer in $CD4^+$ T cells to develop the $CD4^{LVFOXP3}$ as a biotherapeutic for administration in IPEX patients.

Example 2

FOXP3 Gene Transfer into Autologous $CD4^+$ T Cells

[00128] $FOXP3^+$ T regulatory (Treg) cells are a specific $CD4^+$ T cell subset for maintaining immune homeostasis and controlling undesired immune responses. We can convert $CD4^+$ T cells into Treg cells using LV-mediated *FOXP3* gene transfer (Allan SE. et al, Mol Therapy 2008; Passerini L. et al, Sci Transl Med 2013). To obtain $CD4^{LVFOXP3}$ by FOXP3 gene transfer into autologous $CD4^+$ T cells to be used for clinical purposes, we have:

1) modified the vector to make it GMP-compliant, a third-generation bidirectional lentiviral vector containing the full-length cDNA of *FOXP3*, expressed under the EF1α promoter and *NGFR*, a marker gene, expressed under the minimal CMV promoter from the opposite strand, providing a cell surface marker for selection of transduced cells, and
2) developed a robust GMP-compliant method of gene transduction in $CD4^+$ T cells followed by purification and expansion of the $CD4^{LVFOXP3}$.

[00129] The use of a strong EF1α promoter to drive wild-type FOXP3 expression ensures high transgene expression in the transduced cells. The resulting *FOXP3* gene transferred $CD4^{LVFOXP3}$ acquire phenotypic and functional features of Treg cells. $CD4^{LVFOXP3}$ display high FOXP3 expression, and as a consequence they show high CD25 and low CD127, become low proliferative, produce low or no cytokines and, like naturally occurring Treg cells, have the ability to suppress the proliferation and cytokine production by T effector (Teff) cells *in vitro.* In addition, the use of a bidirectional vector gives simultaneous expression of the marker gene, truncated nerve growth factor receptor (NGFR), which enables purification of the modified T cells *in vitro* and their tracing *in vivo*. Similarly to Treg cells, $CD4^{LVFOXP3}$ T cells exert their suppressive activity *in vivo* in the xenogeneic graft-versus-host-disease (GvHD) mouse model. This is an established model to assess the ability of human Treg cells to control Teff cells responses by increasing mice survival and preventing weight loss.

[00130] In addition, we have demonstrated that the $CD4^{LVFOXP3}$ T cells also exert their suppressive activity *in vivo* in a hu-mouse model in which the FOXP3-deficient human stem cells engraft a mouse, and FOXP3-deficient T cells that reconstitute show CD4+ memory T cell proliferation, resembling what occurs in IPEX patients. $CD4^{LVFOXP3}$ T cells can prevent this lymphoproliferation and normalize survival in this model. The data show that $CD4^{LVFOXP3}$ T cells are not impeding an ongoing immune response to pathogens or tumor clearance, supporting the safety of this cell product.

[00131] The pre-clinical data demonstrates that LVFOXP3 provides efficient functional gene transduction in $CD4^+$ obtained from both healthy individuals and IPEX patients with different FOXP3 mutations. In addition, we show functional equivalence using $CD4^{LVFOXP3}$ allogenic or autologous to the responder effector T cells.

[00132] We have applied the *FOXP3* gene transfer technology to obtain Treg cells from $CD4^+$ T cells from several IPEX patients with different *FOXP3* gene mutations, each of which cause severe autoimmune manifestations and immune dysregulation. In IPEX syndrome there are no functional Treg cells and patients have autoimmunity from birth. The IPEX $CD4^{LVFOXP3}$ have identical cellular markers, anergic phenotype, cytokine production and suppressive function to naturally occurring Treg cells obtained from healthy donors, suggesting they could support restoration of immune regulation. $CD4^{LVFOXP3}$ T cells will be of great benefit in IPEX patients.

Example 3

$CD4^{LVFOXP3}$ Cell Product and Characteristics

[00133] $CD4^{LVFOXP3}$ T cells consists of autologous $CD4^+$ T cells that have undergone lentiviral-mediated gene transfer of wild-type FOXP3 leading to persistent high FOXP3 expression and acquisition of regulatory T cell function.

[00134] Adoptive immunotherapy with $CD4^{LVFOXP3}$ T cells is proposed in subjects with IPEX syndrome to control the clinical manifestations of autoimmunity and immune dysregulation.

[00135] Autologous $CD4^{LVFOXP3}$ are infused in suspension by intravenous infusion, according to an escalating dose 3+3 Phase 1 clinical design (doses of 10^6 cells/kg, 3×10^6 cells/kg or 10×10^6 cells/kg).

Example 4

Manufacturing Process

[00136] The manufacturing process for $CD4^{LVFOXP3}$ requires the isolation of patient CD4+ T cells from non-mobilized apheresis product followed by lentiviral-mediated gene transfer of wild-type FOXP3 and truncated nerve growth factor receptor (NGFR) with the recombinant lentiviral vector LVFOXP3. $CD4^{LVFOXP3}$ are manufactured, labeled, tracked and tested according to cGMP and Good Tissue Practice (GTPs) at the LCGM facility.

[00137] $CD4^+$ T cells are isolated from non-mobilized apheresis by immunomagnetic separation using cGMP compliant reagents and devices (Miltenyi Prodigy system) on Day 0. Selected $CD4^+$ T cells are cultured for up to 24 hours in X-VIVO 15 medium in the presence of 5% Human AB Serum, MACS® GMP T Cell TransAct™, IL-2 (100 U/mL) and IL-7 (10 ng/mL). On Day 1, cells are transduced with LVFOXP3. Transduced cells are cultured in X-VIVO 15 medium, or comparable medium suitable for large scale production, supplemented with 5% Human AB Serum, IL-2 (100 U/mL) and IL-15 (10 ng/mL) with periodic media addition and media exchange performed up to Day 8-10. At day 6-8 following activation, the transduced $CD4^+$ T cells are selected for NGFR expression on the CliniMACS Plus instrument using CliniMACS CD271 reagent (NGFR microbeads). Post-enrichment, cells are cultured in X-

VIVO 15 medium supplemented with 5% Human AB Serum, IL-2 (100 U/ml) and IL-15 (10 ng/mL). The transduced selected $CD4^+$ cells are re-stimulated by the addition of MACS® GMP T Cell TransAct™ and cells are split for further expansion. $CD4^{LVFOXP3}$ are harvested between Days 16-21. Cells are washed and re-suspended in CryoStor CS-5 at a density of 5-100x10^6 cells/mL.

[00138] The $CD4^{LVFOXP3}$ manufacturing plan is summarized in **FIG.1** and the reagents used in this process are summarized in **Table 1**.

Table 1: Critical Components and Reagents for $CD4^{LVFOXP3}$ Production

Item	Manufacturer	Application	Standard
LVFOXP3	Lentigen, Gaithersburg, MD	Transduction of patient $CD4^+$ cells for FOXP3 gene transfer	As described below for transfer plasmid components, and per Lentigen Biologics Master File, reference to be included in IND submission
CliniMACS LS Tubing Set	Miltenyi Biotec, Bergisch-Gladbach, Germany	Immunomagnetic cell selection	Per DMF BB-MF 15499
CliniMACS Prodigy TS 520 Tubing Set	Miltenyi Biotec, Bergisch-Gladbach, Germany	Immunomagnetic cell selection	Per DMF BB-MF 16214
CliniMACS PBS-EDTA Buffer	Miltenyi Biotec, Bergisch-Gladbach, Germany	Immunomagnetic cell selection	Per DMF BB-MF-12541
CliniMACS CD4 Reagent (Murine IgG_1)	Miltenyi Biotec, Bergisch-Gladbach, Germany	$CD4^+$ cell selection	Per DMF BB-MF-12541
CliniMACS CD271 Reagent	Miltenyi Biotec, Bergisch-Gladbach, Germany	$NGFR^+$ cell selection	Per DMF BB-MF 15499
Albumin (Human) USP, Albutein® 25%	Nova Biologics, Oceanside, CA	Buffer supplement	USP; manufactured from human plasma units collected in USA plasmapheresis centers licensed by the FDA
X-Vivo 15 Medium	Lonza, Basel, Switzerland	$CD4^+$ cell culture	Manufactured according to GMP standards
MACS® GMP T Cell TransAct™	Miltenyi Biotec Bergisch-Gladbach, Germany	$CD4^+$ cell culture	Per DMF BB-MF 17483
IL-2	Prometheus Laboratories, Inc. San Diego, CA	$CD4^+$ cell culture	Manufactured according to GMP standards
CellGenix® rh IL-7, or equivalent	CellGenix, Freiburg, Germany	$CD4^+$ cell culture	Manufactured according to GMP standards
CellGenix® rh	CellGenix,	$CD4^+$ cell culture	Manufactured according to GMP

IL-15	Freiburg, Germany		standards
CryoStor® CS5	Biolife Solutions, Bothell, WA	Cryopreservative	Per DMF BB-MF-13671

Production and Purification of LVFOXP3

[00139] LVFOXP3, also referred to in previous publications as pCCL.FP3, is a recombinant bi-directional lentiviral vector in which expression of the Human Forkhead Box Protein 3 (FOXP3) therapeutic transgene is under the control of the human elongation factor 1a (EF1α) promoter, and NGFR gene encoding a truncated nerve growth factor receptor expressed under control of a minimal CMV promoter is included as a cell-surface marker for in vitro selection and in vivo tracking of transduced cells. The truncated NGFR was chosen as a marker gene because it is known to be safe and non-immunogenic in humans. A schematic of the vector genome illustrating the bi-directional nature of the respective expression cassettes is shown in FIG. 2A and a summary table of the transfer plasmid, pLVFOXP3, components are provided in FIG 2B.

[00140] Apheresis is performed using either the Spectra Optia or COBE Spectra devices (Terumo BCT, Lakewood, CO) and follows current practices for autologous non-mobilized donor collections according to Standard Operating Procedures for apheresis at Lucille Packard Children's Hospital (LPCH) as described in the clinical protocol. The apheresis product is labeled and transported to the LCGM for manufacturing in a temperature monitored qualified carrier according to SOP-2008 Transport of Products To_From SHC-LPCH and within LCGM.

[00141] $CD4^+$ T cells are isolated from fresh apheresis products collected as described above. All washing, labeling and selection procedures are performed on the CliniMACS Prodigy System according to manufacturer's directions. Total nucleated cell count in the apheresis product is calculated using the Sysmex Analyzer (Beckman Coulter Inc, West Sacramento, CA). An aliquot is sent to the Quality Control unit of the LCGM for determination of the viable $CD3^+$ and $CD4^+$ cells using the LSR Fortessa X-20 or FACSCelesta flow-cytometry device (BD Biosciences, San Jose, CA). The Prodigy calculates the amount of human CD4 beads required for the selection based on the total number of $CD4^+$ cells. The cells are labeled -with anti-human CD4 microbeads as per the manufacturer's protocol. CliniMACS PBS-EDTA buffer supplemented with 0.5% HSA is used for the dilution, wash, incubation and elution steps. Post-selection, a sample of the CD4-enriched T cells are taken for determination of cell purity, yield, viability and recovery as assessed by flow-cytometry-based immunophenotypic analysis.

[00142] Following immunomagnetic $CD4^+$ cell selection, cells are cultured in medium for activation and polyclonal stimulation (Day 0-1). Specifically, $CD4^+$ cells are cultured in X-VIVO

15 medium supplemented with 5% HSA, MACS® GMP T Cell TransAct™, IL-2 (100 U/mL) and IL-7 (10 ng/mL) for up to 24 hours at a cell density of 1-2 E6 cells/mL.

[00143] After overnight stimulation, cells are transduced with LVFOXP3. The current transduction uses an MOI of 20. However, during process development, a titration is performed with GMP-process comparable vector to determine the optimal MOI for transduction. Following transduction, cells are cultured at 1-2E6 cells/mL in conditions as described above to promote cell expansion with demi-depletion performed every 1-2 days up to Day 7-9.

[00144] Following expansion, NGFR expressing $CD4^+$ T cells are enriched from the culture; all washing, labeling and selection procedures are performed on the CliniMACS Plus Instrument according to manufacturer's directions. Total nucleated cell count and viability in the culture is calculated using an automated cell counter. An aliquot is sent to the Quality Control unit of the LCGM for determination of the $NGFR^+$ cells using the LSR Fortessa X-20 flow-cytometry device (BD Biosciences, San Jose, CA). The total amount of human NGFR beads required for the selection is determined based on cell number and NGFR frequency. The cells are labeled with NGFR microbeads as per the manufacturer's protocol. CliniMACS PBS-EDTA buffer supplemented with 0.5% HSA is used for the dilution, wash, incubation and elution steps. Post-selection, a sample of the NGFR enriched T cells is taken for determination of cell purity, yield, viability and recovery as assessed by flow-cytometry-based immunophenotypic analysis.

[00145] Post-enrichment, NGFR expressing T cells are cultured in X-VIVO 15 medium supplemented with 5% HSA, IL-2 (100U/ml) and IL-15 (10 ng/mL). The transduced CD4 cells are re-stimulated by the addition of MACS® GMP T Cell TransAct™. Cells are maintained at a cell concentration of 1-2E6 cells/mL with demi-depletion for medium exchange every 2-3 days.

[00146] After 16-21 days in culture, the $CD4^{LVFOXP3}$ are harvested, pelleted by centrifugation and re-suspended in cryo-protectant medium CryoStor CS5 at a concentration of 5-100 x 10^6 cells/mL in cryo-bags (Saint Gobain, Gathersberg MD), frozen in a controlled rate freezer (Thermo Scientific) and stored in vapor phase liquid nitrogen.

Example 5

Specifications

[00147] Fresh or frozen formulated $CD4^{LVFOXP3}$ are considered final drug product and samples are tested as outlined **in FIG. 1**. $CD4^{LVFOXP3}$ that meets the product release criteria is released for infusion. Testing is designed to establish the purity, identity and safety of each batch of drug product. Based on previous data, the drug substance is expected to be comprised of

≥75% $CD4^+$ T cells of which ≥70% are $FOXP3^+$ at ≥70% viability. Given that stable and high FOXP3 expression of the transduced cells provides the phenotypic characteristics and functional properties of $CD4^{LVFOXP3}$ we will determine the FOXP3 and NGFR expression to confirm the cell product identity. The impact of the percentage of FOXP3 and NGFR double positive cells on *in vitro* suppression is determined to evaluate the potency release criteria for future Phase 2/3 studies.

[00148] The viral genome copy number of the $CD4^{LVFOXP3}$ drug substance is determined using a WPRE-based qPCR assay developed at the LCGM. This assay detects in the range of 0.003 to 33 viral genome copies per cell. The results from this assay is recorded for information only (FIO) during the Phase 1 study, and is used to inform a viral genome copy number per cell release criterion for Phase 2/3 studies.

[00149] Replication competent lentivirus (RCL) testing is performed on cells from production of $CD4^{LVFOXP3}$using a Stanford LCGM-developed VSV-G-based qPCR assay for release of the drug product. The limit of detection in this assay is 10 copies of VSV-G in 100 ng genomic DNA, which has a detection rate of 97.92%. The false positive rate for this assay is 1.42%. Genomic DNA from the drug substance is tested in triplicate, and samples that are undetectable for VSV-G in ≥ 2/3 replicates are released for subject use. A sample of the drug product will also be sent to Indiana University Vector Production Facility for a full cell-based RCL assay. The final drug product will undergo safety testing based on established methods to confirm that the drug product has no detectable bacterial or fungal contamination.

Testing and Release Specifications for $CD4^{LVFOXP3}$

Release Testing	Method	Assay Type	Proposed Specifications	Sample for Testing
Identity	Flow Cytometry	$CD4^+$ $FOXP3^+$	≥ 70%	Drug Substance
Purity	Flow Cytometry	$CD4^+$	≥75%	Drug Substance
Viability	Automated cell counter	Propidium Iodide and Acridine Orange	≥70% viable cells	Drug Substance and Drug Product
Safety	Mycoplasma	PCR	undetected	Drug Product
Safety	Endotoxin	Limulus Amoebocyte Assay	<5 EU/kg body weight per hour	Drug Product
Safety	Sterility	Gram Stain	Undetected	Drug Product
Safety	Sterility	BacTec	No Growth for bacterial and fungal	Drug Product

Safety	Replication Competent Lentivirus	qPCR	Undetectable	Drug Product
		Cell Based	Undetectable (Annual Reporting)	Drug Product
Product Characterization	**Method**	**Assay Type**	**Proposed Specifications**	**Sample for Testing**
Safety	Vector Copy Number	WPRE based qPCR assay	For Information Only (FIO)	Drug Substance
Identity	Flow Cytometry	$CD4^+$ $FOXP3^+$ $NGFR^+$	For Information Only (FIO)	Drug Substance
Potency	*In vitro* Suppression Assay	Flow cytometry	For Information Only (FIO)	Drug Substance

Analytical Procedures

[00150] The Sysmex Analyzer (Beckman Coulter Inc, West Sacramento, CA) is used to determine the total nucleated cell count of the apheresis product. Cell viability of the apheresis product is determined by flow cytometry. The total nucleated cell count and viability of all other samples after the $CD4^+$ selection is determined using an automated cell counter according to manufacturer's directions.

[00151] Identity and purity determinations for phenotypic markers such as CD4, NGFR and FOXP3 is performed according to standard multiparameter flow cytometry procedures using monoclonal antibodies. Briefly, cells are re-suspended in PBS and then stained with a pre-determined amount of monoclonal antibody following manufacturer recommendation or based on titration results. Viability stain is included to ensure cell population identifications are based on viable cells only. Appropriate Control Sample is generated to ensure instrument, reagent and QC analyst proficiency per testing. For the need of detecting FOXP3, intracellular staining procedure is established to reach clear $FOXP3^+$ population separation. If needed, isotype control may be used to determine intracellular staining background. Stained samples are acquired by a BD Fortessa or BD Celesta Analytical Cytometer, and analyzed by BD FACSDiva software.

[00152] The suppressive capacity of $CD4^{LVFOXP3}$ is determined with an *in vitro* suppression assay (non-GMP). This assay is used to analyze the impact of the percentage of $FOXP3^+$ and $NGFR^+$ cells on suppression by $CD4^{LVFOXP3}$ in order to evaluate release criteria for future Phase 2/3 studies. In this assay, Responder $CD4^+$ T cells are labeled with a fluorescent dye, CellTrace™ CFSE, to measure cell proliferation. $CD4^{LVFOXP3}$ are labeled by a second fluorescent dye, CellTrace violet, which also measures cell proliferation. Responder $CD4^+$ T cells and $CD4^{LVFOXP3}$ are co-cultured at different concentrations after activation by Dynebeads

Human T-cell Activator CD3/CD28. 96 hours after stimulation, proliferation of responder $CD4^+$ T cells is evaluated by flow cytometry. The suppression index is calculated as (%proliferation of responder-%proliferation of responder+suppressor) /%proliferation of responder *100.

[00153] The absence of replication competent lentivirus (RCL) in the drug substance is confirmed using two assays: (1) a cell-based assay and (2) a qPCR-based assay that detects VSV-G. The cell-based RCL assay is performed at the Indiana University Vector Production Facility according to standard procedures. The results of the cell-based assay is reported in the IND annual report. The qPCR assay is performed at the LCGM according to standard procedures, and results of this assay is available at the time of product release.

[00154] In the RCL qPCR assay, a standard curve is generated using VSV-G plasmid DNA spiked into control human DNA. The total amount of VSV-G in the final product is interpolated from the standard curve. VSV-G is used as the positive control for RCL because although VSV-G protein is required for transduction of the target cells, VSV-G is co-transfected into the viral packaging cell line as a separate plasmid from that carrying the packaging signals and transgene of the provirus. Therefore, VSV-G DNA would only be present in the target cells in the event that a replication competent infectious lentivirus was formed. Positive control samples to determine the limit of detection are generated by creating a secondary dilution curve down to 50 copies per reaction. The 50 copies/reaction dilution is then added to control human DNA at 10, 8, 6, 5, 4, 3, 2, or 1 copies/reaction.

[00155] The viral genome copy number is determined with a qPCR-based assay that detects the total copies of Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) per cell. WPRE was chosen for this assay as it is not present in untransduced human cells and is frequently used as a post-transcriptional regulatory element for many viral vectors. In this assay, a standard curve is generated using a WPRE-containing plasmid DNA spiked into control human DNA. The total copies of WPRE per cell is interpolated from this standard curve. Positive control samples for this assay are comprised of serial dilutions of genomic DNA from an H9-1C cell line. This cell line has a known single integration of WPRE. Human DNA from non-transduced cells is used as a negative control. Testing for lentiviral genome copies per cell is for information only and does not have a specification for Phase 1 studies, but clinical manufacturing experience is used to define a release criterion for Phase 2/3 studies.

[00156] Bacterial and fungal sterility testing of the cellular products are performed on the end of culture cell supernatant to preserve product for patient infusion by automatic Sterility device, BacTec. Inoculation of cultures Aerobic/F, Anaerobic/F, and Myco/F bottles is performed in the BSC in QC Laboratory in order to minimize handling of the samples outside of the cleanroom environment. All cultures are maintained for 14 (bacterial) or 42 days (fungal) by

the Stanford Healthcare Clinical Microbiology Laboratory with final reports of "no growth detected" documented in the COA of all products in compliance with 21 CFR 610.12.

[00157] As the drug product may be administered to the patient prior to receiving full sterility results, two additional procedures are performed to ensure the safety of the drug product. First, a gram stain is done at the Stanford Healthcare Clinical Microbiology Laboratory according to standard operating procedures. The gram stain is a rapid method for assessing sterility that involves staining the drug product with crystal violet and safranin to detect Gram positive and Gram negative organisms, respectively. Second, an interim sterility sample is taken three days prior to harvest of the final drug product. This sample is tested by BacTec Inoculation of culture bottles as described above. In the unlikely event that a positive sterility test result is obtained after a cell product has been administered to the subject, an action plan is executed to notify the Principal Investigator. The details of this action plan and justification for administration prior to sterility results are available is included in the IND for this drug product.

[00158] Endotoxin testing is performed on the final formulated product. 0.5 ml aliquots of formulated product is assessed using the ENDOSAFE-PTS Chromogenic Assay (Charles River Laboratories, Wilmington, MA). The assay is performed at the facility performing the processing (LCGM). The assay is based on the Limulus Amoebocyte Assay. If bacterial endotoxin is present, a protease cascade is initiated, resulting in the release of a chromogenic substrate that can be detected and measured spectrophotometrically. The infusion rate of the final drug product is adjusted to not exceed 5 EU/kg body weight/hour required for all products in compliance with 21CFR§610.12 and USP <85>.

[00159] Mycoplasma testing is performed on end of culture supernatant and cells from production of $CD4^{LVFOXP3}$ using the Roche MycoTOOL Real-Time PCR Kit. The MycoTOOL PCR assay is FDA cleared, detects >140 species of mycoplasma and provides results in <5 hours from sample isolation using only 1 ml of culture supernatant. The limit of detection in this assay is 10 CFU/ml. Prior to licensing, a comparability study is conducted to demonstrate the rapid mycoplasma detection method is as effective as the 28 day mycoplasma test USP Mycoplasma test and as specified under 21 CFR 610.30.

[00160] Cell number is based on the target dose for the dose cohort requirements. Safety testing specifications are set to show that the product is free of bacterial, fungal, mycoplasma and endotoxin contamination. Specifications for process intermediates and cell substances are based on the results of analysis of materials from at least 3 large scale process engineering runs performed according to the standard operating procedure anticipated to be used in the production of clinical materials. Since healthy donor apheresis is used for all

engineering runs, specifications may be refined as additional data is collected during the clinical trial.

[00161] Appropriate standards and controls are used (where available) to verify the performance of raw materials, reagents, devices and assays used in the production, purification and testing of materials for clinical use.

[00162] Cells are cryopreserved in USP Class VI fluorinated ethylene propylene (FEP) KryoSure containers that are FDA 510(k) cleared (Saint Gobain, Gaithersburg, MD).

[00163] Stability studies are performed on drug products from qualification runs and will cover the maximum period expected for holding cells (up to 12 months). All drug products are tested for viability and identity. The drug product must retain ≥70% viability upon thaw and meet specifications for $CD4^+$ $FOXP3^+$ to be considered stable and infused into the patient.

[00164] These products are administered within 12 months of cryopreservation. We will record viability and identity at the time of thawing, but we do not anticipate storage of these products beyond this time. We therefore will not perform any long-term stability studies.

Example 6

In Vitro Pharmacology

[00165] The human *FOXP3* coding sequence was cloned under the control of a constitutive promoter in a bi-directional lenti-viral-vector (LV) construct. This vector construct allows simultaneous expression of two transgenes under two independent promoters. In $CD4^{LVFOXP3}$, the two transgenes are encoding for the functionally specific full-length FOXP3 and for the cell-surface marker NGFR used to select and track the transduced T cells as originally described in Allan S et al. in which the vector was named pCCL.FP3. pCCL.FP3 is referred to as LVFOXP3 throughout this document. Naïve $CD4^+$ T cells were initially transduced with LVFOXP3 upon TCR-mediated preactivation with anti-CD3/CD28 mAbs or anti-CD3 mAb and antigen presenting cells (APC) in the presence of IL-2 and IL-7. Since FOXP3 expressing cells have reduced proliferative capacity, the transduced cells have to be purified in order to be tested and further expanded. Transduced cells purification can be easily performed by positive selection of $NGFR^+$ cells. Efficiency of transduction was high (86 sd 12%) with the control vector and lower (47 sd 18%) with LVFOXP3 because of the less efficient packaging of the larger viral genome. However, $NGFR^+$ cells purification consistently resulted in a homogeneous cell population to be assessed phenotypically and functionally. Unlike $CD4^+$ cells transduced with the "empty" control vector, LVFOXP3 $CD4^+$ transduced cells ($CD4^{LVFOXP3}$) homogeneously expressed FOXP3 and CD25 at comparable levels as expanded CD25hi Treg cells, and both cell types had low expression of CD127, as expected. The expression of FOXP3 in lentiviral LVFOXP3 transduced cells was persistent overtime after

activation, which was superior to what was previously obtained when retroviral FOXP3 transduction was used. The percentage of FOXP3 positive cells in the $CD4^{LVFOXP3}$ prepared with LVFOXP3 remained high and stable for up to 4 weeks of culture.

[00166] $CD4^{LVFOXP3}$ showed the ability to suppress proliferation and IFN-γ production of activated $CD4^+$ responder T cells. The presence of $CD4^{LVFOXP3}$cells in the co-culture inhibited the effector function of the responder cells in a cell dose dependent fashion and at comparable levels to *ex-vivo* isolated Treg (CD25hi T cells), whereas cells transduced with the control vector without FOXP3 (pCCL) did not acquire suppressive function. The suppressive activity of the $CD4^{LVFOXP3}$ cells was comparable when the transduction was established starting from naïve or memory $CD4^+$ T cells.

[00167] Overall these initial studies demonstrated the powerful and stable effect of the FOXP3 transduction in converting a $CD4^+$ T cell from an effector cell to a suppressor cell. The lentivirus-mediated FOXP3 transduced cells resembled naturally occurring CD25hi Treg cells peripherally isolated and tested fresh or after expansion, in terms of FOXP3, CD25 and CD127 expression and suppressive function. In addition, the lentivirus-mediated FOXP3 transduced cells expressed FOXP3 more persistently overtime and at higher levels as compared to retrovirus-mediated FOXP3 transduced cells or expanded Treg cells.

[00168] Recently, we performed validation and inter-laboratory reproducibility studies in additional healthy donors and IPEX patients. We first used the same LVFOXP3 construct and cell production method, and more recently, have been working towards establishing a more GMP compliant vector construct and cell production method. Briefly, we eliminated the presence of APC during the pre-transduction activation, and the presence of feeder cells during the reactivation for expansion. We have replaced both steps using TransAct, anti-CD3 and anti-CD28 coated nanoparticles (Miltenyi Biotec Inc,), that are commercially available GMP grade. In addition, we have discontinued the use of polybrene during transduction and have shortened the culture period by re-stimulating the purified $NGFR^+$ transduced cells at day 8-11 and expanding them for 8-10 days in order to shorten the total culture time from the original process. The reproducibility of the method with this optimization is demonstrated in Figure 3 in terms of transduction efficiency, purity and vector copy number (Figure 3a), FOXP3, NGFR, CD25 and CD127 expression (Figure 3b) and cytokine production and coinhibitory membrane markers (Figure 3c-e). The data reported are an example of a consistent production. Numbers may slightly change in the final Phase 1 product.

[00169] Consistent with previous results, the $CD4^{LVFOXP3}$, obtained at Stanford with the transferred method (alloAPC) or with the optimized cGMP method (TransAct), exert good suppressive activity when obtained from healthy donor samples (Figure 4a). Functional $CD4^{LVFOXP3}$ were also obtained from two additional IPEX patients (Figure 4b). Notably, IPEX

Patient #37 is a young adult under chronic immunosuppression, whereas Patient #64 has severe acute IPEX with *FOXP3* mutation that completely abrogated protein expression . The use of cells from these two patients shows that functional $CD4^{LVFOXP3}$ can be obtained even from T cells exposed to chronic immunosuppression, as well as from T cells completely lacking endogenous FOXP3.

[00170] Overall the results obtained comparing the two methods indicate the reproducibility and confirm that the use of the GMP compliant activation beads (TransACT™) allow production of conforming $CD4^{LVFOXP3}$. Expression of FOXP3 and NGFR was evaluated 10 days after culture on $CD4^{LVFOXP3}$, after re-stimulation and expansion. Suppressive activity of $CD4^{LVFOXP3}$ was measured at day 10 after re-stimulation, on a 4 days co-culture suppressive assay. We propose to use the data from small scale experiments as the measure of identity of the $CD4^{LVFOXP3}$ product ($FOXP3^+$ cells ≥ 70%) in the proposed trial. In addition, we propose to measure the percentage of $NGFR^+$ cells, which is not always as high as FOXP3, and suppression *in vitro* as an indication of potency of $CD4^{LVFOXP3}$, which may be further validated for use in future Phase 2 trials.

[00171] Moreover, the FOXP3 and NGFR phenotype is consistent when tested on fresh cells at the end of the culture or after freezing and thawing (Figure 5).

[00172] Lastly, the gene transcription profile of the $CD4^{LVFOXP3}$ T cells has been obtained from 3 healthy subjects and has been compared to that of Treg cells isolated from the same subjects. This comparison revealed that the $CD4^{LVFOXP3}$ T cells are overall divergent from Treg cells and more similar to expanded Teff cells but do overlap with Treg cells for a restricted core of 6 genes (plus of course FOXP3) (Figure 6). These core 6 genes are differentially upregulated in Treg and in $CD4^{LVFOXP3}$ T cells, and they are most likely related to FOXP3 overexpression and the acquired suppressive function. In addition, the TCR repertoire of the $CD4^{LVFOXP3}$ T cells remains polyclonal and therefore similar to that of the original cells (Figure 7).

[00173] To test the efficacy of the $CD4^{LVFOXP3}$ *in vivo*, we utilized a widely accepted method for testing functional Treg cells, consisting of the ability of the Treg population to suppress the xenoGVHD reaction triggered by injection of human $CD4^+$ T cells in an immunodeficient mouse (NSG) sublethally irradiated (Hahn SA et al., 2015). Once injected, the human T cells engraft, expand and mount an immune response towards the mouse tissues, which cause weight loss, wasting and death of the mice within 2-3 weeks. In Passerini L. et al. we showed that cotransfer of human $CD4^+$ T cells (Teff) with $CD4^{LVFOXP3}$, but not with the control $CD4^{LVNGFR}$, promoted survival of 75% of the mice. $CD4^{LVFOXP3}$ prevented xenoGVHD reaction in 71% of the mice even when injected at day 6 after Teff cell administration (Figure 8a and b). Therefore $CD4^{LVFOXP3}$ showed the ability to prevent or control xenoGVHD reaction. In this

hu-mouse model, $CD4^{LVFOXP3}$ were detected in the peripheral blood of the mice up to 14 days post injection, as $NGFR^{+}$ cells (Figure 8c). These results indicate that these cells did not expand but rather declined as the Teff expanded in the control mice. In addition, when injected alone, $CD4^{LVFOXP3}$ did not caused xenoGVHD. The xenoGVHD model was also used to assess *in vivo* stability of the FOXP3 transduced T cells.

[00174] Seven days after injection in the late transfer model, and therefore after being exposed to the inflammatory environment caused by irradiation and by expansion of the xenoreactive Teff cells, $CD4^{LVFOXP3}$ were isolated from secondary lymphoid organs and analyzed for FOXP3, CTLA4 and CD25 expression (Figure 10). All these markers were consistently higher in $CD4^{LVFOXP3}$ as compared to the control $CD4^{LVNGFR}$ that were FOXP3 negative and expressed low levels of CD25 and CTLA4. These data indicate the phenotypic stability of the $CD4^{LVFOXP3}$.

[00175] $CD4^{LVFOXP3}$ generated from IPEX patients were also tested *in vivo* and proved to be efficacious and comparable to healthy donor's $CD4^{LVFOXP3}$ in preventing xenoGVHD (Figure 9). In all the experiments previously done *in vivo*, the responder Teff cells were allogeneic to the suppressor cells. However, since we plan to inject autologous $CD4^{LVFOXP3}$ in IPEX patients, we tested in parallel autologous and allogeneic human xenoGVHD.

[00176] We demonstrated that both allogenic and autologous $CD4^{LVFOXP3}$ have the ability to prevent the xenoGVHD. Both had similar survival (14 days). In addition, at sacrifice, we collected the human T cells from the spleen of sacrificed (day 40) mice (NSG) and we showed a significant decrease in activation markers as compared to those expressed by cells obtained from the control mice.

[00177] In addition, the mice injected with $CD4^{LVFOXP3}$ were resistant to further injections (re-challenge) of a second subset of Teff cells, suggesting that $CD4^{LVFOXP3}$ exert an effect on other cells, leading to a decrease in activation and possibly reduced reactivity of other cells (Figure 11). Therefore, these data suggest an "infectious tolerance" effect on the reactive T cells.

[00178] Overall these data show the efficacy of the $CD4^{LVFOXP3}$ *in vitro* and *in vivo*, in autologous as well as in the allogeneic setting.

[00179] We established: i) a method to assess the efficacy of $CD4^{LVFOXP3}$ T cells in preventing and controlling lymphoproliferation of FOXP3 deficient CD4+T cells whereby these cells are obtained from healthy donors CD34+ HSPCs in which FOXP3 has been knocked-down by CRISP-Cas9 RNP specifically targeting FOXP3 or from IPEX patients' CD34+ HSPCs. The FOXP3 deficient HSPCs are then transplanted intrahepatically in 3-5 days old pups of immune deficient mice (NSG or SGM3_NSG) and differentiate into an immune system with reduced/absent FOXP3 expression (Figure 12) . ii) a method to assess the impact of the $CD4^{LVFOXP3}$ T cells on completion and expansion of an immune response to pathogens, including but not limited to fungi (candida albicans), virus (adenovirus), using huPBL-mice

model (Figure 13) ; and iii) a method to assess the impact of $CD4^{LVFOXP3}$ T cells on immunesurvelliance and tumor clearance in a skin sarcoma hu-mouse model (Figure 14).

[00180] $CD4^{LVFOXP3}$ T cells express high and stable levels of CD25 on the membrane that is directly regulated by FOXP3. Therefore, we have successfully demonstrated (Figure 15) that these cells can be eliminated in vivo by administration of anti-CD25 mAb Basiliximab, commercially available and in use in pediatric and adult patients undergoing transplantation.

[00181] $CD4^{LVFOXP3}$ T cells have a stable phenotype even in the presence of rapamycin (Figure 18).

[00182] The immune system of IPEX patients manifests multiple immune defects which have been described in other autoimmune diseases not caused by *FOXP3* mutations. Specifically, increased T lymphocyte numbers, increased autoreactive B cells with autoantibody production, increased IL-17 producing T cells, increased T follicular helper cells, increased T helper cells' cytokines (increased IFNγ, IL-17, IL-23) are all common hallmarks of autoimmunity. Therefore, even if $CD4^{LVFOXP3}$ have not been used *in vivo,* there are data supporting the beneficial effect of the clinical use of Treg cells of different origin to safely regulate uncontrolled immune responses and clinical manifestations commonly observed in IPEX syndrome (Bluestone Science, 2018; Esensten JH JACI, 2018).

[00183] Adoptive transfer of polyclonal $CD4^+CD25^+$ $FOXP3^+$ Treg cells has proven to be safe and showed some level of efficacy in clinical trials. Numerous Treg trials have been recently completed or are currently being conducted in a variety of diseases including Inflammatory Bowel Disease, autoimmune hepatitis, skin autoimmune diseases and allergy (Esensten JH, 2018).

[00184] Several groups have applied polyclonal $CD4^+CD25^+$ Tregs containing a high proportion of $FOXP3^+$ T cells, either freshly isolated or *ex-vivo* expanded, with the aim of preventing GvHD after allogenic-HSCT for onco-haematological diseases. The results showed that the overall procedure is feasible and safe. One trial reported decreased incidence of grade II-IV GvHD as compared to historical controls in patients receiving umbilical-cord-blood-derived Tregs, without increased incidence of infections (Brunstein et al., 2011). Data were confirmed in a more recent trial from the same group, in which the clinical outcome of patients receiving Treg-based cell therapy was compared with that of control patients who received the same conditioning regimen and immunosuppressive treatment but no Tregs. The incidence of grade II-IV acute-GvHD at 100 days was 9% vs 45% in controls, whereas chronic-GvHD at 1 year was zero in treated patients (Brunstein et al., 2016). The Treg treatments were well tolerated without any suspected adverse events, even at the highest cell dose of $100x10^6$ cells/kg, and no increase in the number or severity of infections in the Treg treated cohort was observed.

[00185] In a third trial, patients undergoing allogenic-HSCT who were injected with freshly isolated peripheral Tregs showed low grade GvHD and no development of chronic-GvHD (Di Ianni et al., 2011). More recently, the same group showed reduced incidence of disease relapse in Treg-treated patients (Martelli et al., 2014).

[00186] These initial results encouraged a wider application of Tregs as therapy after solid organ transplantation. Several trials are currently ongoing, although final results are not currently available (Vaikunthanathan et al., 2017b). Among those, in The-ONE-Study, a Phase 1/2 dose-escalation study, several subtypes of Tregs, including *ex-vivo* expanded FOXP3^{+}-Tregs, have been infused in patients undergoing kidney transplant with the goal of avoiding life-long immunosuppression through the induction of active tolerance (NCT02129881) (Leslie, 2011; Vaikunthanathan et al., 2017b). Similarly, a Treg-immunotherapy trial in the setting of liver transplantation, ThRIL (NCT02166177), has been initiated, although safety data are not yet available (Safinia et al., 2016).

[00187] FOXP3^{+}-Treg-based therapy was safely tested also in the context of autoimmune diseases. In a trial limited to a few patients, *ex-vivo* expanded autologous CD4^{+}CD25^{hi}CD127^{-} Tregs were administered to children with recent-onset T1D (Marek-Trzonkowska et al., 2012), and more recently to new-onset adult T1D patients (Bluestone et al., 2015). In both cases the procedure appeared to be feasible and safe, as no toxicities or serious adverse events attributable to the infusion were reported in up to one year follow up, although published data do not provide conclusions on efficacy. In the first trial, patients were treated with 10-20x10^{6} cells/kg, which resulted in a statistically significant lower insulin requirement and higher C peptide laboratory measure when compared to matched control subjects, while 2 of 12 treated patients became insulin independent. Importantly, in the latter trial, safety was demonstrated for transfer of high number of Tregs (up to 2.6 x 10^{9} cells/kg) (Bluestone et al., 2015). Several patients post Treg infusion had stable, improved C peptide levels and insulin use for up to 2 years after a single Treg injection, although the study was not powered to determine efficacy.

[00188] Overall, the data available support the safety of the Treg cell therapy approach. However, the cells manufactured in the previous studies were not stable in inflammatory conditions and their *in vivo* survival was limited and difficult to assess. The potential advantage of the proposed CD4^{LVFOXP3} is that they are phenotypically stable. CD4^{LVFOXP3} can also be traced via cell surface expression of NGFR encoded in the LV that contains the *FOXP3* gene. The efficacy of CD4^{LVFOXP3} is a medical breakthrough in treatment of patients with IPEX and will inform us about the response of clinical manifestations of the disease (T1D and other endocrinopathies, inflammatory bowel disease (IBD), eczema or psoriasis-like dermatitis, cytopenia) over time. This information is key for the design of future indications.

Example 7

Specifications of New LVFOXP3 Construct

[00189] To obtain $CD4^{LVFOXP3}$ by FOXP3 gene transfer into autologous $CD4^+$ T cells we have developed a third-generation bidirectional lentiviral vector containing the full-length cDNA of *FOXP3*, expressed under an EF1α promoter and *NGFR*, a marker gene, expressed under the minimal CMV promoter from the opposite strand, providing a cell surface marker for selection of transduced cells, and a robust method of gene transduction in $CD4^+$ T cells followed by purification and expansion of the $CD4^{LVFOXP3}$.

[00190] The LVFOXP3 vector construct underwent improvements relative to the vector described in the publication Passerini L. et al Sci Transl Med. (2013) 5(215):215ra174) that reduced the size, enhanced efficiency of production, and made this construct suitable and safer for clinical use. These modifications consisted of CAP binding site/AC promoter and HA tag removal; AmpR replacement with KanaR; and replacement of wildtype WPRE with a mutated WPRE, and are shown in Figure 2A and 2B.

[00191] The pre-clinical data available to date demonstrates that LVFOXP3 provides efficient functional gene transduction in $CD4^+$ obtained from both healthy individuals and IPEX patients with different FOXP3 mutations. In addition, we showed functional equivalence using $CD4^{LVFOXP3}$ allogenic or autologous to the responder effector T cells. To improve safety, basiliximab, an anti-CD25 mAb, can be used to eliminate $CD4^{LVFOXP3}$ cells in the event of unacceptable toxicity.

Example 8

Clinical Trial

[00192] We propose that administration of autologous $CD4^+$ T cells converted into $CD4^{LVFOXP3}$ by LV-mediated *FOXP3* gene transfer to patients with IPEX reduces autoreactivity and immune dysregulation, therefore offering an improved therapeutic approach that circumvents the requirement for generalized immunosuppression and avoids the risks of allogenic hematopoietic stem cell transplant (HSCT), or at least would ameliorate the patient's clinical status without additional organ damage, favoring a better outcome for HSCT, when available. We have successfully converted healthy donor $CD4^+$ T cells into Treg cells using the LV-mediated *FOXP3* gene transfer as described above.

[00193] A key advantage to autologous cell therapy is that wild-type *FOXP3* is delivered to the patient's own $CD4^+$ T cells, containing also the pathogenic T cells. These autologous engineered cells are infused to restore immune regulation. In addition, the autologous $CD4^{LVFOXP3}$ are functionally stable, since they constitutively express FOXP3, and they might

alone be sufficient to immune regulate, allowing reduction or avoidance of pharmacologic immunosuppression.

[00194] A standard 3 + 3 dose escalation design is used in the clinical trial. Three to 6 patients per dose-cohort are treated at each dose of $CD4^{LVFOXP3}$ ($1x10^6$ cells/kg, $3x10^6$ cells/kg, and $10x10^6$ cells/kg). The starting dose level of $1x10^6$ cells/kg was chosen based on previous experience with freshly isolated or expanded Treg cell therapy. Indeed, expanded autologous Treg cells administered in T1D patients have been shown to be safe even at doses of 10^9/kg. However, since our cell product is FIH, is genetically modified and we plan to treat acutely ill pediatric patients with very diverse age and weight, we chose this conservative low dose. In addition, a 28 day safety assessment period after the first patient in each dose cohort and prior to escalation to the next dose cohort will occur to evaluate dose limiting toxicities (DLTs). All other infusions in each dose cohort are staggered by at least fourteen (14) days to allow for adequate safety assessment.

[00195] Although this study is a first-in-human trial, because of the lethal nature of this disease, the median age of surviving individuals with IPEX, and the lack of alternative curative options, the safety of $CD4^{LVFOXP3}$ is evaluated in patients of all ages. We propose that the first patient in the first dose cohort is 12 years of age or older, and if tolerated (no dose limiting toxicity observed), all other patients is enrolled irrespective of age. Patients enrolled in the study will not change their current pharmacological therapy, unless receiving steroids, in which case the dose of steroids must be less than 0.5 mg/kg/day in order for the patient to be eligible for participation in this trial.

[00196] The study is designed to evaluate the feasibility of manufacturing $CD4^{LVFOXP3}$ to meet the targeted dose level and established release criteria, and determine the maximum tolerated dose (MTD) of autologous $CD4^{LVFOXP3}$ in subjects with IPEX who are affected by Treg cell deficiency due to FOXP3 mutation. In addition, the impact of $CD4^{LVFOXP3}$ T cell on the clinical manifestations in IPEX patients are assessed. The administration of autologous $CD4^+$ T cells converted into $CD4^{LVFOXP3}$ by LV-mediated *FOXP3* gene transfer administered to patients with IPEX is expected to reduce autoreactivity, inflammation and immune dysregulation; thereby, offering a therapeutic approach that circumvents the requirement for generalized immunosuppression and avoids the risks of allogenic HSCT, or when a suitable donor is available, may stabilize the patient's clinical condition to minimize the risk of transplant. In addition, $CD4^{LVFOXP3}$ could also be given after hematopoietic stem cell transplantation to boost immune regulation and control rejection (disease relapse) that is often observed in IPEX patients.

What is claimed is:

1. A method of producing regulatory T cell (Treg)-like cells for clinical use, the method comprising:

i) obtaining a biological sample comprising $CD4^+$ T lymphocytes from a subject;

ii) transducing the $CD4^+$ T lymphocytes with a recombinant lentiviral vector comprising full-length *FOXP3* cDNA; and

iii) culturing the $CD4^+$ T lymphocytes under conditions suitable for expression of the FOXP3, wherein the $CD4^+$ T lymphocytes are converted into the Treg-like cells ($CD4^{LVFOXP3}$ T cells).

2. The method of claim 1, wherein the lentiviral vector integrates into the genome of the $CD4^+$ T lymphocyte following transduction.

3. The method of claim 1, wherein the lentiviral vector is non-integrated following transduction.

4. The method of any of claims 1-3, wherein the full-length *FOXP3* cDNA is expressed under a ubiquitous constitutive promoter.

5. The method of claim 4, wherein the ubiquitous constitutive promoter is EF1α promoter.

6. The method of any of claims 1-5, wherein the lentiviral vector further comprises *NGFR* expressed under minimal CMV promoter from the opposite strand vector

7. The method of any of claims 1-6, wherein the biological sample comprises peripheral blood lymphocytes.

8. The method of any of claims 1-7, wherein $CD4^+$ T lymphocytes are isolated from the biological sample by immunomagnetic separation.

9. The method of any of claims 1-8, wherein the CD4+ T lymphocytes are cultured for up to 24 hours in medium comprising IL-2 and IL-7 before transduction.

10. The method of any of claims 1-9, wherein step iii comprises culture in medium comprising IL-2 and IL-15 for a period of from 5 to 15 days.

11. The method of any of claims 1-10, comprising substantially purifying $CD4^{LVFOXP3}$ T cells following step iii) by positive selection to obtain a cell population of greater than 85% purity.

12. The method of claim 11, wherein the positive selection comprising immunomagnetic selection for NGFR.

13. The method of any of claims 1-7, wherein transduced selected $CD4^+$ cells are re-stimulated by culture in medium comprising an agent that binds to and activates polyclonal T cells through the T cell receptor in the absence of antigen-presenting cells.

14. A bidirectional lentiviral vector of not more than 11,000 bp comprising full-length *FOXP3* cDNA expressed under an EF1α promoter and *NGFR* expressed under minimal CMV promoter from the opposite strand vector, for use in the method of any of claims 1-8, comprising:

a) a polynucleotide encoding forkhead box protein 3 (FOXP3);
b) an elongation factor 1a (EF1α) promoter, wherein the EF1α promoter is operably linked to the polynucleotide encoding FOXP3;
c) a polynucleotide encoding NGFR;
d) a modified Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) modified to remove a WHx start codon;
e) human immunodeficiency virus (HIV)-derived elements comprising a 5' long terminal repeat (5' LTR), a Ψ packaging signal, a truncated Gag sequence, a rev response element (RRE) sequence, a central polypurine tract (cPPT), a central termination sequence (CTS), a truncated negative regulatory factor (NEF) sequence, and a SIN 3' long terminal repeat (3' LTR);
f) a polyadenylation sequence;
g) an SV40 origin of replication; and
h) a bacterial high copy origin of replication (Ori)
i) kanamycin resistance gene.

15. The recombinant lentiviral vector of claim 9, wherein the NGFR is a truncated nerve growth factor receptor (tNGFR) operably linked to the minimal CMV promoter.

16. The recombinant lentiviral vector of claim 14 or claim 15, wherein the recombinant lentiviral vector comprises components arranged as depicted in the vector map shown in FIG. 2.

17. The recombinant lentiviral vector of any of claims 14-16, wherein the recombinant lentiviral vector comprises the nucleotide sequence of SEQ ID NO:2 or a sequence having at least 80% identity to the sequence of SEQ ID NO:2, wherein the recombinant lentiviral vector is capable of generating a Treg-like cell by transduction of a $CD4^+$ T lymphocyte.

18. A $CD4^{LVFOXP3}$ T cell produced by the method of any of claims 1-13.

19. A composition comprising a unit dose of the Treg-like cell of claim 18 and a pharmaceutically acceptable excipient, for use in treatment of an inflammatory and/or autoimmune condition.

20. The composition of claim 19, wherein the inflammatory and/or autoimmune condition is immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome.

21. The composition of claim 19, wherein the inflammatory and/or autoimmune condition is for example, multiple sclerosis (MS), rheumatoid arthritis (RA), autoimmune thyroid disease (AITD), myasthenia gravis, diabetes mellitus type 1, alopecia areata, atopic dermatitis, autoimmune aplastic anemia, autoimmune enteropathy, autoimmune hepatitis, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, celiac disease, Crohn's disease, eosinophilic fasciitis, eosinophilic gastroenteritis, eosinophilic pneumonia, undifferentiated connective tissue disease, undifferentiated spondyloarthropathy, urticarial vasculitis, vasculitis, vitiligo, autoimmune cardiomyopathy, scleroderma, atherosclerosis, fibrosis, inflammatory bowel disease, inflammatory myopathy, giant cell arteritis (GCA), asthma, allergy, Parkinson's disease and acute respiratory distress syndrome ARDS, e.g. resulting from coronavirus infection.

22. A method of treating an inflammatory and/or autoimmune condition in a subject, the method comprising administering a therapeutically effective amount of the composition of claim 19 to the subject.

23. The method of claim 22, wherein the inflammatory and/or autoimmune condition is caused by Treg deficiency.

24. The method of claim 22, wherein the inflammatory and/or autoimmune condition is caused by Treg numeric or functional deficiency.

25. The method of claim 22, wherein the inflammatory and/or autoimmune condition is controlled or prevented by Treg infusion.

26. The method of claim 17, wherein the inflammatory and/or autoimmune condition is IPEX syndrome.

27. The method of any of claims 22-26 wherein multiple cycles of treatment are administered to the subject.

28. The method of any of claims 22-27, wherein the Treg-like cell is autologous or allogeneic relative to the recipient.

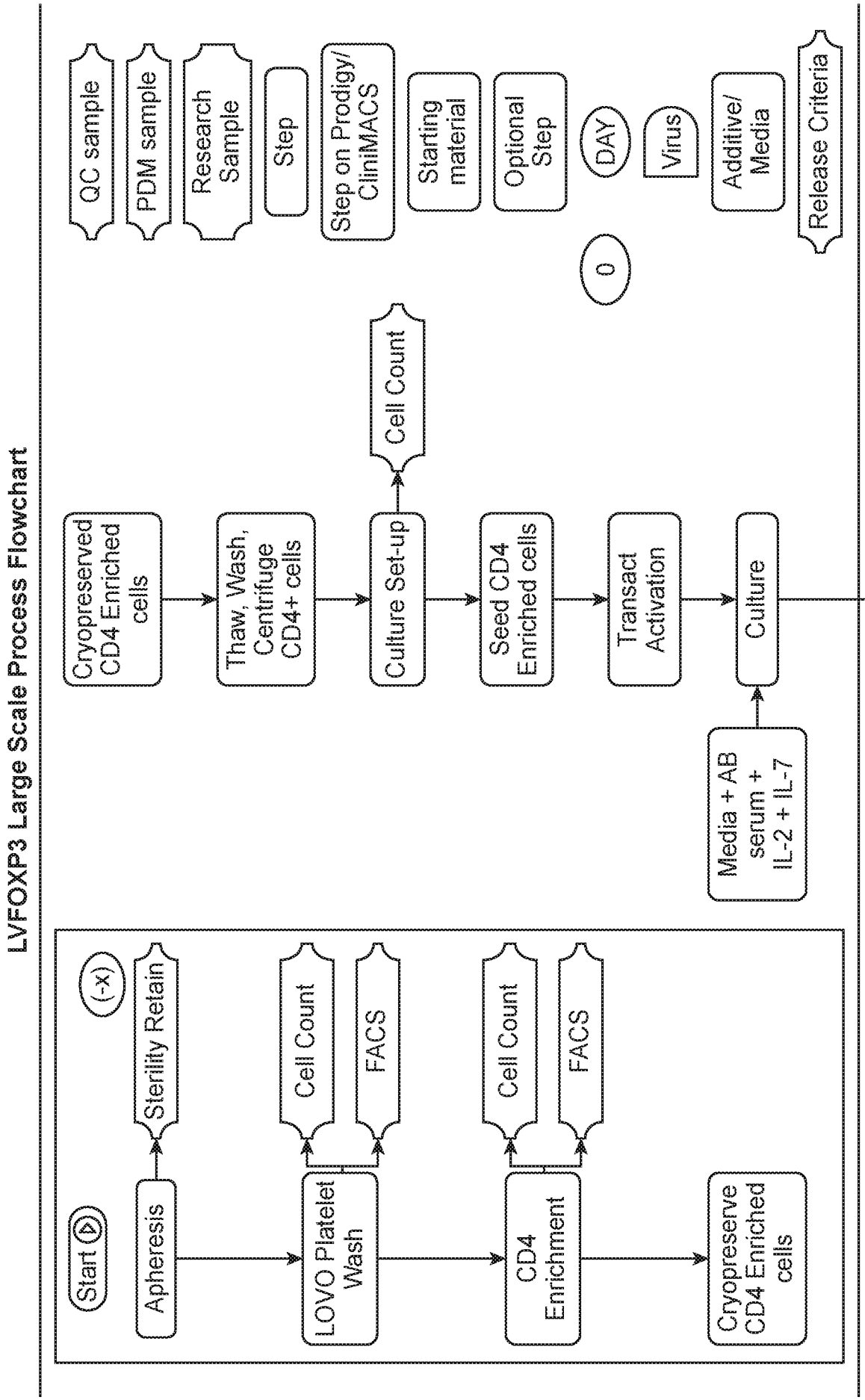

LVFOXP3 Large Scale Process Flowchart
Start
Apheresis
(-x)
Sterility Retain
LOVO Platelet Wash
Cell Count
FACS
CD4 Enrichment
Cell Count
FACS
Cryopreserve CD4 Enriched cells
Cryopreserved CD4 Enriched cells
Thaw, Wash, Centrifuge CD4+ cells
Culture Set-up
Cell Count
Seed CD4 Enriched cells
Transact Activation
Culture
Media + AB serum + IL-2 + IL-7
0
DAY
Virus
Additive/ Media
Release Criteria
QC sample
PDM sample
Research Sample
Step
Step on Prodigy/ CliniMACS
Starting material
Optional Step

FIG. 1

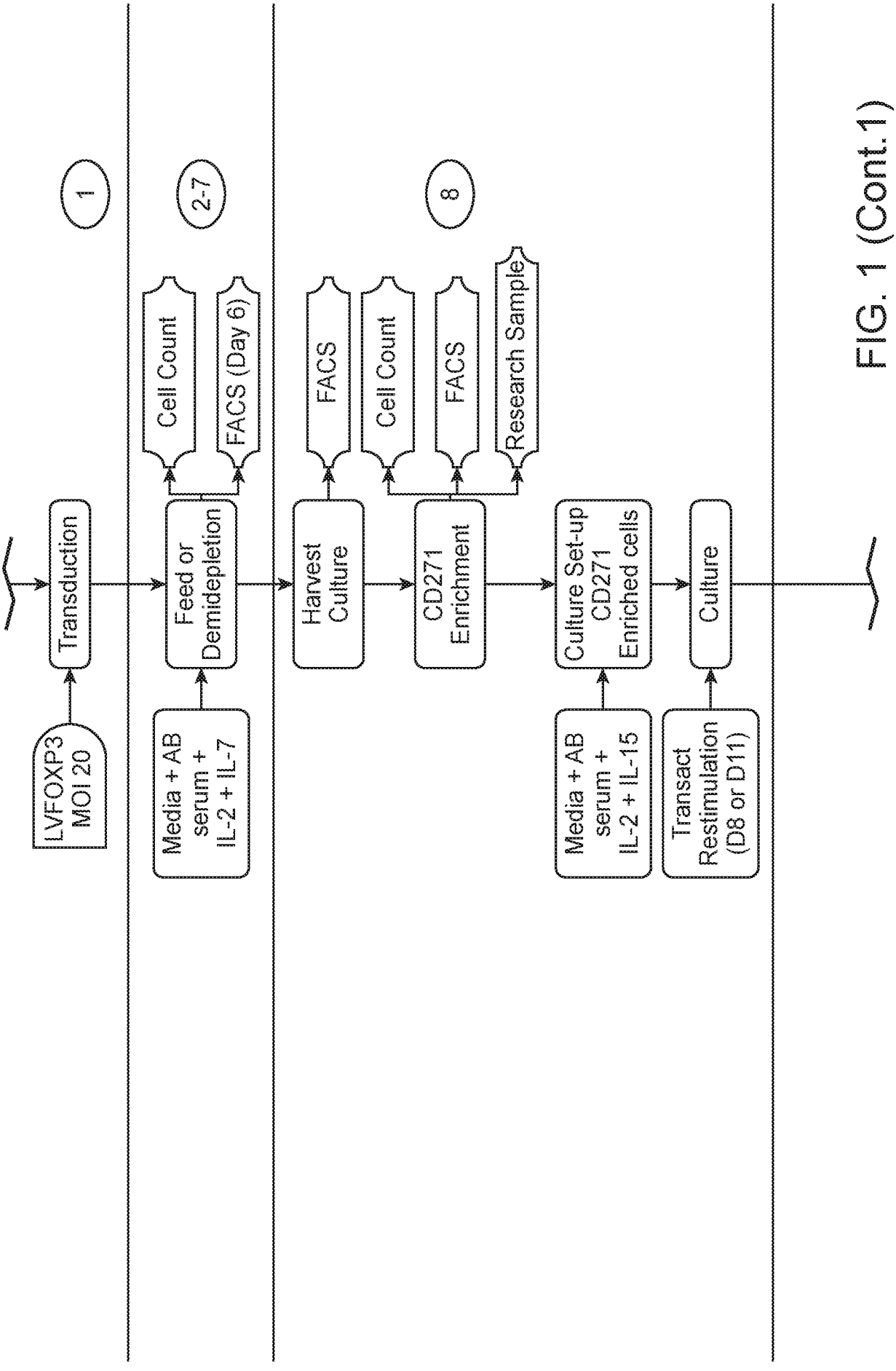

1
2-7
8
LVFOXP3 MOI 20
Transduction
Media + AB serum + IL-2 + IL-7
Feed or Demidepletion
Cell Count
FACS (Day 6)
Harvest Culture
FACS
CD271 Enrichment
Cell Count
FACS
Research Sample
Media + AB serum + IL-2 + IL-15
Culture Set-up CD271 Enriched cells
Transact Restimulation (D8 or D11)
Culture

FIG. 1 (Cont.1)

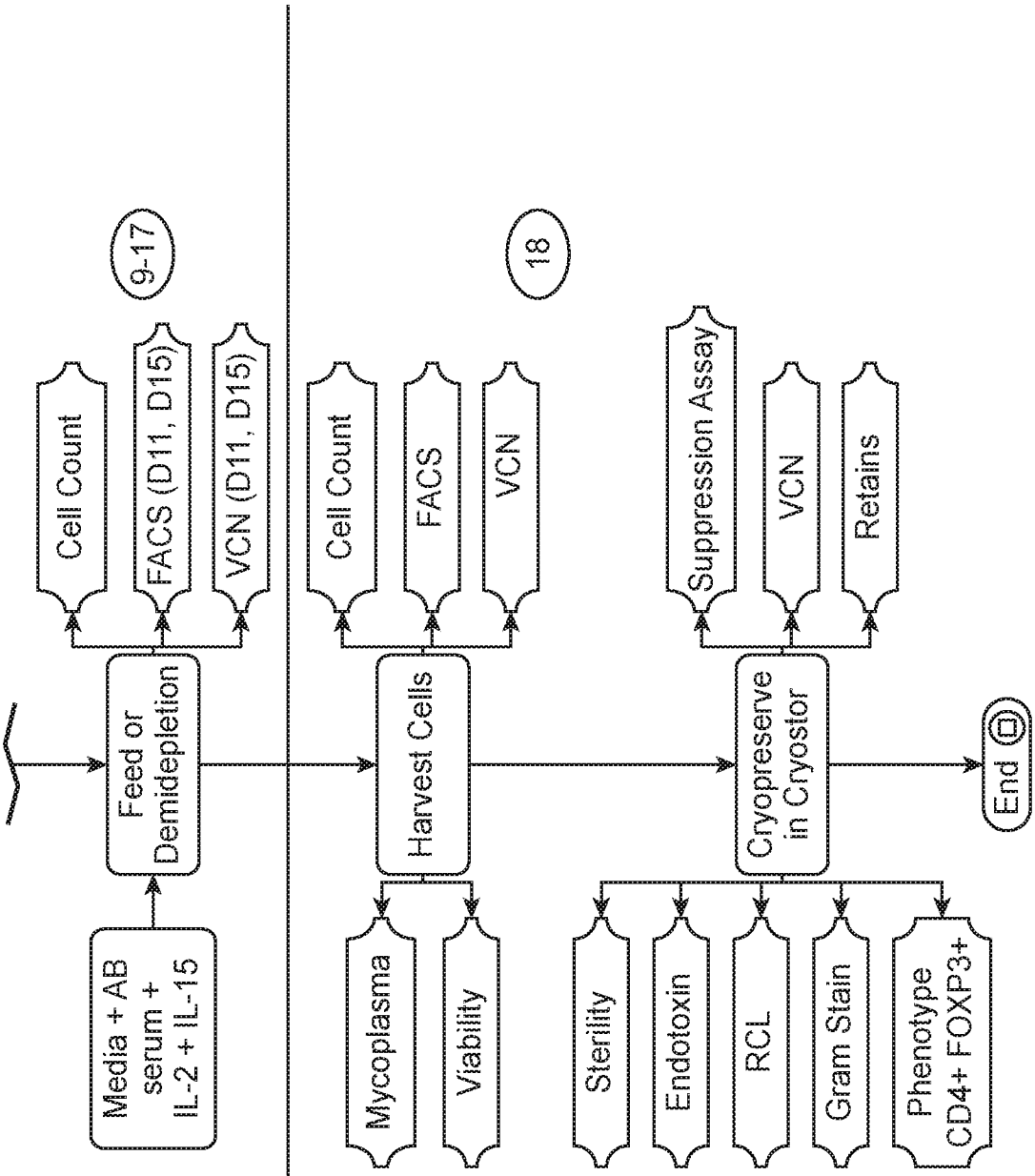

9-17
18
Cell Count
FACS (D11, D15)
VCN (D11, D15)
Cell Count
FACS
VCN
Suppression Assay
VCN
Retains
Feed or Demidepletion
Harvest Cells
Cryopreserve in Cryostor
End
Media + AB serum + IL-2 + IL-15
Mycoplasma
Viability
Sterility
Endotoxin
RCL
Gram Stain
Phenotype CD4+ FOXP3+

FIG. 1 (Cont.2)

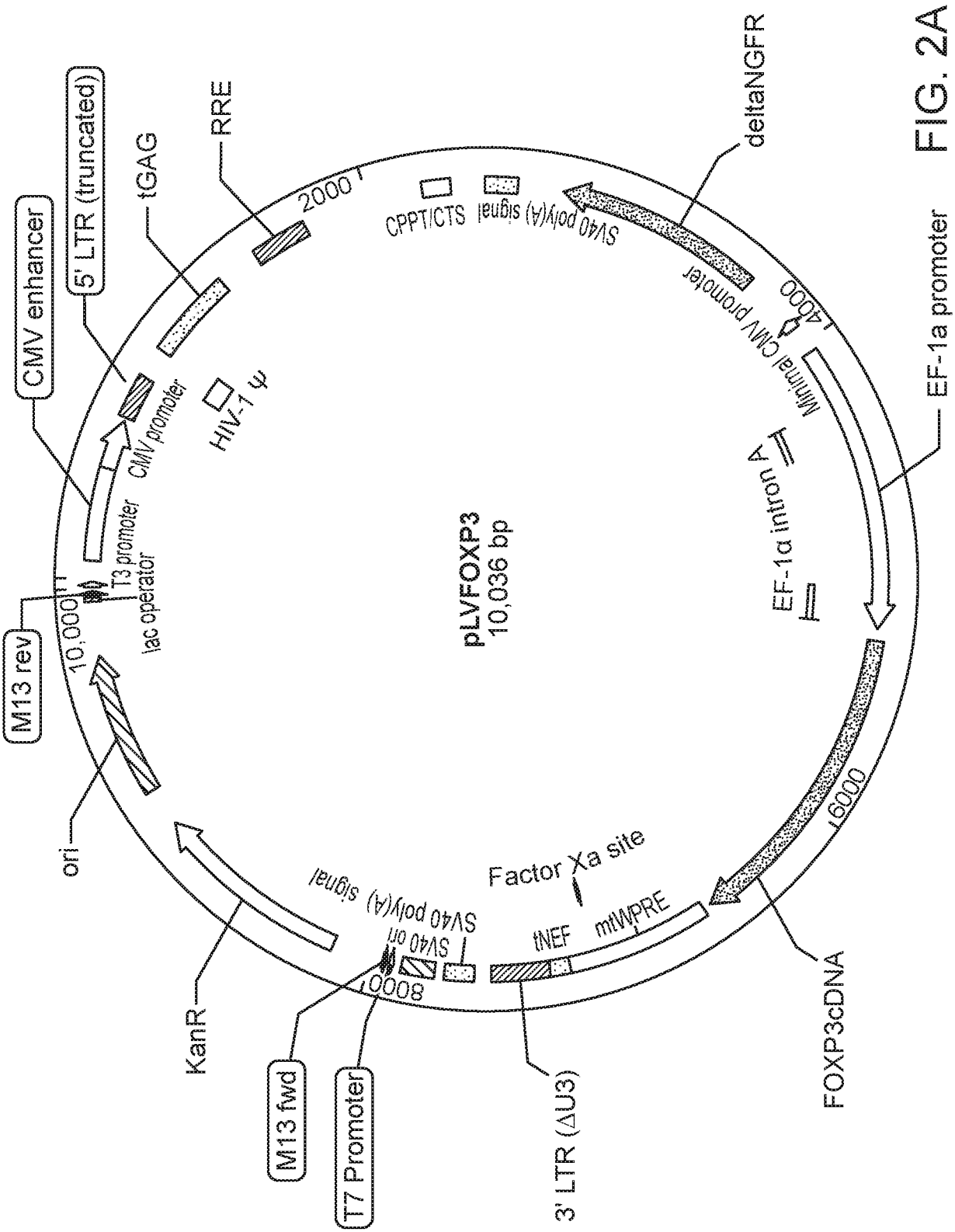

pLVFOXP3
10,036 bp
2000
4000
6000
8000
10,000
CMV enhancer
5' LTR (truncated)
tGAG
RRE
CPPT/CTS
SV40 poly(A) signal
deltaNGFR
Minimal CMV promoter
EF-1a promoter
EF-1α intron A
FOXP3cDNA
3' LTR (ΔU3)
T7 Promoter
M13 fwd
KanR
ori
M13 rev
T3 promoter
lac operator
CMV promoter
HIV-1 ψ
SV40 ori
tNEF
mtWPRE
Factor Xa site

FIG. 2A

Component (position)	Description
CMV enhancer (77-456)	human cytomegalovirus (CMV) promoter enhancer
CMV promoter (457-649)	CMV promoter
5' LTR (675-855)	5' HIV Long Terminal Repeat
HIV-1 ψ (902-1027)	Required for viral gene expression, reverse transcription, and target cell genome integration
tGAG (983-1347)	Required for packaging of genomic transfer RNA
RRE (1520-1753)	HIV Rev Response Element, required for processing and transport of viral RNA
CPPT/CTS (2249-2366)	Central Polypurine Tract and Central Termination Sequence from HIV-1; increases transduction
SV40 PolyA (2503-2637)	Polyadenylation sequence from Simian Virus 40; required for transgene expression
NGFR (2825-3667), also referred to as deltaNGFR	Truncated nerve growth factor receptor; expressed on cell surface and required for selection of transduced cells
Minimal CMV promoter (3889-3927)	Human CMV immediate early promoter; internal promoter required for expression of truncated NGFR marker gene
EF-1a promoter (4042-5219)	Human elongation factor 1a promoter; internal promoter required for expression of therapeutic transgene FOXP3
hFOXP3 cDNA (5269-6554)	Human Forkhead Box Protein 3 (FOXP3) cDNA; Therapeutic Gene of Interest
Mutated WPRE (6596-7184)	Woodchuck hepatitis virus posttranscriptional regulatory element; increases GOI expression efficiency by increased mRNA stability and protein synthesis. The mutated WPRE was modified to remove the WHx start codon, the modification confirmed by sequencing, and verified to not affect expression of the FOXP3 transgene following transduction in cell culture.
tNEF (7188-7270)	Truncated NEF sequence from HIV-1; required for high viral burden
3'LTR (7271-7504)	HIV 3' Long Terminal Repeat
SV40 PolyA (7576-7697)	Polyadenylation sequence from SV40; required for transgene expression
SV40 ori (7737-7872)	SV40 origin of replication
KanR (8161-8976)	Kanamycin Resistance gene for plasmid growth and selection; Aminoglycocide phoshotransferase
Ori (9137-9725)	Bacterial high copy origin of replication for plasmid growth

FIG. 2B

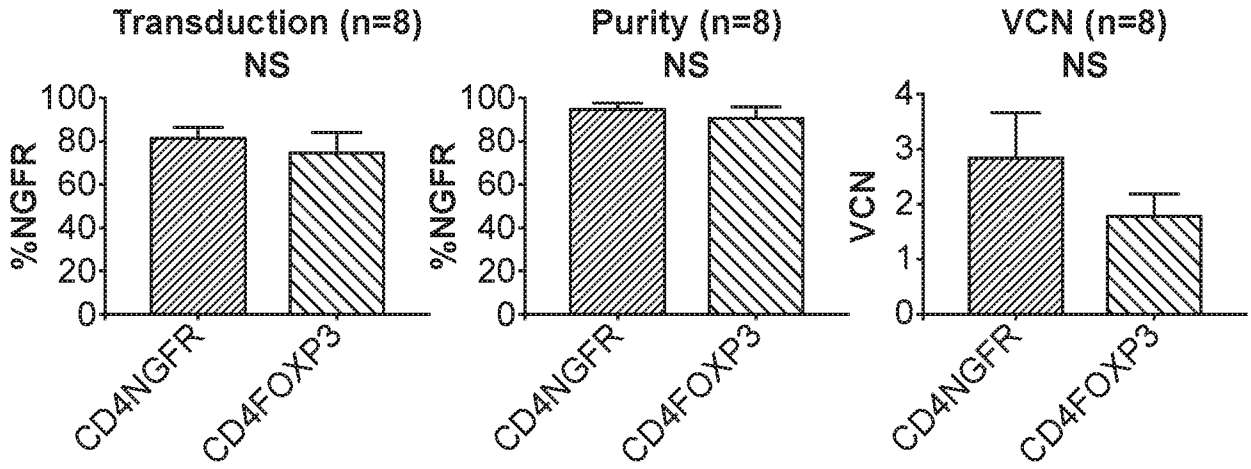


FIG. 3A

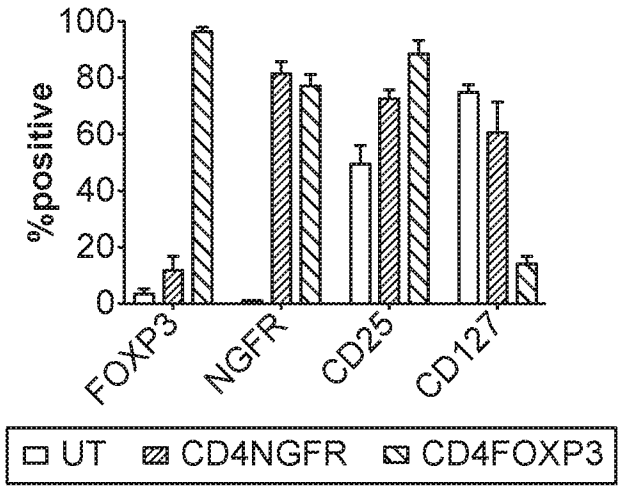


FIG. 3B

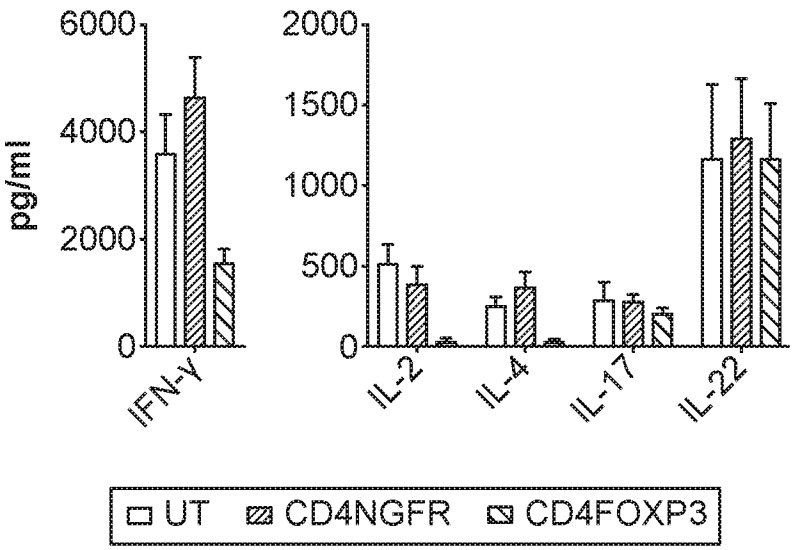


FIG. 3C

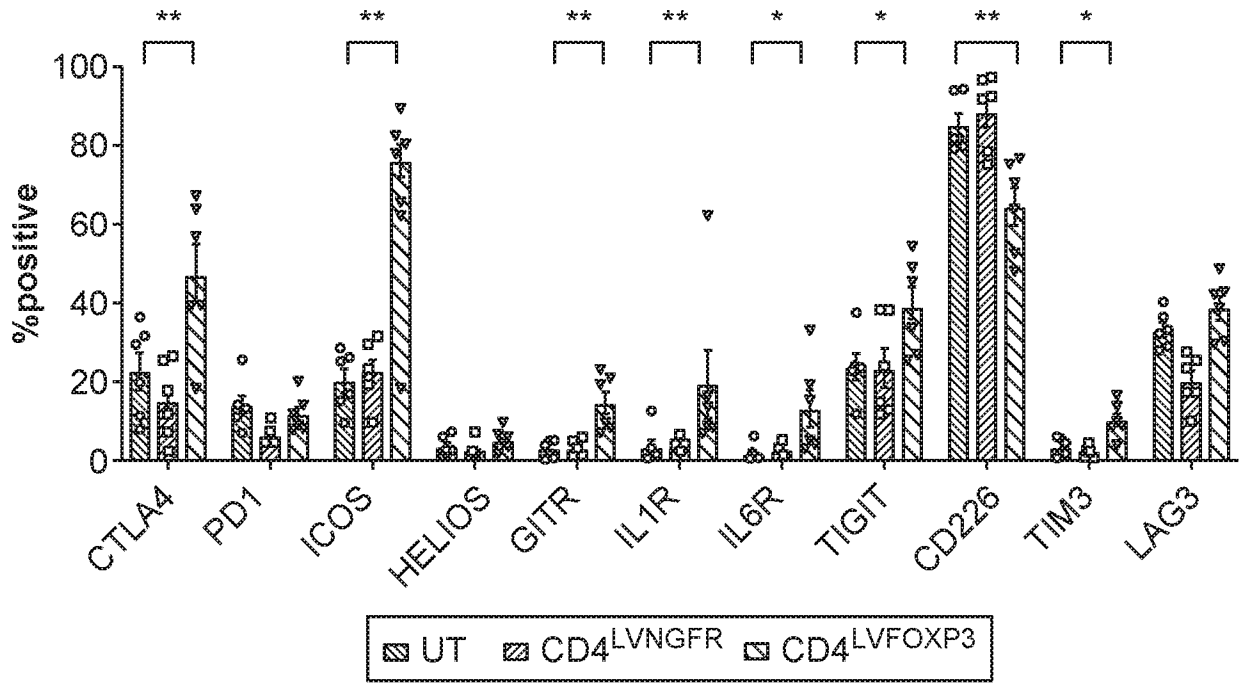

%positive
100
80
60
40
20
0
**
**
**
**
*
*
**
*
CTLA4
PD1
ICOS
HELIOS
GITR
IL1R
IL6R
TIGIT
CD226
TIM3
LAG3
UT
CD4LVNGFR
CD4LVFOXP3

FIG. 3D

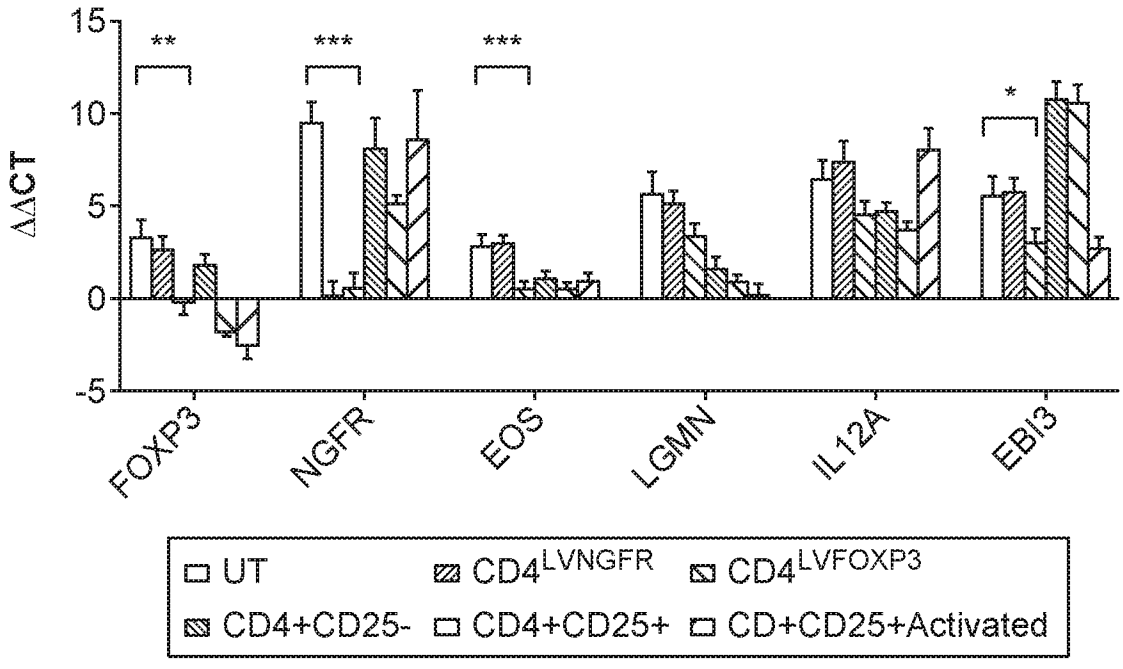

ΔΔCT
15
10
5
0
-5
**
***
***
*
FOXP3
NGFR
EOS
LGMN
IL12A
EBI3
UT
CD4LVNGFR
CD4LVFOXP3
CD4+CD25-
CD4+CD25+
CD+CD25+Activated

FIG. 3E

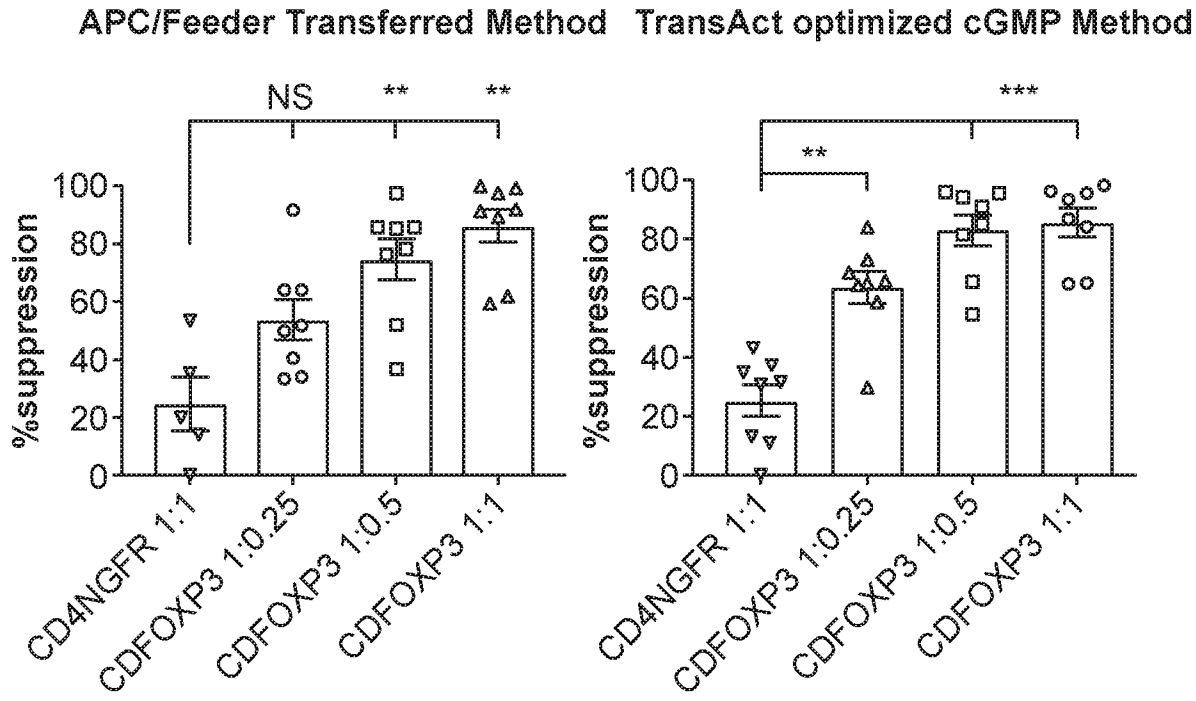

APC/Feeder Transferred Method
TransAct optimized cGMP Method
NS
**
**
**
***
%suppression
100
80
60
40
20
0
CD4NGFR 1:1
CDFOXP3 1:0.25
CDFOXP3 1:0.5
CDFOXP3 1:1

FIG. 4A

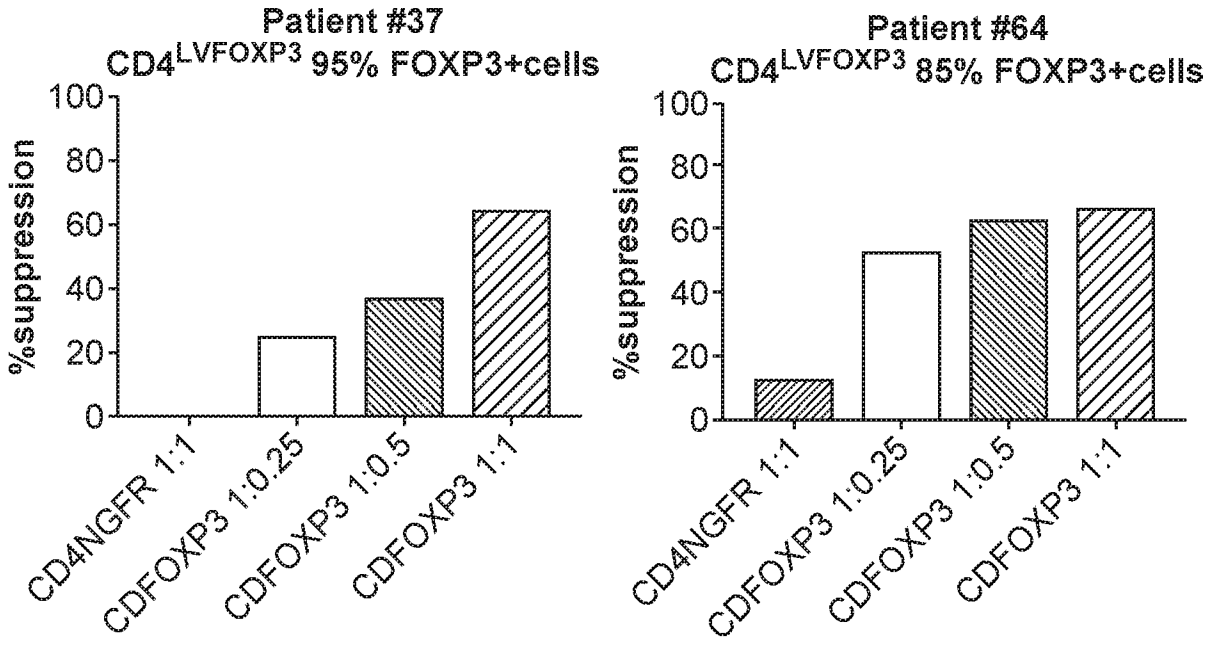
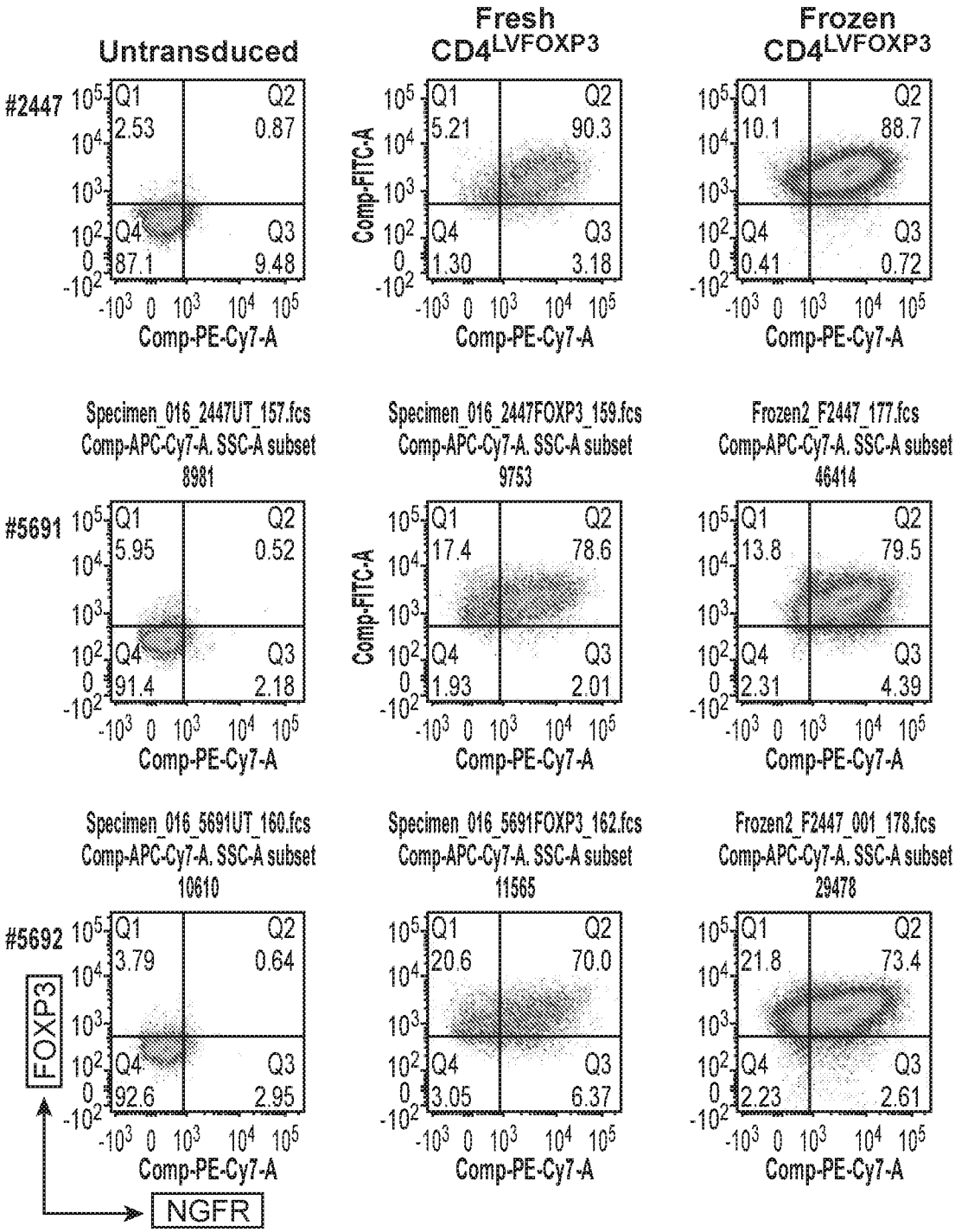

Patient #37
CD4LVFOXP3 95% FOXP3+cells
Patient #64
CD4LVFOXP3 85% FOXP3+cells
%suppression
100
80
60
40
20
0
CD4NGFR 1:1
CDFOXP3 1:0.25
CDFOXP3 1:0.5
CDFOXP3 1:1

FIG. 4B



Small Scale Runs		CD4	FOXP3	NGFR	FOXP3/ NGFR
Subject ID		(% positive)			
2447	fresh	95.7	95.5	93.5	90.3
	frozen	94.7	98.8	89.4	88.7
5691	fresh	95.9	96.0	80.6	78.6
	frozen	93.8	93.3	83.9	79.5
5692	fresh	92.5	90.6	76.4	70.0
	frozen	88.8	95.2	76.0	73.4

FIG. 5

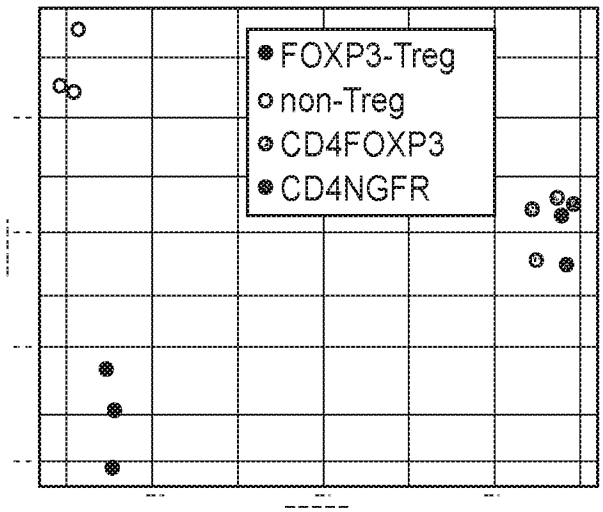


FIG. 6A

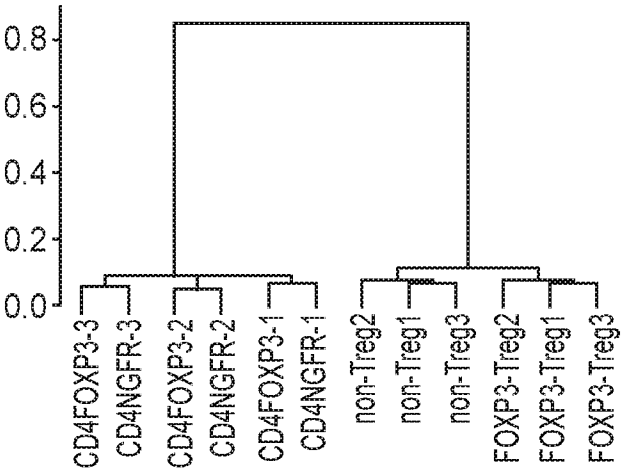


FIG. 6B

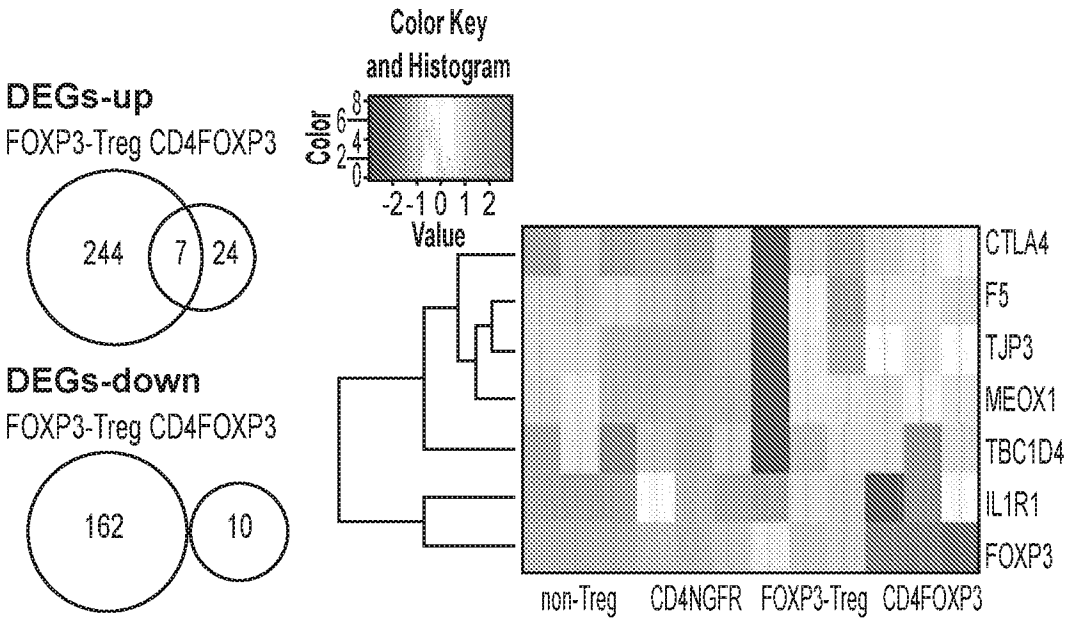


FIG. 6C

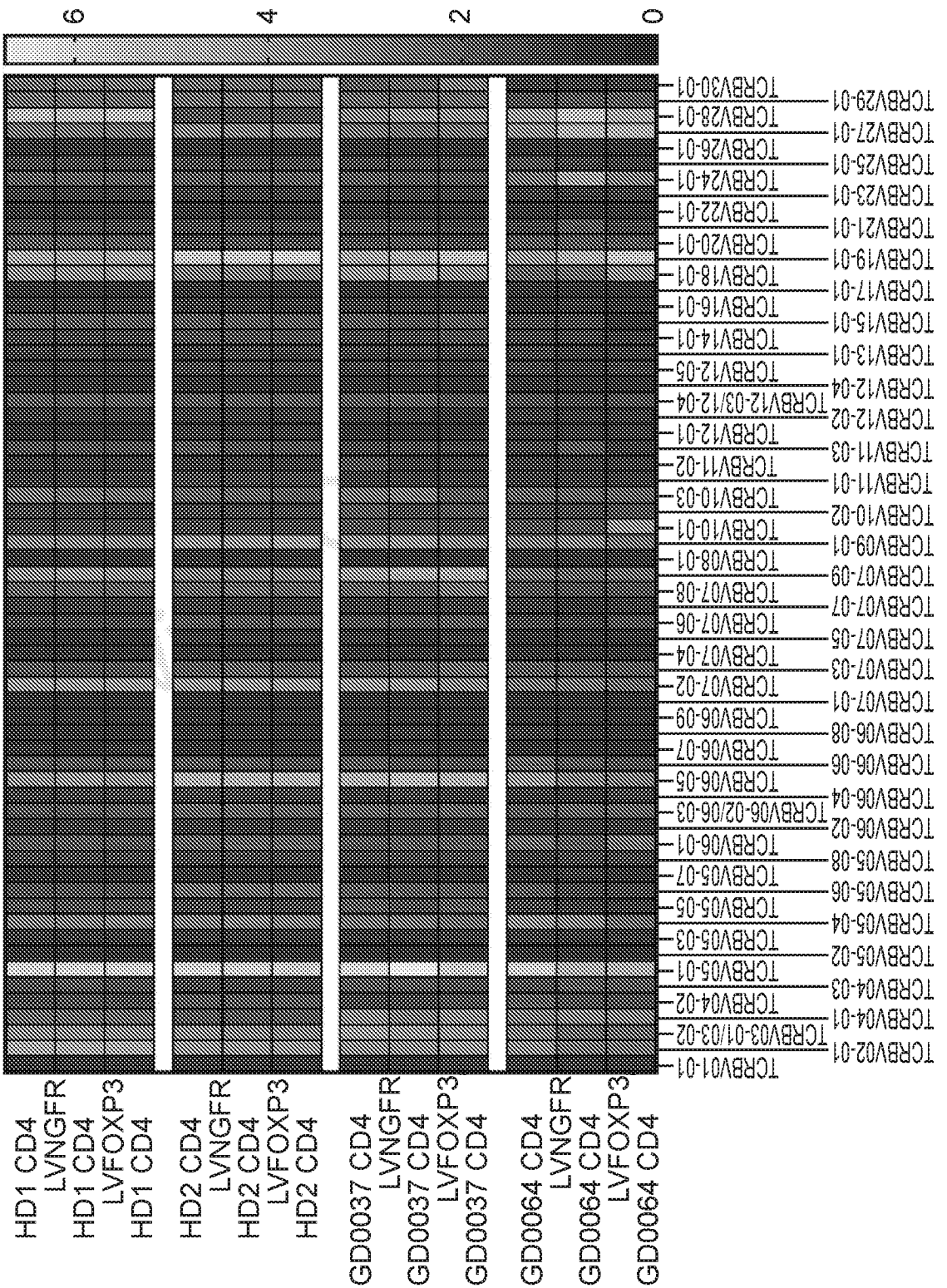

6
4
2
0
HD1 CD4
HD1 CD4 LVNGFR
HD1 CD4 LVFOXP3
HD1 CD4
HD2 CD4
HD2 CD4 LVNGFR
HD2 CD4 LVFOXP3
HD2 CD4
GD0037 CD4
GD0037 CD4 LVNGFR
GD0037 CD4 LVFOXP3
GD0037 CD4
GD0064 CD4
GD0064 CD4 LVNGFR
GD0064 CD4 LVFOXP3
GD0064 CD4
TCRBV01-01
TCRBV02-01
TCRBV03-01/03-02
TCRBV04-01
TCRBV04-02
TCRBV04-03
TCRBV05-01
TCRBV05-02
TCRBV05-03
TCRBV05-04
TCRBV05-05
TCRBV05-06
TCRBV05-07
TCRBV05-08
TCRBV06-01
TCRBV06-02/06-03
TCRBV06-02
TCRBV06-04
TCRBV06-05
TCRBV06-06
TCRBV06-07
TCRBV06-08
TCRBV06-09
TCRBV07-01
TCRBV07-02
TCRBV07-03
TCRBV07-04
TCRBV07-05
TCRBV07-06
TCRBV07-07
TCRBV07-08
TCRBV07-09
TCRBV08-01
TCRBV09-01
TCRBV10-01
TCRBV10-02
TCRBV10-03
TCRBV11-01
TCRBV11-02
TCRBV11-03
TCRBV12-01
TCRBV12-02
TCRBV12-03/12-04
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TCRBV12-05
TCRBV13-01
TCRBV14-01
TCRBV15-01
TCRBV16-01
TCRBV17-01
TCRBV18-01
TCRBV19-01
TCRBV20-01
TCRBV21-01
TCRBV22-01
TCRBV23-01
TCRBV24-01
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TCRBV29-01
TCRBV30-01

FIG. 7

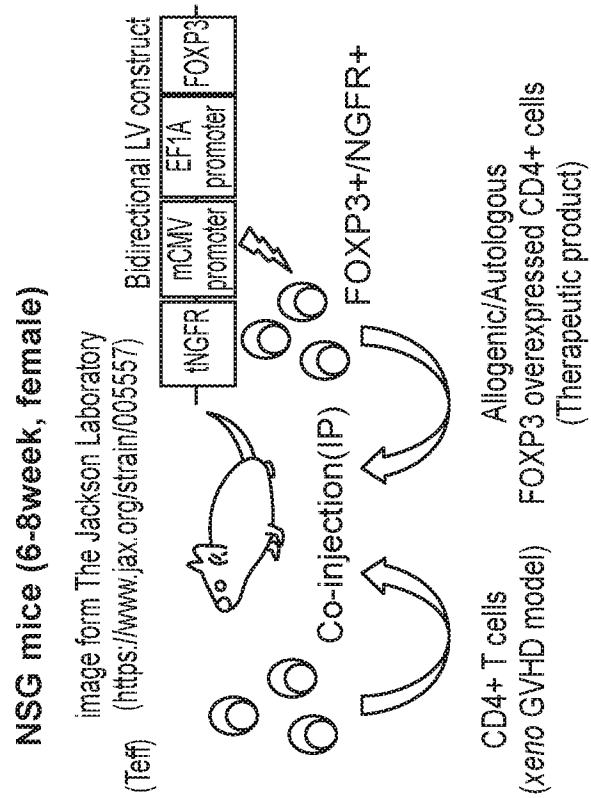


FIG. 8A

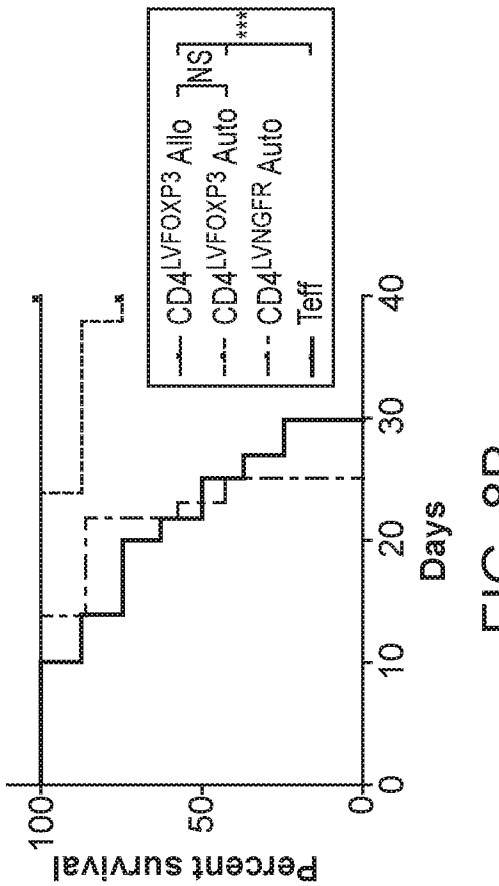


FIG. 8B

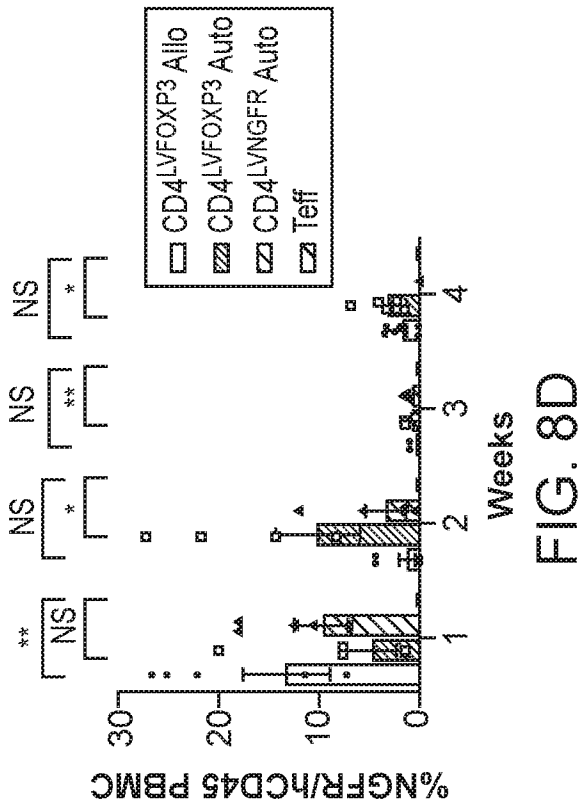


FIG. 8D

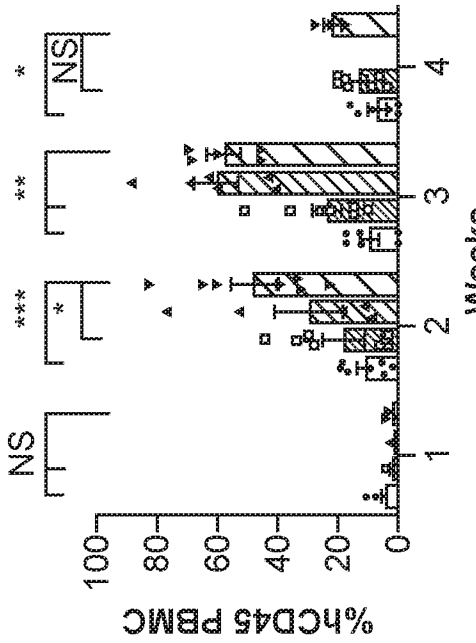


FIG. 8C

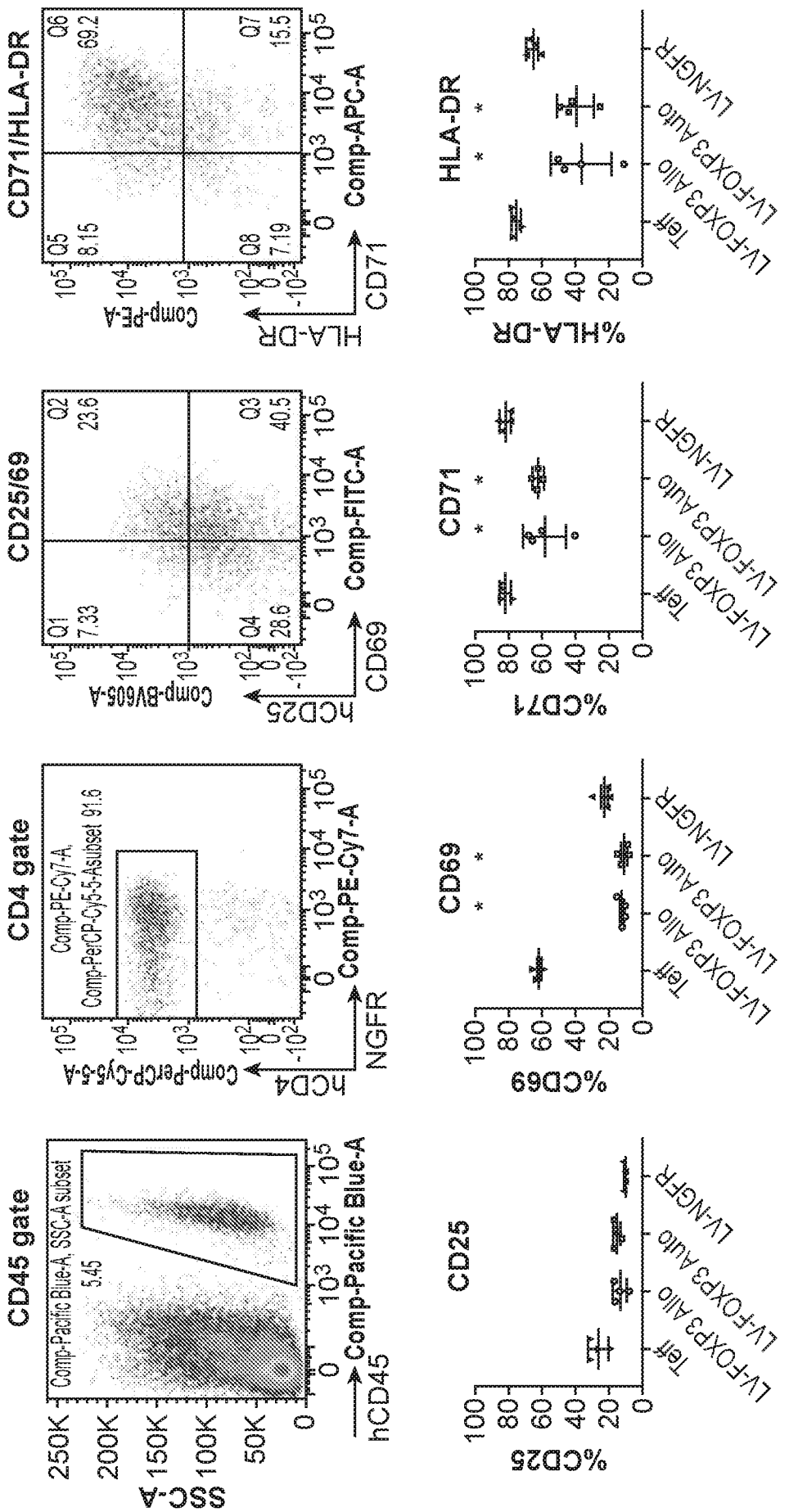

CD45 gate
Comp-Pacific Blue-A, SSC-A subset
5.45
SSC-A
hCD45
Comp-Pacific Blue-A
CD4 gate
Comp-PE-Cy7-A,
Comp-PerCP-Cy5-5-A subset 91.6
hCD4
NGFR
Comp-PE-Cy7-A
CD25/69
Q1 7.33
Q2 23.6
Q4 28.6
Q3 40.5
hCD25
CD69
Comp-BV605-A
Comp-FITC-A
CD71/HLA-DR
Q5 8.15
Q6 69.2
Q8 7.19
Q7 15.5
HLA-DR
CD71
Comp-PE-A
Comp-APC-A
CD25
CD69
%CD71
%HLA-DR
Teff
LV-FOXP3 Allo
LV-FOXP3 Allo
LV-NGFR

FIG. 9

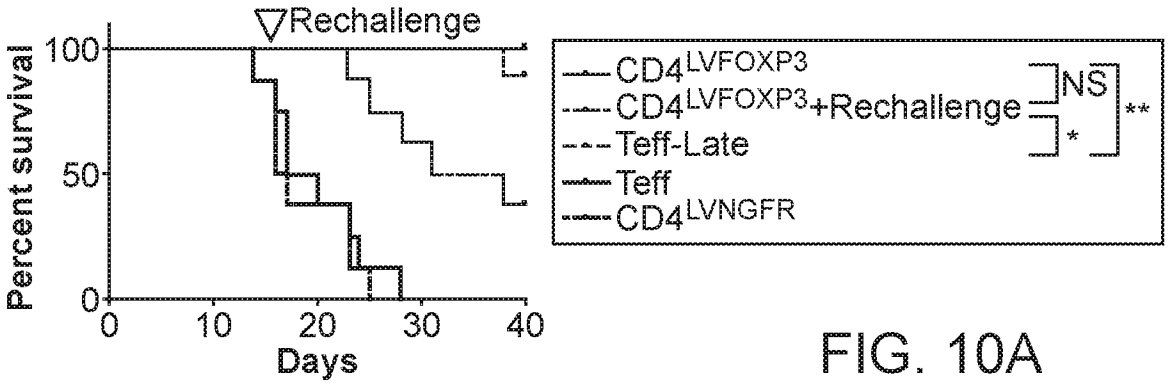


FIG. 10A

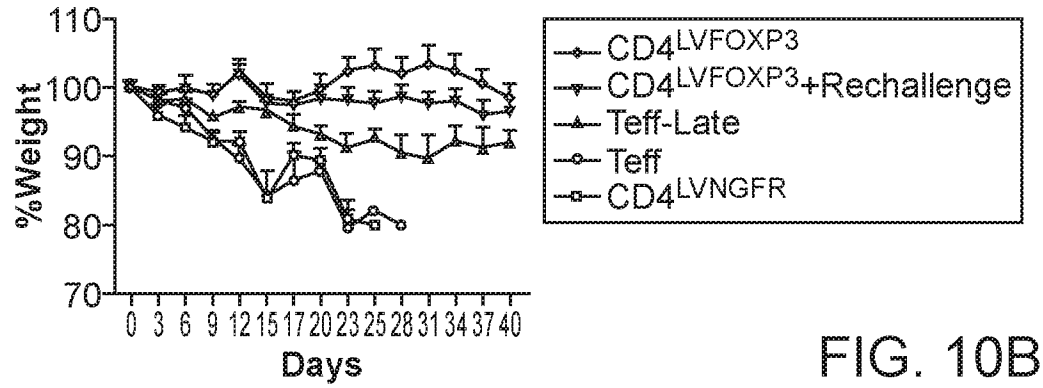


FIG. 10B

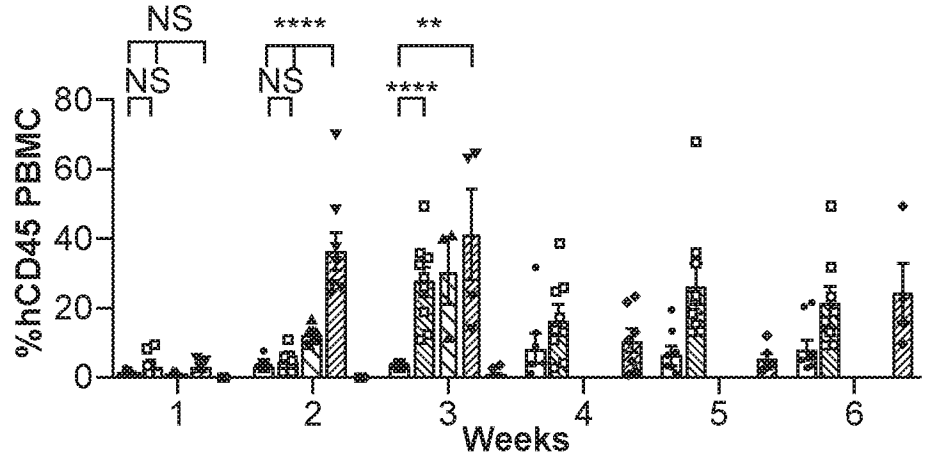


FIG. 10C

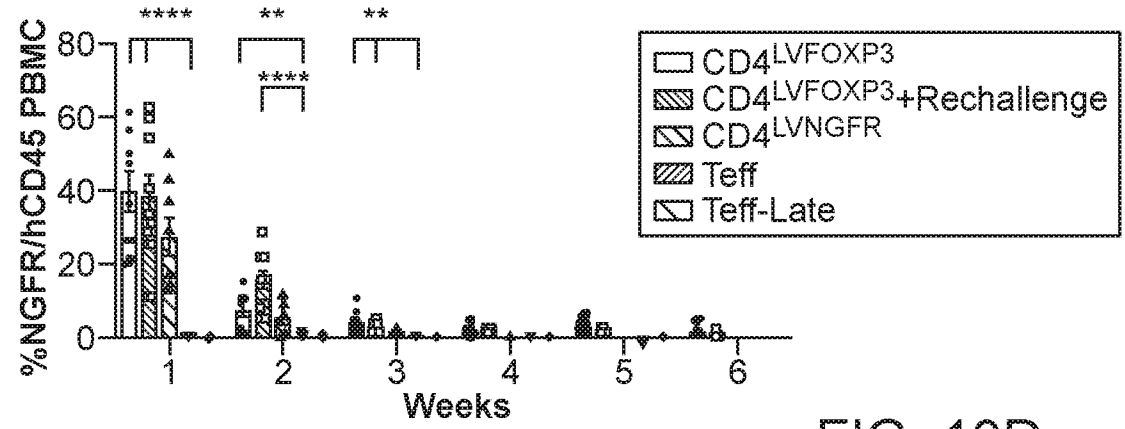


FIG. 10D

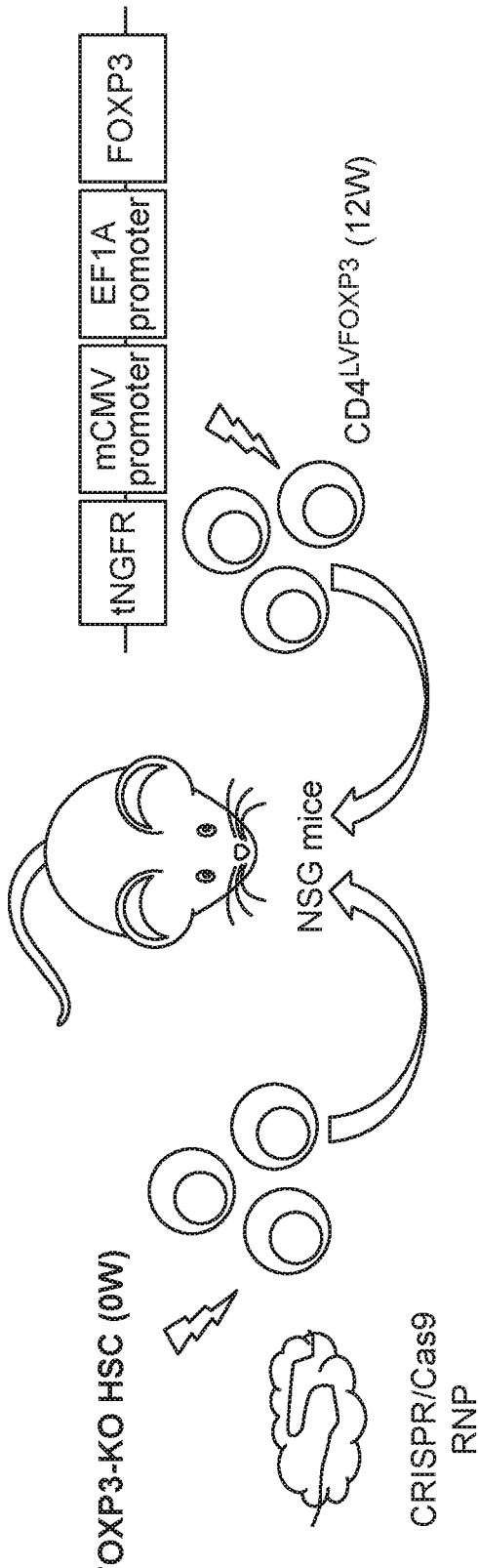


FIG. 11A

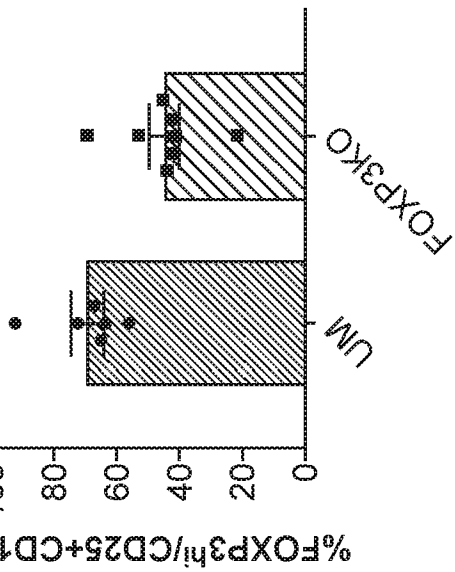
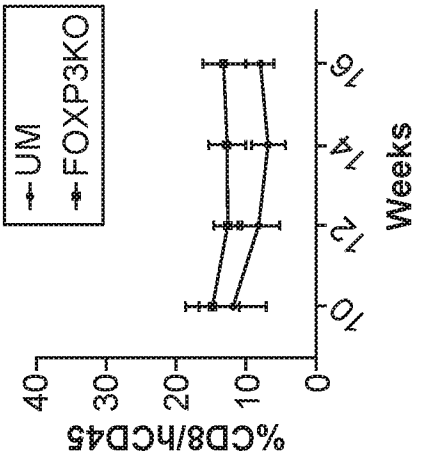


FIG. 11C



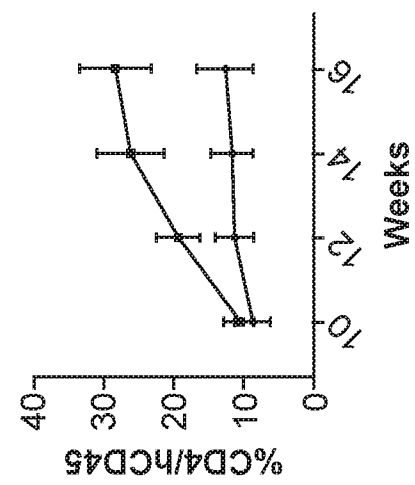


FIG. 11B

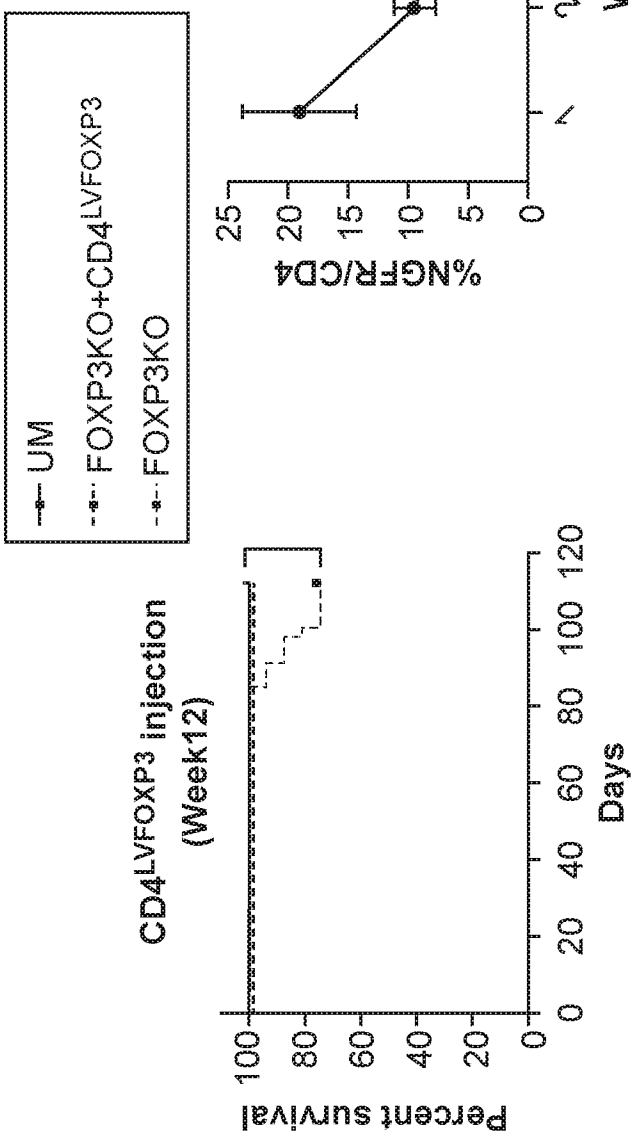


FIG. 12A

FIG. 12B

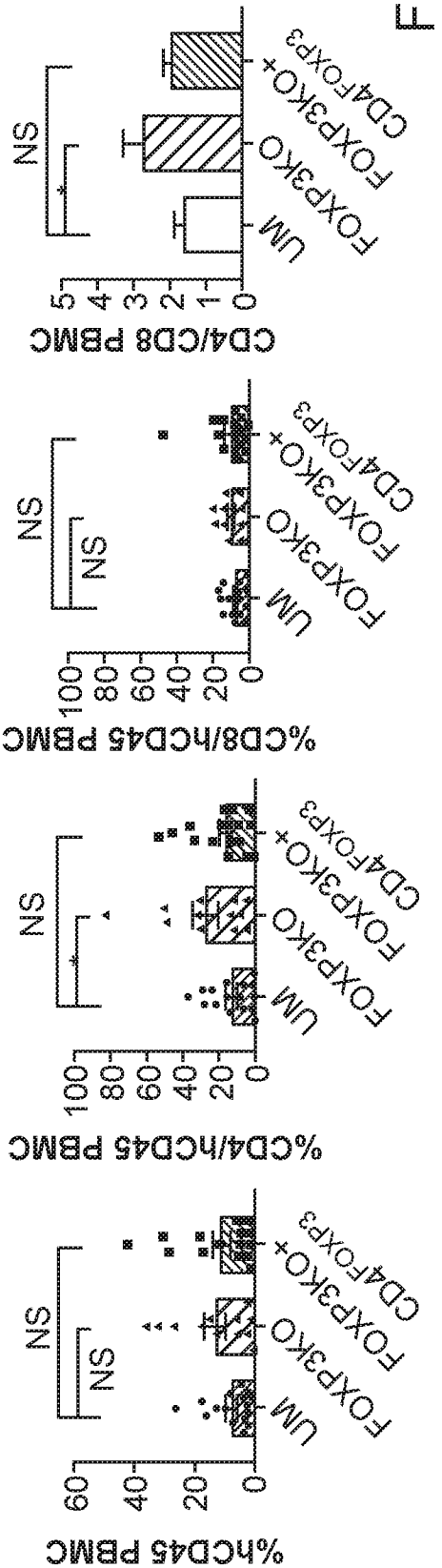


FIG. 12C

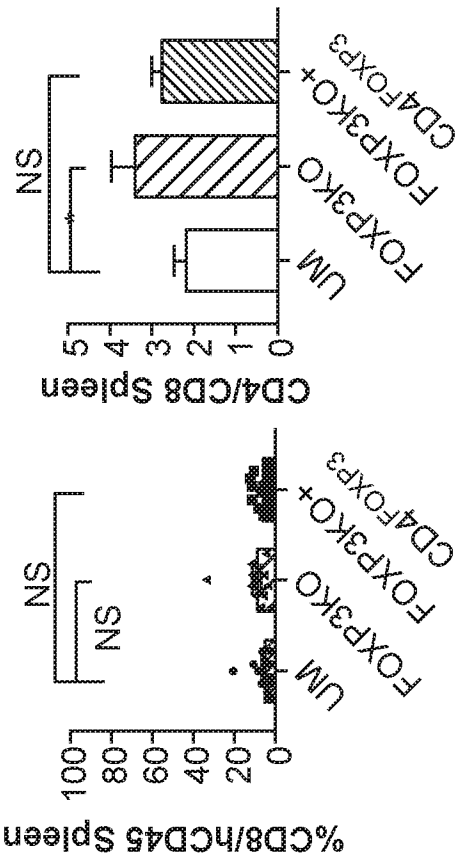


FIG. 12D

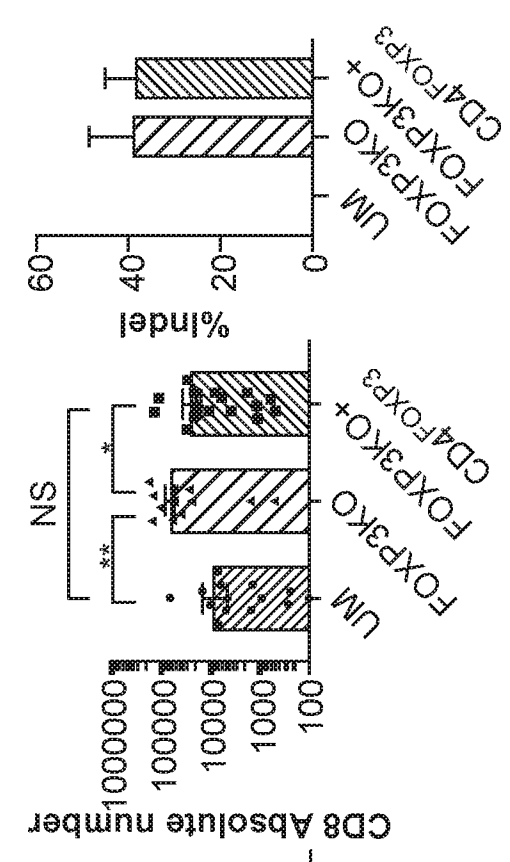


FIG. 12E

FIG. 12F

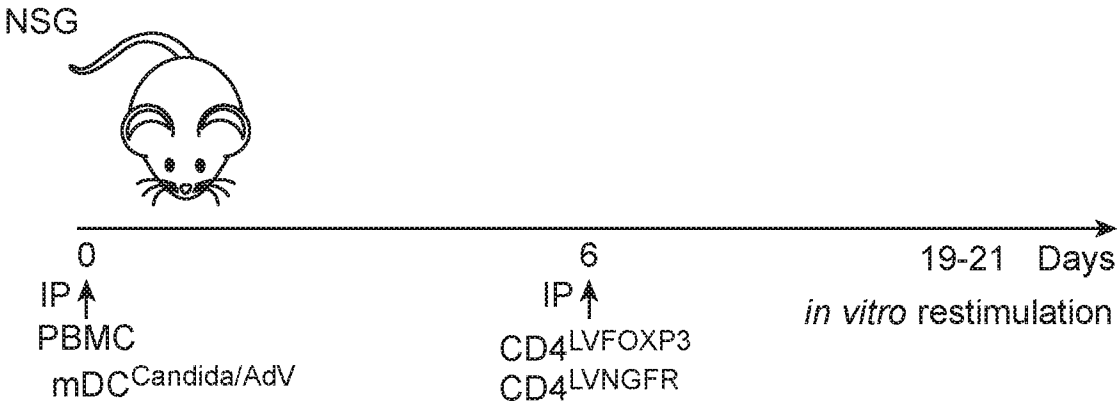


FIG. 13A

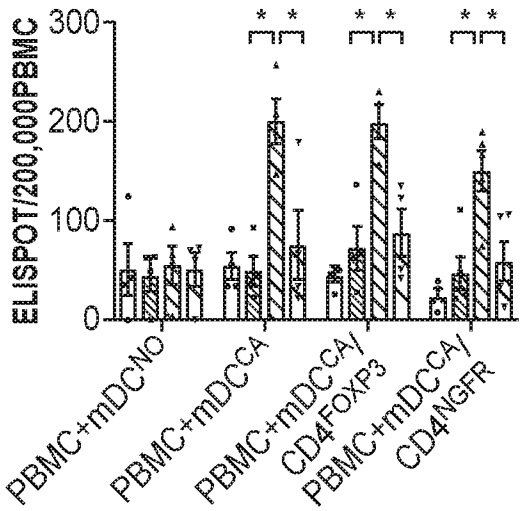


FIG. 13B

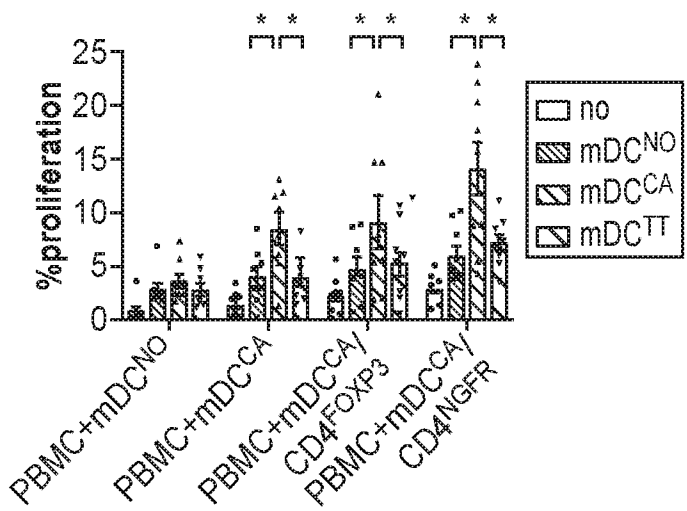


FIG. 13C

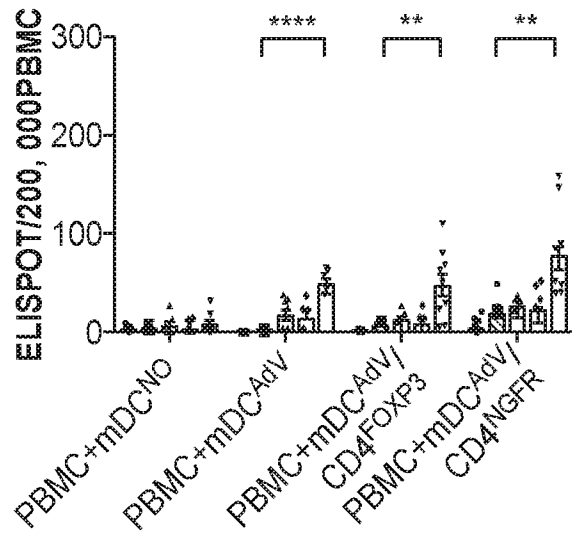


FIG. 13D

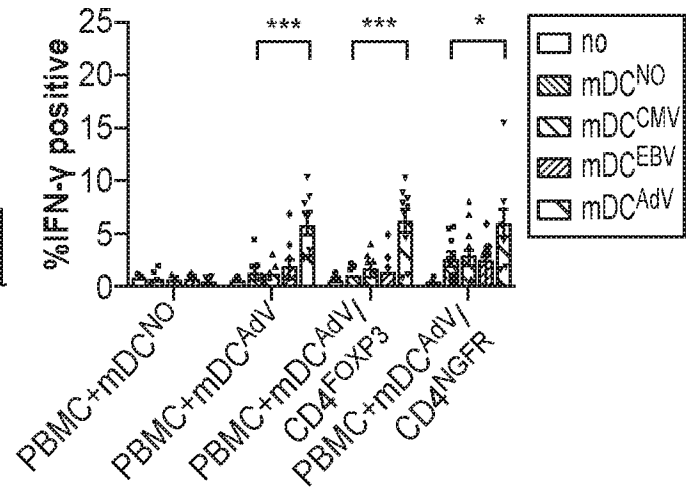


FIG. 13E

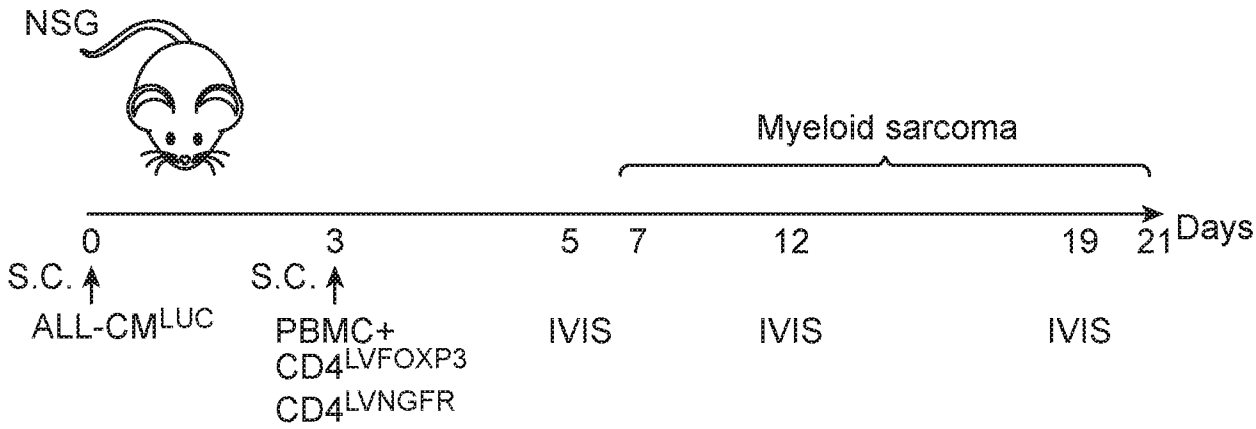
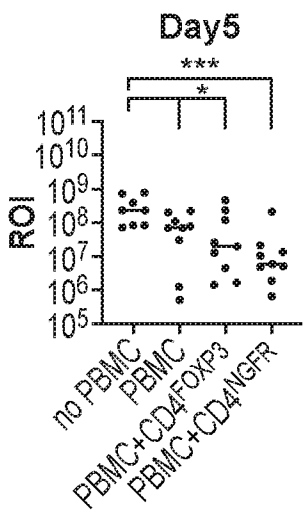
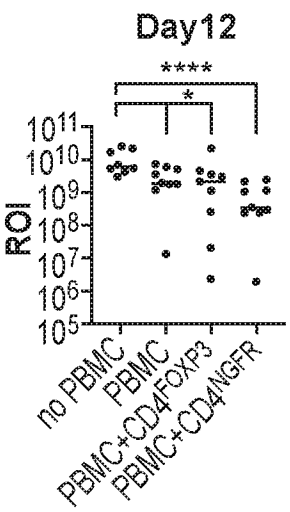


FIG. 14A





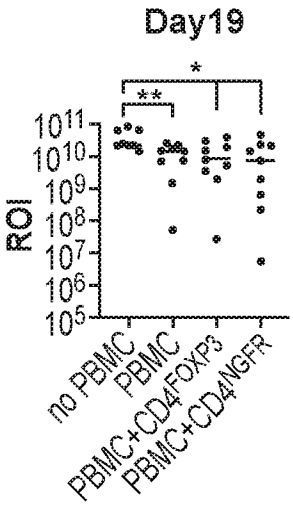


FIG. 14B

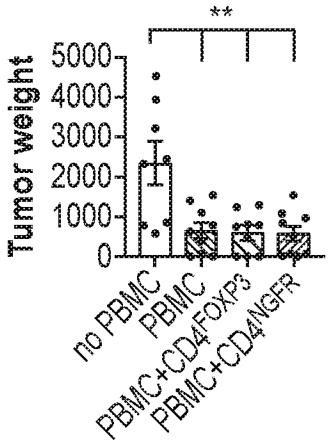


FIG. 14D

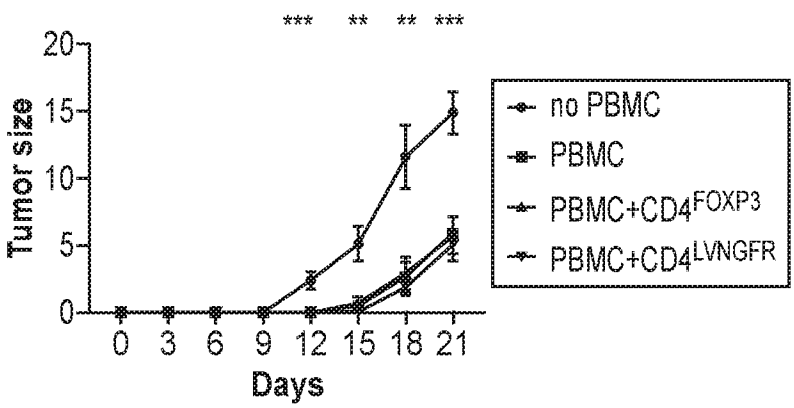


FIG. 14C

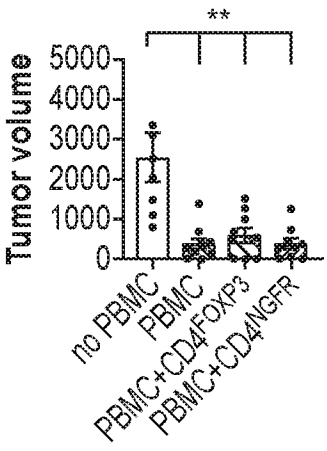


FIG. 14E

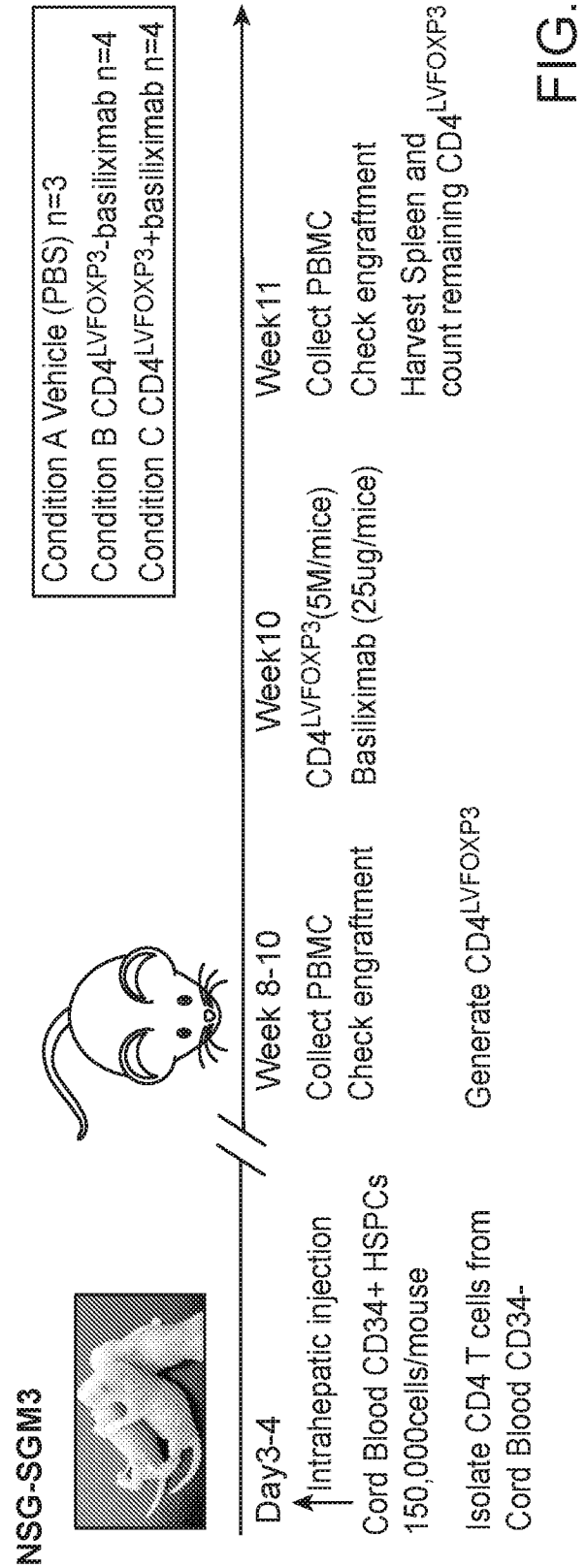


FIG. 15A

FIG. 15B

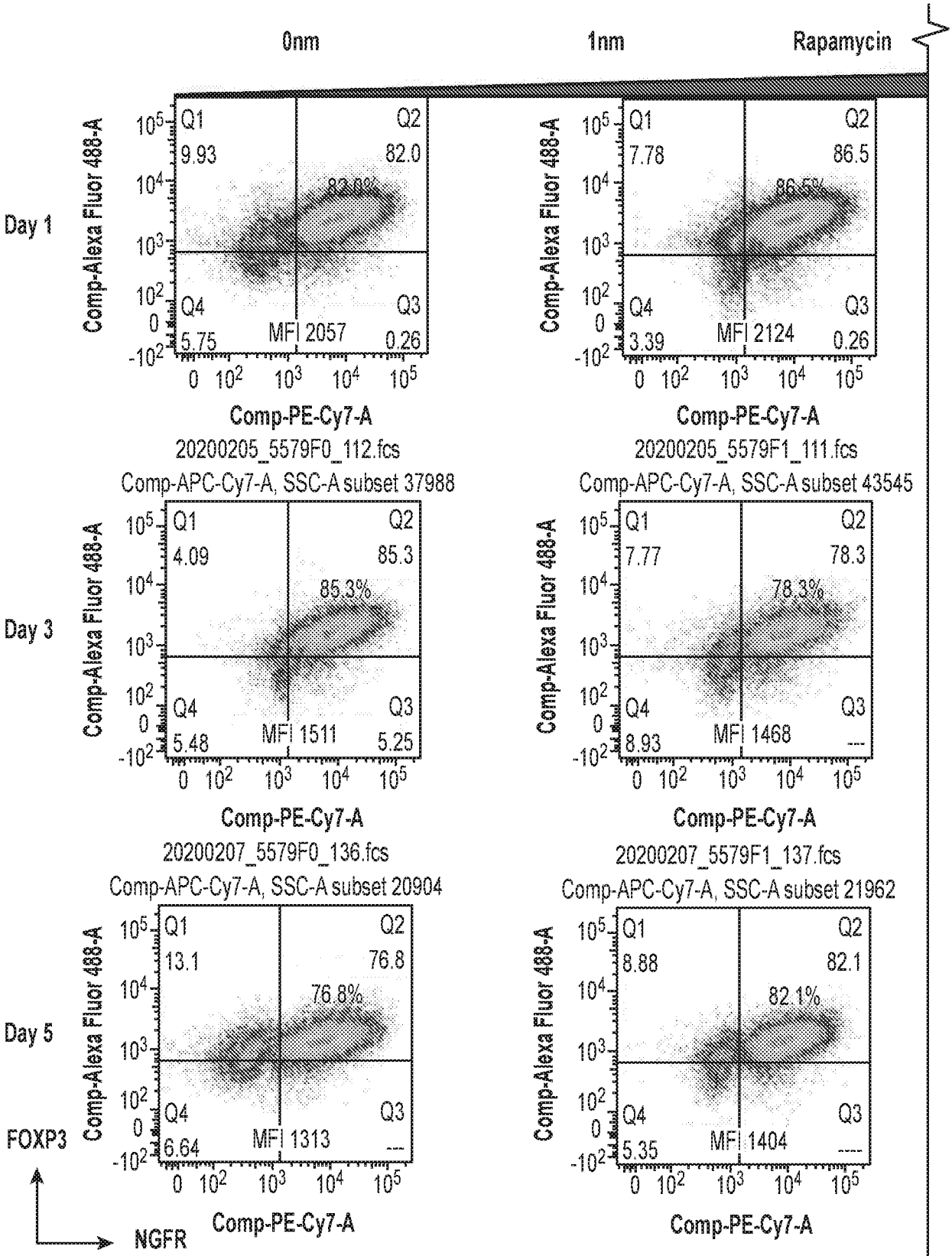

0nm
1nm
Rapamycin
Day 1
Day 3
Day 5
FOXP3
NGFR
Comp-Alexa Fluor 488-A
Comp-PE-Cy7-A
Q1
Q2
Q3
Q4
9.93
82.0
5.75
0.26
MFI 2057
83.8%
7.78
86.5
3.39
0.26
MFI 2124
86.5%
20200205_5579F0_112.fcs
Comp-APC-Cy7-A, SSC-A subset 37988
20200205_5579F1_111.fcs
Comp-APC-Cy7-A, SSC-A subset 43545
4.09
85.3
5.48
5.25
MFI 1511
85.3%
7.77
78.3
8.93
MFI 1468
78.3%
20200207_5579F0_136.fcs
Comp-APC-Cy7-A, SSC-A subset 20904
20200207_5579F1_137.fcs
Comp-APC-Cy7-A, SSC-A subset 21962
13.1
76.8
6.64
MFI 1313
76.8%
8.88
82.1
5.35
MFI 1404
82.1%

FIG. 16

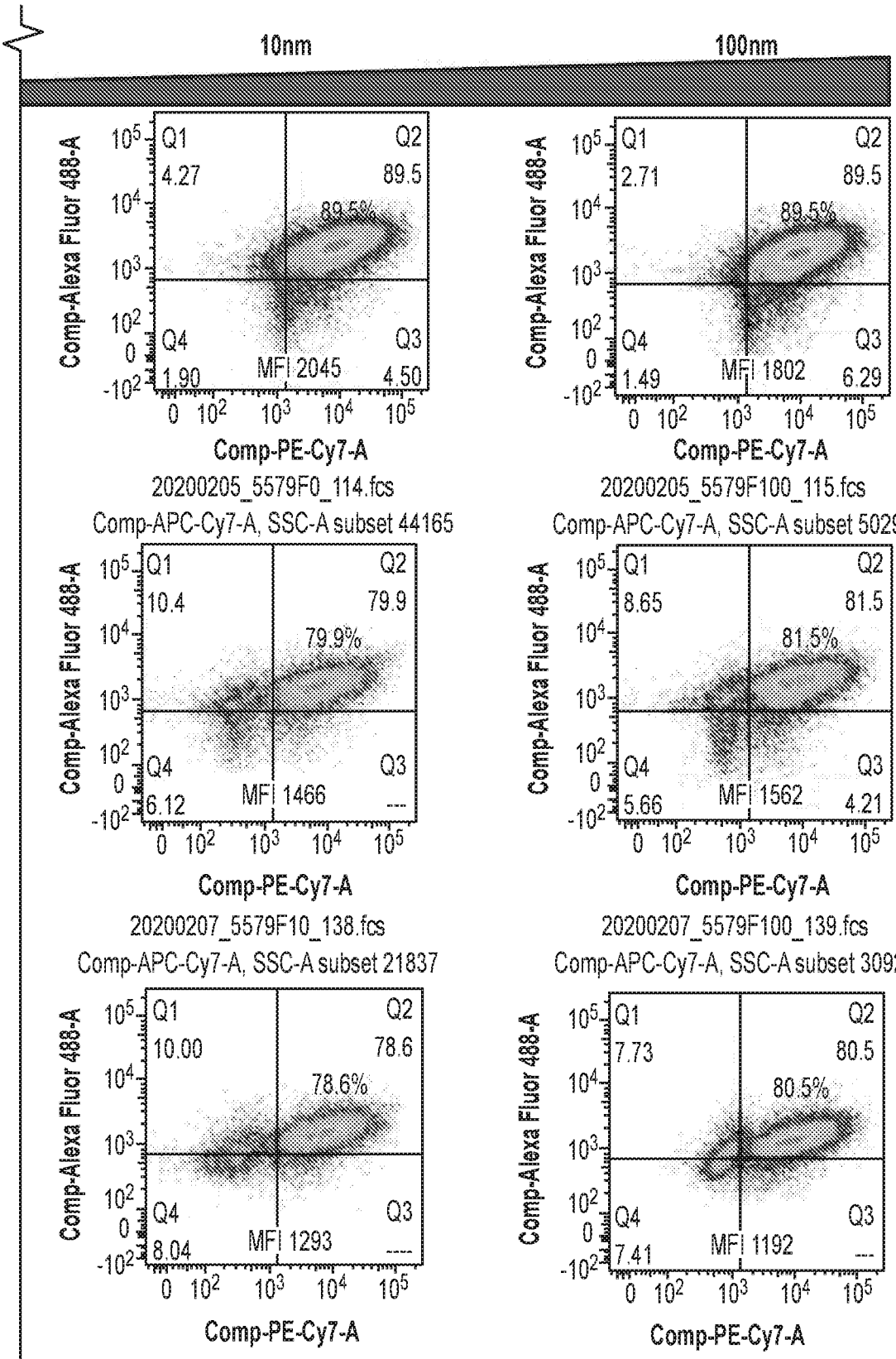

10nm
100nm
Comp-Alexa Fluor 488-A
Comp-PE-Cy7-A
Q1
4.27
Q2
89.5
89.5%
Q4
1.90
MFI 2045
Q3
4.50
20200205_5579F0_114.fcs
Comp-APC-Cy7-A, SSC-A subset 44165
Q1
2.71
Q2
89.5
89.5%
Q4
1.49
MFI 1802
Q3
6.29
20200205_5579F100_115.fcs
Comp-APC-Cy7-A, SSC-A subset 50297
Q1
10.4
Q2
79.9
79.9%
Q4
6.12
MFI 1466
Q3
20200207_5579F10_138.fcs
Comp-APC-Cy7-A, SSC-A subset 21837
Q1
8.65
Q2
81.5
81.5%
Q4
5.66
MFI 1562
Q3
4.21
20200207_5579F100_139.fcs
Comp-APC-Cy7-A, SSC-A subset 30928
Q1
10.00
Q2
78.6
78.6%
Q4
8.04
MFI 1293
Q3
Q1
7.73
Q2
80.5
80.5%
Q4
7.41
MFI 1192
Q3

FIG. 16 (Cont.)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/36401

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 35/17, A61K 35/15 (2020.01)

CPC - G01N 33/505, C12N 5/0636 , G01N 33/5047, G01N 33/56972, A61K 39/001, G01N 2333/70514

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PASSERINI et al. Forkhead-Box-P3 Gene Transfer in Human CD4+ T Conventional Cells for the Generation of Stable and Efficient Regulatory T Cells, Suitable for Immune Modulatory Therapy. Front Immunol., 12 October 2017, vol 8, article 1282, pp 1-8, abstract, pg 3, col 2, para 2-3, Table 1, Fig. 1	1-5
Y	WO 2019/040655 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 February 2019 (28.02.2019) para [0105], [0111], [0130]	1-5
Y	WO 2007/091066 A1 (UCL BUSINESS PLC) 16 August 2007 (16.08.2007) abstract, pg 1, ln 23-25 - pg 2, ln 1-6	3, (4-5)/3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2020	18 SEP 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/36401

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.