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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING AN IMMUNE RESPONSE

(57) Abstract: The invention provides methods of modulating follicular regulatory T (TFR) cell-mediated immune responses, follicular helper T (TFH) cell-mediated immune responses or both, and the use of those methods in the treatment of diseases or conditions mediated by TFR or TFH cells. The invention also provides novel methods for identifying TFR and TFH cells in a population of cells. The invention also provides compositions comprising TFR cells that have enhanced suppressive activity as compared wild type TFR cells. The invention also provides compositions comprising T follicular regulatory (TFR) cells isolated from the peripheral blood of a subject wherein the composition is enriched for TFR cells. Methods of making and using the compositions of the invention to modulate an immune response are also provided.

COMPOSITIONS AND METHODS FOR MODULATING AN IMMUNE RESPONSE

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 61/724,424, filed on November 9, 2012. The entire teachings of the above application are incorporated herein by reference.

GOVERNMENT SUPPORT

This invention was made with government support under grant numbers T32 AI070085; R01 AI40614, P01 78897 and T32 HL007627 awarded by The National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Regulation of immune responses is central for the prevention of inflammatory and autoimmune disorders. While downregulation of the immune system can be achieved by way of immunosuppressive therapy, agents that generally suppress the immune system leave subjects susceptible to other disorders, including infections and cancers. A means for controlling the aberrant activation of an immune response to specific antigens would be a major advance in the treatment of autoimmune disorders, graft versus host disease and the side effects of gene therapy, as it would allow downregulation of the immune response against a particular target antigen, but would otherwise leave the immune system functional against invading pathogens and tumor associated antigens. Conversely, methods of specifically improving immunogenicity of specific antigens to which immune responses are desired would be of tremendous benefit in promoting desired immune responses, for example in the context of vaccination and promoting responsiveness to antigens including tumor antigens.

T helper (Th) cells are a class of CD4+ cells that function to regulate the proliferation of B cells and B cell responses. Th cells play an importance role in humoral immunity and immunopathology. Follicular helper T cells (TFH) are a recently defined subset of CD4+ T cells that are essential for helping cognate B cells form and maintain the germinal center (GC) reaction, and for development of

humoral immune responses. These cells are universally defined by expression of the chemokine receptor CXCR5, which directs them to the B cell follicles via gradients of the chemokine CXCL13 [1]. TFH cells also express the transcription factor *Bcl6* (which represses Blimp-1/*Prdm*1) and high levels of the costimulatory receptor ICOS, which are both critical for their differentiation and maintenance [1-4]. In addition, TFH cells secrete large amounts of IL-21, which aids in GC formation, isotype switching and plasma cell formation [5]. In humans and mice functionally similar TFH cells can be found in secondary lymphoid organs. $CXCR5^+$ TFH cells are also present in peripheral blood and seen at elevated levels in individuals with autoantibodies, including systemic lupus erythematosis, myasthenia gravis and juvenile dermatomyositis patients. However, the function of these circulating TFH remains unclear [6-9].

Regulatory T cells (Tregs) have pluripotent anti-inflammatory effects on multiple cell types. In particular they control the activation of innate and adaptive immune cells. Tregs acting in an antigen- specific manner reduce effector T cell activation and function, for example, after effector T cells have successfully mounted an attack against an invading pathogen, or to suppress reactivity to self-antigen and thereby prevent autoimmune disease.

Two subsets of Tregs are classified according to the location at which they develop *in vivo*. Naturally occurring Tregs (nTreg) develop in the thymus and suppress self -reactive immune responses in the periphery, whereas adaptive Tregs (aTreg) develop in the periphery from conventional $CD4^+$ T cells to ensure tolerance to harmless antigens, including those derived from, for example, food and intestinal flora. Both subsets of Treg cells are characterized by expression of high levels of CD25 and the transcription factor Foxp3. Tregs are thought to inhibit the antigen-specific expansion and/or activation of self-reactive effector T cells and to secrete suppressive cytokines, including TGF or IL-10. Because of their potential to provide antigen- specific immune regulation without generalized immunosuppression, Tregs have been contemplated for use in cell-based therapy for inflammatory or autoimmune disorders.

T follicular regulatory (TFR) cells are newly defined subset of $CD4^+CXCR5^+$ cells which are positive for the transcription factors FoxP3, *Bcl6* and *Prdm1*/Blimp1 and function to inhibit the germinal center response[21-23].

PD-1 has been identified as a receptor which binds to PD-L1 and PD-L2. PD-1 is a member of the immunoglobulin gene superfamily. PD-1 (Ishida et al. (1992) *EMBO J.* 11:3887; Shinohara et al. (1994) *Genomics* 23:704; U.S. Pat. No. 5,698,520) has an extracellular region containing immunoglobulin superfamily domain, a transmembrane domain, and an intracellular region including an immunoreceptor tyrosine-based inhibitory motif (ITIM). PD-1 transmits a negative signal to immune cells, similar to CTLA4. PD-1 ligand proteins are expressed on the surface of antigen presenting cells, and other cell types, and can provide a costimulatory signal to immune cells or can transmit downmodulatory signals to immune cells, depending upon the protein to which they bind. While transmission of an inhibitory signal leads to downmodulation in immune cell responses (and a resulting downmodulation in the overall immune response), the prevention of an inhibitory signal (e.g., by using a non-activating antibody against PD-1) in immune cells leads to upmodulation of immune cell responses (and a resulting upmodulation of an immune response).

TFH cells express high levels of programmed death (PD) 1 receptor (CD279). Signaling through PD-1 attenuates TCR signals and inhibits T cell expansion, cytokine production and cytolytic function. In addition, PD-1 promotes the development of induced regulatory T (iTreg) cells from naïve lymphocytes [10-14]. PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is more widely expressed than PD-L2, but PD-L1 and PD-L2 both can be expressed on GC B cells and dendritic cells [15]. Perturbation studies suggest critical roles for this pathway in regulating humoral immune responses. However, there are conflicting reports as to the function of the PD-1 pathway in controlling humoral immunity. Some studies have found that humoral responses are attenuated [16-18], while others have seen that humoral responses are heightened [19, 20] when PD-1:PD-L interactions are prevented.

PD-1 also is found on TFR cells. These cells originate from natural regulatory T cell precursors, but express similar levels of ICOS, CXCR5 and PD-1 as TFH cells. Since ICOS, CXCR5 and PD-1 have been widely used to identify and purify 'TFH cells', it seems likely that the inability to define clear functions for PD-1 in GC responses derives from experimental systems containing mixtures of stimulatory TFH cells and inhibitory TFR cells.

The present inventors have discovered that PD-1:PD-L1 interactions limit TFR cell differentiation and function. This discovery has elucidated novel approaches to modulating an immune response for use in therapy.

This discovery has also elucidated novel cell markers for identifying and separating TFR and TFH cells from all other cell types. These markers are also useful for selectively modifying TFR-mediated and/or TFH mediated immune responses *in vivo* and *in vitro*.

SUMMARY OF THE INVENTION

The invention is based, in part on the discovery that PD-1 regulates immune responses by inhibiting differentiation and function of TFR cells in both lymph nodes and blood. This discovery has led to strategies for modulating an immune response *in vivo* and *in vitro* by altering the interaction of PD-1 receptors on TFR cells with PD-1 ligands.

The invention is also based in part on the discovery of cell markers on TFH and THR cells that enable such cells to be more easily identified, distinguished and tracked *in vivo* and/or separated into highly purified homogenous cell populations of TFH cells or TFR cells. Such markers make it possible to add specificity and reliability in detecting TFR or TFH cells either in situ, in circulation, or as disseminated cells which is particularly useful for example in monitoring disease burden and in tracking the responses of TFH cells and TFR cells to particular stimuli such as agents intended to modulate TFH and TFR cell-mediated immune responses. These markers are also useful for selectively modifying TFR cell-mediated, and/or TFH cell-mediated immune responses *in vivo* and *in vitro*.

In one embodiment the invention provides TFR cells having enhanced suppressive activity as compared to wild type TFR cells. In one embodiment, the invention provides compositions comprising TFR cells having enhanced suppressive activity. TFR cells having enhanced suppressive activity as compared to wild type TFR cells are characterized by their greater capacity for antibody inhibition as compared to WT TFR, as measured by an *in vivo* or *in vitro* assays for antibody suppression. In one embodiment, the enhanced TFR cells have an increase in capacity of at least 2 fold to inhibit antibody production as compared to WT TFR cells.

In one embodiment the invention provides methods for generating TFR cells having enhanced suppressive activity. In accordance with the invention, TFR cells having enhanced suppressive activity may be prepared, *ex vivo*, from a starting population of cells which comprise TFR cells and/or TFR precursor cells such as FoxP3+ T regulatory (Treg) cells, isolated from the blood, tissues or organs of one or more subjects. The starting population of cells may optionally be sorted based on surface markers for Tcells and Tcell subsets. The starting population of cells is expanded and/or activated in the presence of a PD-1 receptor antagonist or PD-1 ligand inhibitor. The expanded/activated cells may optionally be sorted based on surface markers for T cells and T cell subsets to obtain a composition of cells enriched for a particular T cell subset (e.g. TFR cells or TFH cells).

The present inventors have also discovered that TFR cells derived from the peripheral blood of a subject are potent inhibitors of TFH mediated antibody production but do no inhibit other arms of the immune system. Therefore, the invention provides compositions comprising TFR cells isolated from the peripheral blood of a subject. The compositions may be enriched for the peripheral blood TFR cells by purifying TFR cells from other PMBCs and optionally sorting for TFR cells based on surface markers. Compositions of TFR cells derived from peripheral blood may also be expanded and/or activated to produce a clonal population of TFR cells based on the original population of TFR cells derived from the peripheral blood of the patient.

Compositions comprising TFH cells which are also present in the peripheral blood of a subject may be provided in the same manner. The inventors have discovered that such compositions, particularly when enriched for TFH cells, are capable of rapidly upregulating an antibody response *in vitro* and *in vivo*.

The inventors have also discovered that increasing the ratio of TFR cells to TFH cells in a subject prior to, or during an immune response by the subject, inhibits antibody production. Therefore, the invention provides a method for suppressing an immune response in a subject wherein suppression of an immune response is desired, comprising increasing the ratio of TFR cells to TFH cells by administering a composition enriched with TFR cells derived from the peripheral blood of a subject (or an expanded/activated population thereof) or administering a composition comprising TFR cells having enhanced suppressive activity.

The invention also provides methods of promoting rapid antibody production in a subject comprising increasing the number of TFH cells in a patient by administering a composition enriched with TFH cells derived from the peripheral blood of a subject (or an expanded/activated population thereof).

The compositions of TFR cells and TFH cells of the invention are also useful as adjuvants as a part of a vaccination regimen. When used in this manner, the compositions enhance the efficacy of such vaccines.

The invention further provides compositions and methods for suppressing pathogenic antibody responses in a patient comprising selectively modulating differentially expressed receptors on TFR cells, TFH cells, or both TFR cells and TFH cells with agents capable of modulating such receptors in amounts effective to suppress a pathogenic antibody response.

The invention further provides compositions and methods for enhancing a protective antibody response in a patient comprising selectively modulating differentially expressed receptors on TFR cells, TFH cells, or both TFR cells and TFH cells with agents capable of modulating such receptors in amounts effective to enhance a protective antibody response in a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

FIG. 1. PD-1 signaling in FoxP3 Tregs limits the generation of T follicular regulatory cells. (a) Quantitation of TFR cells. WT mice were immunized with MOG/CFA and 7 days later draining lymph nodes were isolated and immediately stained for $CD4^+FoxP3^+ICOS^+CXCR5^+CD19^-$ T follicular regulatory cells (TFR), $CD4^+FoxP3^-ICOS^+CXCR5^+CD19^-$ T follicular helper cells (TFH), $CD4^+ICOS^-CXCR5^-CD19^-$ cells (naive) or $CD4^+ICOS^+CXCR5^-CD19^-$ cells (ICOS+). Numbers indicate percentages of cells located within each gate. (b) PD-1 expression by flow cytometry on WT naive, ICOS+, TFR and TFH cells. Populations were gated as in

(a). (c) Gating of TFR cells from total FoxP3$^+$ cells in WT and PD-1$^{-/-}$ mice immunized with MOG/CFA and analyzed 7 days later and stained as in (a). (d) Quantitation of WT or PD-1$^{-/-}$ TFR cells gated in (c) and expressed as a percentage of CD4$^+$FoxP3$^+$ (left), percentage of total CD4 T cells (middle), or percentage of CD4$^+$ICOS$^+$CXCR5$^+$CD19$^-$ gate (right). (e) Quantitation of TFH cells as a percentage of total CD4 T cells. Data represent means of 5 mice per group. All error bars indicate standard error. (f) PD-1 on FoxP3$^+$ cells has a cell-intrinsic role in inhibiting TFR differentiation *in vivo*. Schematic design of a transfer assay in which 2D2 TCR transgenic CD4$^+$FoxP3$^+$CXCR5$^-$ non-TFR Tregs were transferred into WT mice which were subsequently immunized with MOG/CFA. Draining lymph nodes were harvested 7 days later and analyzed for TFR cells. (g) Representative gating of TFR cells from transfer experiments described in (f). (h-i) Quantitation of TFR cells from transfer experiments expressed as a percentage of FoxP3 GFP$^+$ cells present on day 7 post immunization (h) or total cell number (i) per lymph node. All data are representative of at least two independent experiments with at least 5 mice per group. All error bars indicate standard error. * $P<0.05$, ** $P<0.005$.

FIG. 2. PD-1 deficient TFR cells have altered expression of activation markers. (a) TFR cell gating strategy. WT or PD-1$^{-/-}$ mice were immunized with MOG/CFA and draining lymph nodes were harvested 7 days later. (b) CD25 expression on WT and PD-1$^{-/-}$ CD4 subsets gated as in (a). Overlay histograms of WT and PD-1$^{-/-}$ TFR cells (left) and mean fluorescence intensity (MFI) in CD4 subsets gated as in (a) (right). Data represent means of 5 mice per group. (c) CD69 expression on WT and PD-1$^{-/-}$ CD4 subsets gated as in (a). Overlay histograms of WT and PD-1$^{-/-}$ TFR cells (left) and MFI (right). Data represent means of 5 mice per group. (d) Intracellular staining of cell cycle marker Ki67 in populations as in (a). Overlay histograms of WT and PD-1$^{-/-}$ TFR cells (left) and percent Ki67 high (right) in CD4 subsets gated as in (a). Ki67 high was defined as the highest intensity peak on WT TFR cells and is denoted by a black bar on the histogram. Data represent means of 5 mice per group. All data are representative of at least two independent experiments. All error bars indicate standard error. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

FIG. 3. PD-1 deficient TFR cells are capable of homing to germinal centers (GCs). (a) Micrographs of draining lymph node sections from WT mice immunized

with MOG/CFA and harvested 7 days later. Sections were cut and stained for GL-7 (green), PNA (red) and IgD (blue). GCs were identified by PNA and GL7 positive, but IgD negative, staining. GCs are indicated with a white dotted line. (b) Ki67 staining in GCs. Sections were stained for the cell cycle marker Ki67 (blue), FoxP3 (red) and GL7 (green). (c) Colocalization of CD4 and FoxP3. Sections were stained for CD4 (blue), FoxP3 (red) and GL7 (green). Box indicates magnification highlighting CD4 positive staining on $FoxP3^+$ cells (d) Colocalization of FoxP3 in the nucleus. Sections were stained with the nuclear stain DAPI (blue), FoxP3 (red) and GL7 (green). Box indicates magnification highlighting FoxP3 protein within DAPI positive nuclei. (e) Comparison of $FoxP3^+$ TFR cells in germinal centers of WT and PD-$1^{-/-}$ mice. Representative GC staining in WT and PD-$1^{-/-}$ lymph nodes 7 days after immunization with MOG/CFA. (f) Average GC area was determined by calculating the area within the dotted lines according to materials and methods. Data represent mean area per lymph node of 5 individual mice. (g) Numbers of $FoxP3^+$ cells contained within GCs. Data represent mean per GC from 5 pooled mice. (h) Quantitation of the distance of each $FoxP3^+$ cell to the GC border. The distance for each $FoxP3^+$ cell in (e) from the GC borders (dotted line in (e)) was calculated as described in materials and methods. (i) CXCR5 expression was quantified on WT and PD-$1^{-/-}$ $CD4^+ICOS^+CXCR5^+FoxP3^-CD19^-$ TFH and $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$ TFR cells by flow cytometry 7 days after MOG/CFA immunization. Data represent means of 5 mice per group. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

FIG. 4. PD-1 deficient TFR cells have enhanced regulatory capacity. (a) TFR cells express high levels of GITR. WT mice were immunized with MOG/CFA and 7 days later lymph node cells were isolated and expression of GITR on TFR ($CD4^+FoxP3^+ICOS^+CXCR5^+CD19^-$, blue) and TFH ($CD4^+FoxP3^-ICOS^+CXCR5^+CD19^-$, red) was quantified as shown by histogram overlays. (b) Expression of FoxP3 mRNA in sorted TFR ($CD4^+GITR^+ICOS^+CXCR5^+CD19^-$), TFH ($CD4^+GITR^-ICOS^+CXCR5^+CD19^-$) and naive ($CD4^+ICOS^-CXCR5^-CD19^-$) cells. Data represent fold change in mRNA normalized to *Hprt*. (c) Bcl6 expression analyzed by intracellular flow cytometry on TFH and TFR cells from WT (blue) and PD-$1^{-/-}$ (green) mice. (d-f) mRNA expression of (d) blimp-1/*Prdm1* (e) *Rorc* and (f) *Irf4* from sorted WT (blue) and PD-$1^{-/-}$ (green) TFR and TFH cells and in

CD4-ICOS$^-$CXCR5$^-$ (naive) cells quantified by qPCR analysis. Data represent means from at least three separate experiments in which cells were sorted from lymph nodes of 10 pooled mice. (g) Design of assay to analyze capacity of TFR cells to inhibit activation of naïve CD4 T cells. WT and PD-1$^{-/-}$ mice were immunized with MOG/CFA and TFR cells were sorted from draining lymph nodes and plated 1:1:1 with CFSE-labeled CD4 naïve WT (CD4$^+$CD62L$^+$FoxP3$^-$) responder cells and WT GL7$^-$B220$^+$ B cells from MOG/CFA immunized mice along with anti-CD3 and anti-IgM for 4 days. 3 days later samples were analyzed by flow cytometry. (h) PD-1$^{-/-}$ TFR cells suppress activation of naïve T cells to a greater extent than WT TFR cells. T responders from suppression assays from (g) were analyzed for CD69 expression (h) and proliferation (i) by measuring CFSE dilution. % divided indicates percent of cells that have gone through at least one division. (j) *In vitro* IgG suppression assay design. TFR cells sorted as in (g) were plated in a 1:1:1 ratio of TFR (CD4$^+$ICOS$^+$CXCR5$^+$GITR$^+$CD19$^-$), TFH (CD4$^+$ICOS$^+$CXCR5$^+$GITR$^-$CD19$^-$), and B (GL-7$^-$B220$^+$) cells from draining lymph nodes of MOG/CFA immunized mice in the presence of anti-CD3 and anti-IgM for 6 days. Total IgG was measured by ELISA from supernatants. (k) Suppression assay using two concentrations of anti-CD3. (l) PD-1 deficient TFR cells suppress IgG production to a greater extent than WT TFR cells at a 1:1 TFR:TFH ratio. Naive (CD4$^+$ICOS$^-$CXCR5$^-$CD19$^-$) cells from immunized mice were included as controls. (m) PD-1 deficient TFR cells suppress IgG production to a greater extent than WT TFR cells at a 1:5 TFR:TFH ratio. Data indicates means +/- standard error of replicate wells and is representative of at least two experiments (h-m). * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

FIG. 5. PD-1 controls circulating blood TFR cells. (a) Gating strategy to identify circulating TFH and TFR cells from blood. WT mice were immunized with MOG/CFA and blood was collected 7 days later by cardiac puncture. TFH and TFR populations were gated as shown. (b) Quantitation of blood TFH and TFR cells following MOG/CFA immunization. Mice were immunized as in (a) and sacrificed on the indicated days. Blood was collected and cells stained and gated as in (a). (c) Ki67 expression in blood and lymph node TFH, TFR and naïve (CD4$^+$ICOS$^-$CXCR5$^-$) cells 7 days after MOG/CFA immunization. (d-f) Comparison of blood TFH and TFR cells in WT and PD-1$^{-/-}$ mice immunized as in (a) and harvested 7 days after immunization. (d) Blood TFH cells are shown gated on FoxP3$^-$CD4$^+$CD19$^-$ (left)

and TFR cells are shown gated on FoxP3$^+$CD4$^+$CD19$^-$ (right). (e) Quantitation of blood TFH and TFR cells from immunized WT and PD-1$^{-/-}$ mice gated as in (d) and expressed as a percent of CD4$^+$CD19$^-$ cells. (f) Quantitation of CXCR5$^-$ FoxP3$^+$ cells from immunized WT and PD-1$^{-/-}$ mice, expressed as a percentage of CXCR5$^-$ CD4$^+$ cells. All data indicates means +/- standard error of 5 mice and is representative of at least two independent experiments. * P<0.05, ** P<0.005, *** P<0.0005.

FIG. 6. PD-L1 but not PD-L2 controls blood TFR cells. (a) PD-L1 and PD-L2 expression on B cell subsets. WT mice were immunized with NP-OVA subcutaneously and 12 days later germinal center B (GC B), GL7$^-$, and plasma cells (PC) from draining lymph nodes were analyzed for PD-L1 (top) and PD-L2 (bottom) expression. (b) PD-L1 and PD-L2 expression on dendritic cells (DC). WT mice were immunized with NP-OVA and 3 days later CD8α$^+$ DC and CD8α$^-$ DC subsets from draining lymph nodes were analyzed for PD-L1 (top) and PD-L2 (bottom) expression. (c) Lymph node and blood TFH and TFR cells in PD-1 ligand deficient mice. WT, PD-L1$^{-/-}$ and PD-L2$^{-/-}$ mice were immunized with MOG/CFA, and 7 days later draining lymph nodes and blood were harvested and analyzed for TFH (c), TFR (d) and CXCR5$^-$ FoxP3$^+$ (e) CD4 T cells. Data represent means of 5 mice per group. All data are representative of at least two independent experiments. * P<0.05, ** P<0.005, *** P<0.0005.

FIG. 7. Blood TFR cells require ICOS and CD28 costimulation. (a) TFH and TFR gating in WT and ICOS$^{-/-}$ mice. Mice were immunized with MOG/CFA and 7 days later draining lymph nodes (dLN) and blood were harvested. TFH cells were gated as CD4$^+$CD44$^+$CXCR5$^+$FoxP3$^-$CD19$^-$, and TFR cells as CD4$^+$CD44$^+$CXCR5$^+$FoxP3$^+$CD19$^-$ cells. TFH (b) and TFR (c) quantitation in lymph nodes (dLN) and blood of WT and ICOS$^{-/-}$ mice as in (a). (d) TFH and TFR gating strategy in WT and CD28$^{-/-}$ mice. Mice were immunized as in (a) and TFH cells were gated as CD4$^+$ICOS$^+$CXCR5$^+$FoxP3$^-$CD19$^-$ and TFR cells as CD4$^+$ICOS$^+$CXCR5$^+$FoxP3$^+$CD19$^-$. TFH (e) and TFR (f) quantitation in lymph nodes and blood of WT and CD28$^{-/-}$ mice gated as in (d). All data are representative of at least two independent experiments. * P<0.05, ** P<0.005, *** P<0.0005.

FIG. 8. PD-1 deficient blood TFR cells more potently regulate antibody production *in vivo*. (a) Experimental strategy to assess blood TFH and TFR cell

function by transfer of blood TFH and/or TFR cells into mice that lack both lymph node and blood TFH/TFR cells. Blood TFH and/or TFR cells were isolated from 20 pooled mice immunized with NP-OVA 8 days previously and $CD4^+CXCR5^+GITR^-CD19^-$ TFH and $CD4^+CXCR5^+GITR^+CD19^-$ TFR cells were purified by cell sorting; recipient $CD28^{-/-}$ or $TCR\alpha^{-/-}$ mice received either no cells, $4x10^4$ TFH cells, or $4x10^4$ TFH plus $2x10^4$ TFR cells. One day later recipients were immunized with NP-OVA. 12 days later sera were collected and NP-specific antibody titers quantified by ELISA. (b) WT blood TFR cells potently suppress antibody production. NP-specific antibody titers from experiments as in (a) in which WT TFH or WT TFH plus WT TFR cells were transferred into $CD28^{-/-}$ recipients. (c) $CD138^+$ plasma cell percentages in draining lymph nodes of $CD28^{-/-}$ recipients following no transfer (Control), Blood TFH transfer (Blood TFH) or Blood TFH plus TFR cell transfer (Blood TFH + TFR) 24 days after immunization. Cells are gated as a percentage of $CD11b^-CD11c^-Ly6c^-$ (dump) cells. (d) Quantitation of CD138 plasma cells as gated in (c) in draining lymph node, spleen and bone marrow. (e) Blood TFH and/or TFR Transfer into $TCR\alpha^{-/-}$ recipients using experimental design as in (a). Comparison of NP-specific antibody titers in (1) WT control mice, (2) $TCR\alpha^{-/-}$ recipients given no cells, (3) $TCR\alpha^{-/-}$ recipients given WT blood TFR cells alone, (4) $TCR\alpha^{-/-}$ recipients given total blood CD4 T cells from $CXCR5^{-/-}$ mice immunized with NP-OVA 8 days previously, (5) $TCR\alpha^{-/-}$ recipients given blood $CD4^+FoxP3^-$ cells from unimmunized FoxP3-GFP mice, (6) $TCR\alpha^{-/-}$ recipients given WT blood TFH cells, and (7) $TCR\alpha^{-/-}$ recipients given WT blood TFH cells plus TFR cells. NP specific IgG levels were determined by ELISA. (f) $CD138^+$ plasma cells from the spleen (gated as a percent of live cells) and (g) $CD4^+FoxP3^-$ TFH cells from the draining lymph node pre-gated on $CD4^+FoxP3^-$ were quantified from experiments in (e) 12 days after secondary immunization. Error bars indicate standard error of at least three separate experiments. (h) Blood TFH cells can have an enhanced ability to stimulate antigen-specific antibody production compared to lymph node TFH cells. Blood TFH cells and draining lymph node TFH cells were isolated from WT mice immunized with NP-OVA 8 days previously and $4x10^6$ cells were transferred into $TCR\alpha^{-/-}$ mice and immunized as in (e). (i) Blood TFR cell suppression is aided by the follicular program. Blood TFH cells were transferred to $TCR\alpha^{-/-}$ mice along with blood

CXCR5- FoxP3 GFP$^+$ cells from FoxP3 reporter mice or blood TFR cells. Antibody titers were quantified 12 days after NP-OVA immunization and NP IgG levels are expressed as a percent of TFH transfer group. Data indicate standard error of at least three independent experiments. (j-k) PD-1 deficient blood TFR cells more potently suppress antibody production *in vivo* compared to WT TFR cells. $4x10^4$ WT blood TFH and $1.5x10^4$ WT or PD-1 deficient blood TFR cells from mice immunized with NP-OVA 8 days previously were transferred into CD28$^{-/-}$ mice (j) or TCR$\alpha^{-/-}$ mice (k). Recipient mice were immunized with NP-OVA, and NP specific antibody titers were measured from serum 12 days later. Data are representative of two independent experiments.

FIG. 9. Blood Tfh and Tfr cells are present in human blood. Peripheral blood mononuclear cells were isolated from the blood of a healthy individual by sucrose density centrifugation and stained for indicated proteins and were analyzed by flow cytometry. Numbers indicate percentages contained within gates.

FIG. 10. PD-1 controls TFR cells in NP-OVA immunized mice. (a) WT or PD-1$^{-/-}$ mice were immunized with NP-OVA emulsified in CFA and 7 days later draining lymph nodes were stained for CD4$^+$FoxP3$^+$ICOS$^+$CXCR5$^+$CD19$^-$ T follicular regulatory cells (TFR) and CD4$^+$FoxP3$^-$ICOS$^+$CXCR5$^+$CD19$^-$ T follicular helper cells (TFH) using gating strategy shown. (b) TFH cells were gated and expressed as a percentage of CD4$^+$FoxP3$^-$ cells. (c) TFR cells were gated and expressed as a percentage of all CD4$^+$ cells. (d) TFR cells were gated and expressed as a percentage of CD4$^+$FoxP3$^+$ cells. (e) TFR cells were gated and expressed as a percentage of CD4$^+$CXCR5$^+$ICOS$^+$ cells.

FIG. 11. Comparison of PD-L1, CD103 and GITR expression on WT and PD-1$^{-/-}$ T Follicular Regulatory Cells. (a-c) WT or PD-1$^{-/-}$ mice were immunized with MOG (35-55) and 7 days later draining lymph nodes were stained. TFR (CD4$^+$ICOS$^+$CXCR5$^+$FoxP3$^+$CD19$^-$) cells were analyzed for surface expression of PD-L1 (a), CD103 (b) and GITR (c).

FIG. 12. WT and PD-1 deficient TFR cells express similar levels of GITR. WT and PD-1$^{-/-}$ mice were immunized with NP-OVA and 7 days later populations were sorted according to gating strategy.

FIG. 13. TFH and TFR cells can bind similar levels of anti-CD3. (**a**) Gating of TFH, TFR and B cells from *in vitro* suppression assays as in FIG. 4k. (b) Cells

gated as in (**a**) were stained with an anti-hamster secondary antibody to quantify the amount of anti-CD3 bound to the surface of the cells.

FIG. 14. Blood TFH and TFR cells express central memory homing markers. WT and PD-$1^{-/-}$ mice were immunized with MOG/CFA and 7 days later populations from blood were analyzed for CD62L (a) and CD44 (b) expression. (**c**) PD-1 expression was compared on WT and PD-1 deficient mice from draining lymph node and blood TFH and TFR cells 7 days after NP-OVA immunization.

FIG. 15. Circulating TFH and TFR cells require dendritic cells for development. (A-B) WT or $CD11c^{DTR}$ bone marrow chimeras were immunized with NP-OVA subcutaneously and diphtheria toxin (DT) was administered (or not) on days 0, 2, 4, and 6 to deplete DCs. Draining lymph node analysis of $CD11c^{+}$MHC II^{+} DCs (A) and quantification of Ki67+ TFH ($CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{-}Ki67^{+}CD19^{-}$) and TFR ($CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{+}Ki67^{+}CD19^{-}$) cells in the draining lymph node and blood (B).

(C) WT or *Ciita*$^{-/-}$ bone marrow derived dendritic cells (BMDCs) were pulsed with NP-OVA in an overnight *in vitro* culture, washed and adoptively transferred subcutaneously to WT mice. 5 days later draining lymph nodes and blood were analyzed for ICOS and CXCR5 expression on CD4 T cells. WT mice that received no transfer were included as controls. Plots are pregated on $CD4^{+}CD19^{-}$. Representative plots are shown.

(D) Quantification of draining lymph node and blood TFH (top) and TFR (bottom) cells in BMDC adoptive transfer experiments as in (C).

(E) mMT mice have normal circulating TFH and TFR cells. WT or *Ighm*$^{-/-}$ "mMT" mice were immunized with NP-OVA subcutaneously and 7 days later $ICOS^{+}CXCR5^{+}$ CD4 T cells were identified. Plots are pregated on $CD4^{+}CD19^{-}$. Representative plots are shown.

(F) Quantification of total lymph node or blood $CD4^{+}ICOS^{+}CXCR5^{+}$ (left; as gated in (D)), TFH (middle), or TFR (right) cells.

(G) Analysis of the blood:lymph node ratio of TFH and TFR cells in WT and μMT mice immunized as in (E). Data are means +/- standard error with 5 mice per group. Data are representative of at least 2 independent experiments.

FIG.16. Circulating TFH and TFR cells depend on S1P signals to exit the lymph node. (A) Analysis of CD69 expression on TFH and TFR cells. Gating of TFH

and TFR cells from the lymph node and blood of WT mice immunized with NP-OVA 7 days previously (left). Plots are pregated on $CD4^+CD19^-$. Histograms (right) show CD69 expression on TFH and TFR cells from lymph node (blue) and blood (red) or on $CD4^+ICOS^-CXCR5^-$ cells.

(B) Quantification of CD69 on lymph node and blood TFH and TFR cells as in (A). $CD4^+ICOS^-CXCR5^-CD19^-$ cells are included as controls.

(C) Quantification of numbers of TFH cells in the lymph node (left; blue) and blood per ml (right; red) after FTY720 treatment for 5 days (on days 2, 4, and 6 post immunization followed by analysis on day 7) are shown.

(D) Quantification of numbers of TFR cells in the lymph node (left; blue) and blood (right; red) after FTY720 treatment for 5 days, as in (C).

(E) Numbers of TFH (left) and TFR (right) cells (per ml blood) after FTY720 treatment during the last 3 or 6 hours of a 7 day immunization.

(F) Representative plots of total $CD4^+ICOS^+CXCR5^+$ cells from the draining lymph node (dLN), efferent lymph (lymph) and blood of WT mice immunized 7 days previously. Plots are pregated on CD4+CD19-.

(G) Quantification of percentages of TFH and TFR cells in the draining lymph node (blue), efferent lymph (green) and blood (red) of WT mice immunized 7 days previously.

(H) Histograms showing ICOS expression on draining lymph node (blue), lymph (green) and blood (red) TFH (left) and TFR (right) cells as in (G). Data are means +/- standard error with 5 mice per group (A-E); data are representative of at least 2 independent experiments.

Data are representative from at least 3 replicates (F-G).

FIG. 17. Circulating TFH and TFR cells migrate to diverse secondary lymphoid organs and tissues. (A) 20 $Actin^{CFP}$-Fox^{GFP} mice were immunized with NP-OVA subcutaneously and 7 days later $2x10^4$ sorted blood $CD4^+CXCR5^+CD19^-$ cells were adoptively transferred into CD28 deficient mice which were immunized with NP-OVA. Organs were harvested 7 days later for analyses. Transferred cells from indicated organs were identified as CFP positive according to gates: iLN; draining inguinal lymph node, aLN; axillary lymph node, cLN; cervical lymph node, mLN; mesenteric lymph node, Skin; skin around site of immunization. Plots are pregated on $CD4^+CD19^-$.

(B) Dual color overlay plots showing ICOS and CXCR5 expression on CFP positive transferred cells (red) or endogenous CD28 deficient CD4 T cells (blue) in organs as in (A). (C) Quantification of the percentage of TFH cells of the $CFP^+CXCR5^+ICOS^+$ transferred cells as in (B). (D) Quantification of FoxP3+ cell percentages of the $CFP^+CXCR5^+ICOS^+$ transferred cells. (E) Quantification of the percentage of FoxP3+ cells from the total transferred CFP^+ population gated as in (A).

(F) Interaction of blood TFH cells with $IgG1^+$ B cells upon homing to lymph nodes. $2x10^4$ blood TFH ($CD4^+ICOS^+CXCR5^+GITR^-CD19^-$) cells from NP-OVA immunized CD45.2 mice were adoptively transferred to CD45.1 mice that were then NP-OVA immunized. 7 days later dLN were stained for CD45.2 (Blood TFH), IgG1 or IgD.

(G) Interaction of blood TFR cells with $IgG1^+$ B cells upon homing to lymph nodes. $1x10^4$ blood TFR ($CD4^+ICOS^+CXCR5^+GITR^+CD19^-$) cells from NP-OVA immunized CD45.2 mice were adoptively transferred to CD45.1 mice that were then NP-OVA immunized. 7 days later dLNs were stained for CD45.2 (Blood TFR), FoxP3 or IgG1. Data are from transfers of TFH or TFR cells from 20 pooled mice into a single mouse recipient and representative of at least 2 individual experiments.

FIG. 18. Circulating TFH and TFR cells show enhanced activation upon reentering lymphoid organs of immunized mice *in vivo*. (A) 20 $Actin^{CFP}$-Fox^{GFP} mice were immunized with NP-OVA subcutaneously and 7 days later $2x10^4$ sorted lymph node or blood $CD4^+CXCR5^+CD19^-$ cells were adoptively transferred to CD28 deficient mice that were then immunized. 7 days later CFP positive cells in draining lymph nodes were analyzed by flow cytometry. CD28 deficient mice that received no transfer ("No Trans") were used as controls. (B) Bcl6 expression in dLN $FoxP3^-CFP^+$ (TFH) cells in $CD28^{-/-}$ recipients of dLN CXCR5 or blood CXCR5 cells as described in (a). WT dLN TFH (CD4+ICOS+CXCR5+$FoxP3^-CD19^-$) cells from WT mice immunized 7 days previously were used as positive controls and $CD28^{-/-}$ CD4 T cells from immunized LN were used as negative controls for Bcl6 expression, and shown for comparison. (C) ICOS expression on lymph node or blood $FoxP3^-CFP^+$ (TFH) cells (assayed population) which originated from either dLN or blood CXCR5 transfers (CXCR5 Transfer) as in (a). Total CD4 T cells, dLN and blood TFH from WT immunized mice were included for comparison. (D) ICOS expression on lymph node or blood $FoxP3^+CFP^+$ (TFR) cells (assayed population) which originated from

either dLN or blood CXCR5 transfers (CXCR5 Transfer) as in (C). (E) CXCR5 expression on lymph node or blood FoxP3$^-$CFP$^+$ (TFH) cells as in (C). (F) CXCR5 expression on lymph node or blood FoxP3$^+$CFP$^+$ (TFR) cells as in (D). (G) Intracellular cytokine production of IL-21, IL-17A or IL-2 by transferred FoxP3$^-$ CFP$^+$ (TFH cells) from dLN or blood CXCR5 transfers as in (A). Data are from transfers of TFH or TFR cells from 20 pooled mice into a single mouse recipient and representative of at least 2 individual experiments.

FIG. 19. Circulating TFH cells Require Dendritic Cells for Restimulation and Have Memory Properties. (A) In vitro activation assay in which draining lymph node (dLN) or blood TFH cells (sorted as CD4$^+$ICOS$^+$CXCR5$^+$GITR$^-$CD19$^-$) were plated with B cells or DCs (isolated from lymph nodes of WT mice immunized 7 days previously) in the presence of anti-IgM and anti-CD3 for 6 days. Cells were stained for CD4+CD19- and intracellularly for Bcl6 and Ki67. Plots are pregated on CD4$^+$CD19$^-$. (B) Quantification of data presented in (A). (C) Intracellular cytokine staining of samples as in (A) except samples were stimulated with PMA/Iono for 4 hours in the presence of golgistop. (D) Blood TFH cells require DCs for expansion *in vivo*. 1.5x10^4 blood CD4+CXCR5+CD19- cells from immunized Actin^{CFP}-Fox^{GFP} mice were adoptively transferred to CD11cDTR bone marrow chimeric mice that were immunized with NP-OVA and treated or not with DT on days 0, 3 and 5. 7 days after immunization the draining lymph nodes were harvested for flow cytometric analysis of total CFP$^+$ cells (left) and expression of CXCR5 on CFP$^+$FoxP3$^-$ cells (right). (E) Circulating Memory blood CXCR5+ cells dominate the germinal center reaction upon homing to dLNs after challenge. Schematic diagram of parabiosis experiments (left). Actin^{CFP} Fox^{GFP} mice were immunized subcutaneously with NP-OVA at d0 or d7. At d0, mice were surgically joined to WT mice and circulatory systems were allowed to freely exchange for 19 days. Mice were then separated and 6 days later the WT "recipient" was immunized with NP-OVA. 7 days later organs were harvested for presence of CFP+ cells. Comparison of CFP+ cells in the non-draining lymph node naïve gate or CXCR5 gate in specified organs (right). (F) Blood TFH and TFR cells persist for 30 days *in vivo*. 2x10^4 blood CFP$^+$ICOS$^+$CXCR5$^+$CD19$^-$ (TFH + TFR) cells were adoptively transferred to WT mice that were immunized with NP-OVA 30 days later (left). Typical flow cytometry plots and quantification of data (right). "End" =

endogenous CD4 T cells. Adoptive transfer recipients are compared to mice that did not receive CFP+ cells, "No Trans". Data indicate means +/- standard error of at least 3 individual experiments (A-C), data from transfer of TFH or TFR cells from 20 pooled mice into 3 recipients (D) data from 3 individual mice/parabiotants (E), or 25 pooled mice transferred to a single mouse recipient and repeated 4 times (F).

FIG. 20. TFR cells suppress TFH activation and B cell class switch recombination in vitro. (A) dLN TFR suppression assays in which dLN TFH CD4+ICOS+CXCR5+GITR-CD19-) and/or TFR (CD4+ICOS+CXCR5+GITR+CD19-) cells were plated with either B cells or DCs (isolated from the dLN of WT mice immunized with NP-OVA 7 days previously) for 5-6 days with anti-CD3 and anti-IgM. TFH cells were gated as $CD4^{+}CD19^{-}FoxP3^{-}$ and stained for surface expression of CXCR5. Representative histograms (left) and quantification (right) are shown. (B-C) TFH cells gated as in (A) were intracellularly stained for (B) Bcl6 or (C) Ki67, with representative histograms (left) and quantification (right) shown. (D) Intracellular cytokine staining in TFH cells from suppression assays in which dLN TFH and/or TFR cells were plated with B cells as in (A). (E) B cells from suppression assays in which dLN TFH and/or TFR cells were plated with B cells as in (A) and quantified for surface expression of GL7. (F) Analyses of B cells from suppression assays in which no TFH or TFR cells were added, TFH cells alone were added, TFH and dLN FoxP3+CXCR5- cells from WT unimmunized mice were added, or TFH and TFR cells were added; B cells were stained for GL7 and CD138. Plots are pregated on $CD19^{+}CD4^{-}$. (G-H) Intracellular expression of (G) Ki67 and (H) IgG1 in B cells from suppression assays in which dLN TFH and/or TFR cells were plated with B cells as in (A). (I) Surface staining of GL7 and intracellular staining of IgG1 in B cells from suppression assays in which B cells were cultured with with dLN TFH cells alone, or with $CD4^{+}FoxP3^{+}CXCR5^{-}$ cells from LN of unimmunized FoxP3 GFP reporter mice, or dLN TFR cells. B cells cultured with $CD4^{+}CXCR5^{-}FoxP3^{-}CD62L^{+}$ naïve cells were included as controls. Data indicate means +/- standard error of replicate wells and representative of at least 3 independent experiments.

FIG. 21. Blood TFR cells are less suppressive than LN TFR cells. (A) GL7 and IgG1 expression in B cells from suppression assays in which dLN or blood TFR (CD4+ICOS+CXCR5+GITR+CD19-) cells from WT mice immunized 7 days

previously were added to dLN TFH cells and B cells as in (FIG. 6A) for 6 days. Gating strategy (left) and quantification (right) are shown. Plots are pregated on $CD4^+CD19^-FoxP3^-$. (B) GL7 expression on B cells from suppression assays with dLN TFR cells and blood TFR cells as in (A). (C) Intracellular Ki67 staining in TFR cells from suppression assays in which dLN TFH cells were plated with either B cells or DCs along with dLN TFR cells or blood TFR cells. Histograms are pregated on CD4+CD19-FoxP3+. (D-E) Blood TFH and TFR cells are capable of eliciting more potent B cell activation *in vivo*. $Actin^{CFP}$-Fox^{GFP} mice were immunized with NP-OVA and 7 days later $2x10^4$ blood or dLN $CD4^+ICOS^+CXCR5^+$ (TFH and TFR) cells (with the same TFH/TFR ratio) were adoptively transferred to CD28 deficient mice that were then immunized with NP-OVA. 7 days later draining lymph nodes were harvested and B cells were stained for (D) GL7 and FAS (plots are pregated on CD19) or (E) CD138 and CD19 (pregated on live cells). Data indicate means +/- standard error of at least 3 independent experiments (A-B) or individual plots that are representative of at least two experiments (C-E).

FIG. 22. TFH and TFR cells from the circulation show decreased expression of CXCR5 and ICOS compared to lymph node TFH and TFR cells. (A) WT mice were immunized or not with NP-OVA in CFA subcutaneously and 7 days later the draining lymph node and blood were collected and analyzed by flow cytometry. Populations of $ICOS^+CXCR5^+$ T follicular helper (TFH) cells in the lymph node (LN; left) and blood (right) of WT mice. Plots are pregated on $CD4^+FoxP3^-CD19$ (B) Quantification of TFH cells in lymph node (left) and blood (right) from plots as in (a). Total CD4+CD19- cells "Total CD4" are included as controls. (C-E) Quantification of (C) CXCR5 expression, (D) ICOS expression and (E) Ki67 expression on lymph node and blood TFH cells gated as in (A). (F) Populations of $ICOS^+CXCR5^+$ T follicular regulatory (TFR) cells in the lymph node and blood of WT mice immunized or not with NP-OVA 7 days previously. Plots are pregated on $CD4^+FoxP3^+CD19^-$.

FIG. 23. Comparison of TFR and Treg gene expression signatures (left) Top hits (oriented top to bottom) and (right) bottom hits (oriented bottom to top) of differentially expressed genes in WT CD4+ICOS+CXCR5+ GITR+ TFR cells (WT) compared to CD4+CXCR5-FoxP3+ non-TFR Tregs (Fox) in microarray analysis. PD-1 deficient TFR cells (PD1) (which have increased suppressive capacity) are also

shown. Each row indicates one gene. Data indicate top 100 and bottom 100 hits comparing WT TFR to Tregs. Black indicates row maximum expression, white indicates row minimum expression.

FIG. 24. Surface receptors differentially expressed by human blood TFR cells. Top 56 hits from a survey of surface receptor expression on human blood CD4+CXCR5+ ICOS+FoxP3+CD19- TFR, CD4+CXCR5+ ICOS+FoxP3-CD19- TFH, CXCR5- CD4 (CD4) and CXCR5- Tregs (Treg) are assessed at the protein level by flow cytometry. Hits are sorted based on receptors differentially expressed by TFR and TFH cells. Id indicates gene name. Value indicates mean fluorescence intensity on TFR cells. Heat map indicates relative high expression (black) or low expression (white).

FIG. 25. Surface receptors differentially expressed by human blood TFH cells. Top 19 hits from a survey of surface receptor expression on human blood CD4+CXCR5+ ICOS+FoxP3+CD19- TFR, CD4+CXCR5+ ICOS+FoxP3-CD19- TFH, CXCR5- CD4 (CD4) and CXCR5- Tregs (Treg) are assessed at the protein level by flow cytometry. Hits are sorted based on receptors differentially expressed by TFH and TFR cells. Id indicates gene name. Fold change indicates MFI fold increase on TFH verus TFR cells. Value indicates mean fluorescence intensity on TFR cells. Heat map indicates relative high expression (black) or low expression (white).

FIG. 26. Blockade of the PD-1 pathway can heighten antibody stimulating capacity of TFH cells. In vitro class switch recombination assay in which murine B cells are plated with sorted murine TFH cells to induce IgG1 class switch recombination. In some wells an anti-PDL1 blocking antibody was added to inhibit the PD-1 pathway.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

So that the invention may be more readily understood, certain terms are first defined.

T follicular regulatory (TFR) cells as used herein include, but are not limited to, the following cell surface markers: CD4+ICOS+CXCR5+FoxP3+CD19-.or CD4+ICOS+CXCR5+GITR+CD19-, or CD4+ICOS+CXCR5+CD25hiCD19-. In

one embodiment, TFR cells have the following cell surface markers: $CD4^+CXCR5^+ICOS^+$ and at least one surface marker selected from: $GITR^+$, $CD25^{hi}$, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86. In one embodiment, TFR cells have the following cell surface markers: $CD4^+CXCR5^+ICOS^+$ and at least one surface marker selected from: CD27, CD278 (ICOS), CD150, Siglec-9, CD140a, CD158b, CD33.

T follicular helper (TFH) cells as used herein include, but are not limited to the following cell surface markers: CD4+ICOS+CXCR5+FoxP3-CD19-. In one embodiment, TFH cells have the following cell surface markers: CD4, CXCR5, ICOS positive and at least one marker selected from CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and CD99 and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86. In one embodiment, TFH cells have the following cell surface markers: CD4, CXCR5, ICOS positive and at least one marker selected from CD163, CD127, CD161, CD6, CD229, CD272, CD100, CD126, PD-1 (CD279), and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

In one embodiment, TFH cells have the following cell surface markers: CD4, CXCR5, ICOS positive and at least one marker selected from CD163, CD127,

CD161, CD6, CD229, CD272, CD100, CD126, PD-1 (CD279), and optionally wherein the following markers are expressed a lower levels on TFH cells as compared to the levels of expression on TFR cells wherein such receptors are selected from: GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD8.

T regulatory cells (Tregs) as used herein include, but are not limited to the following cell surface markers: CD4+ GITR+ $CXCR5^-$ or CD4+FoxP3+ $CXCR5^-$ or CD4+ CD25hi $CXCR5^-$.

Populations of TFH or TFR cells referred to herein as "isolated" or purified" from blood refers to cells that have been removed from the body as part of a sample taken from the peripheral blood, organs or tissues of a subject. "Isolated" and "purified" cell compositions may further be enriched for the desired cell type via known procedures for separating desired cell types from other cell populations in a sample including cell sorting. As used herein" enriched" means that the resulting sample comprises more of the desired cell type than other cell types in the sample.

The terms "inhibit", "inhibition, "suppress" and "suppression" in terms of an immune response includes the decrease, limitation or blockage of, for example a particular action, function or interaction (e.g. antibody suppression).

The terms "enhance", "promote" or "stimulate" in terms of an immune response includes an increase, facilitation, proliferation, for example a particular action, function or interaction associated with an immune response (e.g. increase in antibody production).

As used herein, the term "modulate" includes up-regulation and down-regulation, e.g., enhancing or inhibiting an immune response. The term "modulate" when used with regard to modulatiion of a receptor includes up-regulation or down-regulation of the biological activity associated with that receptor when the receptor is activated, for example, by its ligand or inhibited, for example, with a blocking antibody.

As used herein, the term "immune cell" refers to cells that play a role in the immune response. Immune cells are of hematopoietic origin, and include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

As used herein, the term "T cell" includes CD4+ T cells and CD8+ T cells. The term "antigen presenting cell" includes professional antigen presenting cells (e.g., B lymphocytes, monocytes, dendritic cells, Langerhans cells) as well as other antigen presenting cells (e.g., keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes).

The term "native" cells or "wild-type" cells as used herein with reference to, for example, TFR cells, TFH cells or other cells, means that the cells are essentially phenotypically and functionally the same as those cells of the same cell-type generally found at the original source of the native or wild type cells, such as, for example, TFR cells normally found in the blood, organs or tissue of a subject.

The term TFR cells with "enhanced suppressive capacity" or "enhanced immune suppressive activity" or "enhanced regulatory capacity" refers to TFR cells that have been activated in the presence of a PD-1 or PD-1 ligand antagonist such that they have enhanced immune suppressive activity as compared to native TFR cells. Enhanced immune suppressive activity may be measured by standard *in vivo* and *in vitro* assays such as antibody suppression assays as are known in the art and described herein.

The term TFH cells with "enhanced stimulatory capacity", "enhanced immune stimulatory capacity" or "enhanced antibody stimulatory capacity" refers to TFH cells that have been activated in the presence of a PD-1 or PD-1 ligand antagonist such that they have enhanced stimulatory capacity as compared to native TFR cells. Enhanced stimulatory capacity may be measured, for example, by the novel *in vivo* and *in vitro* antibody proliferation assays of the invention as described herein.

As used herein, the term "immune response" includes T cell mediated and/or B cell mediated immune responses and include those immune responses that are mediated by TFR cells or TFH cells. Exemplary immune responses include T cell responses, e.g., cytokine production, and cellular cytotoxicity. In addition, the term immune response includes immune responses that are indirectly affected by T cell

activation, e.g., antibody production (humoral responses) and activation of cytokine responsive cells, e.g., macrophages.

A "subject" is preferably a human subject but can also be any mammal, including an animal model, in which modulation of an autoimmune reaction is desired. Mammals of interest include, but are not limited to: rodents, e.g. mice, rats; livestock, e.g. pigs, horses, cows, etc., pets, e.g. dogs, cats; and primates. A subject may also be a donor of peripheral blood T cells who is not the subject in which modulation of an autoimmune reaction is desired also referred to herein as a "healthy donor". A subject may also be referred to herein as a "patient".

The terms "treatment" "treat" and "treating" encompasses alleviation, cure or prevention of at least one symptom or other aspect of a disorder, disease, illness or other condition (collectively referred to herein as a "condition"), or reduction of severity of the condition, and the like. A composition of the invention need not affect a complete cure, or eradicate every symptom or manifestation of a disease, to constitute a viable therapeutic agent. As is recognized in the pertinent field, drugs employed as therapeutic agents may reduce the severity of a given disease state, but need not abolish every manifestation of the disease to be regarded as useful therapeutic agents. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilization (i.e., not worsening) of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total, whether detectable or undetectable) and prevention of relapse or recurrence of disease. Similarly, a prophylactically administered treatment need not be completely effective in preventing the onset of a condition in order to constitute a viable prophylactic agent. Simply reducing the impact of a disease (for example, by reducing the number or severity of its symptoms, or by increasing the effectiveness of another treatment, or by producing another beneficial effect), or reducing the likelihood that the disease will occur or worsen in a subject, is sufficient.

"Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. In one embodiment, an indication that a therapeutically effective amount of a composition has been administered to the patient is a sustained improvement over baseline of an indicator that reflects the severity of the particular disorder.

By a "therapeutically effective amount" of a composition of the invention is meant an amount of the composition which confers a therapeutic effect on the treated subject, at a reasonable benefit/risk ratio applicable to any medical treatment. The therapeutic effect is sufficient to “treat” the patient as that term is used herein.

As used herein, “cell therapy” is a method of treatment involving the administration of live cells.

“Adoptive immunotherapy” is a treatment process involving removal of cells from a subject, the processing of the cells in some manner *ex-vivo* and the infusion of the processed cells into the same subject as a therapy.

As used herein, a vaccine is a composition that provides protection against a viral infection, cancer or other disorder or treatment for a viral infection, cancer or other disorder. Protection against a viral infection, cancer or other disorder will either completely prevent infection or the tumor or other disorder or will reduce the severity or duration of infection, tumor or other disorder if subsequently infected or afflicted with the disorder. Treatment will cause an amelioration in one or more symptoms or a decrease in severity or duration. For purposes herein, a vaccine results from co-infusion (either sequentially or simultaneously) of an antigen and a composition of cells produced by the methods herein. As used herein, amelioration of the symptoms of a particular disorder by administration of a particular composition refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein a “vaccination regimen" means a treatment regimen wherein a vaccine comprising an antigen and/or adjuvant is administered to a subject in combination with for example, composition of the invention comprising TFR cells and/or TFH cells, simultaneously, in either separate or combined formulations, or sequentially at different times separated by minutes, hours or days, but in some way act together to provide the desired enhanced immune response to the vaccine in the subject as compared to the subject’s immune response in the absence of a TFR and/or TFH composition in accordance with the invention.

The term "adjuvant" is used in its broadest sense as any substance which enhances, increases, upwardly modulates or otherwise facilitates an immune

response to an antigen. The immune response may be measured by any convenient means such as antibody titre or level of cell-mediated response.

"Immune-related disease" means a disease in which the immune system is involved in the pathogenesis of the disease. Subsets of immune-related diseases are autoimmune diseases. Autoimmune diseases include, but are not limited to, rheumatoid arthritis, myasthenia gravis, multiple sclerosis, psoriasis, systemic lupus erythematosus, autoimmune thyroiditis (Hashimoto's thyroiditis), Graves' disease, inflammatory bowel disease, autoimmune uveoretinitis, polymyositis, and certain types of diabetes. Other immune-related diseases are provided *infra*. In view of the present disclosure, one skilled in the art can readily perceive other autoimmune diseases treatable by the compositions and methods of the present invention.

A disease or condition wherein modulation of, and preferably selective modulation of, TFR cells and/or TFH cells is therapeutic, includes diseases wherein suppression of a pathogenic antibody response is desired and diseases where enhancement of a protective antibody response is desired. In some instances, for example, in a disease in which suppression of a pathogenic antibody response is therapeutic, it is contemplated herein that the disease may be treated by selectively up-regulating TFR cell-mediated antibody suppression while simultaneously selectively down-regulating TFH cell-mediated immune response.

Examples of diseases or conditions wherein suppression of a pathological antibody response is desired include diseases in which antibodies contribute to, or are primarily responsible for pathogenesis. Such diseases or conditions in which antibodies contribute to and/or are primarily responsible for pathogenesis include, but are not limited to, diabetes (Type 1), multiple sclerosis, systemic lupus erythematosus, allergy, asthma, multiple sclerosis, myasthenia gravis, lupus erythematosus, autoimmune hemolytic, scleroderma and systemic sclerosis, Sjogren's syndrom, undifferentiated connective tissue syndrome, antiphospholipid syndrome, vasculitis (polyarteritis nodosa, allergic granulomatosis and angiitis, Wegner's granulomatosis, hypersensitivity vasculitis, polymyositis systemic lupus erythematosus, collagen diseases, autoimmune hepatitis, primary (autoimmune) sclerosing cholangitis or other hepatic diseases, thyroiditis, glomerulonephritis, Devic's disease, autoimmune throbocytopenic purpura, pemphigus vulgaris, vasculitis caused by ANCA, Goodpasture's syndrome, rheumatic fever, Grave's

disease (hyperthyroidism), insulin resistant diabetes, pernicious anemia, celiac disease, hemolytic disease of the newborn, cold aggutinin disease, IgA nephropathology, glomerulonephritis (including post-streptococcal), primary biliary cirhosis, and serum sickness. In one embodiment diseases in which pathogenic antibodies contribute to and/or are primarily responsible for pathogenesis are selected from multiple sclerosis, systemic lupus erythematosis, allergy, myasthenia gravis, collagen diseases, glomerulonephritis, Devic's disease, vasculitis caused by ANCA, and celiac disease.

Examples of diseases or conditions wherein enhancement of a protective antibody response is desired includes those diseases in which the presence of a robust antibody response reduces or eliminates the causes or pathogenesis of the disease. Examples of diseases or conditions benefiting from a protective antibody response include, but are not limited to viral infections and cancer.

While not a disease or condition, enhancement of a protective antibody response is also beneficial in a vaccine or as part of a vaccination regimen as is described herein.

An agent that is an "antagonist" of a cell surface receptor on a TFH cell or a TFR cell is an agent which down regulates or blocks the biological function of the cell surface receptor. As used herein, an agent which is an "antagonist" includes agents that bind or otherwise interfere with ligands of cell surface receptor thereby blocking the ability of the ligand to bind to the cell surface receptor and down-regulate or prevent the biological function of the cell surface receptor.

An agent that is an "agonist" of a cell surface receptor on a TFH cell or a TFR cell is an agent which upregulates or increases the biological function of the cell surface receptor.

II. Starting Population of Cells

In one embodiment TFR cells and TFR precursor cells, for example, T regulatory (Treg) progenitor cells are derived from a mixed cell population containing such cells (e.g. from peripheral blood, tissue or organs). Preferably the mixed cell population containing TFR cells or TFR cell precursors is enriched such that TFR cells or TFR cell precursors comprise more TFR cells than other cell types in the population. In one embodiment, an enriched composition of TFR cells is a

composition wherein the TFR cells make up greater than about 50% (e.g. , 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 99.5%, 99.9% or more) of the cell population in the composition. In some embodiments, the TFR cells comprise about 90%, 95%, 98%, 99%, 99.5%, 99.9% or more of the cells in the composition, and such compositions are referred to herein as "highly purified" or "substantially homogenous" TFR cell compositions.

While a starting population of TFR cells is described above, it is understood that similar procedures may be applied to obtaining a starting population of TFH cells. Accordingly in some embodiments a mixed cell population containing TFH is enriched such that the composition comprises more TFH cells than other cell types in the population. In one embodiment, an enriched composition of TFH cells is a composition wherein the TFH cells make up greater than about 50% (e.g. , 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 99.5%, 99.9% or more) of the cell population in the composition. In some embodiments, the TFH cells comprise about 90%, 95%, 98%, 99%, 99.5%, 99.9% or more of the cells in the composition, and such compositions are referred to herein as "highly purified" or "substantially homogenous" TFH cell compositions. In some embodiments, TFR cells or TFH cells are enriched from a population of cells prior to an activating and/or expanding step. In some embodiments TFR cells or TFH cells are enriched from a population of cells after the activating and/or expanding step.

Such highly purified or substantially homogenous populations of TFR cells or TFH cells may be used for *in-vivo* and *in-vitro* diagnoses and examination of TFR cell-mediated, or TFH cell-mediated diseases.

TFR cells can be enriched by targeting for selection of cell surface markers specific for immune suppressive TFR cells and separating using automated cell sorting such as fluorescence-activated cell sorting (FACS), solid-phase magnetic beads, etc. To enhance enrichment, positive selection may be combined with negative selection against cells comprising surface makers specific to non-T-regulatory cell types, such as depletion of CD8, CD11b, CD16, CD19, CD36 and CD56-bearing cells.

In one embodiment TFR cells are sorted via flow cytometry based on surface markers of $CD4^+CXCR5^+ICOS^+GITR^+$, or $CD4^+CXCR5^+ICOS^+CD25^+$. In one embodiment, TFR cells are sorted via flow cytometry based on the following cell

surface markers: $CD4^+CXCR5^+ICOS^+$ and at least one surface marker selected from one or more of: $GITR^+$, $CD25^{hi}$, CD162, CD27, CD95, CD9, CD43, CD278, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

TFH cells may be sorted based on surface markers of CD4, CXCR5, ICOS positive; GITR negative, CD25 negative. In one embodiment, TFH cells are sorted via flow cytometry based on the following cell surface markers: CD4, CXCR5, ICOS positive and at least one marker selected from: CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and CD99, and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrin?7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

It is believed that these sorting methodologies also contribute to the enhanced suppressive capacity of TFH cells and TFR cells alone or in combination with activating such cells in the presence of an antagonist of PD-1 or PD-1 ligand as is described herein.

In one embodiment, an initial population of TFR cells may also be isolated from the peripheral blood of a subject and further enriched for TFR cells. In one embodiment, an initial population of TFH cells may also be isolated from the peripheral blood of a subject and further enriched for TFH cells. Methods of purifying TFR cells or TFH cells from other PBMCs in the blood, using methods such as differential sedimentation through an appropriate medium, e.g. Ficoll-Hypaque [Pharmacia Biotech, Uppsala, Sweden], and/or methods of cell sorting, are well known and examples of such methods are described herein.

In one embodiment the invention provides a composition of TFR cells derived from the peripheral blood of a subject (also referred to herein as "blood TFR

cells") wherein the composition comprises about 90%, 95%, 98%, 99%, 99.5%, 99.9% or more of the cells in the composition.

In one embodiment the invention provides a composition of TFH cells derived from the peripheral blood of a patient (also referred to herein as "blood TFH cells") wherein the composition comprises about 90%, 95%, 98%, 99%, 99.5%, 99.9% or more of the cells in the composition. Such high purity compositions of blood TFR cells or blood TFH cells may be used directly in methods of modulating the immune system as described herein. Alternatively such compositions may be further activated and/or expanded prior to use in modulating the immune system as described herein.

III. Activation/Expansion of Starting Population of Cells

In one embodiment, the activation of a starting cell population is achieved by contacting the starting population of TFH cells or TFR cells with T cell stimulatory composition and/or in the presence of a PD-1 or PD-1L antagonist. The activating step may further include an expanding step or the cell population may be expanded separately from the activating step. If an expanding step is desired, the cells are preferably expanded at least 50-fold, and preferably at least 100, 200, 300, 500 and 800-fold.

Preferred stimulatory compositions stimulate the T cells by binding and activating the T cell receptor complex on the cells. In one embodiment, stimulatory compositions may include agents capable of binding and activating selective TFR and selective TFH receptors as described herein. In one embodiment the stimulatory compositions comprise physiological antigen presenting cells (APCs), such as CD19+ B cells (preferably autologous from blood) a TCR/CD3 activator such as a multivalent antibody or ligand for TCR/CD3; a TCR costimulator activator such as multivalent antibody or ligand for CD28, GITR, CD5, ICOS, OX40 or CD40L; and optionally an interleukin such as IL-2. In one embodiment, the TCR/CD3 activator is an anti-CD3 antibody, and the TCR costimulator activator is an anti-CD28 antibody. The anti-CD3 and anti-CD28 antibodies are optionally immobilized on beads as are known in the art and provided in a cell:bead ratio of between 1:1 and 1:2.

In certain embodiments, the stimulatory composition may further include one or more additional agents, e.g., a costimulatory agent, a second regulatory T cell stimulatory agent, or agents that generally promote the survival and/or growth of T cells.

In certain embodiments, the costimulatory agent is an antibody or ligand specific for a T cell costimulator, such as CD28 or ICOS, as described below. In particular embodiments, the costimulatory agent is an agonist antibody, such as an agonist antibody which binds to CD28.

The stimulatory composition alternatively comprises a second regulatory T cell stimulatory agent. Exemplary stimulatory agents include granulocyte colony stimulating factor, interleukins such as IL-2, IL-6, IL-7, IL-13, and IL-15, and hepatocyte growth factor (HGF).

In particular embodiments, one or more components of the stimulatory composition is immobilized on a substrate, such as a cell or bead. Cells suitable for use as substrates include artificial antigen-presenting cells (AAPCs) (Kim, J V et al, Nat Biotechnol. April 2004; 22(4):403-10; and Thomas, A K et al, Clin Immunol. December 2002; 105(3):259-72). Beads can be plastic, glass, or any other suitable material, typically in the 1-20 micron range.

Examples of PD-1 and PD-1 ligand antagonists are disclosed in U.S. Patent 7,722,868, incorporated herein by reference. Suitable PD-1 and PD-1 ligand antagonists include a PD1-ligand antibody, an anti-PD-1 antibody, a peptide or a small molecule wherein the agent inhibits the interaction between PD-1 and a PD-1 ligand. Other examples of PD-1 or PD-1 ligand antagonists are disclosed, for example in U.S. Pat. Nos. 8,168,757; 8,114,845; 8,008,449; 7,595,048; 7,488,802; and 7,029,674.

Optimal concentrations of each component of the stimulatory compositions, culture conditions and duration can be determined empirically using routine experimentation.

Populations of TFR cells expanded/activated in the presence of a PD-1 or PD-1 ligand antagonist have enhanced suppressive activity as demonstrated by their ability to inhibit TFH-mediated antibody production *in vitro* and *in vivo* as demonstrated in appropriate assays as are described herein.

Populations TFH cells expanded/activated in the presence of a PD-1 or PD-1 ligand antagonist have enhanced antibody stimulatory activity as demonstrated by their ability to enhance TFH-mediated antibody production *in vitro* and *in vivo* as demonstrated in appropriate assays described herein. Such TFH cells are referred to herein as "TFH cells having enhanced stimulatory capacity".

IV. Modulation of the Immune System

The expanded and/or activated TFR cells and compositions thereof as described herein may be introduced into the subject to treat immune related diseases, for example, by modulating an autoimmune reaction. For example, the subject may be afflicted with a disease or disorder characterized by having an ongoing or recurring autoimmune reaction, such as the diseases/disorders including, but not limited to, lupus erythematosus; myasthenia gravis; autoimmune hepatitis; rheumatoid arthritis; multiple sclerosis; Grave's disease, and graft versus host disease (GVHD).

In one embodiment, if upregulation of an immune response in a subject is desired, such as for example, increasing antibody proliferation in response to a vaccine, an enriched and optionally expanded and/or activated composition comprising a starting population of TFH cells may be administered to a subject.

In one embodiment modulation of the immune system is achieved when upon administration of a composition of the invention, the ratio of TFR cells to TFH cells in a subject is changed as compared to the ratio of TFR cells to TFH cells in a subject prior to administration of a composition of the invention. The ratios of TFR cells to TFH cells in a subject prior to, and after, administration of a composition of the invention may be measured by assaying for the presence of TFR cells or TFH cells in a patient's blood. Examples of suitable assays for measuring the ratio of TFR cells to TFH cells in a patient's blood are described herein.

In one embodiment, if downregulation of a subject's immune response is desired, such as, for example, when the subject has an autoimmune disease and inhibition of an antibody response is desired, a highly purified composition of blood derived TFR cells, or a composition enriched for TFR cells having enhanced suppressive activity may be administered to a patient. After administration of the composition, a blood sample from the patient may be tested to determine if the ratio of TFR cells to TFH cells is high.

In one embodiment, if upregulation of an immune response in a subject is desired, such as for example, increasing antibody proliferation in response to a vaccine, a highly purified composition of blood-derived TFH cells may be administered to a patient. After administration of TFH composition, a blood sample from the patient may be tested to determine if the ratio of TFH cells to TFR cells is high.

Accordingly, the invention provides methods and compositions for adoptive cellular immunotherapy comprising introducing into a patient in need thereof an effective amount of the subject's *ex vivo* expanded/activated TFR cells, for example. These applications generally involve reintroducing expanded/activated TFR cells extracted from the same patient, though the methods are also applicable to adoptive cellular immunotherapy for treatment of graft-versus-host disease associated with transplantation, particularly bone marrow transplantation using TFRs derived from donor tissue, and/or healthy individuals.

In an exemplary adoptive cell transfer protocol comprises a mixed population of cells is initially extracted from a target donor. Depending on the application, the cells may be extracted during a period of remission, or during active disease. Typically this is done by withdrawing whole blood and harvesting PMBCs by, for example, leukapheresis (leukopheresis). For example, large volume leukapherisis (LVL) has been shown to maximize blood leukocyte yield. Harvests reach 20×10^6 cells/L using a continuous flow apheresis device (Spectra, COBE BCT). Symptoms of hypocalcemia are avoided by a continuous infusion of calcium administrated throughout leukapheresis. Typically 15-45 liters of fluid corresponding to about 4 total blood volumes are harvested during a period of time ranging from about 100 to 300 minutes.

The harvested PMBCs may be separated by flow cytometry or other cell separation techniques based on Treg and/or TFR-specific cell markers such as CD4, CD25, CXCR5, ICOS, and GITR and expanded /activated as described herein, and then transfused to a patient, preferably by the intravenous route, typically the cell donor (except in GVHD where the donor and recipient are different), for adoptive immune suppression. Alternatively, the cells may be frozen for storage and/or transport prior to and/or subsequent to expansion.

Effective and optimized dosages and treatment regimens using the expanded and/or enriched and optionally highly pure TFH or TFR cells are known in the art based on previous clinical experience with existing T-cell infusion therapies, and can be further determined empirically.

The preferred route of administration of the TFR and TFH cell compositions to a subject in accordance with the invention is by the intravenous route. However, depending on the application, cell compositions in accordance with the invention may be administered by other routes including, but not limited to, parenteral, oral or by inhalation.

V. Vaccination

The present invention also contemplates a method for enhancing an immune response to an antigen comprising the administration to a subject as part of a vaccination regimen, TFR cells having enhanced suppressive activity, TFR cell compositions derived from peripheral blood and/or TFH cells derived from peripheral blood. The present invention is particularly useful in pharmaceutical vaccines and genetic vaccines in humans.

Adjuvants promote the immune response in a number of ways such as to modify the activities of immune cells that are involved with generating and maintaining the immune response. Additionally, adjuvants modify the presentation of antigen to the immune system. The compositions of the invention may be used as adjuvants in a vaccination regimen.

In one embodiment, compositions of TFR cells in accordance with the invention may be used in a vaccination regimen. Without being limited to a specific theory, it is believed that TFR cells may control germinal center (GC) B cell

differentiation into long-lived plasma cells versus memory B cells thereby enhancing the immune response to the antigen.

In one embodiment compositions of TFH cells, particularly TFH cells derived from the peripheral blood of a patient (also referred to herein as "blood TFH cells") may be used in a vaccination regimen to enhance TFH cell mediated antibody responses. Without being limited to any particular theory, it is believed that TFH cells derived from the blood migrate to lymph nodes and interact with cognate B cells rapidly upon antigen exposure, wherein naïve T cells need at least two to four days to differentiate and upregulate CXCR5. Accordingly, TFH cells derived from blood have greater antibody stimulatory capacity.

In one embodiment, it may be desirable to upregulate an immune response or downregulate an immune response as part of a vaccination regimen. This can be accomplished by adminstering compositions enriched for or highly purified for TFR cells or compositions enriched for or highly purified TFH cells to change the ratio of TFR cells to TFH cells in a subject's blood in combination with the administration of a vaccine.

VI. Novel in-vivo and in vitro assays

The invention also provides *in vivo* and *in vitro* assays to analyze the functions of the compositions of TFR cells and TFH cells in accordance with the invention.

In one exemplary embodiment the invention provides an assay to analyze the capacity of TFR cells to inhibit activation of naïve CD4 T cells. Briefly, WT and PD-$1^{-/-}$ mice are immunized with MOG/CFA and TFR cells are sorted from draining lymph nodes and plated 1:1:1 with CFSE-labeled CD4 naïve WT ($CD4^{+}CD62L^{+}FoxP3^{-}$) responder cells and WT $GL7^{-}B220^{+}$ B cells from MOG/CFA immunized mice along with anti-CD3 and anti-IgM for 4 days. 3 days later samples are analyzed by flow cytometry. It is understood that any suitable antigen/adjuvant combinations may be used to immunize mice and that the cells may be stimulated by any suitable combinations of stimulatory factors for this assay.

In one exemplary embodiment the invention provides an assay to analyze capacity of TFR cells to inhibit activation of naïve CD4 T cells. WT and PD-$1^{-/-}$ mice are immunized with MOG/CFA and TFR cells and sorted from draining lymph

nodes and plated 1:1:1 with CFSE-labeled CD4 naïve WT ($CD4^+CD62L^+FoxP3^-$) responder cells and WT $GL7^-B220^+$ B cells from MOG/CFA immunized mice along with anti-CD3 and anti-IgM for 4 days. 3 days later samples are analyzed by flow cytometry. T responders are analyzed for CD69 expression and proliferation by measuring CFSE dilution.

In one embodiment the invention provides an assay for an in vitro IgG suppression. Briefly, TFR cells are sorted as in the assay to analyze capacity of TFR cells to inhibit activation of naïve CD4 T cells and are plated in a 1:1:1 ratio of TFR ($CD4^+ICOS^+CXCR5^+GITR^+CD19^-$), TFH ($CD4^+ICOS^+CXCR5^+GITR^-CD19^-$), and B ($GL\text{-}7^-B220^+$) cells from draining lymph nodes of MOG/CFA immunized mice in the presence of anti-CD3 and anti-IgM for 6 days. Total IgG was measured by ELISA from supernatants. In one embodiment the in-vitro suppression assay may be performed over a range of concentrations of anti-CD3. Naive ($CD4^+ICOS^-CXCR5^-CD19^-$) cells from immunized mice may be included as controls It is understood that any suitable antigen/adjuvant combinations may be used to immunize mice and that the cells may be stimulated by any suitable combinations of stimulatory factors for this assay.

Novel assays of the invention are useful as a diagnostic tool for measuring a subject's TFR cell function and TFH cell function. Such assays are useful in the identification and typing of autoimmune diseases.

The assays of the invention are also useful in measuring the ratio of TFR cells to TFH cells in a patient's blood prior to or during an immune response and/or prior to and after administration of a composition of the invention. Such assays are useful as a diagnostic to assist in determining whether an immune response in a subject should be upregulated or down-regulated or whether an immune modulating treatment regimen has had the desired effect. This assay also may be useful in the diagnosis or progression of specific diseases.

In accordance with the invention, an exemplary assay comprises a method for assaying the TFR cell function or the TFH cell function or both, in a patient comprising the steps of:

a) Obtaining a sample of peripheral blood from a patient;

b) Isolating a population of TFH cells and TFR cells from the blood sample;

c) Contacting the TFH cells and TFR cells with a stimulatory composition comprising antigen present cells (e.g.B cells) in the presence of T-cell receptor stimulating factors and cofactors such as anti-CD3 and anti-IgM ,for a time period sufficient to allow the production of antibody such as IgG; and

d) measuring the total antibody produced using standard assays (e.g. ELISA).

VII. Modulation of TFR and TFH cell-mediated immune responses via selective TFR and TFH cell surface receptors

The data in Fig. 23 shows that TFR cells are distinct in their gene expression as compared to Treg cells and TFH cells suggesting that TFR cells are capable of independently regulating immune responses. This knowledge may be applied to diagnose, monitor and treat diseases or conditions wherein TFR-immune responses may be selectively modulated such as in those diseases or conditions in which antibodies play a key role in the pathogenesis and enhanced immune suppression is therapeutic. Examples of such diseases are provided herein *supra*.

TFR cell function may be modulated by use of an agent such as an agonist or an antagonist of one or more of TFR cell surface receptors as described herein. Use of such an agent in an amount effective to inhibit or induce the differentiation of TFR cells and/or modulate the biological function of TFR cells can affect the TFR cell-mediated immune response.

In one embodiment, the invention provides a method suppressing a pathogenic antibody response in a patient in need thereof comprising, administering to the patient, an agent which modulates at least one receptor which is differentially expressed on TFR cells as compared to TFH cells at an increased mean fluorescence intensity (MFI) fold change of at least 1.17, and wherein the receptor has an MFI of at least 186 on TFR cells and wherein the agent administered in an amount that is effective to modulate the TFR receptor and increase TFR cell-mediated antibody suppression, as compared to the TFR cell-mediated antibody suppression in the absence of the agent. Such differentially expressed receptors are referred to herein as "selective TFR receptors". In one embodiment at least one selective TFR receptor is selected from one or more of: CD162, CD27, CD95, CD9, CD43, CD278 (ICOS), CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9,

Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86. In one embodiment, selective TFR receptors are selected from one or more of: CD27, CD278 (ICOS), CD150, Siglec-9, CD140a, CD158b, and CD33.

Methods of modulating TFR cell-mediated immune response through antagonizing or agonizing the biological function of a selective TFR receptor are useful in the treatment of diseases and conditions wherein a decreased or increased TFR cell-mediated immune response is useful. Examples of disorders or conditions which may be treated by increasing TFR cell-mediated immune response include those diseases and conditions in which antibodies contribute to and/or are primarily responsible for, pathogenesis such as in those diseases listed previously herein. In one embodiment the disease or condition in which antibodies contribute and/or are primarily responsible for pathogenesis include: multiple sclerosis, systemic lupus erythematosis, allergy, myasthenia gravis, collagen diseases, glomerulonephritis, Devic's disease, vasculitis caused by ANCA, and celiac disease.

In one embodiment, the agent is a blocking antibody capable of blocking/antagonizing a selective TFR receptor, or binding to the ligand of the receptor and thereby blocking its ability to bind its corresponding receptor. The agent may also be a small molecule, or a DNA or RNA molecule (e.g. dsRNA, or antisense molecule) capable of antagonizing or agonizing the biological function of the receptor, for example by causing overexpression of a receptor or by blocking expression of a receptor.

In addition to the known agonists and antagonists of various selective TFR receptors known generally, other agents may be tested for their ability to antagonize or agonize selective TFR receptors using known assays and screens.

Assays and screens of the invention useful for testing various agents for their ability to agonize or antagonize TFR cell surface receptors are used to identify agents of the invention. In one embodiment, the invention provides assays to specifically and sensitively determine the stimulatory function of TFH cells. Complementary assays are also provided which determine the inhibitory capacity of TFR cells. These assays which include both *in vitro* and *in vivo* experiments can be

used to determine the functional consequences of sending agonist and/or antagonist signals through surface receptors on TFH and TFR cells.

In vitro murine TFH stimulation assays are performed by immunizing/vaccinating mice with an antigen/adjuvant. In some cases live or attenuated virus can be used. Seven to ten days later TFH cells defined as (CD4+ICOS+CXCR5+FoxP3+CD19-) or (CD4+ICOS+CXCR5+GITR+CD19-) (or alternative TFH marker) are sorted by flow cytometry. TFH cells are incubated with B cells along with anti-CD3 and anti-IgM (or alternatively with specific antigen). Agonists and/or antagonists for TFH surface receptors are also added into cultures. After 7 days antibody production and class switch recombination is assessed by either staining B cells from the culture for activation markers (B7-1, GL7, etc.) or intracellular for IgG isotypes. Activation status of the TFH cell can also be determined. Alternatively, the supernatants can be assessed for presence of IgGs via ELISA. Examples of this assay are included in FIGs. 4, 20 and 21. Similar assays are performed using human cells isolated from blood or other tissues.

In vivo murine TFH stimulation assays are performed by immunizing/vaccinating mice with an antigen/adjuvant. In some cases live or attenuated virus can be used. Seven to ten days later TFH cells defined as (CD4+ICOS+CXCR5+FoxP3-CD19-) or (CD4+ICOS+CXCR5+GITR-CD19-) (or alternative TFH marker) are sorted by flow cytometry. Cells are either used right away, or incubated in vitro with agonists or antagonists as in in vitro assays. Cells are adoptively transferred intravenously to mice that are vaccinated or likewise challenged with antigen and/or virus. Ten days later serum is collected from mice and IgGs are detected by ELISA. Within these 10 days agonists or antagonists for TFH surface receptors can be administered.

Examples of this assay are in FIGs. 8 and 21. These assays can also be used to determine how TFH cells change disease autoimmune pathology. As an example, mice can be immunized with collagen and then TFH cells can be sorted and transferred to a new mouse that is immunized with collagen. The resulting anti-collagen antibodies will cause arthritis which can be measured to determine how TFH cells function within this specific disease. Additionally, these assays can be used to determine TFH stimulation of B cell antibody production in the context of vaccination by using TFH cells from influenza infected mice and then adoptively

transfer them to a new mouse that is infected with influenza. Extent of viral infection can be measured as a readout for antibody mediated clearance of virus.

In vitro murine TFR suppression assays are performed by immunizing/vaccinating mice with an antigen/adjuvant. In some cases live or attenuated virus can be used. Seven to ten days later TFH cells defined as (CD4+ICOS+CXCR5+FoxP3-CD19-) or (CD4+ICOS+CXCR5+GITR-CD19-) (or alternative TFH marker) and TFR cells defined as (CD4+ICOS+CXCR5+FoxP3+CD19-) or (CD4+ICOS+CXCR5+GITR+CD19-) (or alternative TFR marker) are sorted by flow cytometry. TFH and/or TFR cells are incubated with B cells along with anti-CD3 and anti-IgM (or alternatively with specific antigen). Agonists and/or antagonists for TFR surface receptors are also added into cultures. After 7 days antibody production and class switch recombination is assessed by either staining B cells from the culture for activation markers (B7-1, GL7, etc.) or intracellular for IgG isotypes. Activation status of the TFH cell can also be determined. Alternatively, the supernatants can be assessed for presence of IgGs via ELISA. Examples of this assay are included in FIGs. 4, 20 and 21. Similar assays are performed using human cells isolated from blood or other tissues.

In vivo murine TFR suppression assays are performed by immunizing/vaccinating mice with an antigen/adjuvant. In some cases live or attenuated virus can be used. Seven to ten days later TFH cells defined as (CD4+ICOS+CXCR5+FoxP3+CD19-) or (CD4+ICOS+CXCR5+GITR+CD19-) (or alternative TFH marker) and TFR cells defined as (CD4+ICOS+CXCR5+FoxP3+CD19-) or (CD4+ICOS+CXCR5+GITR+CD19-) (or alternative TFR marker) are sorted by flow cytometry. Cells are either used right away, or incubated in vitro with agonists or antagonists as in in vitro assays. Cells are adoptively transferred intravenously to mice that are vaccinated or likewise challenged with antigen and/or virus. Ten days later serum is collected from mice and IgGs are detected by ELISA. Within these ten days agonists or antagonists for TFR surface receptors can be administered. Examples of this assay are in FIG. 8 and 21. These assays can also be used to determine how TFR cells change disease pathology. As an example, mice can be immunized with collagen and then TFH and TFR cells can be sorted and transferred to a new mouse that is immunized with

collagen. The resulting anti-collagen antibodies will cause arthritis which can be measured to determine how TFR cells function to suppress the TFH mediated disease.

The present invention further provides agents identified in the assays described herein. Such agents are capable of up antagonizing or agonizing a selected TFR receptor and thereby modulate TFR cell-mediated immune function and to further treat diseases as described herein.

Animal model systems which can be used to screen the effectiveness of the selected agents and test agents of the present invention in protecting against or treating the disease are available. Methods for the testing of systemic lupus erythematosus (SLE) in susceptible mice are known in the art (Knight et al. (1978) J. Exp. Med. , 147: 1653 ; Reinersten et al. (1978) New Eng. J. Med., 299: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease with soluble AchR protein from another species (Lindstrom et al. (1988) Adv. Immunol., 42: 233). Arthritis is induced in a susceptible strain of mice by injection of Type II collagen (Stuart et al. (1984) Ann. Rev. Immunol., 42: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has been described (Van Eden et al. (1988) Nature, 331: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron et al. (1980) J. Exp. Med. , 152: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa et al. (1984) Diabetologia, 27: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration of myelin basic protein (see Paterson (1986) Textbook of Immunopathology, Mischer et al., eds., Grune and Stratton, New York, pp. 179-213; McFarlin et al. (1973) Science, 179: 478: and Satoh et al. (1987) J. hnmunol., 138: 179).

Generally, suitable agents identified and tested as described above will be used in purified form together with pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically- acceptable adjuvants, if necessary to keep, for example, a polypeptide complex such as an antibody in suspension, may be

chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates. Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) Remington's Pharmaceutical Sciences, 16th Edition).

The selected agents of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cylcosporine, methotrexate, adriamycin or cisplatinum, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the agents of the present invention.

The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the selected agents can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, via the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

In certain therapeutic applications, an adequate amount to accomplish modulation of a TFR and/or TFH cell-mediated immune response will depend upon the severity of the disease and the general state of the patient's own immune system. Generally, if the agent is a blocking antibody, for example, a range from 0.01 mg-100 mg per kilogram of body weight, with doses of 1-10 mg/kg would be suitable.

A composition containing one or more selected agents according to the present invention may be used in prophylactic and therapeutic settings to aid in the modulation of a TFR and/or TFH cell-mediated response. In addition, the agents described herein may be used extracorporeally or *in vitro* to selectively modulate TFR and/or TFH cell-mediated immune responses.

The invention also provides agents and methods for modulating TFH cell-mediated immune responses. TFH cells are known in the prior art to be a subset of T helper cells that are genetically distinct from other types of T helper cells suggesting that TFR cells may independently regulate immune responses. This knowledge may be applied to diagnose, monitor and treat, for example, diseases or conditions wherein enhancement of a protective antibody response is therapeutic. Such diseases include but are not limited to treating viral infections and treating cancer. Enhancing and preferably selectively enhancing, TFH cell-mediated immune responses are also particularly beneficial as part of a vaccine or vaccination regimen.

TFH cell function may be modulated by use of an agent such as an agonist or an antagonist of one or more of TFH cell surface receptors as described herein. Use of such an agent in an amount effective to inhibit or induce the differentiation of TFH cells and/or modulate the biological function of TFH cells can affect the TFH cell-mediated immune response.

In one embodiment, the invention provides method of increasing a protective antibody response in a patient in need thereof comprising administering to the patient, an agent which modulates at least one receptor which is differentially expressed on TFH cells as compared to TFR cells at an increased mean fluorescence intensity (MFI) fold change of at least 1.17, and wherein the receptor has an MFI of at least 200 on TFH cells and wherein the agent is effective to modulate the receptor and increase the antibody response in the patient as compared to the antibody response when the agent is absent. Such differentially expressed receptors are referred to herein as "selective TFH receptors". In one embodiment at least one selective TFH receptor is selected from one or more of: CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, PD-1 (CD279), CD5, and CD99. In one embodiment, at least one selective TFH receptor is selected from one or more of: CD163, CD127, CD161, CD6, CD229, CD272, CD100, CD126, PD-1 (CD279).

In one embodiment, the agent is a blocking/antagonizing antibody capable of blocking a selective TFH receptor, or binding to the ligand of the receptor and thereby blocking its ability to bind its corresponding receptor. The agent may also

be a small molecule, or a DNA or RNA molecule (e.g. dsRNA, or antisense molecule) capable of antagonizing or agonizing the receptor.

In addition to the known agonists and antagonists the various selective TFH receptors described herein, other agents may be tested for their ability to antagonize or agonize selective TFH receptors using known assays and screens. Assays and screens for testing various agents for their ability to agonize or antagonize TFH cell surface receptors are described previously herein. Animal models may be used to test modulation of selective TFH receptors as described previously herein.

The present invention further provides agents identified in the assays described herein wherein such agents are capable of antagonizing or agonizing a selective TFH receptor and thereby modulate TFH cell-mediated immune function. Agents for modulating selective TFH receptors may be formulated and administered to patients as described above.

In one embodiment, the invention provides a method of decreasing a pathogenic antibody response in a patient in need thereof comprising administering to the patient a first agent capable of modulating a selective TFR receptor in an amount effective to increase TFR cell-mediated antibody suppression in the patient, in combination with an second agent capable of modulating a selective TFH receptor in an amount effective to decrease TFH cell-mediated antibody production, wherein the pathogenic antibody response is decreased as compared to the pathogenic antibody response in the absence of the first or second agents.

In one embodiment, the invention provides a method of increasing a protective antibody response in a patient in need thereof comprising administering to the patient a first agent capable of modulating a selective TFR receptor in an amount effective to decrease TFR cell-mediated antibody suppression in the patient, in combination with a second agent capable of modulating a selective TFH receptor in an amount effective to increase TFH cell-mediated antibody production, wherein the protective antibody response is increased as compared to the protective antibody response in the absence of the first or second agents.

VIII. Modulation of TFR and TFH cell-mediated immune responses via PD-1 receptors

The inventors' discovery that PD-1:PD-L1 interactions limit TFR cell differentiation and function has also elucidated another novel approach to modulating both TFR cell-mediated and TFH cell-mediated immune responses in a patient.

Therefore, in one embodiment the invention provides a method of decreasing a pathogenic antibody response in a patient in need thereof comprising administering to the patient, an agent which modulates the PD-1 receptor on a TFR cell. In one embodiment, the agent is an antagonist of the PD-1 receptor on a TFR cell. In one embodiment, the agent is an antibody capable of blocking the PD-1 receptor on a TFR cell. In one embodiment, the agent is an antibody capable of binding to a ligand selected from PD-L1 or PD-L2 and preventing the ligand from binding to the PD-1 receptor. The agent may also be a small molecule, or a DNA or RNA molecule (e.g., dsRNA, or antisense molecule) capable of antagonizing or agonizing the receptor.

In one embodiment a disease or condition wherein suppression of a pathogenic antibody response is therapeutic includes those diseases listed previously in which antibodies contribute to, or are primarily responsible for pathogenesis.

In another embodiment, the invention provides a method of increasing a protective antibody response in a patient in need thereof comprising administering to the patient, an agent which modulates the PD-1 receptor on a TFH cell. In one embodiment the agent is an agonist of the PD-1 receptor on a TFH cell. The agent may also be a small molecule, or a DNA or RNA molecule (e.g. dsRNA, or antisense molecule) capable of agonizing the PD-1 receptor.

In one embodiment, the invention provides a method of decreasing a pathogenic antibody response in a patient in need thereof comprising administering to the patient a first agent capable of modulating a PD-1 receptor on a TFR cell in an amount effective to increase TFR cell-mediated antibody suppression in the patient, in combination with an second agent capable of modulating a PD-1 receptor on a TFH cell in an amount effective to decrease TFH cell-mediated antibody production, wherein the pathogenic antibody response is decreased as compared to the pathogenic antibody response in the absence of the first or second agents.

In one embodiment, the invention provides a method of increasing a protective antibody response in a patient in need thereof comprising administering to the patient a first agent capable of modulating a PD-1 receptor on a TFR cell in an amount effective to decrease TFR cell-mediated antibody suppression in the patient, in combination with a second agent capable of modulating a PD-1 receptor on a TFH receptor in an amount effective to increase TFH cell-mediated antibody production, wherein the protective antibody response is increased as compared to the protective antibody response in the absence of the first or second agents.

In addition to the known agonists and antagonists of the PD1 receptor generally, other agents may be tested for their ability to antagonize or agonize PD-1 on TFH and TFR cells using known assays and screens. Assays and screens for testing various agents for their ability to agonize or antagonize PD-1 receptors are previously described. Animal models may be used to test modulation of selective TFH receptors as described above.

The present invention further provides agents identified in the assays described herein wherein such agents are capable of antagonizing or agonizing PD-1 receptors and thereby modulate TFR or TFH cell-mediated immune function or both.

Agents for modulating PD-1 receptors on TFR or TFH cells may be formulated and administered to patients as described above.

IX. Examples

Example 1 Introduction

Follicular helper T cells (TFH) are a recently defined subset of CD4 T cells that are essential for helping cognate B cells form and maintain the germinal center (GC) reaction, and for development of humoral immune responses. These cells are universally defined by expression of the chemokine receptor CXCR5, which directs them to the B cell follicles via gradients of the chemokine CXCL13 [1]. TFH cells also express the transcription factor *Bcl6* (which represses Blimp-1/*Prdm*1) and high levels of the costimulatory receptor ICOS, which are both critical for their differentiation and maintenance [1-4]. In addition, TFH cells secrete large amounts of IL-21, which aids in GC formation, isotype switching and plasma cell formation [5]. In humans and mice functionally similar TFH cells can be found in secondary

lymphoid organs. Significantly, $CXCR5^+$ TFH cells are also present in peripheral blood and seen at elevated levels in individuals with autoantibodies, including systemic lupus erythematosis, myasthenia gravis and juvenile dermatomyositis patients. However, the function of these circulating TFH remains unclear [6-9].

TFH cells also express high levels of programmed death (PD) 1 receptor (CD279). Signaling through PD-1 attenuates TCR signals and inhibits T cell expansion, cytokine production and cytolytic function. In addition, PD-1 promotes the development of induced regulatory T (iTreg) cells from naïve lymphocytes [10-14]. PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is more widely expressed than PD-L2, but PD-L1 and PD-L2 both can be expressed on GC B cells and dendritic cells [15]. Perturbation studies suggest critical roles for this pathway in regulating humoral immune responses. However, there are conflicting reports as to the function of the PD-1 pathway in controlling humoral immunity. Some studies have found that humoral responses are attenuated [16-18], while others have seen that humoral responses are heightened [19, 20] when PD-1:PD-L interactions are prevented.

PD-1 also is found on a newly defined subset of $CD4^+CXCR5^+$ cells called T follicular regulatory (TFR) cells, which are positive for the transcription factors FoxP3, *Bcl6* and *Prdm1*/Blimp1 and function to inhibit the germinal center response [21-23]. These cells originate from natural regulatory T cell precursors, but express similar levels of ICOS, CXCR5 and PD-1 as TFH cells. Since ICOS, CXCR5 and PD-1 have been widely used to identify and purify 'TFH cells', it seems likely that the inability to define clear functions for PD-1 in GC responses derives from experimental systems containing mixtures of stimulatory TFH cells and inhibitory TFR cells. We provide a separate analyses of the function of PD-1 on TFH and TFR cells to elucidate how PD-1 controls humoral immunity and to gain insight into the individual roles of TFR cells and TFH cells in regulating antibody production.

Here we demonstrate that PD-1:PD-L1 interactions inhibit TFR, but not TFH, cell numbers in lymph nodes. PD-1 deficient mice have increased numbers of lymph node TFR cells compared to wild type mice. PD-1 deficient lymph node TFR cells have enhanced ability to suppress activation of naïve T cells, as well as antibody production *in vitro*. In addition, we show for the first time that TFR cells are present in the peripheral blood of mice, and that these circulating cells can potently regulate humoral immune responses *in vivo*. Using transfer approaches, we demonstrate that

blood TFH cells can promote antibody production, while blood TFR cells can strongly inhibit antibody production *in vivo*. We further show that the PD-1 pathway inhibits blood TFR cell function and PD-1 deficient blood TFR cells have enhanced suppressive capacity *in vivo*. Taken together, our studies reveal a new immunoregulatory role for the PD-1:PD-L1 pathway in limiting TFR cell differentiation and function, and further demonstrate the dynamic control of humoral immune responses by migration of TFR cells from the circulation into lymph nodes to control antibody production *in vivo*.

Methods

Mice. 6-10 week old mice were used for all experiments. WT C57BL/6 and TCR$\alpha^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PD-$1^{-/-}$, PD-$L1^{-/-}$, and PD-$L2^{-/-}$ mice on the C57BL/6 background were generated in our laboratory [32-34]. $ICOS^{-/-}$ [35] and $CD28^{-/-}$ mice [36] were generated as described. 2D2 TCR Tg mice Foxp3-IRES-GFP knockin mice (Foxp3.GFP; [37] were generated in our laboratory by crossing 2D2 TCR Tg mice [38] with Foxp3.GFP reporter mice. All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Animal Healthcare Guidelines. Animal protocols were approved by the Harvard Medical School Standing Committee on Animals.

Immunizations. For MOG 35-55 immunizations (referred to as "MOG/CFA"), mice were injected subcutaneously with 100μg of MOG 35-55 (UCLA Biopolymers Facility) emulsified in a 1:1 emulsion of H37RA CFA (Sigma) on the mouse flanks. Seven days later mice were euthanized and inguinal lymph nodes (dLN) and/or spleen were harvested for flow cytometric analyses. Blood was collected via cardiac puncture with a 1cc syringe and immune cells were isolated by sucrose density centrifugation using Lymphocyte Separation Media (LSM). For NP-OVA immunizations, 100μg NP_{18}-OVA (Biosearch Technologies) was used in a 1:1 H37RA CFA emulsion and injected similarly as MOG/CFA.

ELISA. For *in vitro* quantitation of antibody production, supernatants were taken from cultures and total IgG was quantified using pan-IgG capture antibody (Southern Biotech) and alkaline phosphatase conjugated pan-IgG detection antibody (Southern Biotech). To assess *in vivo* antibody production, sera were collected from mice at indicated time points. NP-specific antibody titers were measured by coating

ELISA plates with NP_{16}-BSA (Biosearch Technologies), and incubating serum for 1hr followed by alkaline phosphatase-conjugated IgG detection antibodies. A standard curve was generated using antibody from an NP-specific IgG1 hybridoma (a kind gift of Dr. Michael Carroll). This standard curve was used to approximate all IgG subtype antibody levels in the linear range of detection using a Spectramax Elisa plate reader (Molecular Devices).

Flow Cytometry. Cells from lymphoid organs were isolated and resuspended in staining buffer (PBS containing 1% fetal calf serum and 2mM EDTA) and stained with directly labeled antibodies from Biolegend against CD4 (RM4-5), ICOS (15F9), CD19 (6D5), PD-1 (RMP1-30), PD-L1 (10F.9G2), CD69 (H1.2F3), from eBioscience against FoxP3 (FJK-16S), Bcl6 (mGI191E), and from BD bioscience against FAS (Jo2), GL7, Ki67 (B56). For CXCR5 staining, biotinylated anti-CXCR5 (2G8, BD Biosciences) was used followed by streptavidin-brilliant violet 421 (Biolegend). For intracellular staining, the FoxP3 fix/perm kit was used (eBioscience) after surface staining was accomplished. All flow cytometry was analyzed with an LSR II (BD biosciences) using standard filter sets.

Confocal Microscopy. Draining lymph nodes were embedded in OCT and 8μm sections were cut, fixed with 4% paraformaldehyde, and stained before imaging on a Zeiss LSM 510 confocal microscope by acquiring z-stacks of 0.5um with a 40x oil objective. Germinal center quantitation was calculated by drawing outlines around $GL7^{+}IgD^{-}$ areas present within the B cell zone. FoxP3 quantitation was performed by determining germinal center zones and scrolling through z-stack images to identify large FoxP3 positive spots. Axiovision (Zeiss) software was used to measure distances from germinal center borders. For micrograph panels, single z slices were linearly contrasted and merged images were made in Adobe photoshop.

Quantitative PCR. Q-PCR was performed using standard TaqMAN probes (Applied Biosystems) and an ABI FAST9500 QPCR machine according to the manufacturer's instructions. mRNA levels were normalized to HPRT or β2M, and the $2^{-deltadeltaCT}$ method was used to quantitate mRNA. Each bar graph represents mean values from more than three individual experiments consisting of cells sorted from 5-10 pooled mice.

***In vitro* Suppression Assay.** Cell populations were sorted to 99% purity on an Aria II flow cytometer. For TFR suppression assays, sorted cells were counted on

an Accuri cytometer (BD biosciences) by gating live cells only, and $1x10^5$ $GL7^-$ B cells from dLNs of WT mice immunized with MOG/CFA 7 days previously, $1x10^5$ CFSE labeled $CD4^+CD62L^+FoxP3^-$ T responder cells from unimmunized WT FoxP3 GFP reporter mice, and $1x10^5$ TFR cells from the draining dLN of 10 pooled mice immunized with MOG/CFA 7 days previously were plated with 2μg/ml soluble anti-CD3 (2C11, BioXcell) and 5μg/ml anti-IgM (Jackson Immunoresearch). After 3 days, cells were harvested and stained for CD4 and CD19. T cell responders were identified as CFSE positive, and percent divided was gated as the percent of cells with CFSE diluted compared to unstimulated T responders.

Adoptive Transfers. For blood TFH/TFR adoptive transfers, 20 to 30 WT mice were immunized with NP-OVA subcutaneously as described above, and 8 days later blood was collected by cardiac puncture. TFH and TFR cells were sorted as described. $4x10^4$ blood TFH cells alone or together with $2x10^4$ blood TFR cells were transferred into $CD28^{-/-}$ or $TCR\alpha^{-/-}$ mice unless specified otherwise. These recipient mice were immunized with NP-OVA as described above. Serum and organs were harvested at indicated times and analyzed by ELISA or flow cytometry.

Statistical Analysis. Unpaired Student's t test was used for all comparisons, data represented as mean +/- SD or SE are shown. P values < 0.05 were considered statistically significant. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

Results

PD-1 Controls T Follicular Regulatory Cells

To analyze the role of PD-1 in controlling T follicular regulatory (TFR) cells, we first compared PD-1 expression on CD4 T cell subsets in draining lymph nodes (dLN) of WT C57BL/6 mice subcutaneously immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 emulsified in CFA (from herein simply referred to as "MOG/CFA"), an immunization that breaks tolerance and also results in effective TFH cell generation [24]. TFR cells were defined as $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$, a gating strategy that separates TFR cells from $CD4^+ICOS^+CXCR5^+FoxP3^-CD19^-$ TFH cells, the cell type that was until recently thought to solely comprise the $CD4^+CXCR5^+$ gate (FIG. 1a). TFH cells showed higher expression of PD-1 compared to $ICOS^+CXCR5^-$ effector-like cells and $ICOS^-CXCR5^-$ naïve (referred to as naïve) cells in the draining lymph node on day 7 after

immunization. Strikingly, TFR cells had even higher PD-1 expression when compared to the other CD4 T cell subsets examined, including TFH cells (FIG. 1b).

To determine the functional significance of PD-1 expression on TFR cells, we immunized WT and PD-$1^{-/-}$ mice and analyzed TFR cells 7 days later. The percentage of TFR cells contained within the $CD4^{+}FoxP3^{+}$ gate was about 4 percent in WT lymph nodes and less than 1 percent of all CD4 T cells. In marked contrast, the percentage of TFR cells in PD-$1^{-/-}$ mice was about 10 percent of the $CD4^{+}FoxP3^{+}$ gate and greater than 2 percent of all CD4 T cells (FIG. 1c-d). Because total numbers of CD4 T cells are typically about two fold higher in the lymph nodes of PD-1 deficient animals, a two-fold increase in TFR cell frequency translates into a ~4-fold increase in absolute numbers of TFR cells (data not shown). When expressed as a percentage of all $CD4^{+}ICOS^{+}CXCR5^{+}$ cells (and therefore the percentage of CD4 T cells that respond to CXCL13 and migrate to the B cell zone), PD-$1^{-/-}$ TFR cells comprised half of this population, whereas WT TFR cells comprised only about 20 percent (FIG. 1d). The dramatic increase in the percentage of TFR cells in PD-$1^{-/-}$ mice also was observed when other classical B cell antigens, such as 4-hydroxy-3-nitrophenylacetyl hapten conjugated to ovalbumin (NP-OVA), were used (FIG. 10). We did not find a significant difference in the percentage of $FoxP3^{-}$ TFH (from hereon called "TFH") cells when expressed as a percentage of all CD4 T cells in WT and PD-$1^{-/-}$ mice on day 7 post immunization (FIG. 1e).

Since PD-1 can be expressed by a number of hematopoietic cell types including T cells, B cells, macrophages and some dendritic cells [15], we next investigated whether PD-1 regulates TFR cells directly by controlling their generation from $FoxP3^{+}$ T regulatory cells (Treg). To track the fate of $FoxP3^{+}$ cells following transfer into WT mouse recipients, we used antigen-specific $FoxP3^{+}$ T cells from TCR transgenic mice for these studies. We sorted $FoxP3^{+}$ Tregs from WT or PD-$1^{-/-}$ 2D2 (MOG–specific) TCR transgenic FoxP3 GFP reporter mice and transferred $2x10^{5}$ 2D2 WT or PD-$1^{-/-}$ $CD4^{+}CXCR5^{-}FoxP3^{+}$ cells into WT recipient mice. We immunized these recipients with MOG/CFA and analyzed cells in the draining lymph node seven days later (FIG. 1f). There were a greater percentage (FIG. 1h) and absolute number (FIG. 1i) of 2D2 PD-$1^{-/-}$ Tregs upregulating CXCR5 and thus differentiating into TFR cells, compared to 2D2 WT Tregs. The increased percentage of PD-$1^{-/-}$ TFR cells in the immunized transfer recipients was similar, but

less pronounced, than the increased percentage in TFR cells seen in immunized intact PD-1 deficient mice (FIG. 1d, h). These results demonstrate that PD-1 controls differentiation of FoxP3$^+$ Tregs into TFR cells.

Since CD25 (the alpha chain of the IL-2 receptor) is frequently used as a marker for Tregs, we next compared CD25 expression on WT and PD-1$^{-/-}$ TFR cells directly *ex vivo* (FIG. 2a). PD-1$^{-/-}$ TFR cells expressed less CD25 than WT TFR cells (FIG. 2b). The attenuated CD25 expression in PD-1 deficient TFR cells is not likely due to decreased activation because the expression of the early activation marker CD69 was virtually identical on WT and PD-1$^{-/-}$ TFR cells (FIG. 2c). To compare the proportion of WT and PD-1$^{-/-}$ TFR cells proliferating at day 7 post immunization, we examined Ki67 expression, a marker widely used to identify cells that are actively dividing. WT ICOS$^+$ CXCR5$^-$ effectors, TFH and TFR gated cells had high expression of Ki67. In contrast, the WT CXCR5$^-$ICOS$^-$ "naïve" cells, lacking CD69 and CD25 expression, had no Ki67 staining consistent with their designation as naïve (FIG. 2d). WT TFR cells expressed significantly higher levels of Ki67 compared to PD-1$^{-/-}$ TFR cells, suggesting that the increased numbers of TFR cells in PD-1 deficient animals reflect increased differentiation, and not maintenance, of TFR cells. Ki67 expression was similarly greater in WT ICOS$^+$ effectors and TFH cells compared to PD-1$^{-/-}$ ICOS$^+$ CXCR5$^-$ effectors and TFH cells. These points to an overall decrease in cell cycling in PD-1$^{-/-}$ effector cells at 7 days after immunization. Other Treg markers such as CD103 and GITR were not altered on TFR cells in PD-1 deficient mice (FIG. 11). Additionally, there was low, but significant expression of PD-L1 on WT and PD-1$^{-/-}$ TFR cells. Together, these data indicate that PD-1 is important in regulating numbers of TFR cells *in vivo*.

PD-1 Deficient TFR cells are Capable of Homing to Germinal Centers

We next compared the capacity of TFR cells from WT and PD-1 deficient animals to enter the germinal center (GC) in order to inhibit the GC response. First, we evaluated GC formation in lymph node sections harvested 7 days after MOG/CFA immunization. GCs were identified by the presence of PNA/GL7 positively stained and IgD negatively stained B cell zones (FIG. 3a). These GCs were determined to be active, based on robust expression of the cell cycle marker Ki67 (FIG. 3b). Similar to previous reports [21, 22], CD4$^+$FoxP3$^+$ TFR cells could be

found within GCs of immunized mice (FIG. 3c). The FoxP3 protein within the TFR cells was judged to be largely nuclear based on its co-localization with the DAPI staining (FIG. 3d).

We then investigated whether the phenotypically distinct TFR cells from PD-1 deficient mice were able to migrate to GCs similarly to WT TFR cells, because PD-1 blockade can prolong the TCR stop signal and decrease T cell migration [25]. We immunized WT and PD-1 deficient mice with MOG/CFA and 7 days later analyzed lymph node sections for IgD, GL7 and FoxP3 expression (FIG. 3e). Although the average germinal center area (FIG. 3f) and numbers of germinal centers per lymph node (data not shown) were equivalent in WT and PD-$1^{-/-}$ mice, there were slightly more $FoxP3^{+}$ cells (and therefore TFR cells) located within the GC borders in PD-$1^{-/-}$ mice as in WT mice (FIG. 3g). However, since this increase is proportional to the larger numbers of TFR cells in PD-1 deficient mice determined by flow cytometry, these data demonstrate that PD-1 deficient TFR cells are not defective in homing to GCs and can enter the GC similarly to WT TFR cells.

The relative location of $FoxP3^{+}$ TFR cells within the GC did not differ significantly between WT and PD-$1^{-/-}$ TFR cells (FIG. 3h). In both WT and PD-$1^{-/-}$ mice the $FoxP3^{+}$ cells tended to reside close to the GC border, with more than half of the $FoxP3^{+}$ nuclei being positioned within 10μm of the border. Furthermore, when CXCR5 fluorescence was quantified by flow cytometry in TFR cells, there was similar CXCR5 expression on TFR cells in the WT and PD-$1^{-/-}$ mice, indicating similar potential for these cells to respond to chemokine cues to migrate to GCs (FIG. 3i). Taken together, these data indicate that TFR cells are increased in lymph nodes of immunized PD-$1^{-/-}$ mice, and these PD-$1^{-/-}$ TFR cells are capable of migrating into GCs to regulate B cell responses.

PD-1 deficient TFR cells More Potently Inhibit T Cell Activation

We next compared the function of TFR cells from WT and PD-$1^{-/-}$ mice. TFR cells express higher levels of GITR on the cell surface than do TFH cells, which allows for separation of the TFH and TFR cells in a similar manner to intracellular staining for FoxP3 (FIG. 4a). For functional studies, we sorted TFR cells from immunized mice by taking the lymph node $CD4^{+}ICOS^{+}CXCR5^{+}CD19^{-}GITR^{+}$ population as TFR cells and the $CD4^{+}ICOS^{+}CXCR5^{+}CD19^{-}GITR^{-}$ population as TFH

cells (FIG. 4b). Sorting in this fashion shows robust mRNA for FoxP3 in the GITR$^+$ (TFR) population, but essentially no FoxP3 mRNA in the GITR$^-$ (TFH) population, validating the use of this gating strategy to isolate TFR and TFH cells for functional assays. Furthermore, this sorting strategy can be used to compare WT and PD-1 deficient TFR cells since GITR expression is identical on WT and PD-1 deficient TFR cells (FIG. 12).

TFR cells express high Blimp1/*Prdm1* and moderate levels of Bcl6 [21]. Bcl6 and Blimp1 reciprocally modulate each other [2]; Bcl6 inhibition of Blimp1 is essential for maintenance of the TFH phenotype, whereas Blimp1 is important in Treg homeostasis in general [26, 27]. Since relative expression of Bcl6 and Blimp1 determines function of TFH subsets, we compared Bcl6 expression in TFR cells from WT and PD-1$^{-/-}$ mice using flow cytometry to analyze intracellular Bcl6 expression at the protein level. Although TFR cells expressed less Bcl6 at the protein level than TFH cells, WT and PD-1$^{-/-}$ TFR had similar Bcl6 levels (FIG. 4c). We next compared the expression of Blimp1 (encoded by *Prdm1*) on TFR cells from WT and PD-1$^{-/-}$ mice. At the mRNA level, we did not find any consistent differences in Blimp1/*Prdm1* expression between WT and PD-1$^{-/-}$ TFR cells (FIG. 4d). Since FoxP3 can directly interact with and negatively regulate the function of Rorγt [28], we also examined *Rorc* (which encodes Rorγt) in WT and PD-1$^{-/-}$ TFR cells. *Rorc* mRNA levels were lower in TFR cells compared to TFH cells, but *Rorc* expression was increased in PD-1$^{-/-}$ TFR cells relative to WT TFR cells (FIG. 4e). In addition, we compared expression of the transcription factor IRF4 in WT and PD-1$^{-/-}$ TFR cells, since Blimp1 and IRF4 synergistically control the differentiation and effector functions of regulatory T cells [26]. We found an increase in *IRF4* mRNA in PD-1$^{-/-}$ TFR cells compared to WT TFR cells (FIG. 4f).

IRF4 is essential for the suppressive capacity of regulatory T cells [26]. To determine if increased IRF4 mRNA in PD-1$^{-/-}$ TFR cells translates into an increase in suppression of naïve T cell proliferation, we set up an *in vitro* suppression assay in which we cultured sorted WT GL7$^-$ B cells, CFSE labeled WT naïve CD4$^+$CD62L$^+$FoxP3$^-$ responder T cells, and either WT or PD-1$^{-/-}$ TFR cells sorted from mice immunized with MOG/CFA together with anti-CD3 and anti-IgM (FIG. 4g). The responder T cells highly upregulated CD69 after 3 days of culture with WT B cells. However, when WT TFR cells were added in a 1:1:1 ratio, the CD69

expression on the responder T cells was much lower, consistent with the function of TFR cells in suppressing T cell activation (FIG. 4h). CD69 upregulation was inhibited to an even greater extent in responder T cells that were cultured with PD-1$^{-/-}$ TFR cells. Moreover, PD-1$^{-/-}$ TFR attenuated the proliferation of responder T cells (FIG. 4i), in contrast to WT TFR cells, which did not inhibit the proliferation of responder T cells during the day 3 culture period.

Although TFR cells are thought to inhibit the germinal response *in vivo,* it is unclear whether TFR cells directly inhibit T cell differentiation, TFH cell function, B cell activation or all three. To assess the capability of TFR cells to suppress B cell antibody production *in vitro*, we cultured WT GL7^{-} B cells with WT FoxP3^{-} TFH cells for 6 days in the presence or absence of TFR cells, anti-IgM and anti-CD3 (FIG. 4j). WT B cells produced large amounts of IgG when cultured with WT FoxP3^{-} TFH cells plus anti-IgM and anti-CD3 (FIG. 4k). No significant IgG was present when CD4^{+}FoxP3^{-} naïve T cells were used in these experiments (FIG. 4l). When TFR cells were added to the wells along with TFH cells, almost no IgG was produced. The TFR-mediated suppression was not due to sequestering of anti-CD3 because there was equally good suppression at the two doses of anti-CD3 tested (FIG. 4k), and the anti-CD3 could still be found on the surface of the TFH cells at the end of the suppression assay (FIG. 13). PD-1$^{-/-}$ TFR cells suppressed IgG production more than WT TFR cells at both a 1:1 (FIG. 4l) and a 1:5 (FIG. 4m) TFR:TFH ratio, with PD-1 deficient TFR cells resulting in a 50% greater reduction in IgG production compared to WT TFR cells. Taken together, these data demonstrate not only that are there increased TFR cells in PD-1$^{-/-}$ mice, but that these PD-1$^{-/-}$ TFR cells have increased suppressive capacity.

PD-1 Controls Blood T Follicular Regulatory Cells

One possible explanation for the increase in TFR cells in lymph nodes of immunized PD-1 deficient mice is that PD-1$^{-/-}$ TFR cells are unable to exit the lymph node. Studies have demonstrated that functional TFH cells can be found in the blood of humans as well as mice [6, 7, 9], but whether TFR cells circulate in the blood of humans or mice is not yet known. Strikingly, we found a significant population of TFH cells, as well as a smaller population of TFR cells, in the blood of WT mice immunized with MOG/CFA (FIG. 5a-b). When we compared the kinetics of TFH

and TFR cell expansion in the lymph node and blood of mice following MOG/CFA immunization, we found that both TFR and TFH cells increase in the draining lymph node of WT immunized mice over a 10 day period, and that TFH cells, but not TFR cells, increase substantially by percentage in the blood over this time (FIG. 5b). Thus, without antigenic stimulus, the blood TFR:TFH ratio is fairly high (sometimes greater than 1:1) but upon addition of a stimulus, blood TFH cells expand more than blood TFR cells so that the TFR:TFH ratio is about 1:5. To investigate whether WT blood TFH and TFR cells are quiescent or are actively in cell cycle, we compared Ki67 expression in draining lymph node and blood TFH and TFR cells 7 days after immunization. TFH cells from the draining lymph node had higher Ki67 expression than those found in the blood (FIG. 5c). Blood TFH and TFR and draining lymph node TFR cells expressed similar levels of Ki67.

Next we investigated whether TFR cells in the blood were inhibited to the same degree by PD-1 signaling as lymph node TFR cells. We immunized WT and PD-$1^{-/-}$ mice with MOG/CFA and 7 days later analyzed the blood for TFH and TFR cells. In WT mice ~2-3 percent of $CD4^{+}FoxP3^{-}CD19^{-}$ cells in the blood were TFH cells, but in the PD-$1^{-/-}$ mice this increased to ~4-5 percent (FIG. 5d). This increase in PD-$1^{-/-}$ TFH cells in blood markedly contrasts with the lymph node, where PD-$1^{-/-}$ mice have similar, if not less, TFH cells compared to WT mice (FIG. 1e). Importantly, TFR cells comprised ~3 percent of all FoxP3 positive cells in the blood of WT mice, but more than 7 percent of FoxP3 positive cells in the blood of PD-$1^{-/-}$ mice (FIG. 5d-e). The increase in $FoxP3^{+}$ cells seems to be specific to the blood TFR subset, as the percentage of $FoxP3^{+}$ cells in the $ICOS^{+}CXCR5^{-}$ ($ICOS^{+}$) and $ICOS^{-}CXCR5^{-}$ naïve cell gates were not increased in PD-$1^{-/-}$ mice (FIG. 5F). Taken together, these data indicate that both TFR and TFH cells are present in the blood of mice, and both subsets are repressed by PD-1 signals.

To investigate whether blood TFH and TFR cells have a central memory phenotype, we analyzed surface expression of CD62L and CD44. About 60% of WT and PD-$1^{-/-}$ blood TFR cells had high expression of CD62L (FIG. 14). This contrasts with the greater than 90% of $ICOS^{-}CXCR5^{-}$ naïve cells that had high CD62L expression. PD-$1^{-/-}$ TFH cells had lower CD62L compared to WT TFH cells. CD44 was highly expressed on all WT and PD-$1^{-/-}$ blood TFR cells, but PD-$1^{-/-}$ blood TFR cells had slightly lower surface expression (FIG. 14). Furthermore, PD-1 was

expressed at lower levels on blood TFR cells than lymph node TFR cells (FIG. 14). Taken together, these data indicate that blood TFR cells can have a central memory homing phenotype.

The increase in TFR cells in PD-$1^{-/-}$ mice led us to investigate which PD-1 ligand is critical for controlling lymph node and blood TFR generation. We first compared PD-L1 and PD-L2 expression on B cells and DCs present in dLNs of immunized WT mice because both B cells and dendritic cells (DCs) contribute to proper TFH differentiation and maintenance in the lymph node [1]. It is not yet clear whether B cells, DC or both are needed for TFR differentiation and/or maintenance. To study GC B cells, we immunized mice with NP-OVA subcutaneously and 12 days later compared PD-L1 and PD-L2 expression on $FAS^{+}GL7^{+}CD19^{+}$ GC B cells, as well as $CD138^{+}$ positive plasma cells (PC) from the dLN. We found that all B cell subsets expressed high levels of PD-L1, but only GC B cells expressed high levels of PD-L2 (FIG. 6a). To quantify PD-L1 and PD-L2 expression on DCs, we immunized mice with NP-OVA and analyzed DC populations from the draining lymph node 3 days later. Both $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC populations expressed high levels of PD-L1 and moderate levels of PD-L2 (FIG. 6b). A subpopulation of $CD8\alpha^{-}$ DCs expressed high levels of PD-L2.

To determine which ligand is important for TFH and TFR generation, we immunized WT, PD-$L1^{-/-}$ and PD-$L2^{-/-}$ mice with MOG/CFA, and analyzed TFH and TFR cells 7 days post-immunization in the draining lymph node and blood. The percentages of lymph node TFH cells in PD-$L1^{-/-}$ and PD-$L2^{-/-}$ mice were comparable to WT mice (FIG. 6c) and PD-$1^{-/-}$ mice (FIG. 1e). PD-$L1^{-/-}$, but not PD-$L2^{-/-}$ mice, had greater blood TFH cell numbers, which was similar to PD-$1^{-/-}$ mice (FIG. 5e). TFR cells, however, were increased in the lymph nodes as well as the blood of PD-$L1^{-/-}$, but not PD-$L2^{-/-}$ mice (FIG. 6d). Similar to PD-$1^{-/-}$ mice, PD-$L1^{-/-}$ mice did not exhibit any increases in non-TFR $FoxP3^{+}$ effector cells within the blood (FIG. 6e). These studies demonstrate that PD-L1, but not PD-L2, is responsible for controlling lymph node and blood TFR cells.

Blood TFH and TFR cells require CD28 and ICOS Signals

We further investigated the costimulatory requirements for blood TFR cells, focusing on the effects of CD28 and ICOS costimulation on $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$ TFR populations in the blood due to the important roles of these costimulatory receptors in controlling lymph node TFH and TFR cells. $CD28^{-/-}$ mice are deficient in lymph node TFH and TFR cells [21]. $ICOS^{-/-}$ mice are deficient in lymph node TFH cells [24]. We analyzed CD28 and ICOS deficient animals for the presence of TFR cells in the lymph nodes and blood 7 days after immunization with MOG/CFA. In WT mice, there were fewer TFH and TFR cells in the blood compared to the draining lymph node (FIG. 7a-b). Numbers of TFR (and TFH) cells were greatly attenuated in the blood, as well as lymph nodes, of ICOS deficient mice (FIG. 7a-c). CD28 deficient mice had similar severe deficiencies in TFR and TFH cell percentages in both lymph nodes and blood (FIG. 7e-f). Thus, ICOS and CD28 supply essential costimulatory signals for TFR and TFH cells in the blood as well as the lymph nodes.

PD-1 deficient blood TFR cells more potently regulate antibody production *in vivo*

We next investigated the function of blood TFR cells in humoral immune responses. Because TFH cells in human blood can function in B cell activation and antibody production *in vitro* [6, 7], we analyzed whether circulating blood TFR cells contribute to suppression of antibody production *in vivo*. To assess this, we designed transfer experiments in which we immunized >20 WT donor mice with NP-OVA subcutaneously and 8 days later sorted TFR ($CD4^+ICOS^+CXCR5^+GITR^+CD19^-$) and TFH ($CD4^+ICOS^+CXCR5^+GITR^-CD19^-$) cells from the blood (FIG. 8a). We transferred these cells into $CD28^{-/-}$ or $TCR\alpha^{-/-}$ mice because they lack both blood and lymph node TFH and TFR cells. This approach enabled us to determine if blood TFR and TFH cells could regulate humoral responses. Since the transferred blood TFH and TFR cells are the only follicular T cells in $CD28^{-/-}$ and $TCR\alpha^{-/-}$ recipients, any responses in the draining lymph node would be due to trafficking of the blood TFR and TFH cells.

Initially, we adoptively transferred $4x10^4$ TFH cells alone or together with $2x10^4$ TFR cells into $CD28^{-/-}$ mice (approximately a two-fold higher ratio of TFR:TFH cells than is found in blood after immunization). We immunized recipients 1 day later with NP-OVA and analyzed NP-specific IgG titers 12 days after immunization (FIG. 8a). Without blood TFH or TFR cell transfer, $CD28^{-/-}$ mice were unable to produce significant amounts of NP-specific IgG (FIG. 8b). The transfer of blood TFH cells alone resulted in a substantial increase in NP-specific IgG titers. Transfer of blood TFH cells led to substantial production of IgG1, but also smaller increases in other isotypes (data not shown). Significantly, transfer of blood TFR cells along with blood TFH cells resulted in robust inhibition of NP-specific antibody production, demonstrating the potent regulatory capacity of blood TFR cells in suppressing antibody production (FIG. 8b). To evaluate the impact of TFR cells on plasma cell generation, draining lymph nodes, spleens and bone marrow were harvested 24 days after immunization and plasma cells were quantified. $CD138^+$ plasma cells were absent from the lymph nodes of immunized $CD28^{-/-}$ mice (FIG. 8c-d). Transfer of blood TFH cells resulted in a sizable population of plasma cells in the draining lymph node, spleen and bone marrow (FIG. 8c-d). Blood TFR cells almost completely prevented plasma cell formation/survival in all organs tested.

Next we transferred blood TFH and/or TFR cells into $TCR\alpha^{-/-}$ recipients. Transfer of $4x10^4$ TFH cells resulted in high levels of NP-specific IgG and at a greater titer than an immunized WT mouse in most experiments (FIG. 8e). The robust antibody production elicited by blood TFH cells depends on the "follicular program" because transfer of total CD4 T cells from $CXCR5^{-/-}$ mice or $CD4^+CXCR5^-FoxP3^-$ naïve cells resulted in near background levels of antibody production in these experiments (FIG. 8e). When blood TFR cells were transferred together with blood TFH cells, the NP-specific antibody titers were markedly reduced, demonstrating the suppressive capacity of these cells (FIG. 8e). The blood TFR cells resulted in both lower plasma cell percentages (FIG. 8f), as well as lower percentages of TFH cells within the lymph node (FIG. 8g). When we compared the functions of blood TFH cells and draining lymph node TFH cells following transfer into $TCR\alpha^{-/-}$ recipients, we found that blood TFH cells have an increased capacity to promote NP-specific IgG production (FIG. 8h). TFR suppression of TFH cells also depends on the "follicular program" in these cells because neither blood

$CD25^+CD62L^+$ Tregs from $CXCR5^{-/-}$ mice (data not shown) nor blood $CXCR5^-$ FoxP3 GFP^+ Tregs from FoxP3 reporter mice possess the same suppressive capacity as WT blood TFR cells (FIG. 8i).

Finally, we investigated the suppressive capacity of PD-1 deficient blood TFR cells *in vivo* since we have found that PD-1 deficient lymph node TFR cells more potently suppress antibody production *in vitro*. We adoptively transferred $4x10^4$ blood TFH cells alone or together with 1.5 $x10^4$ blood TFR cells from WT or PD-1 deficient mice into either $CD28^{-/-}$ or $TCR\alpha^{-/-}$ recipients and immunized as in FIG. 8a. PD-1 deficient TFR cells inhibited antibody production to a greater extent than WT TFR cells in $CD28^{-/-}$ (FIG. 8j) as well as $TCR\alpha^{-/-}$ (FIG. 8k) recipients, demonstrating that they have increased suppressive capacity. Together, these data show that blood TFR cells potently inhibit antibody production *in vivo* and PD-1 deficiency results in enhanced TFR cell suppressive capacity.

Discussion

A mechanistic understanding of TFR cell differentiation and function is provided to gain insight into how humoral immune responses are regulated by TFH and TFR cells. Although TFR cells originate from different precursors than TFH cells, TFR and TFH cells have nearly identical surface receptors. The shared expression of ICOS, CXCR5 and PD-1 by TFR and TFH cells means that functional studies of TFH cells have, in fact, examined mixtures of stimulatory TFH cells and inhibitory TFR cells. The PD-1 pathway regulates many effector arms of the immune response, however biological complexity has led to inconsistencies regarding the role of this pathway in humoral immune responses [16-20]. In this Example 1, we identify a new mechanism by which PD-1 regulates humoral immunity: PD-1 controls the generation and function of suppressive TFR cells. We found that lack of PD-1, or its ligand PD-L1, resulted in greater numbers of TFR cells in the draining lymph node of immunized mice. These $PD\text{-}1^{-/-}$ lymph node TFR cells expressed more *IRF4* and showed an enhanced ability to suppress antibody production. We also discovered that TFR cells are present in the blood of mice, and that PD-1 controls the numbers of blood TFR cells, as evidenced by the substantial increases in PD-1 deficient mice. Importantly, we demonstrated a functional role for blood TFH cells in promoting antibody production and blood TFR cells in suppressing antibody production *in vivo*.

PD-1 deficient blood TFR cells more potently suppress antibody production compared to WT blood TFR cells. Thus, PD-1 limits the development and function of TFR cells in lymph nodes and in the circulation.

We have found that PD-1 signaling inhibits the numbers of TFR cells, but not TFH cells, in the lymph node, skewing the TFR to TFH ratio. It is possible that the greater suppressive capacity of PD-1$^{-/-}$ TFR cells, together with the increased ratio of TFR to TFH cells in PD-1$^{-/-}$ mice, results in inhibition of PD-1$^{-/-}$ TFH cells. Alternatively, there may be alterations in PD-1$^{-/-}$ TFH cells that promote their departure from the lymph node and homing to other sites to perform effector functions. These hypotheses are not mutually exclusive. Some studies have described increases in lymph node/spleen TFH cells in PD-1 deficient mice; however the contribution of TFR cells in these studies was not assessed [16, 18, 19]. It is likely that increased TFR cells in PD-1/PD-L1 knockout mice may have contributed to the increases in TFH cells observed in these studies, and may explain, at least in part, conflicting data regarding the role of PD-1 in regulating TFH cells and germinal center reactions. For example, Kawamoto et al. described increased CD4^{+}CXCR5^{+} (TFH) cells in the Peyer's patches of PD-1 deficient mice. However, upon transfer, these cells were non-functional in supporting IgA production. Increases in TFR cells contained within the TFH gate in PD-1 deficient Peyer's patches may explain these data.

TFR cells depend on SAP, CD28 and *Bcl6* for differentiation [21, 22]. However, the pathways that limit TFR cell differentiation are less clear. To date, only the transcription factor Blimp1 has been shown to inhibit TFR cell differentiation [21]. Here we identify PD-1 as the first surface receptor that inhibits TFR cell development and function. We also show that PD-1 predominantly interacts with PD-L1, and not PD-L2, to inhibit TFR generation. Our adoptive transfer studies demonstrate a cell intrinsic role for PD-1 in TFR cell differentiation from FoxP3^{+} Treg cells. Therefore, the increase in TFR cells in PD-1 deficient mice appears to reflect increased differentiation and not maintenance. We observed a general trend for a decrease in cell cycling of CD4 PD-1$^{-/-}$ effector cells, because ICOS^{+}CXCR5^{+}FoxP3^{-} and ICOS^{+}CXCR5^{-} effector cells also had diminished Ki67 expression, at least at day 7 after immunization, which may temporally correspond to a maintenance phase. Based on these data, we believe that, rather than simply

inhibiting responses, the PD-1:PD-L pathway can act as a molecular switch that controls cell fate decisions in naïve CD4 T cells. Integration of signals through PD-1, the TCR and cytokine receptors may direct CD4 T cell subset differentiation. Likewise, PD-1 may limit differentiated effector T cell expansion, cytokine production and/or survival depending on how signals through the TCR, PD-1 and cytokine receptors are integrated. Thus, the PD-1 pathway can influence CD4 T cell lineage commitment in distinct ways, depending on molecular cues and the local environment. For instance, PD-L1 can promote induced Treg (iTreg) differentiation from naïve T cell precursors [10-14, 29]. However, we find that PD-1 inhibits differentiation of TFR cells. TFR cells arise from natural T regulatory cell (nTreg) precursors (shown here and previously [21]). Therefore, our studies suggest that PD-1 may have distinct roles in iTreg and nTreg differentiation. In addition genetic background may contribute to the effects of PD-1 deficiency.

Because of the recent discovery of TFR cells, there is a lack of fundamental knowledge about TFR cell biology, so we developed novel assays to analyze mechanisms by which PD-1 regulates TFR cell function. TFR cells have the potential to directly inhibit activation of naïve T cells, TFH cell function, and/or B cell activation. TFR cells might regulate TFH or B cell responses either inside the B cell follicle and/or control activation and differentiation of T cells outside the B cell follicle. Here, we present the first specific assays for TFR cell function *in vitro* and show that sorted wild type TFR cells from the lymph node are extremely potent at inhibiting antibody production, but not very effective at suppressing activation of naïve T cells. PD-$1^{-/-}$ TFR cells inhibit naïve T cell activation and attenuate antibody production *in vitro* to a greater extent than WT TFR cells. Our studies also demonstrate the dynamic control of antibody production by lymph node TFH and TFR cells. Initially, our attempts to activate B cells *in vitro* with total $CD4^{+}CXCR5^{+}$ cells resulted in little IgG secretion. However, when we separated TFH cells from TFR cells and used these TFH cells in such experiments, we could detect robust IgG production. Of note, during an immune response to peptide/CFA, the *in vivo* dLN TFR:TFH ratio is ~1:5. When we cultured TFR and TFH cells at this ratio, little antibody production was observed.

TFR cells tend to be present predominantly at the borders of germinal centers, which may be explained by their relatively lower expression of CXCR5 compared to

TFH cells, though other chemokines also may have roles. It is possible that close proximity of TFR cells to germinal center borders enables them to interact with TFH cells as they enter. This could make TFR cells the "gate-keepers" of the germinal center, inhibiting TFH cells as they enter and gain access to B cells undergoing somatic hypermutation and class switch recombination. Furthermore, our studies suggest that the balance between TFR and TFH cells within the germinal center itself may modulate the type and extent of humoral responses. The relative roles of TFR and TFH cells also may depend on the source or strength of antigenic stimulus, cytokine milieu, and tissue microenvironment, and further work is needed to investigate these issues.

Surprisingly, we found substantial populations of TFR cells in the blood of mice. There are a number of reports describing TFH cells in the circulation of humans [6, 7] and one in mice [9]. To our knowledge, our work is the first description of TFR cells in the blood of any organism. In humans, blood TFH cells have been shown to provide B cell help for the production of antibody *in vitro.* Some studies show more efficient B cell antibody production by blood TFH cells compared to blood $CXCR5^-$ cells [6, 7] whereas other studies find no differences between blood TFH and $CXCR5^-$ cells [30]. Differences might relate to mixtures of blood TFH and TFR cells and their relative ratios in these experiments. Since most work describing blood TFH cells was done in humans, little is known about the requirements for blood TFH differentiation and function. Here we show that murine blood TFH and TFR cell generation requires signals through ICOS and CD28, two costimulatory receptors essential for controlling TFH cells in the lymph node. Previous work showed that CD28 is essential for TFR cells in lymph nodes [21]. Here we demonstrate lymph node TFR cell generation also requires ICOS signaling.

Our transfer studies show that blood TFR cells are functional and can regulate antibody production *in vivo*. To study TFH and TFR function, we transferred blood TFH cells alone or with TFR cells into $CD28^{-/-}$ or $TCR\alpha^{-/-}$ mice, which lack both blood and lymph node TFH and TFR cells. This approach allowed us to analyze TFH and TFR cell function separately from differentiation. Our transfer studies demonstrate effective and specific control of humoral responses by blood TFH and TFR cells. Blood TFR cells are extremely potent at inhibiting TFH cell mediated antibody production, even when relatively few cells are transferred. We hypothesize

that blood TFR cells may represent a central memory pool that can be utilized to modulate humoral immunity, analogous to recently reported FoxP3$^+$ cells with regulatory memory to self-antigens [31] and similar to a proposed role for blood TFH cells [7]. High expression of CD62L and CD44 on blood TFR cells along with their ability to home back to lymph nodes strongly support this idea. Blood TFH cells may migrate to lymph nodes and interact with cognate B cells rapidly upon antigen exposure, whereas naïve T cells need at least two to four days to differentiate and upregulate CXCR5. Additionally, blood TFR cells homing to lymph nodes would be able to suppress early B cell responses, before dLN nTregs could fully differentiate into TFR cells.

Beyond their ability to directly suppress antibody responses, TFR cells may be instrumental in determining B cell fates and control whether an immune response generates long-lived plasma cells or memory B cells. For example, cytokines produced by TFR cells may direct GC B cell differentiation into plasma cells versus memory B cells. PD-1 and PD-1 ligand deficiency result in decreased numbers of long-lived plasma cells [16], and further work is needed to determine if this is related to the enhanced PD-1$^{-/-}$ TFR cell numbers and suppressive capacity. If TFR cells can direct B cell fates, this would have implications for rational design of vaccines. We have also identified some of the potential relative roles of TFH and TFR cells in autoimmunity. For example, PD-1 deficiency on autoimmune-prone backgrounds accelerates disease pathologies. It is possible that autoimmune-prone backgrounds may lead to inhibition of TFR differentiation and function. This information will be important for therapeutic strategies using TFR cells. By expanding either TFH or TFR cells from patient blood *in vitro*, it may be possible to enhance antibody responses by transferring TFH cells or to inhibit systemic autoimmunity by transferring TFR cells.

In summary, we define a new role for PD-1 in regulating immune responses, by inhibiting differentiation and function of T follicular regulatory cells in both lymph node and blood. Our research has provided a better understanding of TFR and TFH interactions, and has the potential to provide novel insights into mechanisms that regulate humoral immunity and applications of those mechanisms in, for example, a vaccination regimen. Our understanding of how PD-1 regulates humoral

immunity suggests suggest strategies for manipulating this pathway to enhance protective immunity and long-term memory or to inhibit systemic autoimmunity.

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Example 2. T Follicular Regulatory cell (TFR) Cellular Therapy Strategies

1.) Enrichment and Injection of TFR cells from Blood. In order to skew the TFH:TFR balance, TFR cells will be purified from peripheral blood. First, lymphocytes from peripheral blood will be isolated by sucrose density centrifugation and stained with fluorescently labeled antibodies. TFR cells will be purified and separated from TFH cells by flow cytometry. TFR cells will be sorted via flow cytometry based on surface markers of $CD4^+CXCR5^+ICOS^+GITR^+$ and then administered intravenously to patients. These patients include a) individuals with autoimmune diseases with pathogenic antibodies (such as systemic lupus erythematosis) as a means to inhibit immunopathology related to these pathogenic antibodies, b) individuals undergoing transplantation (to prevent pathogenic effects of antibodies during transplant rejection), c) patients undergoing gene therapy or stem cell therapy to prevent development of antibodies that would abrogate the intended beneficial effects of the transferred gene or cell.

2.) Enrichment/Activation and Injection of TFR cells from Blood. In order to skew the TFH:TFR balance and to promote heightened suppressive function of TFR cells, TFR cells will be sorted and activated in vitro prior to administration into patients. Lymphocytes from peripheral blood will be isolated by sucrose density centrifugation and stained with fluorescently labeled antibodies. TFR cells will be sorted via flow cytometry based on surface markers of $CD4^+CXCR5^+ICOS^+GITR^+$ and activated *in vitro* by using a soluble anti-CD3 antibody along with autologous CD19+ B cells from blood to trigger TCR signaling and activation. After activation, TFR cells will be given to patients *intravenously*.

3.) Enhanced activation of TFR cells by blocking PD-1 pathway. In order to skew the TFH:TFR balance and to promote heightened suppressive function of TFR cells, TFR cells will be sorted and activated in vitro in the presence of PD-1 or PD-L1 antagonists, which should enhance TFR cell numbers and suppressive capacity. Lymphocytes from peripheral blood will be isolated by sucrose density centrifugation and stained with fluorescently labeled antibodies. TFR cells will be sorted via flow cytometry based on surface markers of $CD4^+CXCR5^+ICOS^+GITR^+$ and activated *in vitro* by using a soluble anti-CD3 antibody along with autologous CD19+ B cells from blood to trigger TCR signaling. PD-1 antagonists (monoclonal antibodies and/or small molecule inhibitors) are added during activation to enhance activation of TFR cells. After activation, TFR cells are administered to patients *intravenously*.

4.) Enrichment and Injection of Tfr cells to Induce Long Term Memory During Vaccination. The goal of this strategy is to skew TFH:TFR balance in the blood during B cell memory development and preferentially promote development of long lived B cell memory cells by inhibiting plasma cell differentiation. TFR cells will be sorted via flow cytometry and injected intravenously into individuals at the time of vaccination. Lymphocytes from peripheral blood will be isolated by sucrose density centrifugation and stained with fluorescently labeled antibodies. TFR cells will be sorted via flow cytometry based on surface markers of $CD4^+CXCR5^+ICOS^+GITR^+$ and reinjected *intravenously*.

5.) Induction of TFR cells from nTreg precursors by blocking PD-1 pathway *in vitro*. In order to generate de novo TFR cells from Treg progenitors, naïve Tregs are sorted from human blood by CD4+CXCR5-GITR+ (or CD25+). These cells are activated with autologous CD19+ B cells with anti-CD3. During this activation, PD-1 antagonists (blocking antibodies or small molecule inhibitors) are used to block the PD-1 pathway. TFR cells are then sorted by flow cytometry as CD4+CXCR5+ICOS+GITR+ or and injected into patients.

Example 3. Summary

T Follicular Regulatory (TFR) cells inhibit whereas T Follicular Helper (TFH) cells stimulate antibody production. Both TFR and TFH cells are found in blood, however relatively little is known about the developmental signals for these cells or

their functions. Here we demonstrate a new mechanism of control of antibody responses mediated by TFR and TFH cells. We find that circulating TFH and TFR cells are distinct from lymph node effector TFR/TFH cells, and represent memory-like cells that have specialized function to patrol diverse sites of potential antigen encounter. Circulating TFH cells can be potently activated by DCs, home to germinal centers and produce large amounts of cytokines. Although memory-like circulating TFR cells can be potently activated in lymph nodes, they have a lower capacity to suppress B cell responses than lymph node TFR cells. Therefore, memory-like circulating TFH cells are able to provide quick and robust systemic B cell help.

Follicular helper T (TFH) cells are a subset of $CD4^+$ T cells that stimulate and maintain the germinal center reaction, enabling B cells to produce high affinity antibodies. TFH cells are defined by CXCR5, the chemokine receptor which directs them to the B cell zone via gradients of the chemokine CXCL13 (Breitfeld et al., 2000; Crotty, 2011). TFH cells express the transcription factor Bcl6 which facilitates CXCR5 expression, as well as production of IL-21, a key cytokine that helps B cells to undergo affinity maturation and produce antibody (Johnston et al., 2009; Nurieva et al., 2008; Nurieva et al., 2009). In addition, TFH cells can produce other cytokines including IFNγ IL-17 and IL-4 that are thought to influence the germinal center reaction by helping with selection of antibody isotypes during class switch recombination. Significant plasticity in cytokine production exists within in the TFH lineage, so distinguishing TFH from Th1, Th2 and Th17 cells can be challenging (Cannons et al.). Lymph node (LN) TFH differentiation is thought to be a two step process: T cells are initially primed by dendritic cells and subsequently interact with B cells in the germinal center, culminating in full effector potential (Crotty, 2011; Goenka et al., 2011; Haynes et al., 2007; Poholek et al., 2010).

Follicular regulatory T (TFR) cells are a newly defined population of $CXCR5^+$ CD4 T cells that until recently were hidden within TFH gating strategies. Like TFH cells, TFR cells express high levels of CXCR5, the costimulatory molecule ICOS, and the coinhibitory molecule PD-1 (Chung et al., 2011; Linterman et al., 2011; Sage et al., 2013; Wollenberg et al., 2011). Importantly, TFR cells are thought to originate from thymic Treg (tTreg) precursors, in contrast to TFH cells which develop from naïve T cells (Chung et al., 2011; Sage et al., 2013).

TFR and TFH cells have diametrically opposing roles in regulating humoral immune responses; TFH cells stimulate humoral immunity, whereas TFR cells potently suppress humoral immune responses (Chung et al., 2011; Linterman et al., 2011; Sage et al., 2013; Wollenberg et al., 2011). The mechanisms by which TFR cells suppress the germinal center reaction are still unclear. It is not known whether TFR cells suppress TFH cells, germinal center B cells, or both. Understanding how TFR cells inhibit humoral immunity has the potential to enable improved vaccination strategies.

TFH and TFR cells are not only present in LNs, but also in the circulation. Circulating TFH cells from humans can provide help to B cells in vitro (Morita et al., 2011; Schaerli et al., 2000), and circulating TFH cells from mice can potently stimulate B cells *in vivo* (Sage et al., 2013). A subset of human blood TFH cells with CXCR5 expression comparable to lymph node TFH cells but lower levels of PD-1 and ICOS expression has been postulated to represent memory cells (Breitfeld et al., 2000; Morita et al., 2011; Rasheed et al., 2006). Therefore, it is possible that circulating TFH cells may give rise to memory TFH cells (Fazilleau et al., 2007; Hale et al., 2013; Sage et al., 2013). Although there is evidence for the presence of memory TFH cells, the origin and function of these cells is not yet clear (Fazilleau et al., 2007; Hale et al., 2013; Luthje et al.,; MacLeod et al., 2011; Marshall et al., 2011). If blood TFH and TFR cells possess properties of memory cells, then they could serve as attractive candidates for immune monitoring and cellular therapy (Morita et al., 2011; Sage et al., 2013; Saito et al., 2005; Schaerli et al., 2000; Simpson et al., 2010).

Elucidating the relationships between lymph node TFH and TFR cells and circulating TFH and TFR cells may provide insights into TFH (and TFR) memory cell development and function (Crotty, 2011). Although lymph node TFR and TFH cells depend on CD28, ICOS and B cells for development, the specific cues for blood TFH and TFR cell development and maintenance are not yet clear. Circulating TFH cells in humans appear to differ from lymph node TFH cells by microarray analysis, however these differences may be due to decreased activation in the blood or contaminating TFR cells (Rasheed et al., 2006). The most straightforward explanation for the presence of TFH and TFR cells in the circulation is that some TFH and TFR cells from the germinal center "decide" to leave the lymph node. If this

hypothesis is true, then circulating TFH and TFR cells would require lymph node TFH and TFR cells for their development. In support of this hypothesis, TFH and TFR cells are completely missing from the lymph nodes and blood of CD28 and ICOS deficient mice (Bauquet et al., 2009; Bossaller et al., 2006; Sage et al., 2013). However, there are data that are inconsistent with this hypothesis. PD-1 deficient mice have increased TFH cells in the blood, but not in the lymph node (Sage et al., 2013). In addition, lymph node TFH and TFR cells develop with similar kinetics as blood TFH and TFR cells (Sage et al., 2013). Moreover, recent tracking experiments suggest that GC TFH cells are unable to gain access to the circulation (Shulman et al., 2013). These findings suggest that circulating and lymph node TFH and TFR cells may develop independently.

Here we demonstrate that "memory-like" blood TFR and TFH cells are distinct and specialized cellular subsets that potently and systemically control antibody production. Memory-like blood TFH and TFR populations differ from lymph node TFR and TFH "effector" cells in several ways. Memory-like blood TFH and TFR cells can circulate throughout the body patrolling for antigen, in contrast to LN TFH and TFR cells. After homing to lymph nodes, blood TFH cells require repriming with dendritic cells to be completely functional; these activated TFH cells produce enhanced amounts of cytokines and dominate the CXCR5 population compared to lymph node TFH cells. In addition, we find that blood TFR cells have decreased suppressive capacity in vitro, compared to lymph node TFR cells. Since blood TFH cells are more potently activated and blood TFR cells are less suppressive, the blood $CD4^{+}CXCR5^{+}$ population as a whole is poised to form a quick memory-like response throughout the body wherever re-exposure to antigen may occur. Taken together, these studies identify a novel mechanism by which circulating TFH cells control B cell responses *in vivo*.

Results

Differentiation of Circulating TFH and TFR cells Require Priming by Dendritic Cells

We recently demonstrated that T follicular regulatory (TFR) and T follicular helper (TFH) cells are present in the blood of mice. To determine if blood and lymph node TFH and TFR cells have phenotypic differences, we first compared the expression of CXCR5 and other cell surface markers on murine TFH and TFR cells from lymph node and blood. Initially, we analyzed CXCR5 and ICOS expression on circulating and lymph node cells 7 days after subcutaneous immunization with NP-OVA in CFA, an immunization that causes robust differentiation of both TFH and TFR cells (Sage et al., 2013). TFH cells, defined as $CD4^+ICOS^+CXCR5^+FoxP3^-CD19^-$, greatly expanded in the draining lymph nodes (dLN) and blood of immunized mice compared to unimmunized controls (FIG. 15A-B). The blood TFH cells had lower, but still substantial, expression of CXCR5 (FIG. 22). Expression of the essential TFH costimulatory molecule ICOS was much lower on blood TFH compared to LN TFH cells (FIG. 22). Intracellular staining of the cell cycle marker Ki67 revealed that fewer blood TFH cells were in cell cycle compared to dLN TFH cells. However, blood TFH cells had much higher Ki67 expression than the total CD4 T cell population (FIG. 22).

TFR cells, defined as $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$ cells, differentiated significantly in both the dLN and blood after immunization (FIG. 22). Draining LN TFR cells expressed lower levels of CXCR5 than dLN TFH cells. Blood TFR cells expressed even lower levels of CXCR5 than dLN TFR cells (FIG. 22). ICOS expression was also greatly attenuated in blood TFR cells compared to dLN TFR cells (FIG. 22). However, Ki67 intracellular staining revealed that there were similar proportions of dLN and blood TFR cells in cell cycle (FIG. 22). Taken together, these data indicate that circulating TFH and TFR cells are phenotypically distinct from dLN TFH and TFR cells and express less CXCR5 and ICOS.

Next we analyzed the developmental cues necessary for circulating TFH and TFR cells. LN TFH cells depend on dendritic cells for initial differentiation (Goenka et al.) and require B cells for maintenance of the TFH phenotype, as well as optimal CXCR5 and ICOS expression (Choi et al., 2011; Kerfoot et al., 2011; Poholek et al., 2010). LN TFR cells also depend on B cells for differentiation (Linterman et al., 2011). To determine if dendritic cells are necessary for the generation of circulating TFH and TFR cells, we utilized CD11c DTR bone marrow chimeric mice to deplete dendritic cells. We used bone marrow chimeric mice for these in vivo studies to circumvent the lethality related to stromal expression of CD11c in the brain. We administered diphtheria toxin every 2 days starting on day 0 during an NP-OVA immunization, and compared the generation of blood and dLN TFH and TFR cells 7 days post immunization. Diphtheria toxin administration resulted in depletion of most, but not all, dendritic cells in the dLN of CD11cDTR mice (FIG. 15A). Depletion of dendritic cells led to significantly lower percentages of $Ki67^+$ TFH and TFR cells in both the dLN and blood, suggesting that dendritic cells are key for differentiation of both dLN and blood TFH and TFR cells (FIG. 15B).

Next, we investigated whether antigen presentation by dendritic cells was sufficient for development of circulating TFH and TFR cells by comparing antigen presentation by WT and MHC II deficient (*Ciita*$^{-/-}$) dendritic cells using adoptive transfer approaches. We differentiated WT or *Ciita*$^{-/-}$ bone marrow derived dendritic cells (BMDCs) with GM-CSF, activated them with LPS and pulsed them overnight with NP-OVA. After washing the cells thoroughly, we adoptively transferred these Ag-pulsed BMDCs subcutaneously into WT mice, and analyzed the dLN and blood for TFH and TFR cell development 5 days later. When NP-OVA-pulsed WT BMDCs were adoptively transferred, there were substantial increases in percentages of total $CD4^+CXCR5^+$ cells in both the dLN and blood (FIG. 15C). When TFH cells were quantified, we found that transfer of antigen-pulsed WT BMDCs caused a significant increase in the proportions of TFH cells in both the lymph node and blood (FIG. 15D). In contrast, adoptive transfer of NP-OVA-pulsed MHC II-deficient BMDCs resulted in modest increases in percentages of $CXCR5^+$ cells in the dLN and blood (FIG. 15C-D). Antigen-pulsed WT BMDCs also stimulated a significant increase in the proportion of TFR cells in the lymph node. However, Ag-pulsed WT BMDC did not result in increased blood TFR cells, suggesting that cues for blood

TFR cell generation can be supplied by subcutaneous immunization but not DC transfer approaches.

Since B cells are required for full differentiation of dLN TFH and TFR cells, we next tested whether B cells are required for the generation of circulating TFH and TFR cells. We immunized WT and "μMT" mice (that lack mature B cells due to the lack of surface IgM expression) with NP-OVA, and compared dLN and circulating TFH and TFR cells. Similar to previous reports, the percentages of TFH cells and TFR cells in the dLN were severely attenuated in μMT mice compared to WT mice (FIG. 15E-F) (Poholek et al., 2010). Surprisingly, the percentages of TFH and TFR cells within the blood of μMT mice were equivalent to those of WT mice, suggesting that blood TFH and TFR cells require distinct cues for development compared to LN TFH and TFR cells. Furthermore, since blood TFH and TFR cell numbers did not increase in the μMT mice compared to WT mice, it is unlikely that the absence of dLN TFH and TFR cells is due to migration of these cells into the blood. Since the lack of mature B cells attenuated TFH and TFR numbers in the dLN but not the blood, the μMT mice have a blood:LN TFH ratio 6 times higher than that of WT mice and a blood:LN TFR ratio 2 fold higher than WT mice (FIG. 15G). Taken together, these data indicate that circulating TFH and TFR cells require signals from DCs for their generation.

Blood TFH and TFR Cells Exit the Lymph Node via S1P Signals

To further understand the relationship between LN and blood TFH and TFR cells, we next investigated the cues responsible for exit of TFH and TFR cells from the dLN and entry into the circulation. Sphingosine-1-phosphate (S1P) levels control T cell exit from the lymph node into the efferent lymph (Matloubian et al., 2004). High levels of S1P in blood and lymph act as a chemoattractant for T cells. This directed migration can be blocked by expression of CD69 on newly activated cells, which downregulates S1P receptors (Cyster and Schwab, 2011; Shiow et al., 2006). Based on these data, we hypothesized that blood TFH and TFR cells would have low CD69 expression. To test this hypothesis, we compared CD69 expression on TFH and TFR cells from the dLN and blood of mice that were immunized with

NP-OVA 7 days previously. Blood TFH and TFR cells had ~2-3 fold lower CD69 expression compared to TFH and TFR cells from the lymph node (FIG. 16A-B).

To test whether TFH and TFR cells utilize S1P signals to exit the lymph node via the efferent lymphatic system, we used FTY720 which prevents cells from responding to S1P gradients and prevents lymphocyte egress from lymphoid tissues (Cyster, 2005; Cyster and Schwab, 2011). We immunized mice with NP-OVA and then administered FTY720 to mice every 2 days (starting on day 2) and harvested organs 7 days after NP-OVA immunization. Blood TFH cells were virtually absent in mice that received the FTY720 treatment (FIG. 16C). TFR cells showed a similar dependence on S1P signals and very few were present in the blood after FTY720 administration (FIG. 16D). However, the numbers of dLN TFH and TFR cells were not significantly changed by FTY720 treatment. To investigate the kinetics of blood TFH and TFR circulation, we immunized mice with NP-OVA and administered FTY720 on day 7 after immunization and analyzed TFH and TFR cells after 3 or 6 hours after FTY720 administration. When we treated mice with FTY720 only for 3 or 6 hours prior to analysis, there was also a marked attenuation of both TFH and TFR numbers, demonstrating TFH and TFR cells exit the blood quickly, within only a few hours (FIG. 16E).

To analyze TFH and TFR cell exit from the dLN and entry into efferent lymph, we conducted experiments in which the efferent lymph was collected via a thoracic duct cannulation method (Massberg et al., 2007). We detected robust percentages of both TFH and TFR cells in the lymph (FIG. 16F-G). The percentages of TFH cells in lymph and dLN were similar and the percentages of TFR cells in lymph and blood were similar (FIG. 16F-G). Lymph TFH and TFR cells had lower levels of ICOS compared to dLN TFH and TFR cells, but levels similar to blood TFH and TFR cells (FIG. 16H). Thus, both TFH and TFR cells have low ICOS expression upon exiting the dLN and entering the lymph, but do not further downregulate ICOS after entry into the circulation. Taken together, these data indicate that both TFH and TFR cells use S1P signals to leave the dLN, and their circulation in the blood is transient, on the order of hours.

Circulating TFH and TFR cells Migrate to Diverse Lymph Nodes and Tissues

To test the hypothesis that circulating TFH and TFR cells represent a memory pool of cells that can migrate to diverse secondary lymphoid organs where they can be reactivated to become effector cells, we next investigated blood TFH and TFR cell trafficking in vivo. To monitor blood TFH and TFR cell behavior in vivo we crossed Actin-CFP mice with FoxP3-IRES-GFP mice to create mice that report FoxP3 expression but can also be tracked in vivo by CFP expression (these mice are referred to as $Actin^{CFP}$-$FoxP3^{GFP}$ mice). We immunized 20 $Actin^{CFP}$-$FoxP3^{GFP}$ mice with NP-OVA and 7 days later sorted total $CD4^{+}ICOS^{+}CXCR5^{+}CD19^{-}$ cells to keep blood TFH and TFR cells in endogenous proportions. We transferred these cells into $CD28^{-/-}$ recipients, which cannot generate dLN or blood TFH and TFR cells. Transfer into $CD28^{-/-}$ recipients allowed us to transfer very small numbers ($2x10^{4}$) of cells, yet avoid homeostatic proliferation since $CD28^{-/-}$ mice have substantial T cell populations (Sage et al., 2013). We immunized $CD28^{-/-}$ recipients with NP-OVA subcutaneously and 7 days later isolated lymphoid organs and other tissues and analyzed transferred cells. We found significant populations of transferred cells in all lymph nodes tested, demonstrating that blood TFH and TFR cells have homing properties similar to central memory cells (FIG. 17A). Surprisingly, more than half of the CD4 T cells in the skin around the immunization site were transferred blood $CXCR5^{+}$ cells, demonstrating that circulating TFH and TFR cells can home to peripheral tissues as well as lymphoid organs. When we examined the transferred cells for ICOS and CXCR5 expression we found that the transferred cells in the lymph nodes and skin highly upregulated ICOS surface expression. However, ICOS expression remained low in the blood and spleen, similar to levels of ICOS expression on sorted blood $CXCR5^{+}$ cells prior to transfer (FIG. 11, 17A). In comparison, $CD28^{-/-}$ recipient CD4 T cells had very low ICOS expression. CXCR5 expression was maintained on about 40 percent of transferred cells in the draining (inguinal) lymph node. Other organs, particularly the blood, showed lower CXCR5 expression.

To determine specifically where TFH and TFR cells homed, we used FoxP3^{GFP} to identify TFR cells in the CFP^{+} transferred population. The FoxP3^{-} TFH cells predominated in the transferred population (FIG. 17C). Compared to the percentage of TFR cells in the input population, the percentage of TFR cells in the CXCR5^{+}CFP^{+} gate in the various lymphoid organs was much lower (FIG. 17D). Therefore, blood TFR cells are more short-lived or proliferate less after homing to various tissues than blood TFH cells. The highest proportion of TFR to TFH cells in the recipients was in the mesenteric lymph node. The overall reduction in TFR cells was not due to CXCR5 downregulation on TFR cells, since this decrease was also seen when FoxP3^{+} cells were gated on the total CFP transferred population (FIG. 17E).

To determine if blood TFH and TFR cells are capable of migrating to the B cell zone in order to perform effector functions after homing to lymph nodes, we analyzed the location of transferred cells in dLNs of recipient mice. We immunized CD45.2 WT mice with NP-OVA, transferred sorted blood TFH cells (CD4+ICOS+CXCR5+GITR-CD19-, a gating strategy we have established previously (Sage et al., 2013)) to CD45.1 recipients that were subsequently immunized with NP-OVA. 7 days later we found CD45.2+ blood TFH cells interacting with IgG1+ class switched B cells both within germinal centers and outside germinal centers in the interfollicular zone of the dLN of recipient mice (FIG. 17F). Likewise, when we transferred blood TFR cells (CD4+ICOS+CXCR5+GITR+CD19-) into recipient mice that were subsequently immunized, we found transferred blood TFR cells interacting with IgG1^{+} class switched B cells in the interfollicular zone, and sometimes directly in the germinal centers of the dLN of recipient mice (FIG. 17G). Taken together, these data indicate the circulating TFH and TFR cells are capable of homing to numerous secondary lymphoid organs and tissues to perform effector functions.

Circulating TFH and TFR cells are More Potently Activated After Transfer *in vivo*

Next we compared the function of circulating TFH and TFR cells with LN TFH and TFR cells. We hypothesized that if blood TFH and TFR cells have characteristics of memory cells, they would have increased ability to be activated in

vivo compared to dLN TFH and TFR cells. To investigate this issue, we immunized Actin^{CFP}-FoxP3^{GFP} mice with NP-OVA and 7 days later sorted total CD4^{+}CXCR5^{+}CD19^{-} cells from the dLN and blood and adoptively transferred these cells intravenously into CD28$^{-/-}$ recipients, which were immunized with NP OVA. As in FIG. 18A, we could readily detect small populations of transferred cells in the dLN of recipients 7 days later based on the presence of CFP (FIG. 18A).

Since Bcl6 expression is essential for TFH cell function, we investigated if blood TFH cells could upregulate Bcl6 after entering and being activated in the dLN. We found slightly higher Bcl6 expression in transferred blood TFH cells compared to transferred dLN cells (FIG. 18B). These findings indicate that circulating TFH cells can become effector TFH cells upon homing back to a lymph node during an immune response. Next we compared ICOS expression on transferred dLN and blood cells ("CXCR5 Transfer") in the dLN or blood (the "assayed population") of immunized CD28$^{-/-}$ recipients. We also compared ICOS expression on dLN and blood TFH and TFR cells from immunized WT mice ("endogenous WT") in order to facilitate comparisons. ICOS is expressed at a very low level on endogenous WT blood TFH cells from WT mice (FIG. 18C). ICOS expression was dramatically increased on blood TFH cells after homing back to the lymph node (dLN TFH assayed population, blood CXCR5 transfer) compared both to endogenous WT blood TFH cells and even dLN transferred TFH cells (dLN TFH assayed population, dLN CXCR5 transfer) (FIG. 18C). The increased ICOS expression on circulating TFH cells was not unique to the TFH subset, because blood TFR cells that were found in the dLN also greatly upregulated ICOS expression compared to endogenous blood TFR cells and transferred LN TFR cells (FIG. 18D). CXCR5 expression was similar in blood TFH cells upon rehoming to the lymph node and transferred lymph node TFH and TFR cells in the lymph node (FIG. 18E). Blood TFR cells had slightly lower CXCR5 upon rehoming to the lymph node compared to transferred LN TFR cells in the LN (FIG. 18F).

To determine if the increase in Bcl6 and ICOS translated into an increase in effector cytokine production by the circulating TFH cells, we compared cytokines produced by the transferred dLN or blood TFH cells found in the dLN after immunization. We found no difference in the very low, but detectable, intracellular IL-21 expression in the transferred blood TFH cells and transferred dLN TFH cells.

In contrast, there was a substantial increase in intracellular IL-17A produced by transferred circulating TFH cells compared to transferred dLN TFH cells (FIG. 18G). Intracellular IL-2 expression was also ~2 fold higher in transferred circulating TFH cells, compared to transferred dLN TFH cells (FIG. 18G). Taken together, these data indicate that circulating TFH cells (and TFR cells) can be potently re-activated upon homing to secondary lymphoid organs, consistent with a memory phenotype.

Circulating TFH cells Require Dendritic Cells for Restimulation and Have Memory Properties

Next we investigated the signals required for reactivation of blood TFH cells. We determined if dendritic cells and/or B cells were necessary for reactivation of circulating TFH cells using in vitro systems to sensitively evaluate activation and effector functions of TFH cells. We immunized 20 WT mice with NP-OVA subcutaneously and 7 days later purified dLN and circulating TFH cells by sorting $CD4^+ICOS^+CXCR5^+GITR^-CD19^-$ cells, as we previously reported (Sage et al., 2013)). We compared the reactivation of these TFH cells following culture with B cells or dendritic cells (also sorted from dLN of immunized mice) in the presence of anti-CD3 and anti-IgM. We evaluated Bcl6 and Ki67 expression to compare the activation and effector potential of dLN and circulating TFH cells. When cultured with B cells, dLN TFH cells had a higher percentage of $Bcl6^+Ki67^+$ cells than circulating TFH cells (FIG. 19A-B). Culturing dLN TFH cells with DCs only modestly increased the percentage of $Bcl6^+Ki67^+$ cells, as compared to culture with B cells. In contrast, culture of circulating TFH cells with DCs led to a greatly enhanced percentage of $Bcl6^+Ki67^+$ cells, as compared to circulating TFH cells cultured with B cells or lymph node TFH cells cultured with DCs (FIG. 19B). We next investigated if the increased Bcl6 expression in the circulating TFH cells cultured with DCs was associated with enhanced cytokine production. We found profound increases in intracellular IFNγ as well as smaller increases in IL-21, IL-17A, IL-2 and IL-4 in circulating TFH cells compared to lymph node TFH cells cultured with DCs (FIG. 19C).

To determine if blood TFH cells require DCs for persistence in vivo, we adoptively transferred $1.5x10^4$ blood $CD4^+CXCR5^+CD19^-$ cells from immunized $Actin^{CFP}$-$FoxP3^{GFP}$ mice into CD11cDTR bone marrow chimeric mice that were

immunized with NP-OVA. We administered diphtheria toxin (DT) on days 0, 3 and 5 during a 7 day immunization to deplete DCs and measured the number of transferred cells 7 days later. We found that DC depletion resulted in fewer CFP^+ cells persisting, as well as a lower percentage of cells expressing CXCR5 on the transferred cells (FIG. 19D). Taken together, these data indicate that circulating TFH cells need to be restimulated by dendritic cells and these TFH cells have a superior ability to produce TFH cytokines.

We next determined if blood TFH and TFR cells could persist in vivo similarly to memory T cells and dominate a GC reaction upon re-exposure to antigen by conducting parabiosis experiments. $Actin^{CFP}$-Fox^{GFP} mice were immunized with NP-OVA and the circulatory systems of these mice were surgically joined to WT mice, allowing transfer of blood TFH and TFR cells to the adjoining mouse (FIG. 19E). After being joined for 19 days, chimerism was confirmed, and mice were separated. The WT "non-immunized" mouse was then immunized with NP-OVA and 7 days later organs were harvested and analyzed. We compared the percent of CFP+ cells in the naïve as well as the CXCR5+ICOS+ populations. The CFP+ population in the naïve gate (from the non-draining lymph node) is indicative of baseline chimerism, and contributions above this percentage in the CXCR5+ICOS+ gate are indicative of increased blood TFH/TFR cells in the germinal center. We found significantly enhanced CFP+ cells in the CXCR5+ICOS+ gate in the draining lymph node and less so in the non-draining (cervical) lymph node (FIG. 19E). We also found substantial increases in the CFP population in the spleen, blood and (more dramatically) in the skin. Together these data demonstrate that the blood memory-like CXCR5+ population can out compete and dominate the GC reaction upon re-exposure to antigen.

Next we used adoptive transfer approaches to analyze TFH and TFR cells at memory time points. We transferred total blood CXCR5+ cells from NP-OVA immunized $Actin^{CFP}$-Fox^{GFP} mice to WT mice. After 30 days, WT recipients were immunized with NP-OVA and draining lymph nodes were harvested 7 days later. We found a small, but substantial population of CFP positive cells in the draining lymph node (FIG. 19F). Although the majority of these cells were TFH cells, there was a very small population of TFR cells that persisted. The TFH cells had high ICOS expression and a sizeable population that maintained CXCR5 expression,

suggesting functionality. Taken together these data indicate that blood TFH and TFR cells need to be reactivated by DCs, can dominate the GC reaction upon exposure to antigen, and can persist in vivo similar to memory cells.

TFR cells Potently Suppress T Cell and B Cell Activation

Next we compared the suppressive functions of lymph node and blood Tfr cells. Since the mechanisms by which TFR cells exert their suppressive effects are not well understood, we first developed a series of sensitive *in vitro* suppression assays for analyzing TFR cell suppression. We immunized more than 20 WT mice with NP-OVA subcutaneously and 7 days later sorted TFH ($CD4^+ICOS^+CXCR5^+GITR^-CD19^-$) and TFR ($CD4^+ICOS^+CXCR5^+GITR^+CD19^-$) cells from the dLN. We cultured TFH cells (with or without TFR cells) together with total B cells or dendritic cells (sorted from the dLN of immunized mice) for 6 days in the presence of anti-IgM and anti-CD3. CXCR5 remained highly expressed on TFH cells whether cultured with B cells or DCs (FIG. 20A). The addition of TFR cells did not cause downregulation of CXCR5 on TFH cells, suggesting that TFR cells do not suppress TFH cells by diverting them away from the germinal center by downregulation of chemokine receptors (FIG. 20A). Bcl6 expression was highly expressed on the TFH cells when cultured with dendritic cells or B cells (FIG. 20B). However, Bcl6 expression in the TFH cells was attenuated profoundly in the presence of TFR cells and DCs (but not B cells), suggesting that TFR cell repriming by DCs may increase TFR suppressive capacity. TFH Ki67 expression also was strongly suppressed by TFR cells in both B cell and DC culture conditions, suggesting that a key TFR cell suppression mechanism is inhibition of cell cycling in TFH cells (FIG. 20C).

To determine if TFH cytokine production was also suppressed by TFR cells, we analyzed TFH cells from in vitro cultures for cytokine production by intracellular staining. We found that TFR cells suppressed IFNg, IL-21, IL-10 and TNFα (FIG. 20D). TFR cells had no effect on IL-17A or IL-2 and caused an increase in IL-4. Thus, TFR cells are selective in the TFH cytokines that they suppress, at least in this in vitro model. Together, these studies suggest that TFR cells exert their suppressive effects by inhibiting TFH cell proliferation and cytokine production.

We next investigated whether TFR cells also affect B cell function. We cultured dLN TFH and B cells with or without TFR cells in the presence of anti-CD3 and anti-IgM for 6 days and then analyzed the B cells from these cultures. The germinal center B cell marker GL7 was highly upregulated on B cells when cultured with TFH cells, but markedly reduced when TFR cells were present in co-cultures (FIG. 20E-F). Importantly, this in vitro suppression depends on the "TFR program" as FoxP3+CXCR5- cells sorted from unimmunized lymph nodes did not attenuate the expression of GL7 on B cells in contrast to TFR cells (FIG. 20F). Furthermore, Ki67 expression was greatly attenuated in B cells when cocultured with TFH and TFR cells as compared to culture with TFH cells alone, demonstrating potent suppression of B cell activation by TFR cells (FIG. 20G). TFR cells also inhibited IgG1 expression in B cells. A substantial portion of B cells class switched to IgG1 when cultured with TFH cells but this was abrogated in the presence of TFR cells (FIG. 20H-I). B cell class switch recombination suppression was dependent on the TFR program, since non-TFR Tregs could not suppress IgG1 levels on B cells (FIG. 20I). Taken together these data indicate that lymph node TFR cells can suppress TFH cell activation, as well as B cell activation and antibody production *in vitro*.

Weaker B cell Suppression by Blood TFR cells

Finally, since blood TFH cells can be reactivated more potently than LN TFH cells, we compared the suppressive functions of blood TFR cells and dLN TFR cells. We compared the abilities of dLN TFR and blood TFR cells to suppress dLN TFH cells and B cells using the assays described above. TFH cells promoted IgG1 and GL7 expression in B cells (FIG. 21A). Blood TFR cells were able to greatly suppress IgG1 expression in B cells, but were less potent than dLN TFR cells. Additionally, blood TFR cells were able to attenuate GL7 expression, but this was not as robust as the attenuation by dLN TFH cells (FIG. 21B). Thus, dLN TFR cells potently suppress B cell activation but blood TFR cells suppress B cell responses to a somewhat lesser extent than dLN TFR cells. Additionally, a lower percentage of blood TFR cells expressed Ki67, compared to dLN TFR cells, regardless of culture with B cells or DCs (FIG. 21C). Therefore, unlike blood TFH cells, blood TFR cells are not more potently activated by DCs than dLN TFR cells.

Since blood TFH cells are more potent than LN TFH cells and blood TFR cells are less inhibitory than LN TFR cells in vitro, we next compared the effect of the total blood CXCR5 (TFH/TFR cells) population on B cell activation in vivo compared to dLN CXCR5 cells. We sorted total CXCR5+ cells from dLNs or blood of immunized $Actin^{CFP}$ Fox^{GFP} mice (which had the same TFH/TFR ratios) and adoptively transferred these cells to $CD28^{-/-}$ recipient mice that were immunized as in FIG. 18A. When we analyzed the draining lymph node 10 days after adoptive transfer, we detected a greater percentage of both GC B cells and plasma cells in the mice that received blood CXCR5+ cells (FIG. 21D-E). Taken together, these data indicate that stimulatory blood TFH cells are poised to provide a quick and robust memory-like response in secondary lymphoid organs.

Discussion

In this study we have determined that "memory-like" blood TFH and TFR cells differ from "effector" dLN TFH and TFR cells because they provide systemic B cell control in secondary lymphoid organs and not just at the site of differentiation. We demonstrate that blood TFH and TFR cells have distinct phenotypes and require different cues for generation compared to lymph node TFH and TFR cells. Additionally, we show that circulating TFH and TFR cells can be potently activated by DCs in diverse secondary lymphoid organs and tissues. Finally, beyond their ability to home to and patrol diverse sites of possible antigen encounter, we find that blood TFH and TFR cells may provide effector functions differing from LN TFH and TFR cells upon reactivation. Therefore, blood TFH and TFR cells constitute distinct subsets that share many hallmarks of memory cells and the blood TFH cells can provide a quick and robust response to stimulate humoral immunity.

We find that dendritic cell signals are important for blood TFH and TFR development, but TFH and TFR cell numbers are not altered in mice that lack mature B cells. This contrasts with lymph node TFH and TFR cells that are lacking in μMT mice (Haynes et al., 2007; Johnston et al., 2009; Linterman et al., 2011; Poholek et al., 2010). However, it is important to note that the μMT mouse has an altered lymph node structure and blood TFH/TFR cell behavior may be a consequence of these alterations. Nevertheless, our studies suggest that circulating memory-like TFH

and TFR cells are not derived from lymph node TFH and TFR cells, but differentiate in parallel with lymph node TFH and TFR cells.

The signals that elicit dLN versus circulating memory-like TFH and TFR differentiation are not clear. Full T cell activation in general is thought to occur through multiple serial contacts with dendritic cells leading to full activation (Mempel et al., 2004). We hypothesize that two fates emerge for follicular CD4 T cells during differentiation with DCs. Subsets of TFH and TFR cells that have lower CXCR5 expression and low CD69 after priming exit the lymph node via S1P gradients and are destined to become blood memory cells. TFH and TFR cells with higher CXCR5 and high CD69 after priming follow CXCL13 gradients and migrate to the B cell zone, where full differentiation and maintenance of the TFH and TFR effector phenotype occurs. Therefore, within the "window" for TFH and TFR differentiation, subtle differences in TCR and/or costimulation can push the fate of the cells towards an effector or memory TFH/TFR cell phenotype. Importantly, when TFH and TFR cells enter the efferent lymph, they already have a surface phenotype similar to blood TFH and TFR cells, suggesting that the circulating/memory TFH and TFR cell phenotype is acquired during differentiation and not after entry into the circulation. These data also demonstrate that circulating TFH and TFR cells do not originate from GC TFH cells. This is consistent with a recent study which demonstrated that GC TFH cells can move between the GC and interfollicular regions, but do not enter the circulation (Shulman et al., 2013).

Our studies with the S1P agonist FTY720, as well as our transfer and tracking experiments, suggest that TFH and TFR cells recirculate from the blood to secondary lymphoid organs and tissues rather quickly, on the order of hours. We hypothesize that the function of circulating TFH cells is to probe various organs for antigen and provide specialized and robust B cell help during secondary antigen exposure or systemic infections. Relatively lower expression of CXCR5 on circulating TFH and TFR cells may allow these cells to better patrol T cell zones for DCs presenting antigen, while LN TFH and TFR cells may get "stuck" in the B cell follicle due to high CXCL13 levels present there. Circulating TFR cells may help to regulate circulating TFH responses to ensure that antigen-specific B cell responses are generated, but limit hyperactivation or antibody production in distal secondary lymphoid organs.

We previously showed that blood TFH cells stimulate more IgG production in vivo than dLN TFH cells (Sage et al., 2013). Here we demonstrate that upon reactivation by dendritic cells (but not B cells), blood TFH cells produce more cytokines, particularly IFNγ compared to dLN TFH cells. Classical experiments have demonstrated that IFNγ can inhibit B cell class switching from IgG1 to IgG2a, and recent studies have suggested that directed IFNγ production, presumably by TFH cells, has a similar effect (Reinhardt et al., 2009). However, in our experiments, blood TFH cells do not skew antibody isotypes away from IgG1 to any particular isotype in vivo or in vitro (data not shown). Although IFNγ is the cytokine that is most highly expressed by blood TFH cells compared to dLN TFH cells, there are slight increases in other cytokines as well. This suggests that blood TFH cells have an increased effector function upon restimulation by DCs and are not simply Th1-skewed TFH cells. The distinct cytokine profile of blood TFH cells may lead to specialized differentiation and/or function of germinal center B cells or plasma cells.

Far less is known about the signals necessary for the differentiation and function of TFR cells than TFH cells (Chung et al., 2011; Linterman et al., 2011; Sage et al., 2013; Wollenberg et al., 2011). LN TFR cell development follows many of the same cues as TFH cells, such as the B cell requirement in the lymph node as well as CD28 and ICOS costimulation (Linterman et al., 2011; Sage et al., 2013). It has been suggested that subsets of Treg cells (such as TFR cells) may use T helper transcription factors that are not normally expressed by Tregs to help position themselves near T helper cells and/or to program T helper specific suppression (Josefowicz et al., 2012). For example, adipose tissue-resident Tregs, similar to other cells present in adipose tissue, depend on PPAR-γ (Cipolletta et al., 2012). We previously showed that the TFR program is essential for suppression of antibody production as CXCR5$^-$ Tregs could not suppress antibody production in vivo (Sage et al., 2013). The exact mechanisms of TFR cell suppression are not known (Josefowicz et al., 2012). Here we show that lymph node TFR cells potently suppress activation of TFH cells, but do not suppress CXCR5 or Bcl6 expression in TFH cells cultured with B cells. Draining LN TFR cells can also suppress IFNγ, IL21, IL10 and TNFα expression by TFH cells. Interestingly, dLN TFR cells were unable to suppress IL17A or IL2 production in dLN TFH cells, at least in in vitro models. In

addition, we find that dLN TFR cells potently suppress dLN TFH-mediated B cell activation, class switch recombination and germinal center markers. Importantly, non-TFR FoxP3$^+$ Tregs were not able to suppress GL7 or class switching to IgG1. Since these in vitro assays do not depend on distinct T and B cell zones, the inability of non-TFR cells to suppress B cells suggests a "TFR program" beyond CXCR5 expression that is essential for TFR cell suppressive functions.

It is not currently known if T regulatory cells can possess immunological memory. One report showed that self-reactive Tregs can possess some form of regulatory memory at least within the skin (Rosenblum et al., 2011). Here we demonstrate that memory-like blood TFR cells can home to diverse secondary lymphoid organs, including the skin. Within the skin TFR cells may inhibit effector T cells or provide specific suppression of memory-like TFH cells and antibody secreting cells (ASCs). It also is possible that TFR cells may suppress formation of ectopic follicles that can arise during certain inflammatory conditions within the tissue (Peters et al.). We hypothesize that the main role of blood TFR cells is to provide systemic control of humoral immunity during an immune response by migrating throughout the body to organs that may be exposed to antigens. Blood TFR cells may function to ensure proper TFH control and thereby limit excessive or inappropriate B cell help. Additionally, blood TFR cells may prevent humoral autoimmunity by raising the threshold for B cell activation in situations where an abundance of proinflammatory cytokines might bypass the need for TFH help.

Since blood TFR cells require distinct cues compared to lymph node TFR cells, it is likely that blood TFR cells may be programmed differently than lymph node TFR cells. We show here that both lymph node and blood TFR cells are able to attenuate B cell activation but blood TFR cells are less suppressive. This difference may be due to differential programming during development. Further experiments are needed to investigate these issues. Importantly, the TFH:TFR ratio, which is important in controlling humoral immunity (Sage et al., 2013), is roughly similar between LN and blood TFH:TFR cells. Therefore, functional differences between blood and LN CXCR5$^+$ cells can be attributed to cell intrinsic differences in the TFH and TFR compartments. By interrogating these cells individually, we show that blood TFH cells are more potently activated upon restimulation (compared to LN TFH cells) and blood TFR cells are less suppressive (compared to LN TFR cells).

Additionally, in memory experiments, the TFH to TFR ratio is skewed towards the TFH cell. The combination of these cell intrinsic and extrinsic differences allow circulating memory TFH cells to quickly respond to antigen and activate B cells in lymph nodes after a secondary exposure to antigen. We show in parabiosis experiments that the CXCR5+ population of the non-immunized parabiotic mouse is dominated by blood derived CXCR5+ cells after immunization, demonstrating that the blood-derived CXCR5+ population controls the GC response more potently than newly generated LN CXCR5 cells. We hypothesize this is a key mechanism by which quick and efficient secondary responses are generated upon re-exposure to antigen in distant tissues. Understanding the mechanisms of TFH and TFR cell memory should provide insights into strategies for developing long-lasting B cell memory to vaccines and for controlling pathogenic B cell responses.

Experimental Procedures

Mice. 6-8 week old mice were used for all experiments. WT C57BL/6, *CIITA*$^{-/-}$, *Itax*-DTR (CD11c-DTR), *Ighm*$^{-/-}$ (mMT) and Actin^{CFP} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *CD28*$^{-/-}$ mice were generated as described (Shahinian et al., 1993). Actin^{CFP} Fox^{GFP} mice were generated by crossing Actin^{CFP} mice with Fox-IRES-GFP mice (Bettelli et al., 2006). For CD11c-DTR experiments, bone marrow chimeras were made by irradiating WT recipient mice with 600 rads twice, separated by 3 hours, and adoptively transferring 7x10^6 bone marrow cells. Mice were used after 8 weeks of reconstitution, and reconstitution was confirmed by flow cytometry. Mice were injected with 1mg diphtheria toxin (Sigma) intraperitoneally every 2 days starting at day 0 after immunization and harvested on day 7. In some experiments WT mice were injected intraperitoneally with 25mg/kg FTY720 (Cayman Chemical) every 2 days starting at day 2 after immunization. Alternatively, mice were injected with the FTY720 3 or 6 hours before harvesting organs. Thoracic duct cannulation was performed as described previously (Massberg et al., 2007). All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Animal Healthcare Guidelines. Animal protocols were approved by the Harvard Medical School Standing Committee on Animals.

Immunizations

For NP-OVA immunizations, 100μg NP_{18}-OVA (Biosearch Technologies) in a 1:1 H37RA CFA (DIFCO) emulsion was injected subcutaneously on the mouse flanks. Seven days later mice were euthanized and inguinal lymph nodes (dLN) were harvested, blood was collected via cardiac puncture with a 1cc syringe and immune cells were isolated by sucrose density centrifugation using Lymphocyte Separation Media (LSM).

Bone Marrow Derived Dendritic Cells

Bone marrow derived dendritic cells (BMDCs) were made by culturing total bone marrow cells in the presence of 30ng/ml GM-CSF (Peprotech) for 7 days. During the last 24 hours, 20ng/ml of LPS (sigma) and 20μg/ml of NP-OVA were added to the cultures. Cells were harvested, washed extensively and counted on an Accuri cytometer. $1x10^6$ cells were subcutaneously injected in 100ul PBS on the flank of WT mice. 5 days later the inguinal lymph node and blood were harvested and analyzed for TFH and TFR cells.

Flow Cytometry

Cells from lymphoid organs were isolated and resuspended in staining buffer (PBS containing 1% fetal calf serum and 2mM EDTA) and stained with directly labeled antibodies from Biolegend against CD4 (RM4-5), ICOS (15F9), CD19 (6D5), CD69 (H1.2F3) CD11c (N418), MHC II (M5/114.15.2), IL17A (TC11-18H10.1), IL2 (JES6-5H4), IFNg (XMG1.2), IL4 (11B11), TNFα (MP6-XT22), CD138 (281-2), from eBioscience against FoxP3 (FJK-16S), Bcl6 (mGI191E), IL21 (FFA21), from BD bioscience against GL7, Ki67 (B56) and IgG1 (A85-1), and from Abcam, goat anti-GFP. For CXCR5 staining, biotinylated anti-CXCR5 (2G8, BD Biosciences) was used followed by streptavidin-brilliant violet 421 (Biolegend). For intracellular staining of transcription factors and Ki67, the FoxP3 fix/perm kit was used (eBioscience) after surface staining was accomplished. For intracellular cytokine staining, cells were incubated with 1μg/ml ionomycin (Sigma) and 500ng/ml PMA (Sigma) in the presence of Golgistop (BD biosciences) for 4 hours prior to staining. All flow cytometry was analyzed with an LSR II (BD biosciences) using standard filter sets.

Adoptive Transfers

For $CXCR5^+$ T cell transfers, 20 $Actin^{CFP}$ Fox^{GFP} mice were immunized with NP-OVA subcutaneously as described above, and 7 days later dLN or blood was collected. $2x10^5$ $CFP^+CD4^+ICOS^+CXCR5^+$ cells were adoptively transferred to *CD28*$^{-/-}$ or WT mice that were then immunized subcutaneously with NP-OVA. For memory experiments, recipients were immunized after 30 days. 7 days later organs were harvested. Skin from the immunization site was digested with 500 U/ml collagenase (Worthington Biochemical). Transferred cells were identified by either CFP expression or intracellularly with an anti-GFP polyclonal antibody (Abcam).

Parabiosis

For parabiosis, age- and sex-matched Actin-^{CFP} Fox-^{GFP} mice and C57Bl/6 mice were anesthetized by ketamine/xylazine (i.p.) and surgically joined at the olecranons and knees as described previously (Massberg et al., 2007). Briefly, the lateral sides of each mouse were shaved and wiped with depilatory cream, and incisions were made along the lateral aspect of each mouse from the olecranon to the knee. The subcutaneous fascia was bluntly dissected to expose loose ventral and dorsal skin flaps for connection. Mice were first joined together at the olecranons and knees by a double ligature using 4-0 braided silk suture. Next, the ventral and dorsal skin flaps overlying the olecranon and knee region were joined by continuous 6-0 braided silk suture. Finally, the remaining dorsal and ventral skin flaps between the olecranons and knees were connected by staples. Parabiotic mice were separated after 19 days by reversal of the above procedure. Mice were immunized with NP-OVA subcutaneously on the non-joined side 6 days later. Chimerism among different leukocyte populations was calculated as the average of the percent of CFP+ cells in the WT mouse in the non-draining lymph nodes.

***In vitro* Suppression Assay**

All populations were sorted to 98% purity on a FACS Aria using standard detectors. Cells were counted on an Accuri cytometer (BD biosciences) by gating live cells only. For TFH stimulation assays, $2x10^4$ dLN or blood $CD4^+ICOS^+CXCR5^+CD19^-GITR^-$ TFH cells were plated with $5x10^4$ $CD19^+$ B cells or $2x10^4$ $CD3^-CD19^-CD11c^+$ DCs, all from dLNs of WT mice immunized with NP-

OVA 7 days previously. For DCs, dLNs were digested with 500 U/ml Collagenase I (Worthington Biochemical) before isolation. TFH plus B or DCs were cultured in the presence of 2μg/ml soluble anti-CD3 (2C11, BioXcell) and 5μg/ml anti-IgM (Jackson Immunoresearch) for 5-6 days. Cells were then harvested and stained for flow cytometry. Intracellular expression of Bcl6 and Ki67 were used as readouts for TFH cell activation and functional potential. Intracellular expression of IgG1 was used as a readout for B cell class switch recombination. For TFR suppression assays, $1x10^4$ $CD4^+ICOS^+CXCR5^+CD19^-GITR^+$ TFR cells from either the dLN or blood of WT mice immunized with NP-OVA 7 days previously were added to the wells of TFH stimulation assays. Cells were harvested 5-6 days later.

Confocal Microscopy

Confocal microscopy was performed as described previously (Sage et al., 2013). Briefly, organs were harvested and embedded and frozen in OCT medium (Tissue-Tek). 10um sections were cut, fixed and stained using the FoxP3 kit (ebioscience). Samples were imaged using an Olympus confocal microscope using standard configurations.

Statistical Analysis

Unpaired Student's t test was used for all comparisons, data represented as mean +/- SD or SE are shown. P values < 0.05 were considered statistically significant. * P<0.05, ** P<0.005, *** P<0.0005.

(G) Quantification of lymph node and blood TFR cells from plots in (F). Total $CD4^+FoxP3^+CD19^-$ cells "Total FoxP3+" are included as controls.

(H-J) CXCR5 (H), ICOS (I), Ki67 (J) expression on lymph node and blood TFR cells gated as in (F). Data are means +/- standard error with 5 mice per group. Data are representative of at least 3 independent experiments.

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Example 4. Comparison of TFR and Treg gene expression signatures

TFR (CD4+ICOS+CXCR5+GITR+CD19-) cells were sorted from the lymph nodes of 20 pooled, WT or PD-1 deficient mice 7 days after subcutaneous immunization with NP-OVA in CFA. Similarly, CD4+CXCR5-FoxP3+ Tregs were sorted from the lymph nodes of unimmunized FoxP3 reporter mice. Each sort was performed in triplicate. RNA was isolated using standard procedures, was amplified and reverse transcription was performed. Samples were run on mouse affymetrix gene expression arrays. Data was then normalized. Top 100 hits were determined by comparing WT TFR to Tregs and ranked by signal-to-noise. PD-1 deficient TFR

cells, which have enhanced suppressive capacity, are added as an additional signature of TFR cells. Results are shown in FIG. 23.

Example 5. Surface receptors differentially expressed by human blood TFR cells

Fresh human peripheral blood mononuclear cells were isolated by sucrose density centrifugation from normal human blood. Cells were surface stained with anti-CD4, anti-CXCR5, and anti-CD19. Cells were then plated on 96-well plates that contained antibodies to surface receptors in the screen. Cells were then fixed, permeabilized and intracellularly stained with anti-FoxP3. Populations were analyzed by flow cytometry as CD4 (CD4+CXCR5-FoxP3-CD19-), Treg (CD4+CXCR5-FoxP3+CD19-), TFH (CD4+CXCR5+FoxP3-CD19-) and TFR (CD4+CXCR5+FoxP3+CD19-). Top 56 hits (of TFR versus TFH cell with a fold increase of at least 1.2) are shown in FIG. 24, sorted on mean fluorescence intensity on TFR cells (value).

Example 6. Surface receptors differentially expressed by human blood TFH cells

Fresh human peripheral blood mononuclear cells were isolated by sucrose density centrifugation from normal human blood. Cells were surface stained with anti-CD4, anti-CXCR5, and anti-CD19. Cells were then plated on 96-well plates that contained antibodies to surface receptors in the screen. Cells were then fixed, permeabilized and intracellularly stained with anti-FoxP3. Populations were analyzed by flow cytometry as CD4 (CD4+CXCR5-FoxP3-CD19-), Treg (CD4+CXCR5-FoxP3+CD19-), TFH (CD4+CXCR5+FoxP3-CD19-) and TFR (CD4+CXCR5+FoxP3+CD19-). Top 19 hits (TFH versus TFR) are shown in Fig. 25, sorted by fold increase of expression on TFH versus TFR cell. Value indicates expression (in mean fluorescence intensity) on TFR cells.

Example 7. Blockade of the PD-1 pathway can heighten antibody stimulating capacity of TFH cells

Murine CD4+ICOS+CXCR5+GITR-CD19- TFH cells were sorted from the lymph nodes of WT mice (that were immunized subcutaneously with NP-OVA in CFA 7 days previously) on an Aria cell sorter. TFH cells were plated with CD19+ B cells (isolated from lymph nodes of mice immunized with NP-OVA 7 days

previously) in the presence of 5ug/ml anti-IgM and 2ug/ml anti-CD3 for 6 days. Anti-PDL1 was also added to some wells. After 6 days, cells from cultures were harvested, surface stained with anti-CD4, -CD19, -GL7, and -I-A followed by fixation/permeabilization, and intracellularly stained with anti-IgG1. Cells were analyzed by flow cytometric analysis. B cells were identified by surface expression of CD19 and IA. Ig class switched B cells were identified by coexpression of GL7 and IgG1. Results are shown in Fig. 26.

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims. It should also be understood that the embodiments described herein are not mutually exclusive and that features from the various embodiments may be combined in whole or in part in accordance with the invention.

CLAIMS

What is claimed is:

1. A composition comprising T follicular regulatory (TFR) cells having enhanced immune suppressive activity.

2. The composition of claim 1, wherein the TFR cells inhibit antibody production *in vitro* and *in vivo*.

3. The composition of claim 1, wherein the expanded TFR cells inhibit T-follicular helper (TFH) cell function *in vitro* and *in vivo*.

4. The composition of claim 1, wherein the TFR having enhanced suppressive activity are characterized by their ability to more potently inhibit an antibody response *in vitro a*nd *in vivo* as compared to native TFR cells.

5. The composition of claim 1, wherein the TFR cells having enhanced suppressive activity are at least two-fold more potent than native TFR.

6. The composition of claim 2, wherein the TFR cells inhibit T follicular helper (TFH) cell-mediated antibody production *in vitro* and *in vivo*.

7. A method of preparing a composition comprising TFR cells having enhanced immune suppressive activity comprising the steps of:

a) obtaining an initial population of cells comprising TFR cells, T regulatory (Treg) progenitor cells or both;

b) activating the cells *ex vivo* in the presence of a PD-1 antagonist or PD-1 ligand antagonist, or both; and

c) isolating activated TFR cells from the population wherein the isolated TFR cells have enhanced immune suppressive activity.

8. The method of claim 7, wherein the initial population of cells is isolated from the peripheral blood, tissues or organs of one or more subjects.

9. The method of claim 7, further comprising sorting TFR cells and Treg progenitor cells from the cell population prior to activation of the cell population or after activation of the cell population.

10. The method of claim 7, wherein the activation step comprises expanding the cell population.

11. The method of claim 7, wherein the activation step comprises contacting the cell population with a stimulatory composition.

12. The method of claim 11, wherein the stimulatory composition comprises an anti-CD3 antibody, physiological antigen presenting cells (APCs), purified CD19+ B cells, and anti-CD28 antibody.

13. The method of claim 11, wherein stimulatory composition comprises beads coated with CD3 antibody, the anti-CD28 antibody or any combination thereof.

14. The method of claim 7, wherein the PD-1 antagonist or PD-1ligand antagonist is selected from: a PD1-ligand antibody, an anti-PD-1 antibody, a peptide or a small molecule or any combination thereof, wherein the antagonist inhibits the interaction between PD-1 and a PD-1 ligand.

15. The method of claim 7, wherein the isolating step comprises sorting TFR cells based on surface markers of $CD4^+CXCR5^+ICOS^+GITR^+$ or $CD4^+CXCR5^+ICOS^+CD25^{hi}$.

16. A method of modulating an immune response in a subject comprising administering to the subject, an effective amount of a composition comprising TFR cells having enhanced immune suppressive activity or a composition comprising TFR cells isolated from the peripheral blood of a subject and enriched for TFR cells.

17. The method of claim 16, wherein after administration of the composition, the ratio of TFR cells to TFH cells found in the peripheral blood of the subject is higher as compared to the ratio of TFR cells to TFH cells in the patient prior to the administration of the composition.

18. The method of claim 16, wherein an immune response in the subject is suppressed.

19. The method of claim 16, wherein the subject has a disease or disorder selected from graft versus host disease (GVHD), organ rejection, autoimmune disease, and side effects of gene therapy.

20. The method of claim 16, wherein the composition is administered to the subject intravenously.

21. A method of modulating an immune response in a subject comprising administering to the subject, an effective amount of a composition comprising TFR cells having enhanced immune suppressive activity; a composition comprising TFR cells isolated from the peripheral blood of a subject; a composition comprising TFH cells isolated from the peripheral blood of a subject, TFH cells having enhanced stimulatory capacity or any combination thereof.

22. The method of claim 21, wherein the method further comprises administering a vaccine to the subject.

23. The method of claim 22, comprising upregulating an immune response comprising co-administering a composition comprising TFH cells and a vaccine.

24. The method of claim 21, wherein the composition is administered intravenously.

25. An adjuvant comprising a composition of TFR cells having enhanced immune suppressive activity; a composition comprising TFR cells purified from the

peripheral blood of a subject; a composition comprising TFH cells purified from the peripheral blood of a subject, a composition comprising TFH cells having enhanced stimulatory capacity or any combination thereof.

26. The adjuvant of claim 25, comprising TFH cells isolated from the peripheral blood of a subject.

27. The adjuvant of claim 26, comprising a highly purified composition of TFH cells.

28. A composition comprising TFR cells isolated from the peripheral blood of a subject.

29. The composition of claim 28, wherein the composition is enriched for TFR cells.

30. The composition of claim 28, wherein the composition is a highly purified composition of TFR cells.

31. A method of downregulating an immune response in a subject comprising administering to the subject, an effective amount of a composition of claim 30.

32. The method of claim 31, wherein after administration of the composition, the ratio of TFR cells to TFH cells found in the peripheral blood of the subject is higher as compared to the ratio of TFR cells to TFH cells in the patient prior to the administration of the composition.

33. The method of claim 31, wherein TFH-mediated antibody response in the subject is suppressed.

34. The method of claim 31, wherein the subject has a disease or disorder selected from graft versus host disease (GVHD), organ rejection, autoimmune disease, and side effects of gene therapy.

35. The method of claim 31, wherein the composition is administered to the subject intravenously.

36. A composition comprising TFH cells isolated from the peripheral blood of a subject.

37. The composition of claim 36, wherein the composition is enriched for TFH cells.

38. The composition of claim 37, wherein the composition is a highly purified composition of TFH cells.

39. A method of upregulating an immune response in a subject comprising administering to the subject, an effective amount of a composition of claim 38.

40. The method of claim 39, wherein after administration of the composition, the ratio of TFH cells to TFR cells found in the peripheral blood of the subject is higher as compared to the ratio of TFH cells to TFR cells in the patient prior to the administration of the composition.

41. The method of claim 39, wherein TFH-mediated antibody response in the subject is upregulated.

42. The method of claim 39, wherein the composition is administered to the subject intravenously.

43. A composition comprising TFH cells having enhanced stimulatory activity.

44. A method of preparing a composition comprising TFH cells having enhanced stimulatory capacity comprising the steps of:

a) obtaining an initial population of cells comprising TFH cells;

b) activating the cells *ex vivo* in the presence of a PD-1 antagonist or PD-1 ligand antagonist, or both; and

c) isolating activated TFH cells from the population wherein the isolated TFH cells have enhanced immune stimulatory activity.

45. The method of claim 44, wherein the initial population of cells comprises cells isolated from the peripheral blood, tissues or organs of one or more subjects.

46. The method of claim 44, further comprising sorting TFH cells from the cell population prior to activation of the cell population or after activation of the cell population.

47. The method of claim 44, wherein the activation step comprises expanding the cell population.

48. The method of claim 44, wherein the activation step comprises contacting the cell population with a stimulatory composition.

49. The method of claim 44, wherein the PD-1 antagonist or PD-1 ligand antagonist is selected from: a PD1-ligand antibody, an anti-PD-1 antibody, a peptide, a small molecule or any combination thereof, wherein the antagonist inhibits the interaction between PD-1 and a PD-1 ligand.

50. The method of claim 44, wherein the isolating step comprises sorting TFH cells based on surface markers of CD4, CXCR5, ICOS positive; GITR negative, CD25 negative.

51. A method of modulating an immune response in a subject comprising administering to the subject, an effective amount of a composition comprising TFH cells having enhanced immune stimulatory capacity or a composition comprising TFH cells isolated from the peripheral blood of a subject.

52. The method of claim 51, wherein after administration of the composition, the ratio of TFH cells to TFR cells found in the peripheral blood of the subject is higher as compared to the ratio of TFH cells to TFR cells in the subject prior to the administration of the composition.

53. A composition comprising blood T follicular regulatory (TFR) cells having enhanced immune suppressive activity.

54. A method for assaying the TFR cell function, the TFH cell function or both, in a subject comprising the steps of:

a) obtaining a sample of peripheral blood from a patient;

b) isolating a population of TFH cells and TFR cells from the blood sample;

c) contacting the TFH cells and TFR cells with a stimulatory composition comprising B cells in the presence of anti-CD3 and anti-IgM for a time period sufficient to allow the production of IgG; and

d) measuring the total IgG produced.

55. The method of claim 7 or 54, wherein the isolating step comprises sorting TFR cells based on surface markers of $CD4^{+}CXCR5^{+}ICOS^{+}$ and at least one surface marker selected from: $GITR^{+}$, $CD25^{hi}$, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrin?7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

56. The method of claim 44 or 54, wherein the isolating step comprises sorting TFH cells based on surface markers of CD4, CXCR5, ICOS positive and at least one marker selected from CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and CD99 and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13,

CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrin?7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

57. A method of suppressing a pathogenic antibody response in a patient in need thereof comprising, administering to the patient, an agent which modulates at least one receptor which is differentially expressed on TFR cells as compared to TFH cells at an increased mean fluorescence intensity (MFI) fold change of at least 1.17, and wherein the receptor has an MFI of at least 186 on TFR cells and wherein the agent is effective to modulate the TFR receptor and increase TFR cell-mediated antibody suppression, as compared to the TFR cell-mediated antibody suppression in the absence of the agent.

58. The method of claim 57, wherein the at least one receptor is selected from: CD162, CD27, CD95, CD9, CD43, CD278 (ICOS), CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

59. The method of claim 57, wherein the agent is an agonist of at least one receptor.

60. The method of claim 57, wherein the agent is an antagonist of at least one receptor.

61. The method of claim 57, wherein the agent is an antibody capable of blocking at least one receptor.

62. The method of claim 57, wherein the agent is an antibody capable of binding to a ligand of at least one receptor and preventing the ligand from binding to the receptor.

63. The method of claim 57, wherein the agent is a small molecule, a dsRNA or an antisense molecule.

64. The method of claim 57, wherein the receptor is selected from one or more of: CD27, CD278 (ICOS), CD150, Siglec-9, CD140a, CD158b, and CD33.

65. A method of increasing a protective antibody response in a patient in need thereof comprising administering to the patient, an agent which modulates at least one receptor which is differentially expressed on TFH cells as compared to TFR cells at an increased mean fluorescence intensity (MFI) fold change of at least 1.17, and wherein the receptor has an MFI of at least 200 on TFH cells and wherein the agent is effective to modulate the receptor and increase the antibody response in the patient as compared to the antibody response when the agent is absent.

66. The method of claim 65, wherein the at least one receptor is selected from: CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and CD99.

67. The method of claim 65, wherein the agent is an agonist of at least one receptor.

68. The method of claim 65, wherein the agent is an antagonist of at least one receptor.

69. The method of claim 65, wherein the agent is an antibody capable of blocking at least one receptor.

70. The method of claim 65, wherein the agent is an antibody capable of binding to a ligand of at least one receptor and preventing the ligand from binding to the receptor.

71. The method of claim 65, wherein the agent is a small molecule, a dsRNA or an antisense molecule.

72. The method of claim 65, wherein the receptor is selected from one or more of: CD163, CD127, CD161, CD6, CD229, CD272, CD100, CD126, PD-1 (CD279).

73. The method of claim 57, wherein the patient has a disease or condition selected from the group consisting of multiple sclerosis, systemic lupus erythematosis, allergy, myasthenia gravis, collagen diseases, glomerulonephritis, Devic's disease, vasculitis caused by ANCA, and celiac disease.

74. A method of selectively modulating a TFR cell-mediated immune response in a patient in need thereof comprising administering to the patient, an agent which modulates a PD-1 receptor on a TFR cell.

75. The method of claim 74, wherein the agent is an antagonist of the PD-1 receptor on a TFR cell.

76. The method of claim 74, wherein the agent is an agonist of the PD-1 receptor on a TFR cell.

77. The method of claim 74, wherein the agent is an antibody capable of blocking the PD-1 receptor on a TFR cell.

78. The method of claim 74, wherein the agent is an antibody capable of binding to a ligand selected from PDL-1 or PDL-2 and preventing the ligand from binding to the PD-1 receptor.

79. The method of claim 74, wherein the agent is a small molecule, a dsRNA or an antisense molecule.

80. A method of suppressing a pathogenic antibody response in a patient in need thereof comprising administering to the patient, an agent which modulates the PD-1 receptor on a TFR cell wherein the agent is administered in an amount effective to modulate the TFR receptor and increase TFR cell-mediated antibody suppression as compared to the TFR cell-mediated antibody suppression in the absence of the agent.

81. The method of claim 80, wherein the patient has a disease or condition selected from the group consisting of multiple sclerosis, systemic lupus erythematosis, allergy, myasthenia gravis, collagen diseases, glomerulonephritis, Devic's disease, vasculitis caused by ANCA, and celiac disease.

82. The method of claim 80, wherein the agent is an antagonist of the PD-1 receptor.

83. A method of preparing a composition comprising TFR cells comprising the steps of:

a) obtaining an initial population of cells comprising T cells;

b) isolating TFR cells from the population wherein the TFR cells are sorted based on surface markers of $CD4^+CXCR5^+ICOS^+$ and at least one surface marker selected from: $GITR^+$, $CD25^{hi}$, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

84. The method of claim 83, wherein the initial population of cells is isolated from the peripheral blood, tissues or organs of one or more subjects.

85. The method of claim 83, wherein the TFR cells comprise about 90% or more of the total T cells present in the composition.

86. A method of preparing a composition comprising TFH cells comprising the steps of:

a) obtaining an initial population of cells comprising T cells; and

b) isolating TFH cells from the population wherein the TFH cells are sorted based on surface markers of CD4, CXCR5, ICOS positive and at least one marker selected from CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and CD99 and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

87. The method of claim 86, wherein the initial population of cells is isolated from the peripheral blood, tissues or organs of one or more subjects.

88. The method of claim 83, wherein the TFR cells comprise about 90% or more of the total T cells present in the composition.

89. A composition of TFR cells wherein about 90% or more of the cells in the composition are TFR cells.

90. The composition of claim 89, wherein about 99% or more of the cells in the compositions are TFR cells.

91. A composition of TFH cells wherein about 90% or more of the cells in the composition are TFH cells.

92. The composition of claim 91, wherein about 99% or more of the cells in the composition are TFH cells.

93. A method for assaying TFR cell function in a subject comprising the steps of:

a) obtaining a sample of mixed cells from a patient;

b) isolating a population of TFR cells from the sample;

c) contacting the TFR cells with a stimulatory composition comprising B cells in the presence of anti-CD3 and anti-IgM for a time period sufficient to allow the production of IgG; and

d) measuring the total IgG produced.

94. The method of claim 93, wherein the isolating step comprises sorting TFR cells based on surface markers of $CD4^{+}CXCR5^{+}ICOS$ and at least one surface marker selected from: $GITR^{+}$, $CD25^{hi}$, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

95. A method for assaying the TFH cell function in a subject comprising the steps of:

a) obtaining a sample of mixed cells from a patient;

b) Isolating a population of TFH cells from the sample;

c) Contacting the TFH cells with a stimulatory composition comprising B cells in the presence of anti-CD3 and anti-IgM for a time period sufficient to allow the production of IgG; and

d) measuring the total IgG produced.

96. The method of claim 95, wherein the isolating step comprises sorting TFH cells based on surface markers of CD4, CXCR5, ICOS+and at least one marker selected from CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96,

CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

97. A method of identifying TFR cells in a population of mixed cells comprising the step of sorting the TFR cells based on surface markers of $CD4^{+}CXCR5^{+}ICOS^{+}$ and at least one surface marker selected from: $GITR^{+}$, $CD25^{hi}$, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

98. A method of identifying TFH cells in a population of mixed cells comprising the step of sorting the TFH cells based on surface markers of CD4, CXCR5, ICOS positive and at least one marker selected from CD163, CD127, CD8a, CD89, CD197, CD161, CD6, CD229, CD96, CD272, CD148, CD107a, CD100, CD82, CD126, CD45RO, CD279, CD5, and CD99 and optionally wherein the TFH cells are negative for one or more of the following receptors GITR, CD25, CD162, CD27, CD95, CD9, CD43, CD50, CD45RB, CD102, CD61, CD58, CD196, CD38, CD31, CD15, CD25, CD13, CD66a/c/e, CD11b CD63, CD32, CD97, HLA-HQ, CD150, Siglec-9, Integrinβ7, CD71, CD180, CD218a, CD193, CD235ab, CD35, CD140a, CD158b, CD33, CD210, HLA-G, CD167a, CD119, CX3CR1, CD146, HLA-DR, CD85, CD172b, SSEA-1, CD49c, CD170, CD66b, and CD86.

99. A method of decreasing a pathogenic antibody response in a patient in need thereof comprising administering to the patient a first agent capable of modulating a selective TFR receptor in an amount effective to increase TFR cell-mediated

antibody suppression in the patient, in combination with an second agent capable of modulating a selective TFH receptor in an amount effective to decrease TFH cell-mediated antibody production, wherein the pathogenic antibody response is decreased as compared to the pathogenic antibody response in the absence of the first or second agents.

100. A method of increasing a protective antibody response in a patient in need thereof comprising administering to the patient a first agent capable of modulating a selective TFR receptor in an amount effective to decrease TFR cell-mediated antibody suppression in the patient, in combination with a second agent capable of modulating a selective TFH receptor in an amount effective to increase TFH cell-mediated antibody production, wherein the protective antibody response is increased as compared to the protective antibody response in the absence of the first or second agents.

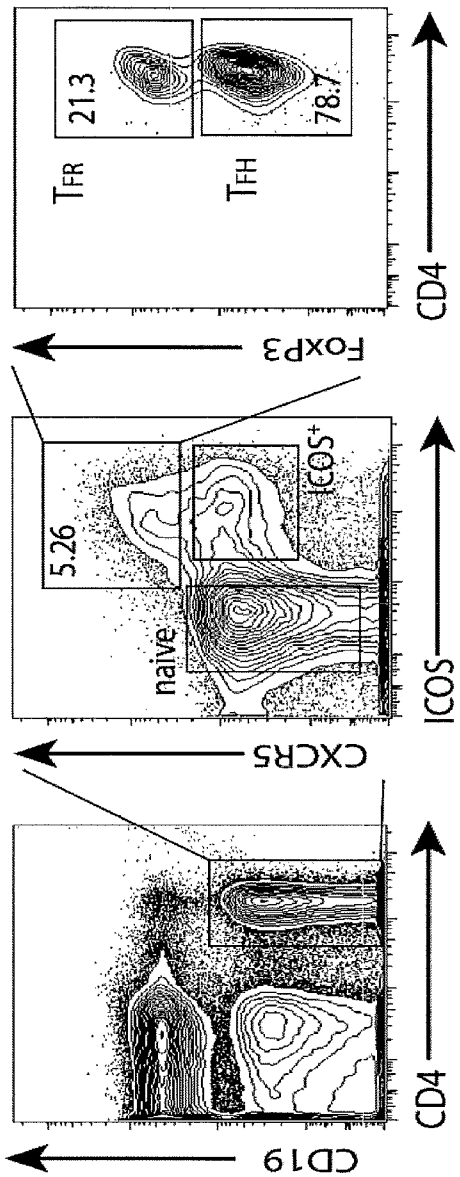


FIG. 1a

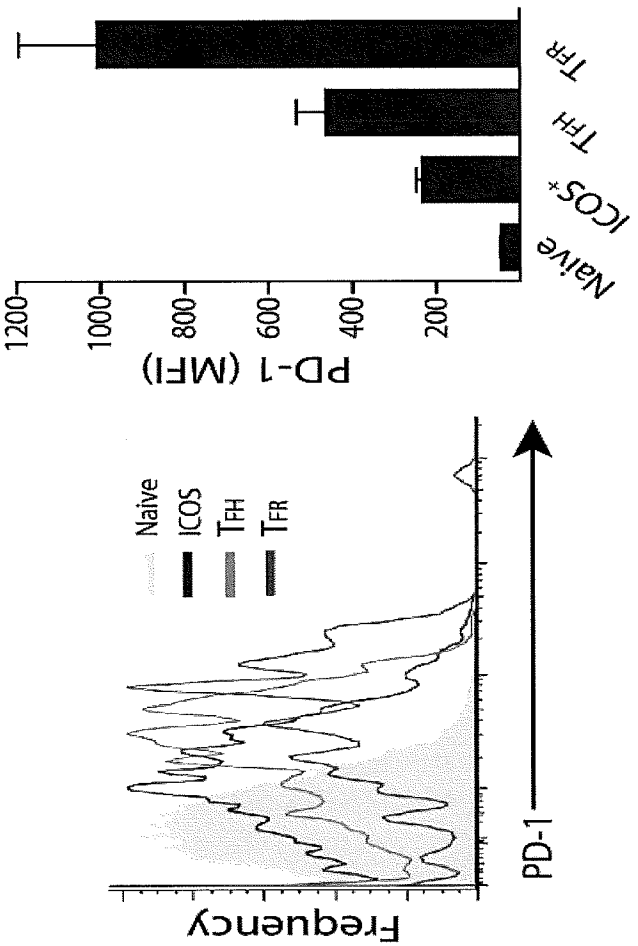

PD-1 (MFI)
1200
1000
800
600
400
200
Naive
ICOS+
TFH
TFR
Frequency
PD-1
Naive
ICOS
TFH
TFR

FIG. 1b

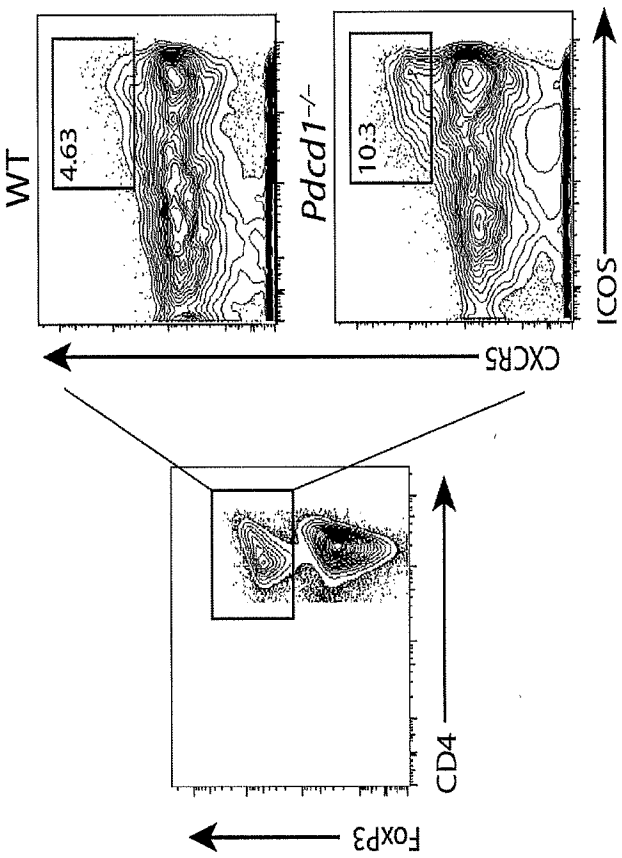


FIG. 1c

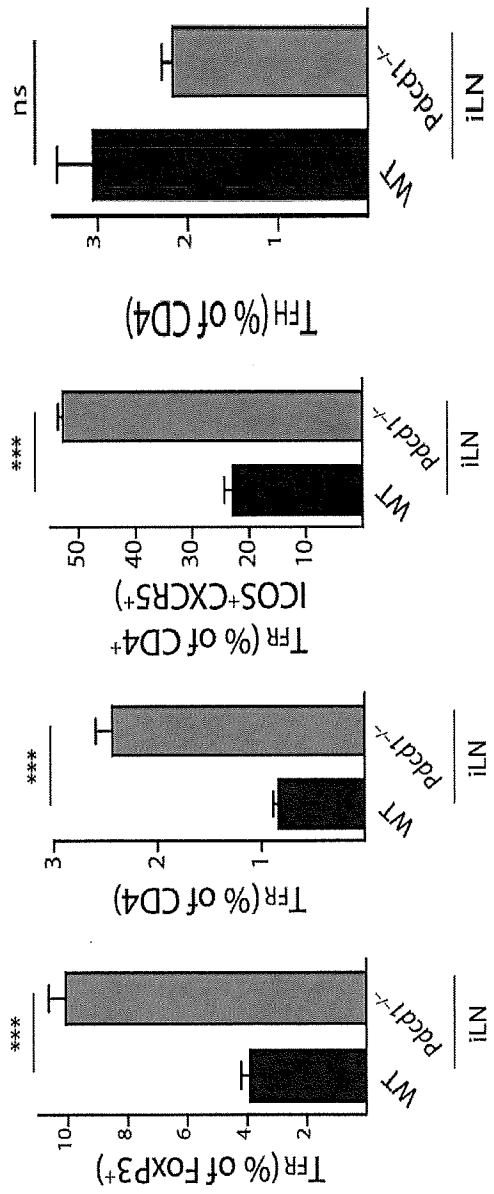

TFR (% of FoxP3+)
TFR (% of CD4)
TFR (% of CD4+ ICOS+CXCR5+)
TFH (% of CD4)
WT
Pdcd1-/-
iLN
***
ns

FIG. 1d

FIG. 1e

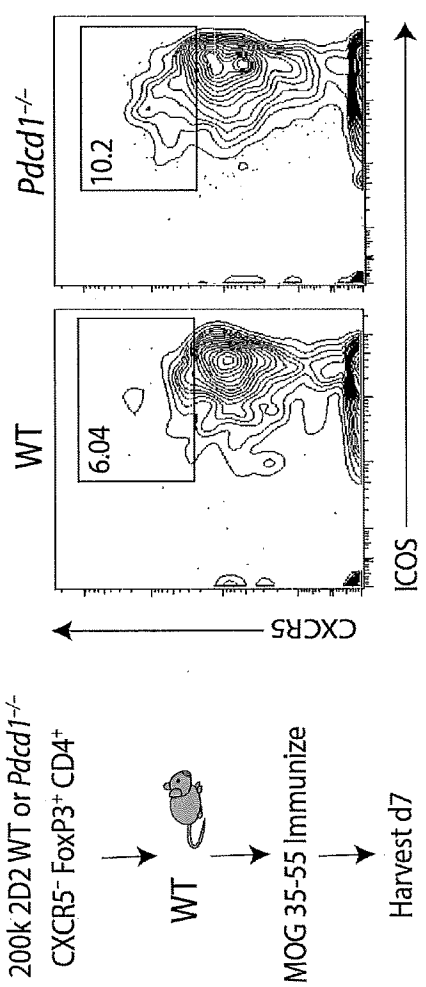

200k 2D2 WT or Pdcd1-/-
CXCR5- FoxP3+ CD4+
WT
MOG 35-55 Immunize
Harvest d7
Gated on CD4+FoxP3.GFP+
WT
Pdcd1-/-
6.04
10.2
CXCR5
ICOS

FIG. 1f

FIG. 1g

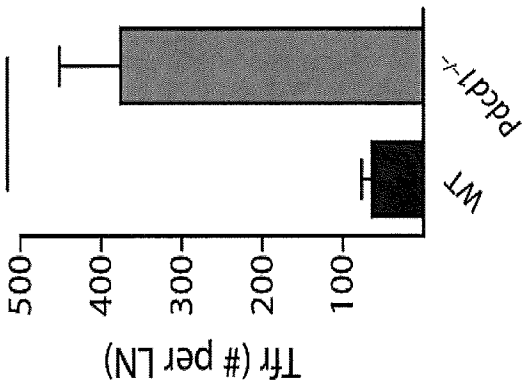

Tfr (# per LN)
500
400
300
200
100
**
WT
pdcd1-/-

FIG. 1i

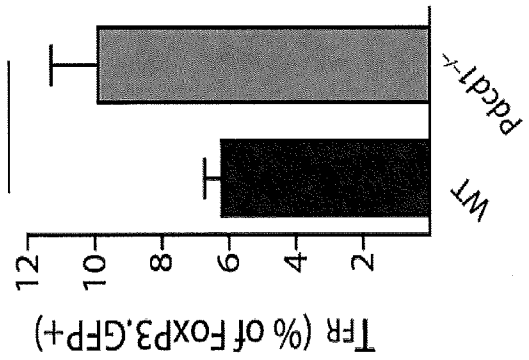

TFR (% of FoxP3.GFP+)
12
10
8
6
4
2
*
WT
pdcd1-/-

FIG. 1h

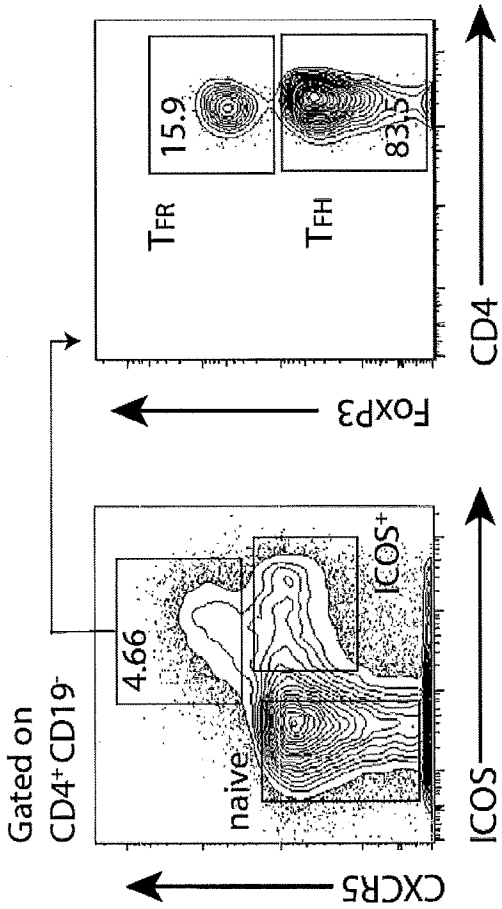

Gated on
CD4+CD19-
naive
4.66
ICOS+
CXCR5
ICOS
TFR
15.9
TFH
83.5
FoxP3
CD4

FIG. 2a

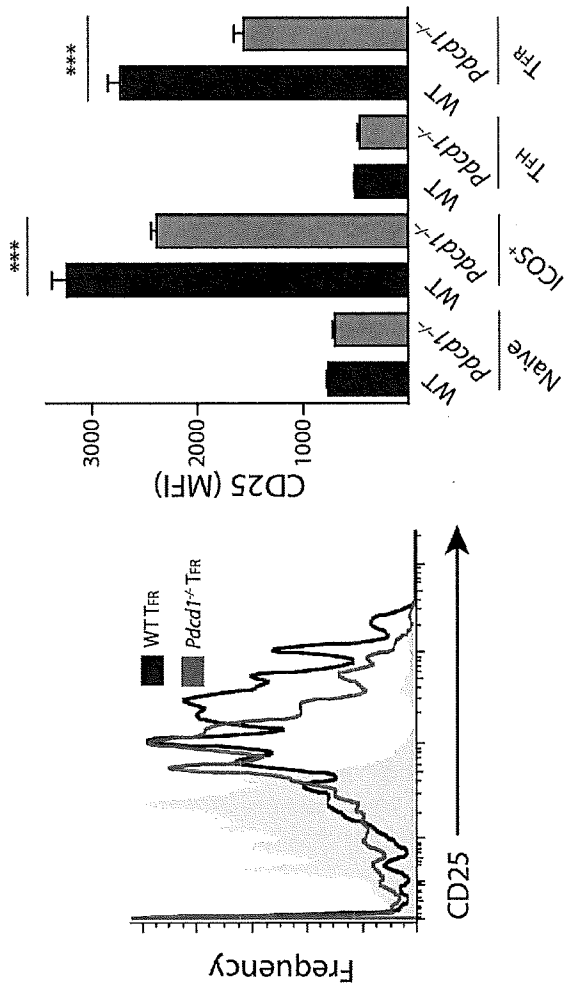

CD25 (MFI)
3000
2000
1000
***
***
Naive
ICOS+
TFH
TFR
WT
Pdcd1-/-
WT
Pdcd1-/-
WT
Pdcd1-/-
WT
Pdcd1-/-
WT TFR
Pdcd1-/- TFR
Frequency
CD25

FIG. 2b

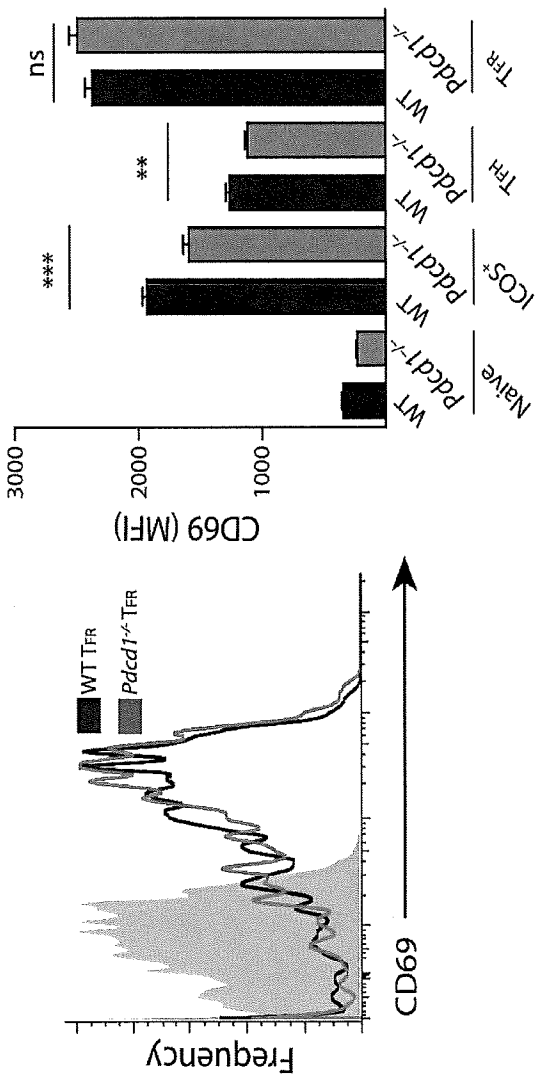

CD69 (MFI)
3000
2000
1000
Naive
ICOS+
TFH
TFR
WT
Pdcd1-/-
***
**
ns
WT TFR
Pdcd1-/- TFR
Frequency
CD69

FIG. 2c

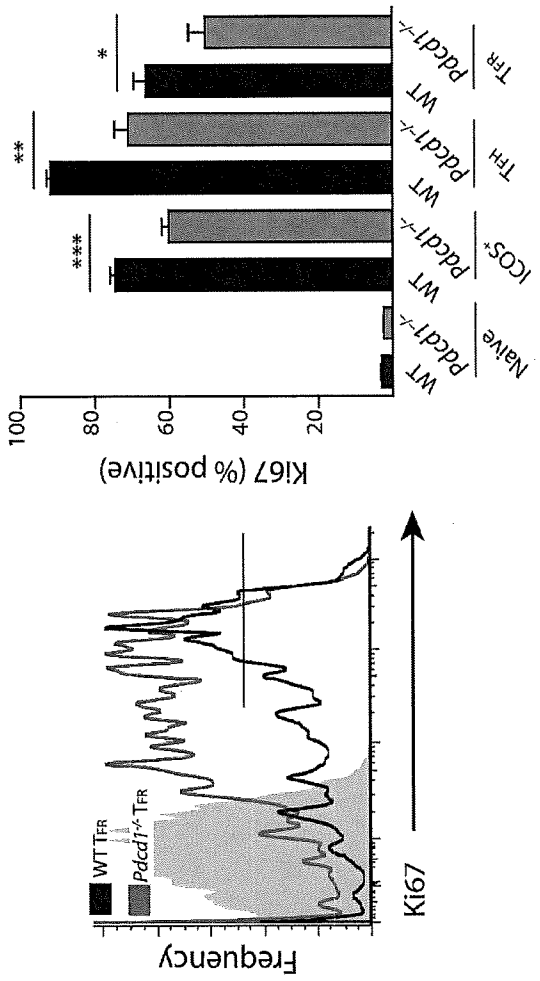

Ki67 (% positive)
100
80
60
40
20
Naive
ICOS+
TFH
TFR
WT
Pdcd1-/-
***
**
*
Frequency
WT TFR
Pdcd1-/- TFR
Ki67

FIG. 2d

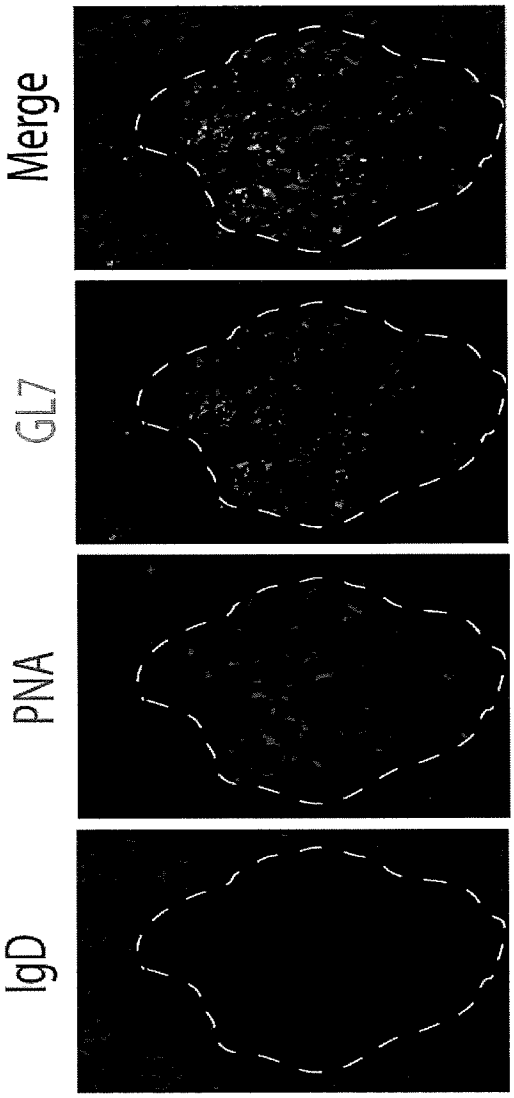


FIG. 3a

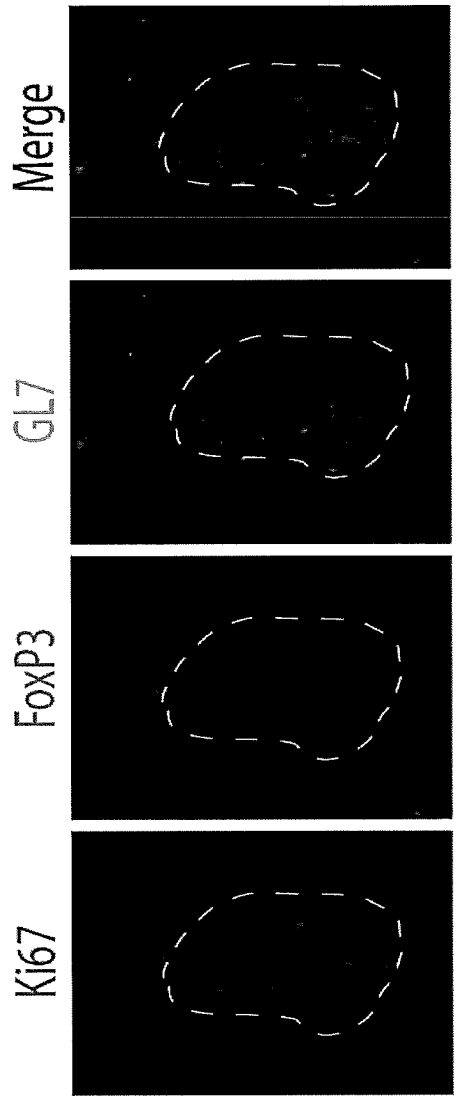


FIG. 3b

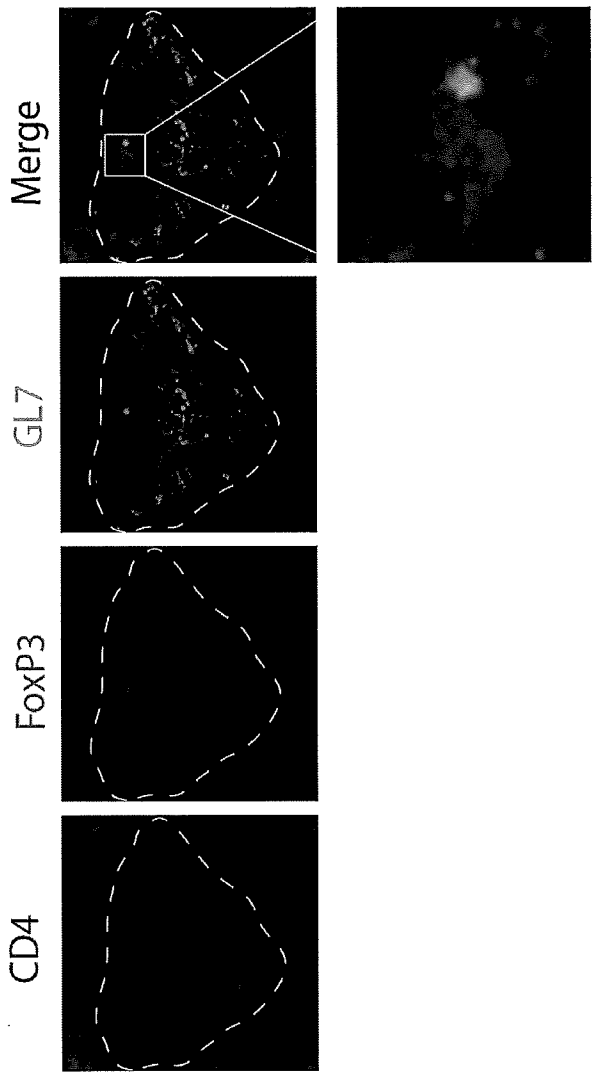


FIG. 3c

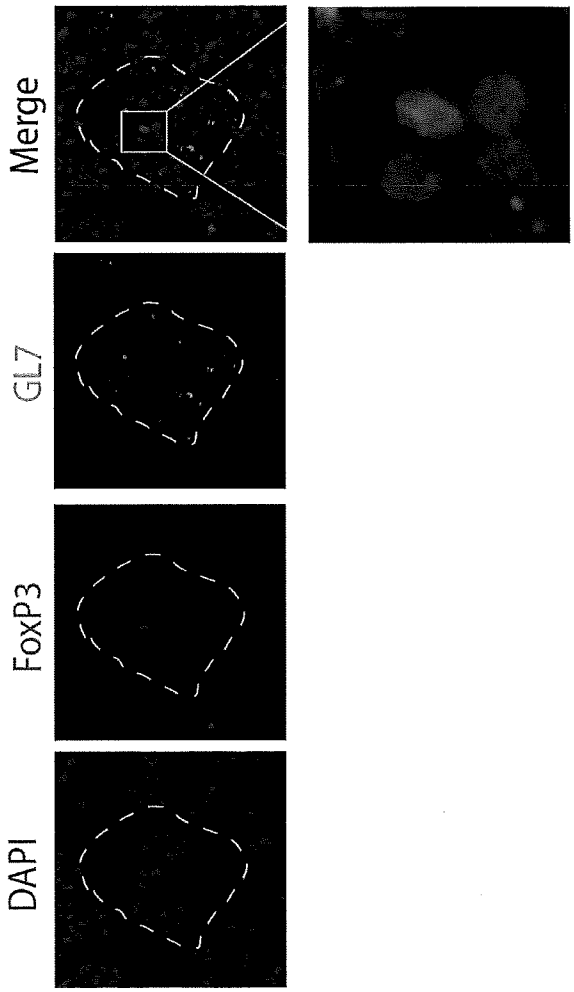


FIG. 3d

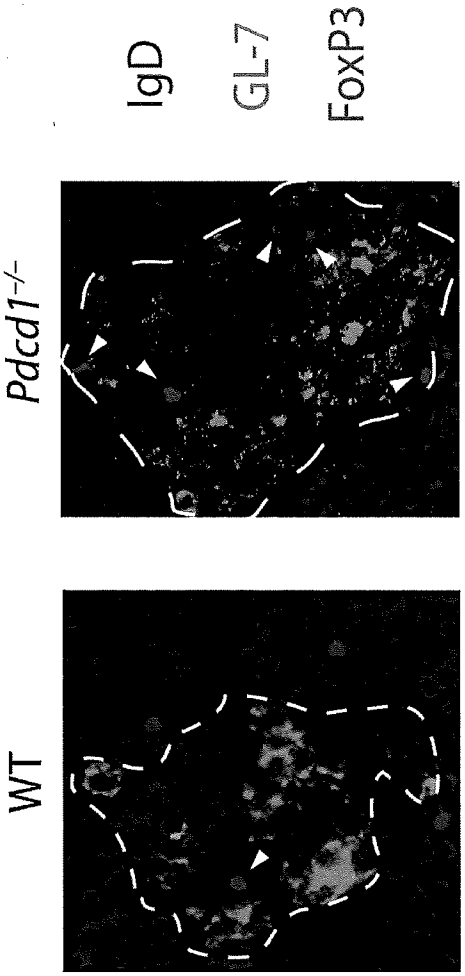


FIG. 3e

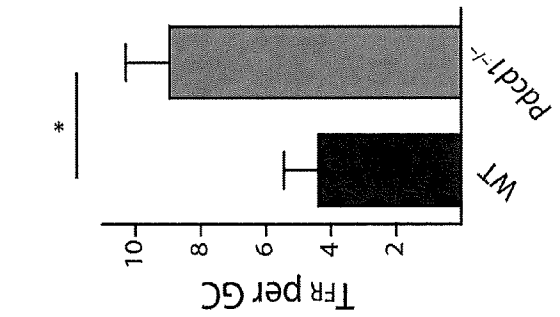

TFR per GC
10
8
6
4
2
*
WT
Pdcd1-/-

FIG. 3g

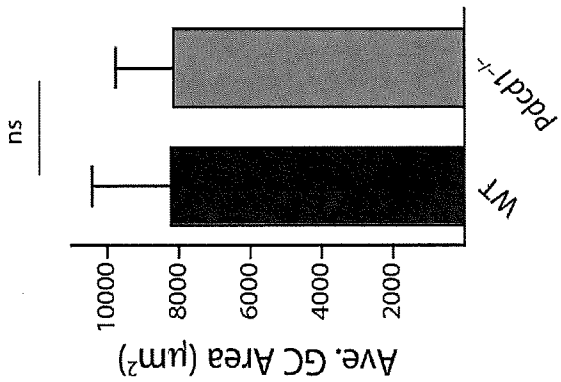

Ave. GC Area (μm²)
10000
8000
6000
4000
2000
ns
WT
Pdcd1-/-

FIG. 3f

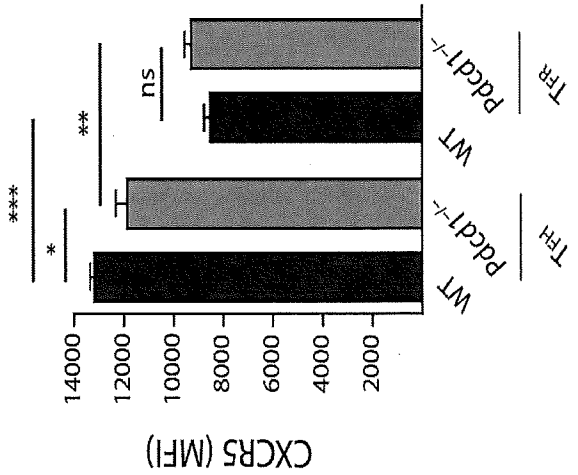

CXCR5 (MFI)
14000
12000
10000
8000
6000
4000
2000
***
*
**
ns
WT
Pdcd1-/-
WT
Pdcd1-/-
TFH
TFR

FIG. 3i

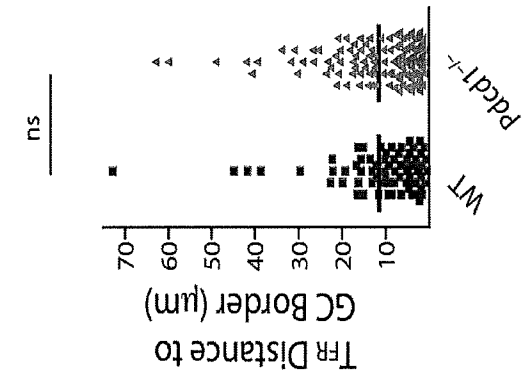

TFR Distance to
GC Border (μm)
70
60
50
40
30
20
10
ns
WT
Pdcd1-/-

FIG. 3h

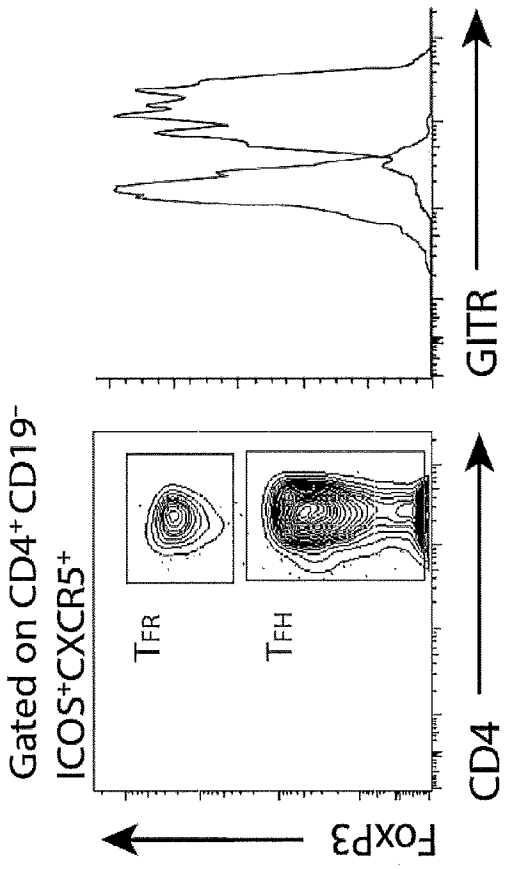


FIG. 4a

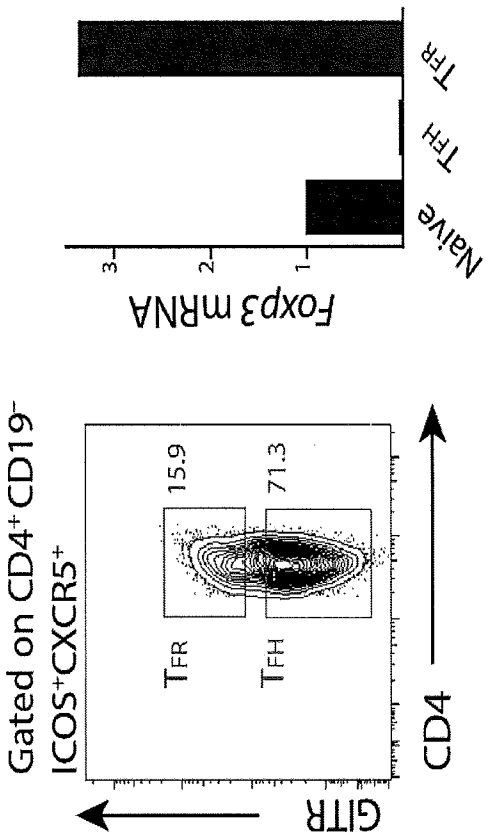

Foxp3 mRNA
3
2
1
Naive
TFH
TFR
Gated on CD4+ CD19-
ICOS+CXCR5+
15.9
71.3
TFR
TFH
GITR
CD4

FIG. 4b

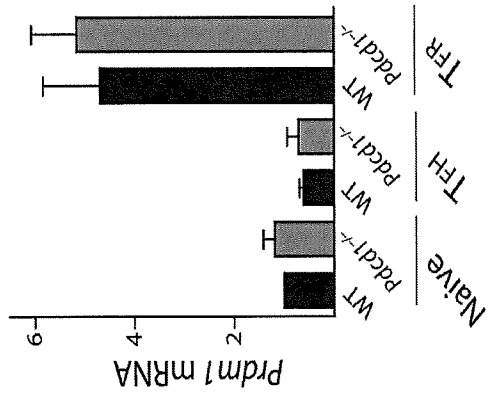

Prdm1 mRNA
6
4
2
Naive
TFH
TFR
WT
Pdcd1-/-

FIG. 4d

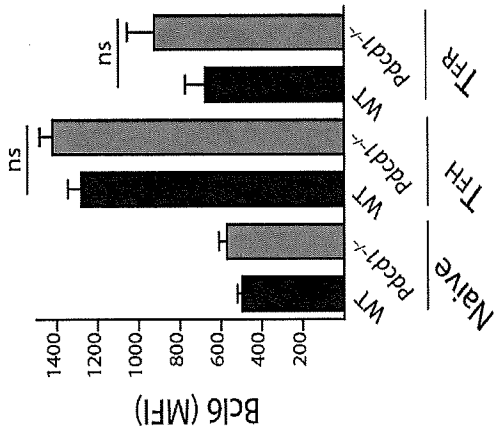

Bcl6 (MFI)
1400
1200
1000
800
600
400
200
ns
ns
Naive
TFH
TFR
WT
Pdcd1-/-

FIG. 4c

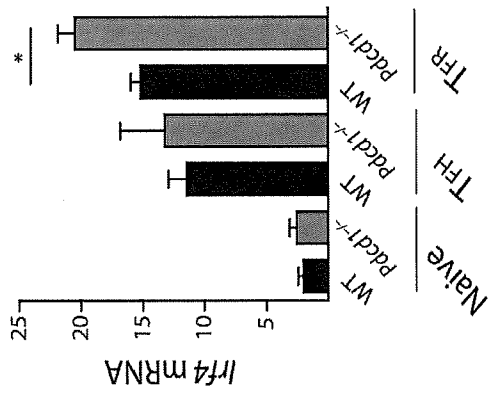

Irf4 mRNA
25
20
15
10
5
*
Naive
TFH
TFR
WT
Pdcd1-/-

FIG. 4f

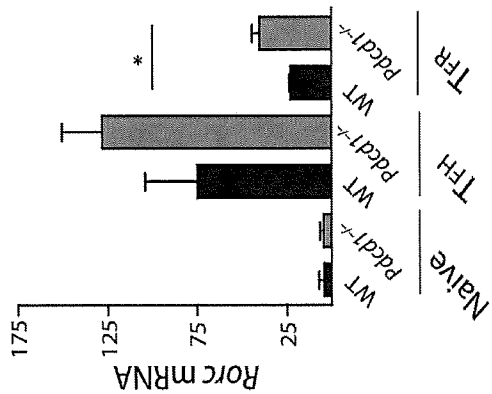

Rorc mRNA
175
125
75
25
*
Naive
TFH
TFR
WT
Pdcd1-/-

FIG. 4e

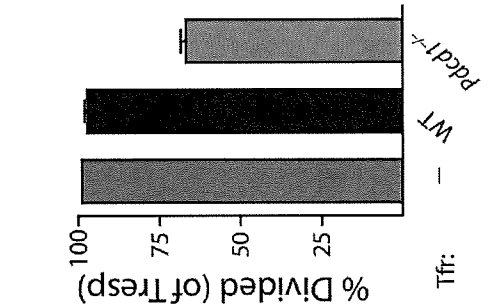


FIG. 4i

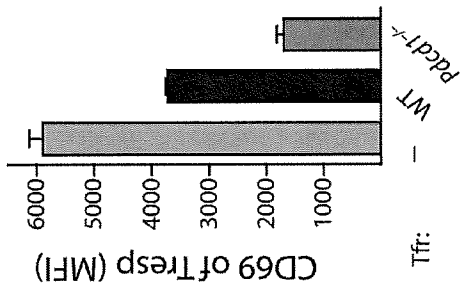


FIG. 4h

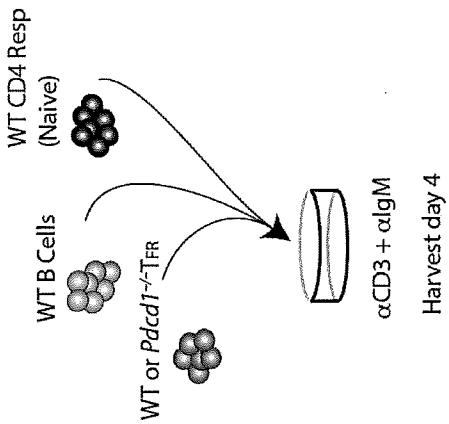


FIG. 4g

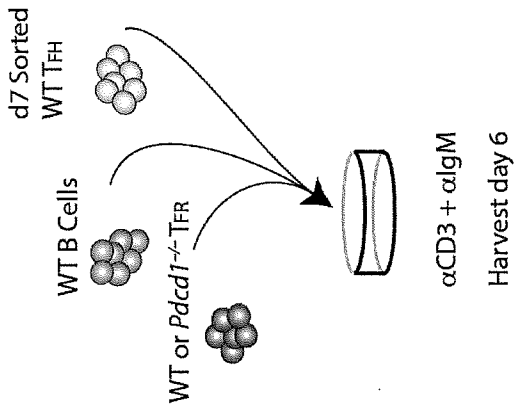

d7 Sorted WT TFH
WT B Cells
WT or Pdcd1-/- TFR
αCD3 + αIgM
Harvest day 6

FIG. 4j

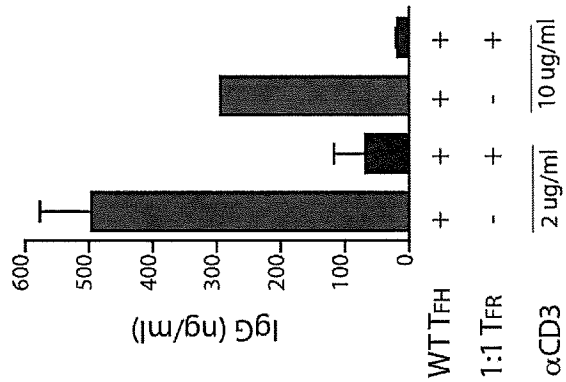

IgG (ng/ml)
600
500
400
300
200
100
0
WT TFH + + + +
1:1 TFR - + - +
αCD3 2 ug/ml 10 ug/ml

FIG. 4k

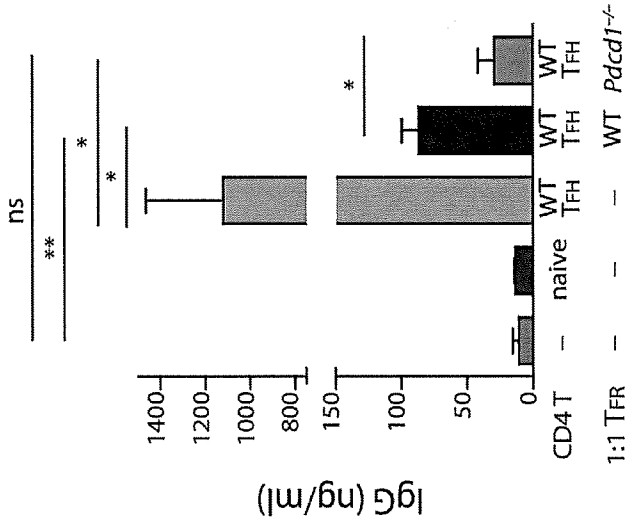

IgG (ng/ml)
1400
1200
1000
800
150
100
50
0
ns
**
*
*
*
CD4 T
–
naive
WT TFH
–
WT TFH
Pdcd1-/-
WT TFH
1:1 TFR
–
–
–
WT
WT TFH

FIG. 4l

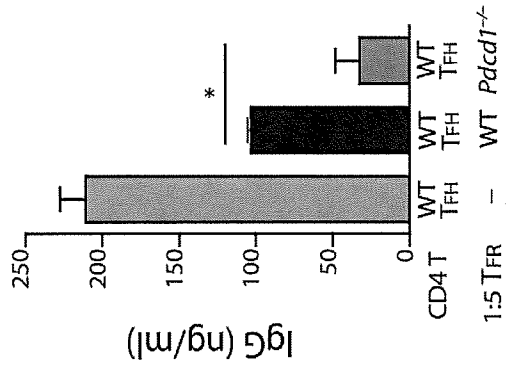

IgG (ng/ml)
250
200
150
100
50
0
*
CD4 T
WT TFH
WT TFH
WT
Pdcd1-/-
WT TFH
1:5 TFR
–
–
WT TFH

FIG. 4m

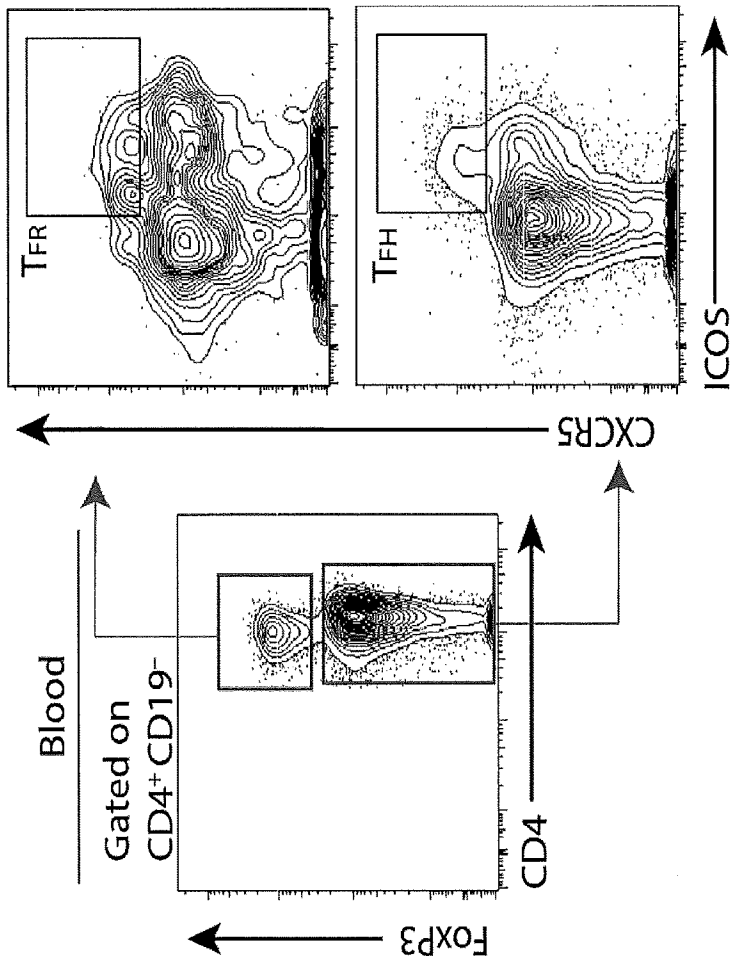


FIG. 5a

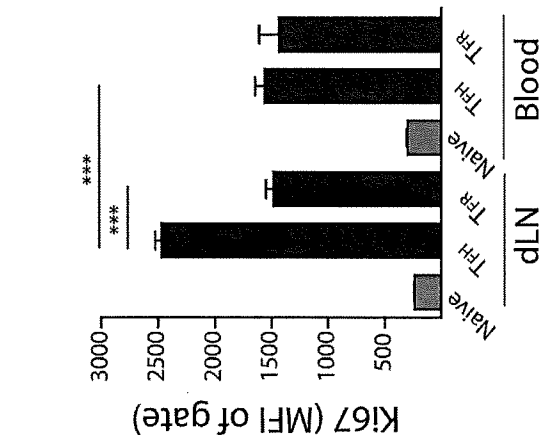

Ki67 (MFI of gate)
3000
2500
2000
1500
1000
500
***
***
Naive
TFH
TFR
Naive
TFH
TFR
dLN
Blood

FIG. 5c

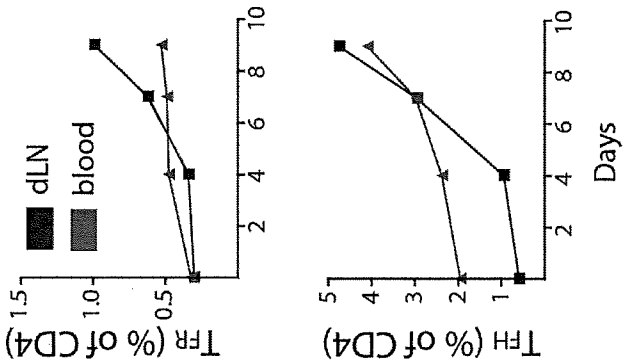

TFR (% of CD4)
1.5
1.0
0.5
dLN
blood
2
4
6
8
10
TFH (% of CD4)
5
4
3
2
1
Days

FIG. 5b

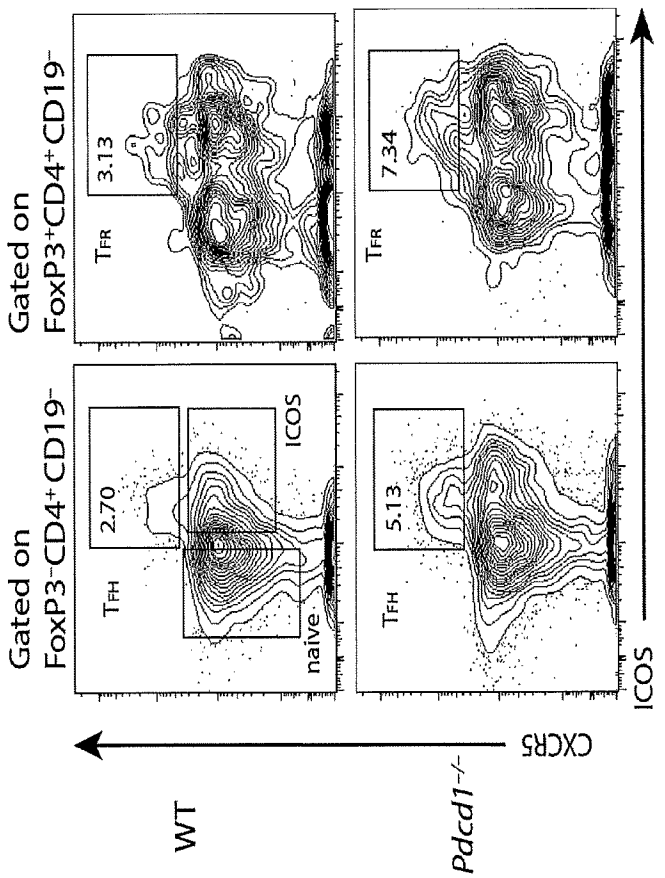

Gated on
FoxP3⁻CD4⁺CD19⁻
Gated on
FoxP3⁺CD4⁺CD19⁻
TFH
TFR
2.70
3.13
5.13
7.34
naive
ICOS
CXCR5
WT
Pdcd1⁻/⁻

FIG. 5d


TFH (% of CD4)
4
3
2
1
***
WT
Pdcd1-/-
TFR (% of CD4)
1.0
0.8
0.6
0.4
0.2
**
WT
Pdcd1-/-
FoxP3+ (%)
25
20
15
10
5
ns
ns
WT
Pdcd1-/-
WT
Pdcd1-/-
Gate:
Naive
ICOS

FIG. 5e

FIG. 5f

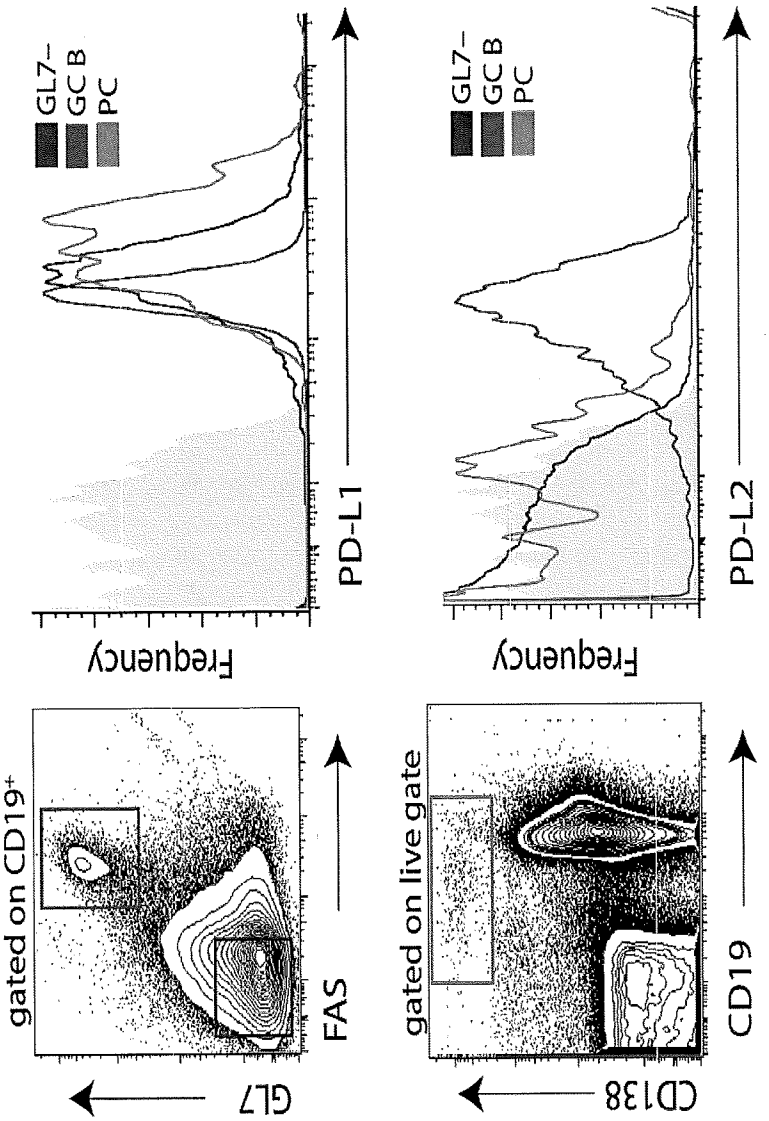

GL7
FAS
gated on CD19+
Frequency
GL7-
GC B
PC
PD-L1
CD138
CD19
gated on live gate
Frequency
GL7-
GC B
PC
PD-L2

FIG. 6a

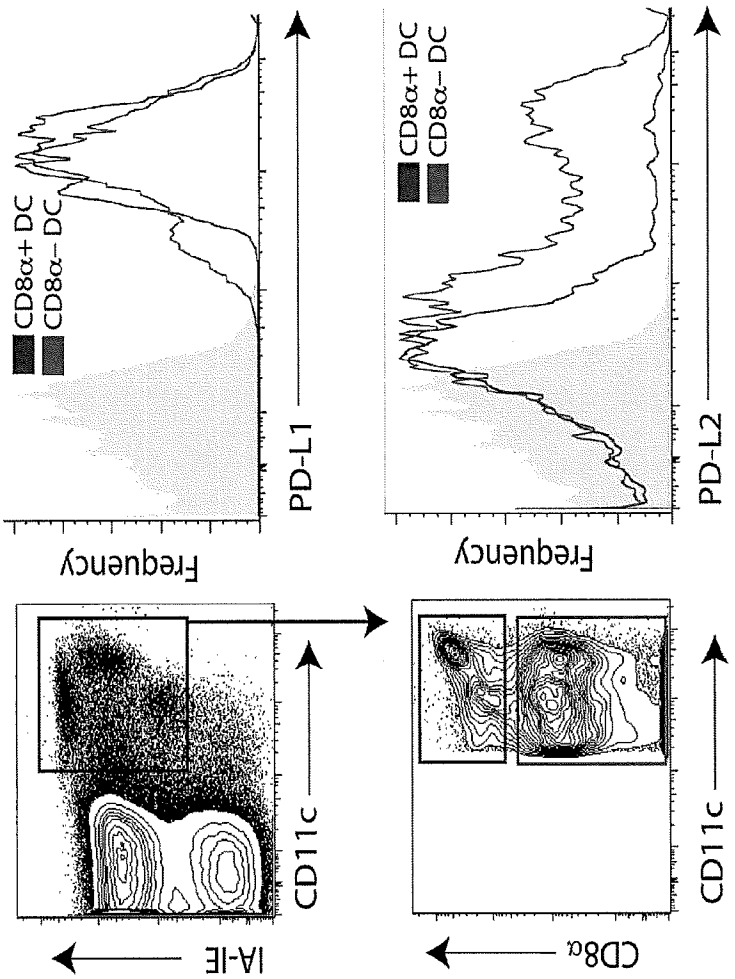

CD8α+ DC
CD8α− DC
Frequency
PD-L1
CD8α+ DC
CD8α− DC
Frequency
PD-L2
IA-IE
CD11c
CD8α
CD11c

FIG. 6b

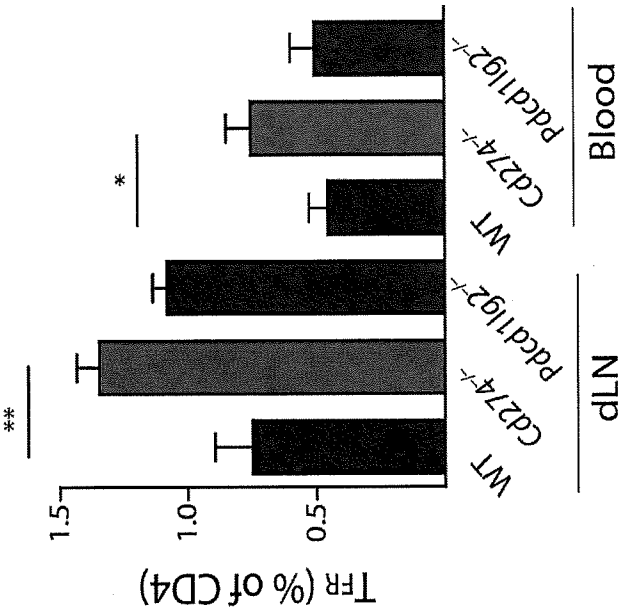

TFR (% of CD4)
1.5
1.0
0.5
**
*
WT
Cd274-/-
Pdcd1lg2-/-
WT
Cd274-/-
Pdcd1lg2-/-
dLN
Blood

FIG. 6d

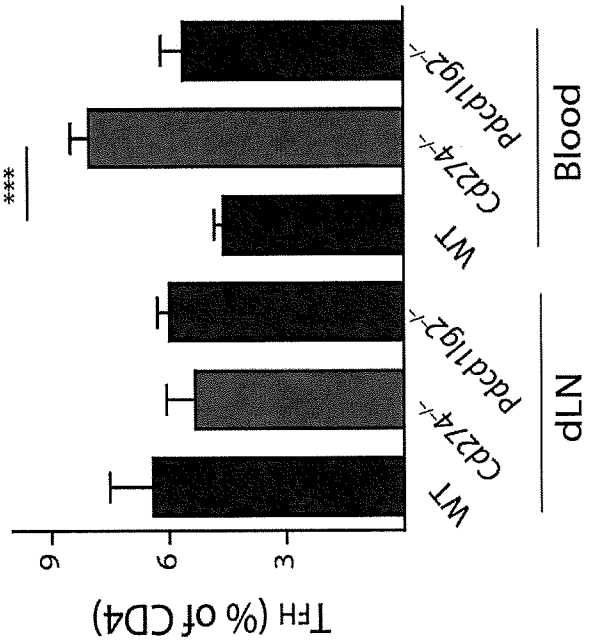

TFH (% of CD4)
9
6
3
***
WT
Cd274-/-
Pdcd1lg2-/-
WT
Cd274-/-
Pdcd1lg2-/-
dLN
Blood

FIG. 6c

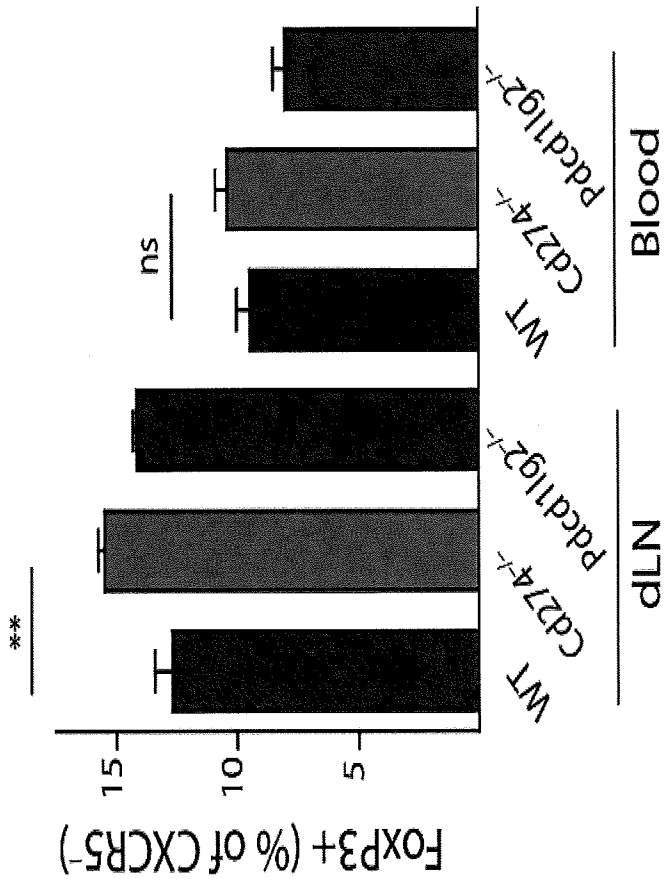

FoxP3+ (% of CXCR5-)
15
10
5
**
ns
WT
Cd27-/-
pdcd1lg2-/-
WT
Cd27-/-
pdcd1lg2-/-
dLN
Blood

FIG. 6e

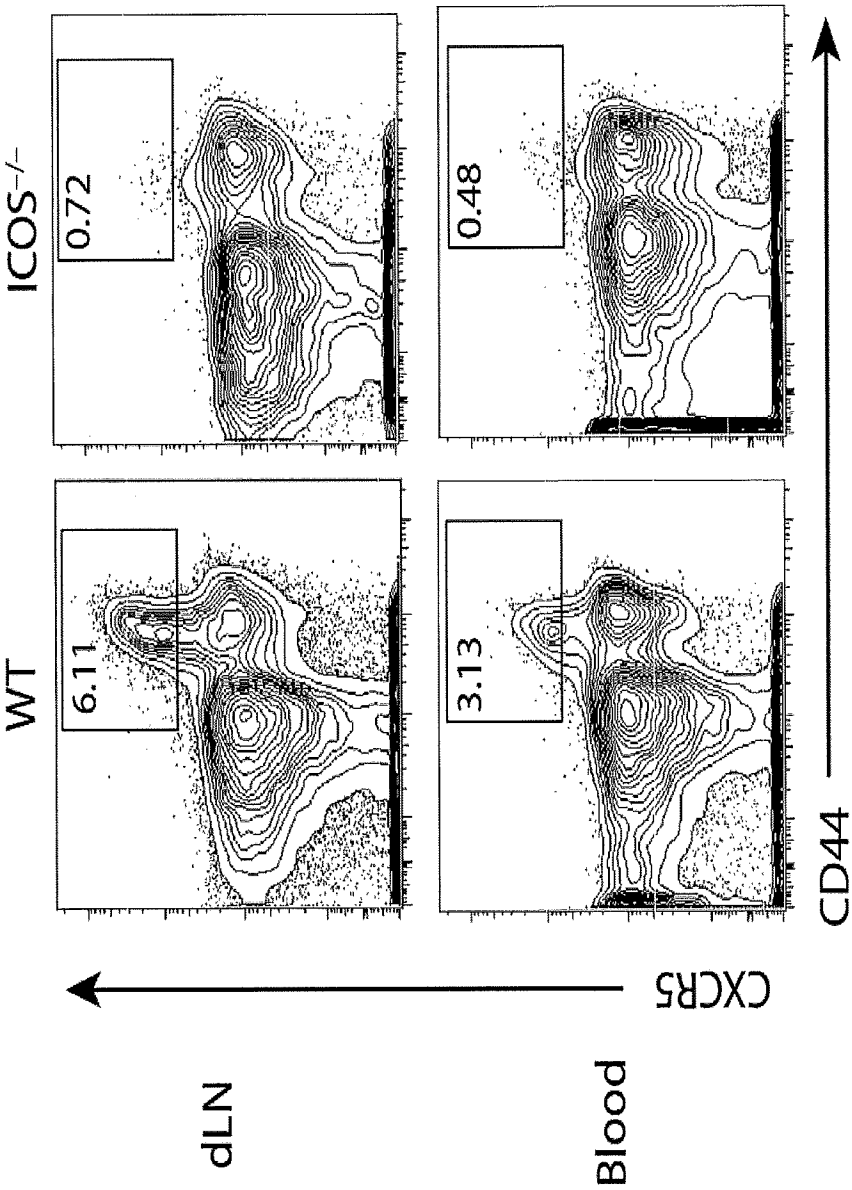


FIG. 7a

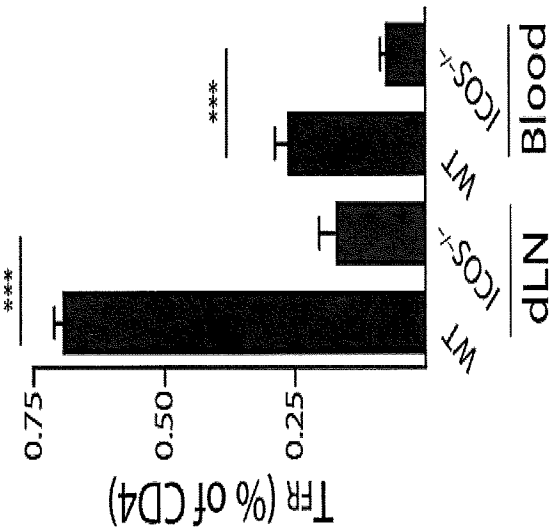

TFR (% of CD4)
0.75
0.50
0.25
***
***
WT
ICOS-/-
WT
ICOS-/-
dLN
Blood

FIG. 7c

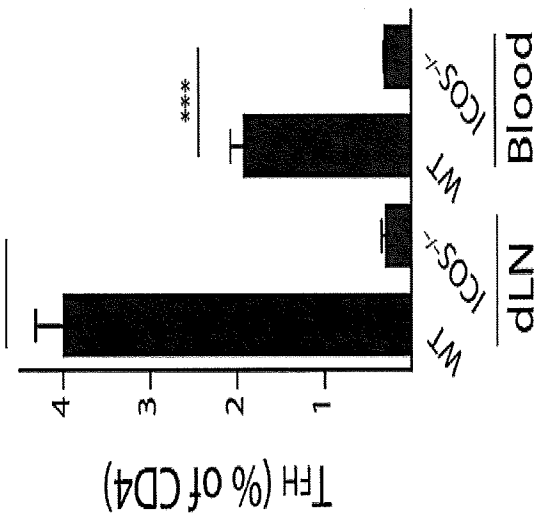

TFH (% of CD4)
4
3
2
1
***
***
WT
ICOS-/-
WT
ICOS-/-
dLN
Blood

FIG. 7b

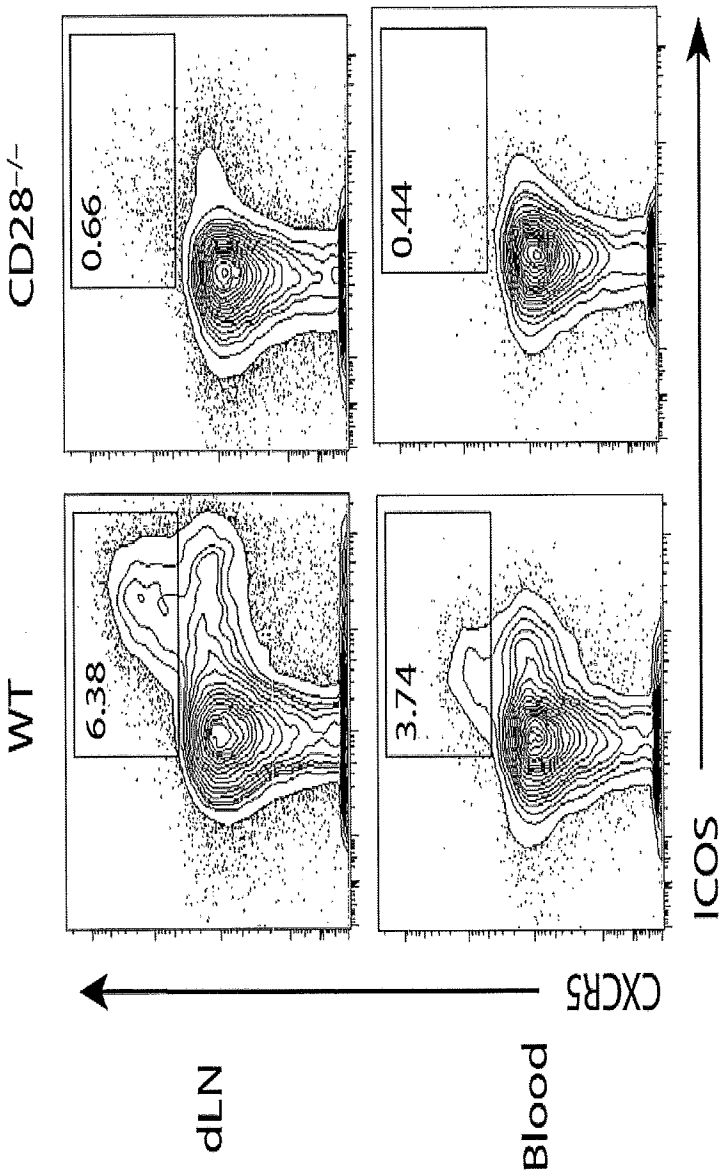


FIG. 7d

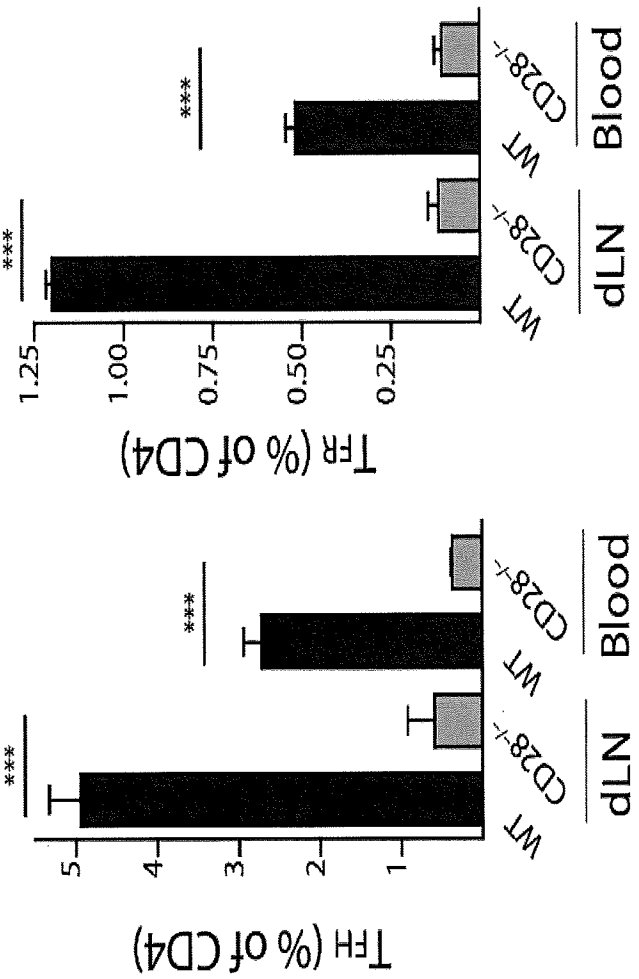

TFR (% of CD4)
1.25
1.00
0.75
0.50
0.25
***
***
WT
CD28-/-
WT
CD28-/-
dLN
Blood

FIG 7f

TFH (% of CD4)
5
4
3
2
1
***
***
WT
CD28-/-
WT
CD28-/-
dLN
Blood

FIG. 7e

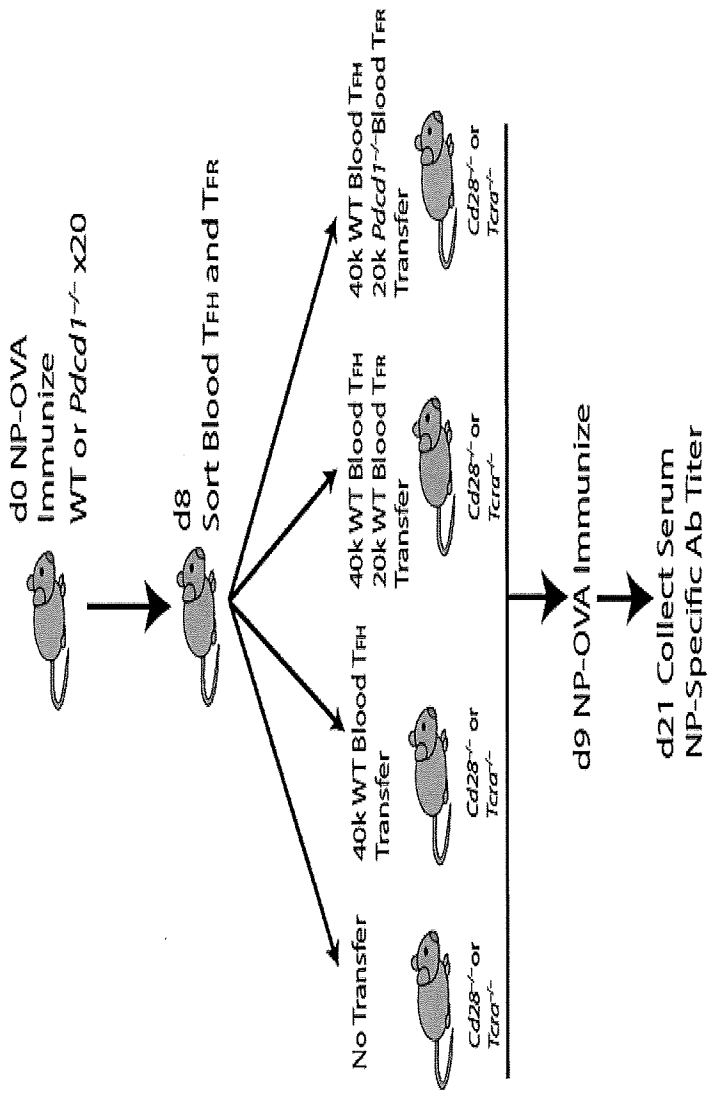

d0 NP-OVA
Immunize
WT or Pdcd1−/− x20
d8
Sort Blood TFH and TFR
No Transfer
40k WT Blood TFH
Transfer
40k WT Blood TFH
20k WT Blood TFR
Transfer
40k WT Blood TFH
20k Pdcd1−/− Blood TFR
Transfer
Cd28−/− or
Tcra−/−
d9 NP-OVA Immunize
d21 Collect Serum
NP-Specific Ab Titer

FIG. 8a

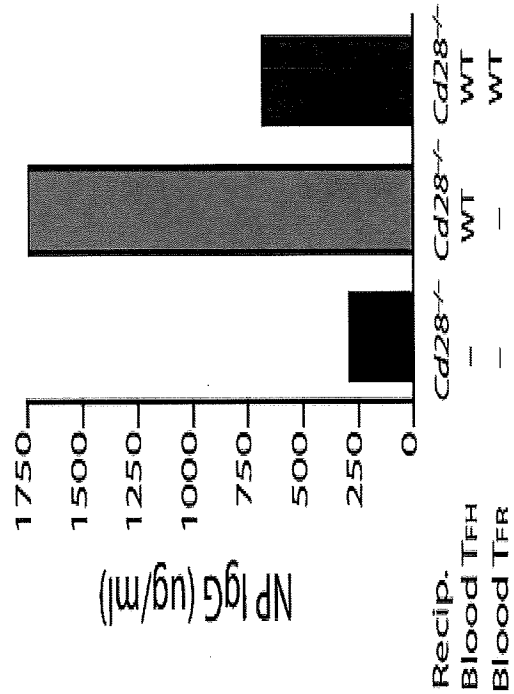

NP IgG (ug/ml)
1750
1500
1250
1000
750
500
250
0
Recip.
Blood TFH
Blood TFR
Cd28-/-
—
—
Cd28-/-
WT
—
Cd28-/-
WT
WT

FIG 8b

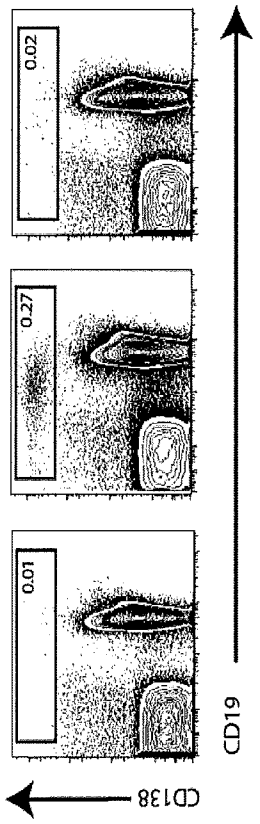

Control
Blood TFH
Blood TFH + TFR
0.01
0.27
0.02
CD138
CD19

FIG. 8c

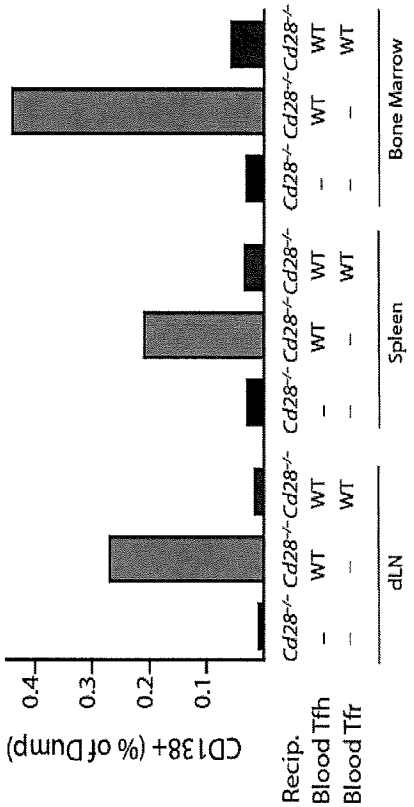

CD138+ (% of Dump)
0.4
0.3
0.2
0.1
Recip.
Blood Tfh
Blood Tfr
Cd28-/-
Cd28-/- WT
Cd28-/-
–
WT
dLN
Spleen
Bone Marrow

FIG. 8d

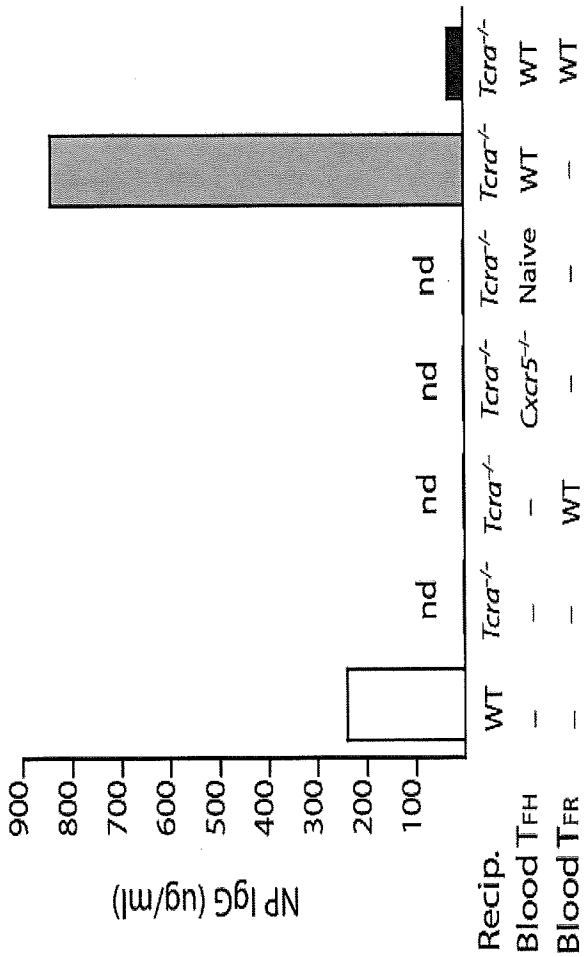

NP IgG (ug/ml)
900
800
700
600
500
400
300
200
100
nd
nd
nd
nd
Recip.
Blood TFH
Blood TFR
WT
Tcra-/-
Tcra-/-
Tcra-/-
Tcra-/-
Tcra-/-
Tcra-/-
Tcra-/-
–
–
–
–
–
–
–
–
–
WT
Cxcr5-/-
Naive
WT
WT
–
–
–
–
–
–
–
WT
WT
–

FIG. 8e

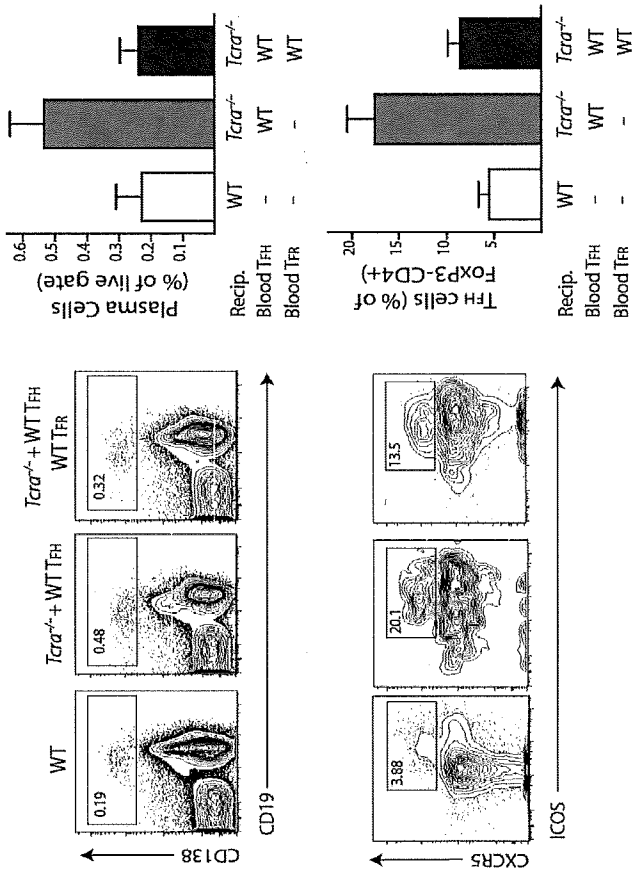

WT
Tcra-/- + WT TFH
Tcra-/- + WT TFH WT TFR
0.19
0.48
0.32
CD138
CD19
Plasma Cells (% of live gate)
0.6
0.5
0.4
0.3
0.2
0.1
Recip.
Blood TFH
Blood TFR
WT
Tcra-/-
TFH cells (% of FoxP3-CD4+)
20
15
10
5
3.88
20.1
13.5
CXCR5
ICOS

FIG. 8f

FIG. 8g

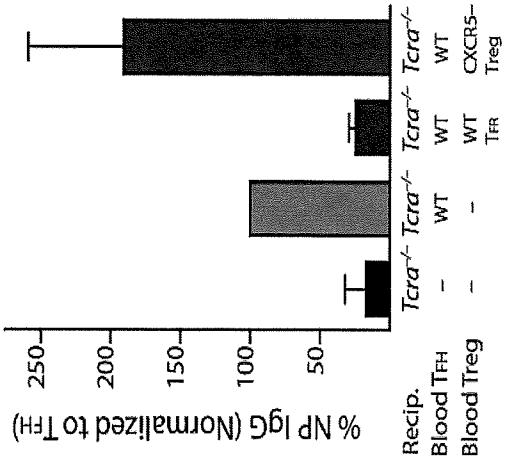

% NP IgG (Normalized to TFH)
250
200
150
100
50
Recip.
Blood TFH
Blood Treg
Tcra-/-
–
–
Tcra-/-
WT
–
Tcra-/-
WT
WT
Tcra-/-
WT
CXCR5–
Treg

FIG. 8i

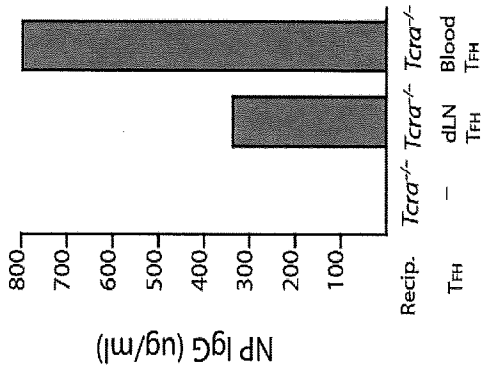

NP IgG (ug/ml)
800
700
600
500
400
300
200
100
Recip.
TFH
Tcra-/-
–
Tcra-/-
dLN TFH
Tcra-/-
Blood TFH

FIG. 8h

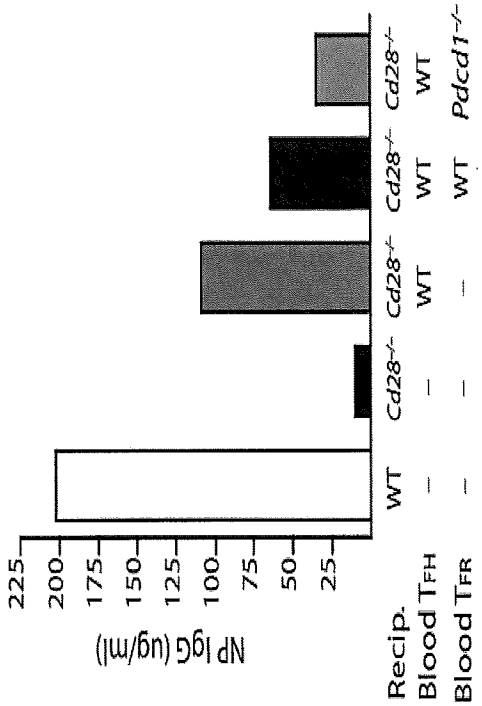

NP IgG (ug/ml)
225
200
175
150
125
100
75
50
25
Recip.
Blood TFH
Blood TFR
WT
—
—
Cd28-/-
—
—
Cd28-/-
WT
—
Cd28-/-
WT
WT
Cd28-/-
WT
WT
Pdcd1-/-
Cd28-/-

FIG. 8j

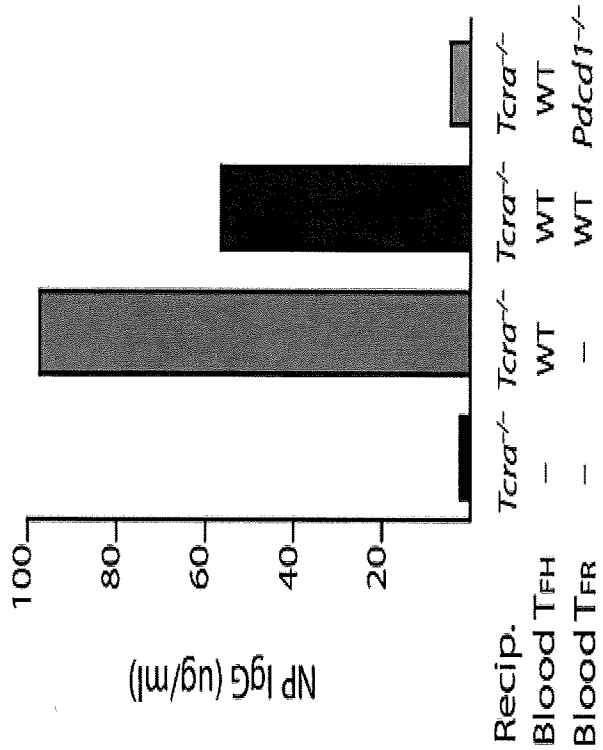

NP IgG (ug/ml)
100
80
60
40
20
Recip.
Blood TFH
Blood TFR
Tcra-/-
–
–
Tcra-/-
WT
–
Tcra-/-
WT
WT
Tcra-/-
WT
Pdcd1-/-

FIG. 8k

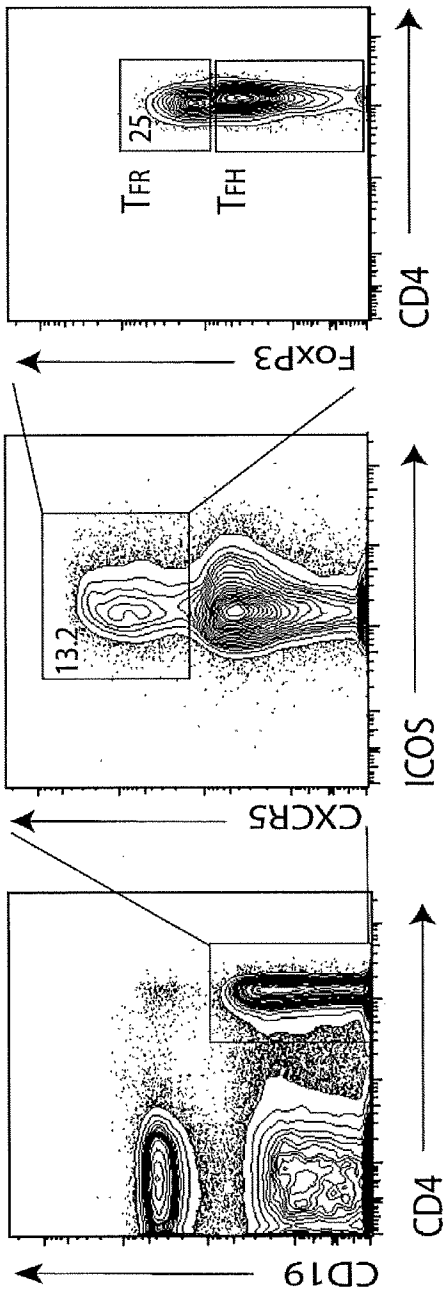


FIG. 9

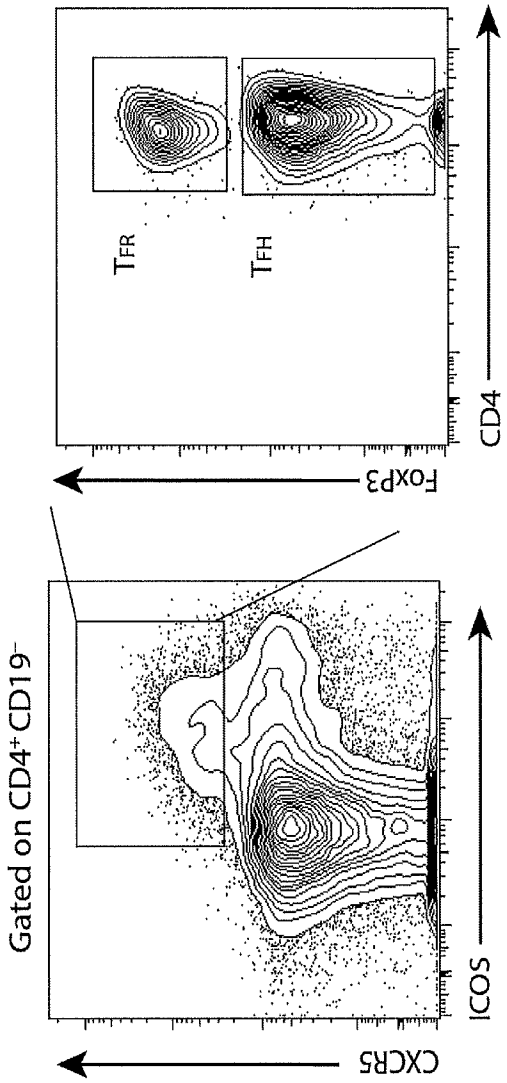


FIG. 10a

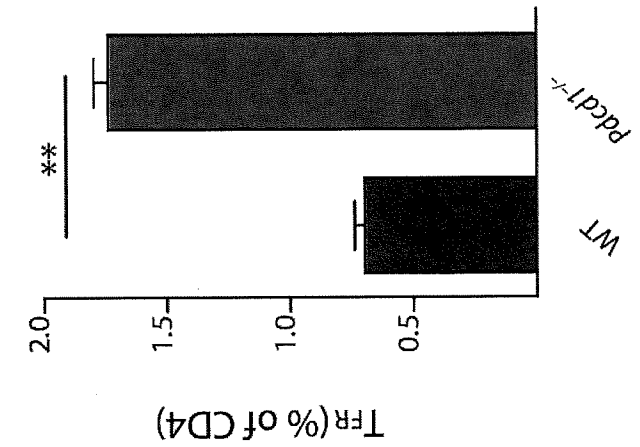


FIG. 10c

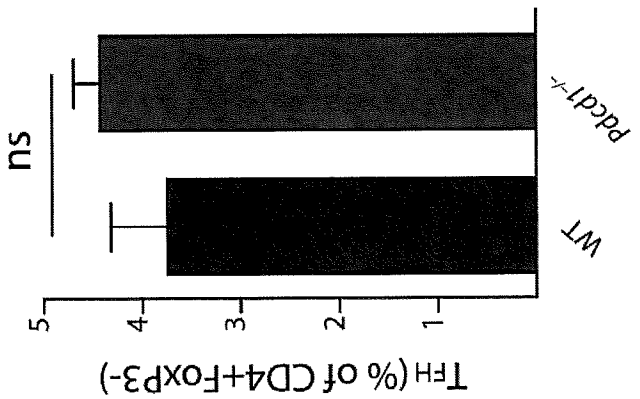


FIG. 10b

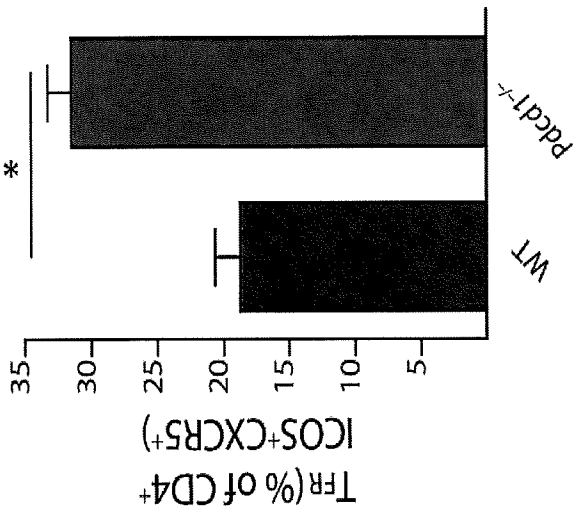


FIG. 10e

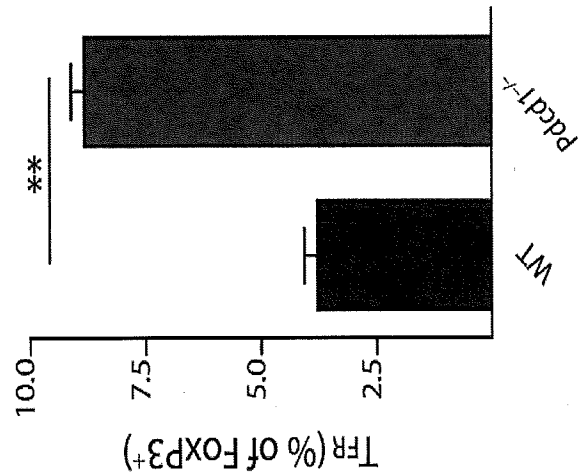


FIG. 10d

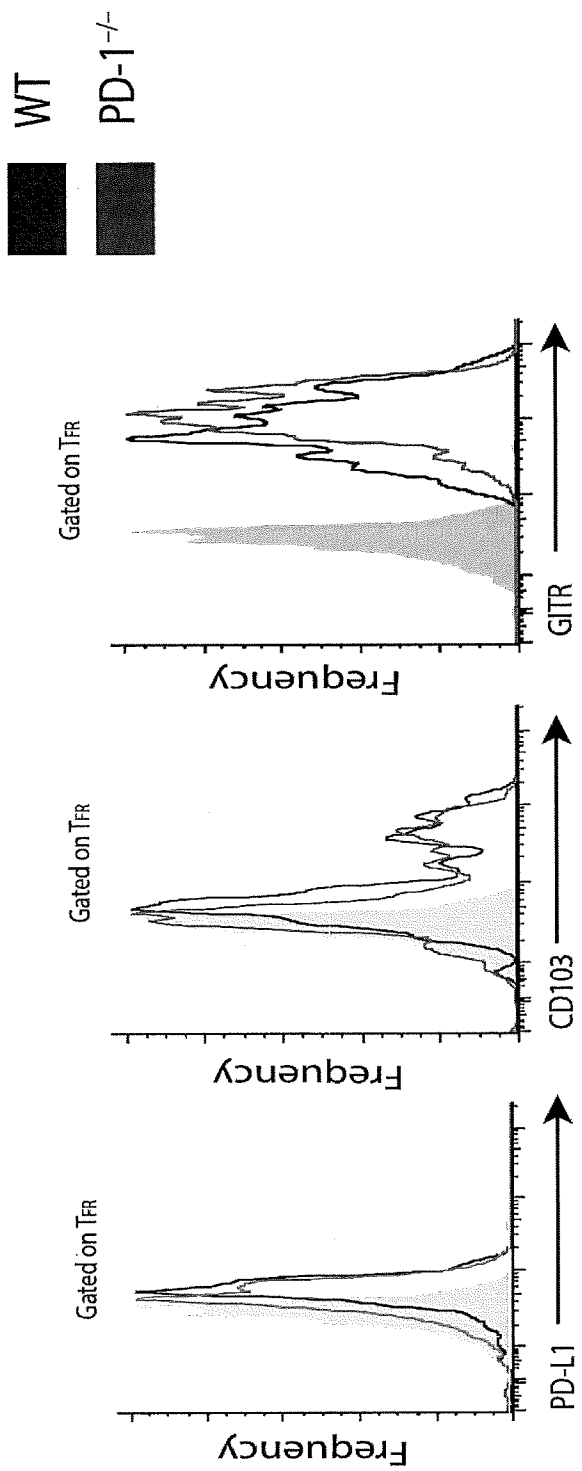

WT
PD-1-/-
Gated on TFR
Frequency
GITR
Gated on TFR
Frequency
CD103
Gated on TFR
Frequency
PD-L1

FIG 11

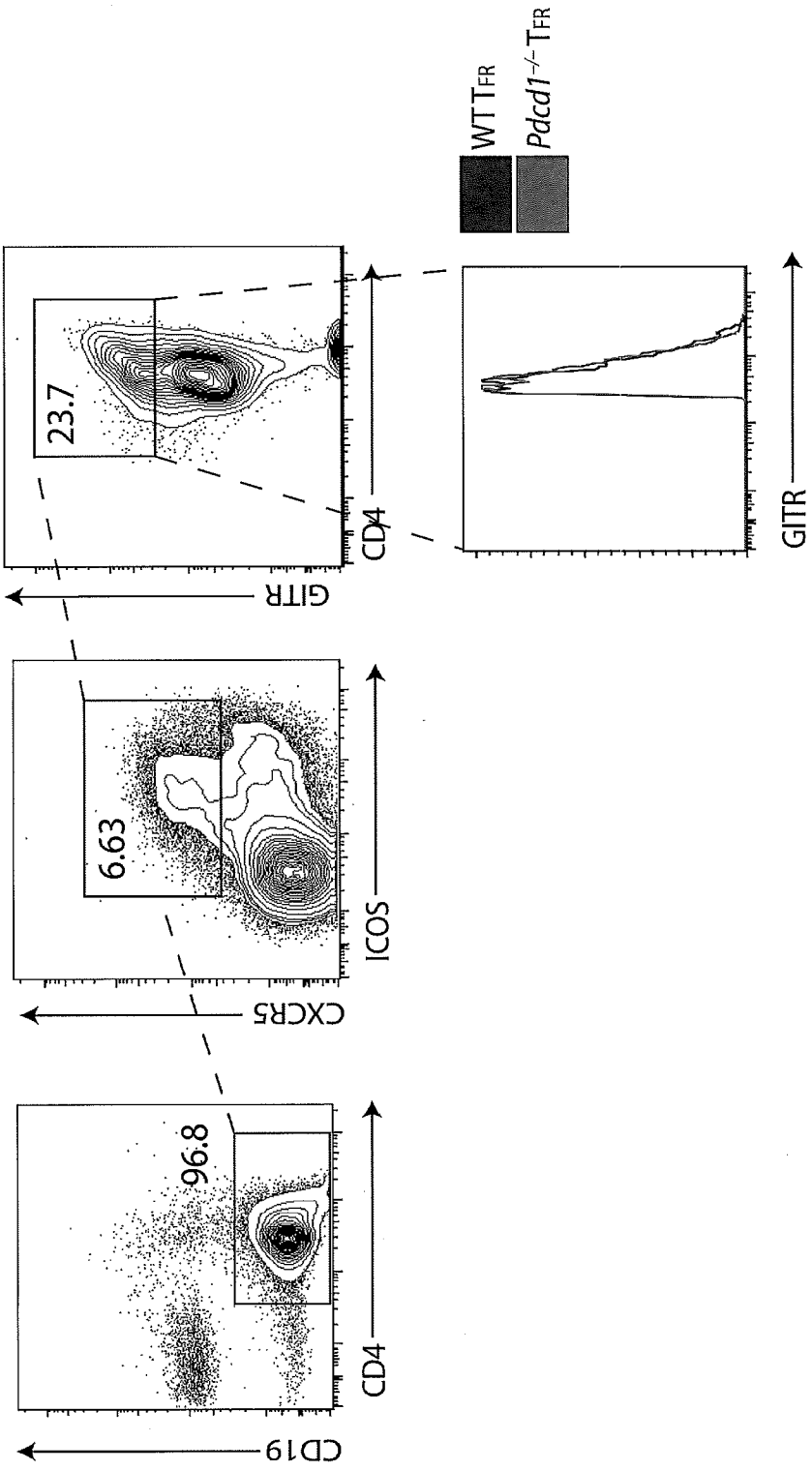


FIG. 12

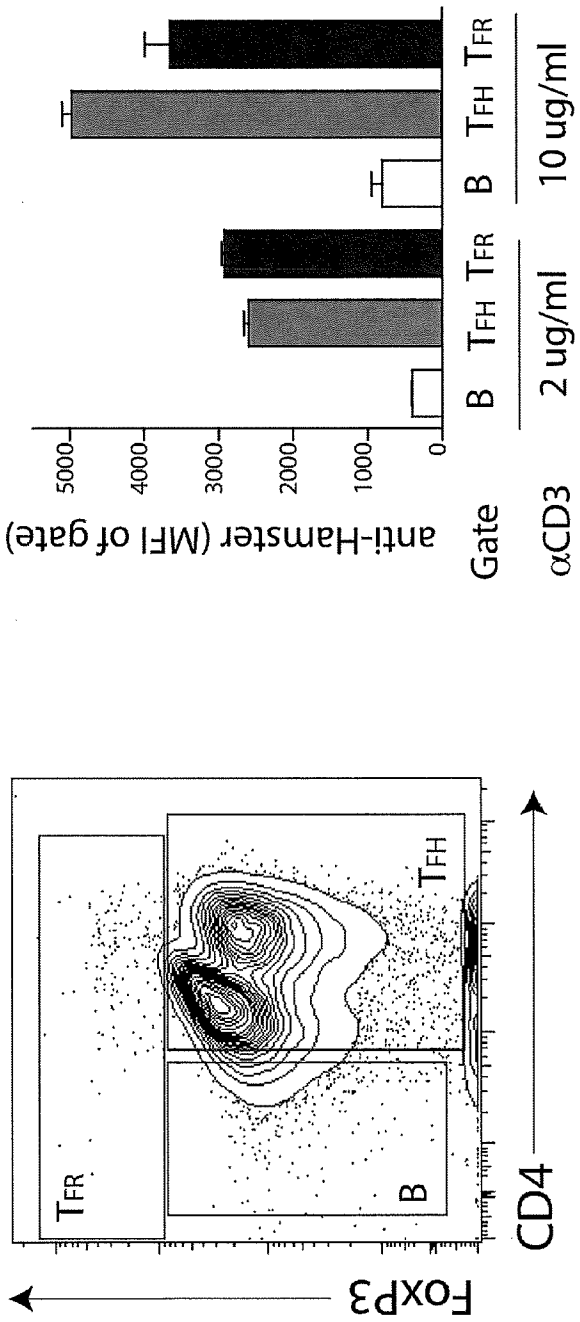

anti-Hamster (MFI of gate)
5000
4000
3000
2000
1000
0
Gate
B
TFH
TFR
B
TFH
TFR
αCD3
2 ug/ml
10 ug/ml
FoxP3
CD4
TFR
B
TFH

FIG. 13

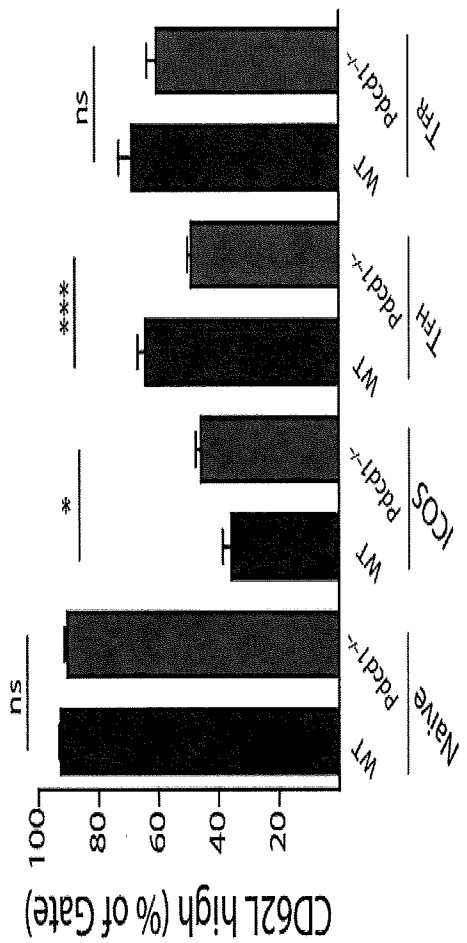

CD62L high (% of Gate)
100
80
60
40
20
ns
*
***
ns
WT
Pdcd1-/-
Naive
ICOS
TH
TFR

FIG. 14a

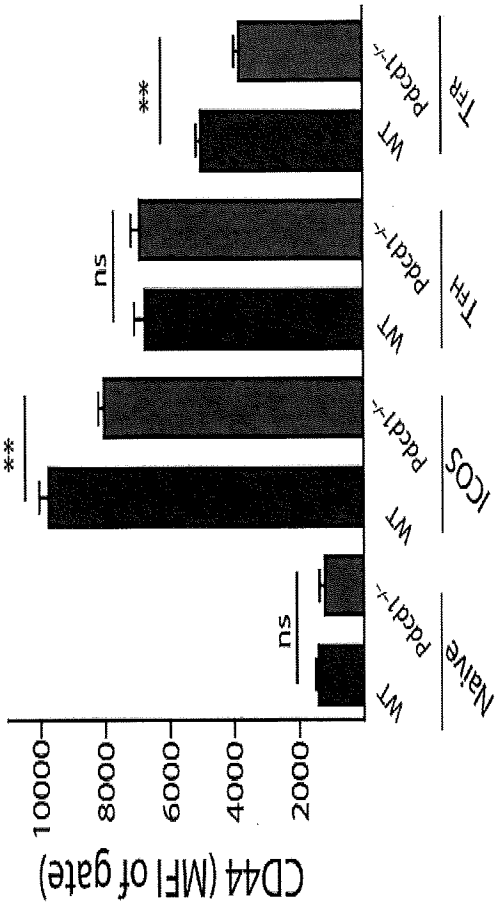

CD44 (MFI of gate)
10000
8000
6000
4000
2000
ns
**
ns
**
WT
Pdcd1-/-
Naive
ICOS
TFH
TFR

FIG. 14b

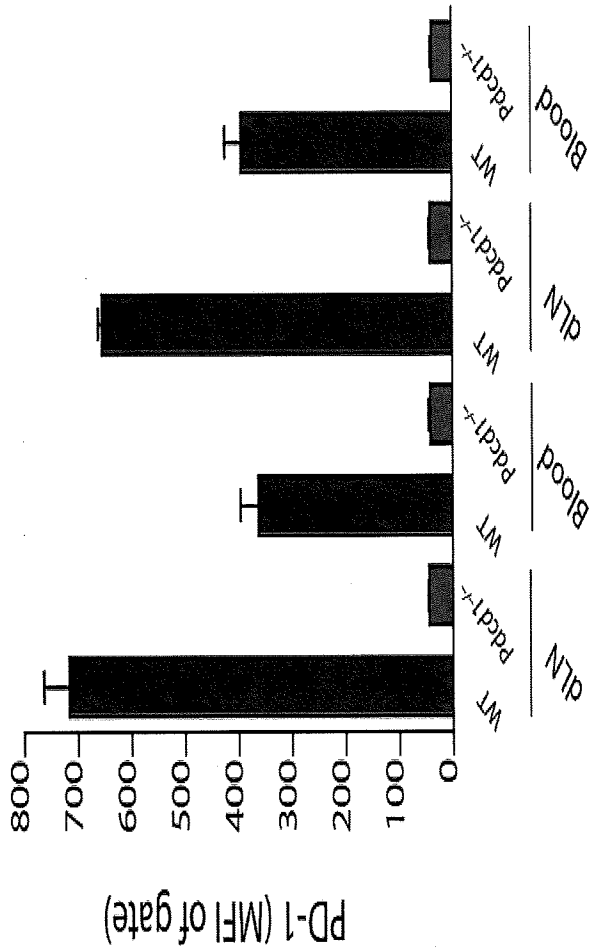

PD-1 (MFI of gate)
800
700
600
500
400
300
200
100
0
WT
Pdcd1-/-
dLN
WT
Pdcd1-/-
Blood
WT
Pdcd1-/-
dLN
WT
Pdcd1-/-
Blood

FIG. 14c

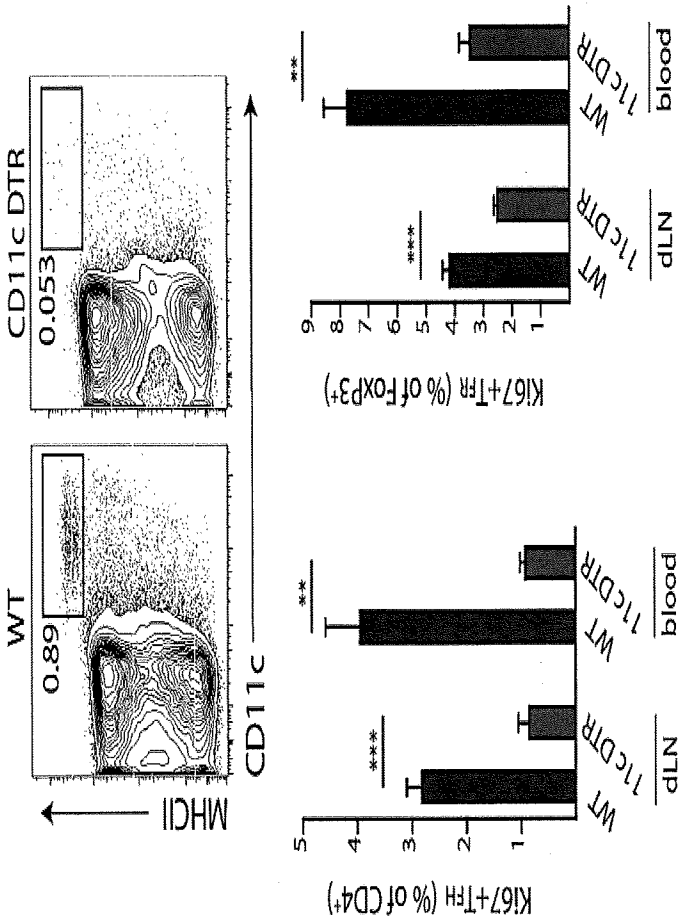


FIG. 15a

FIG. 15b

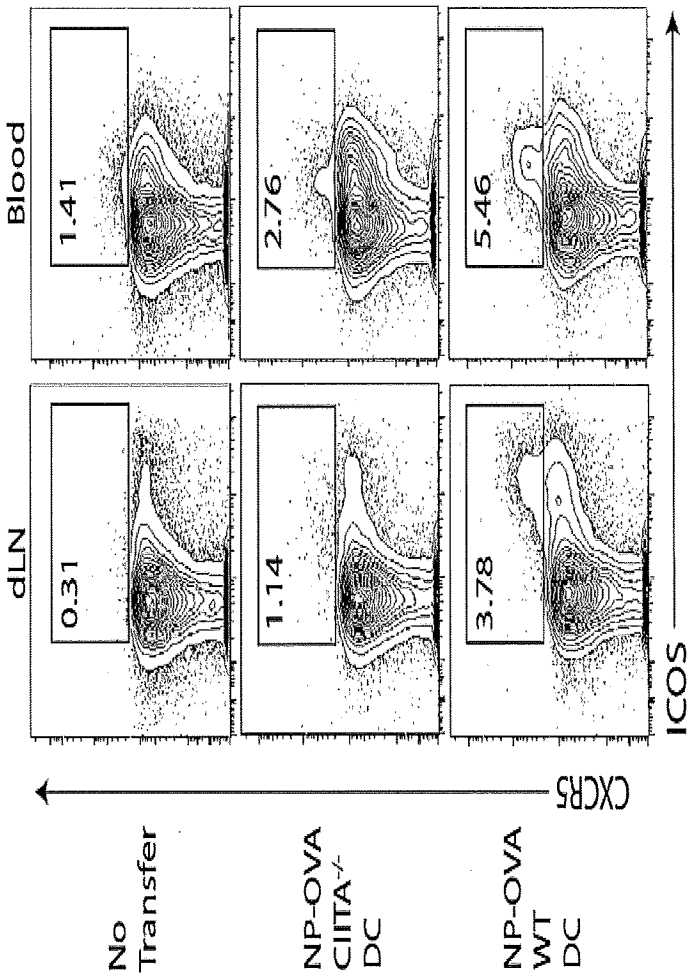

Blood
dLN
1.41
2.76
5.46
0.31
1.14
3.78
CXCR5
ICOS
No Transfer
NP-OVA CIITA-/- DC
NP-OVA WT DC

FIG. 15c

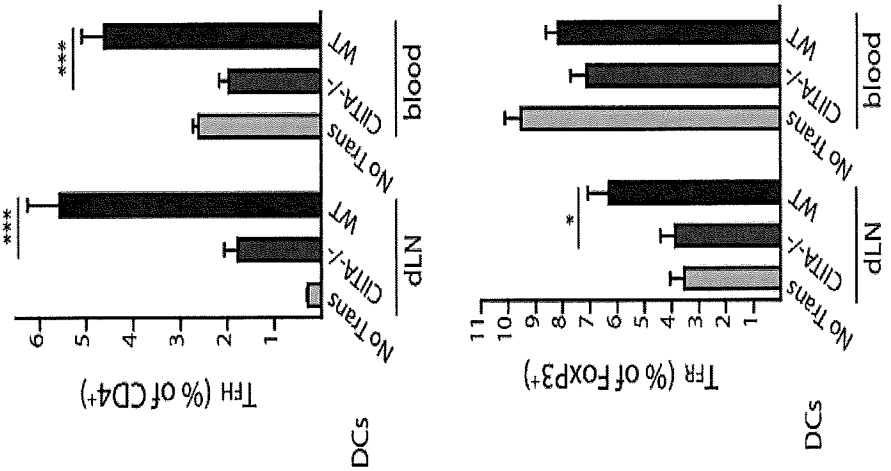


FIG. 15d

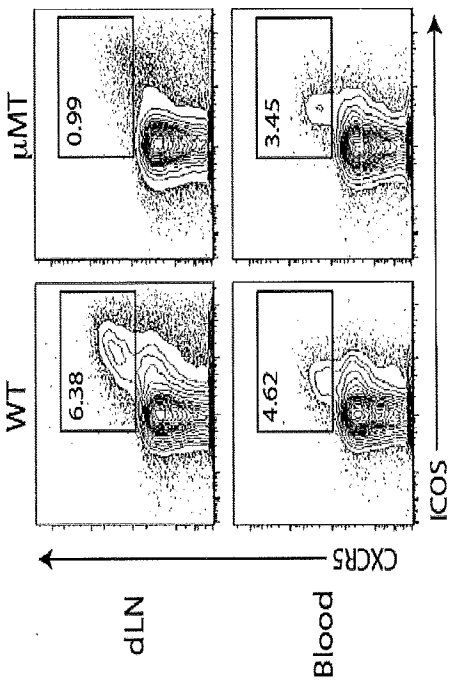


FIG 15e

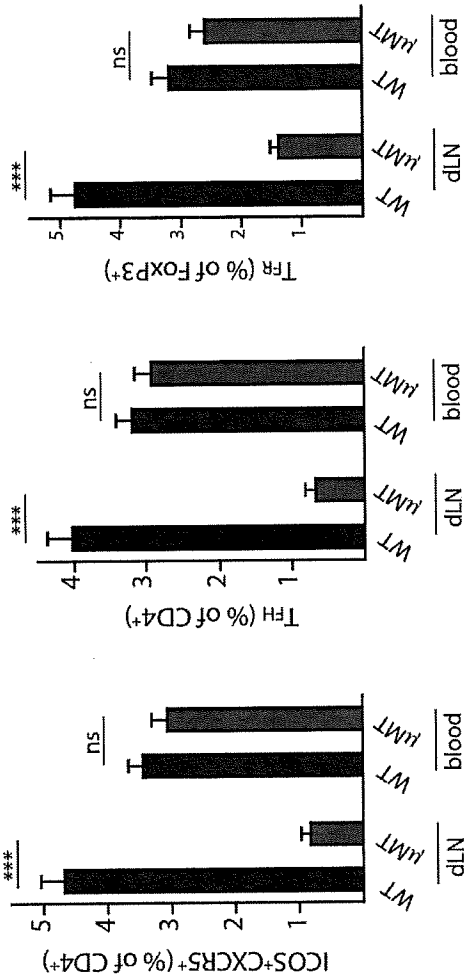


FIG. 15f

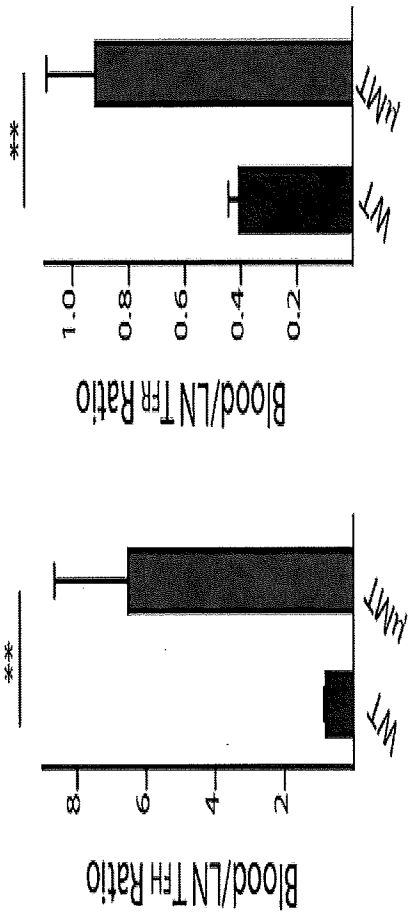

Blood/LN TFR Ratio
1.0
0.8
0.6
0.4
0.2
**
WT
MMT
Blood/LN TFH Ratio
8
6
4
2
**
WT
MMT

FIG. 15g

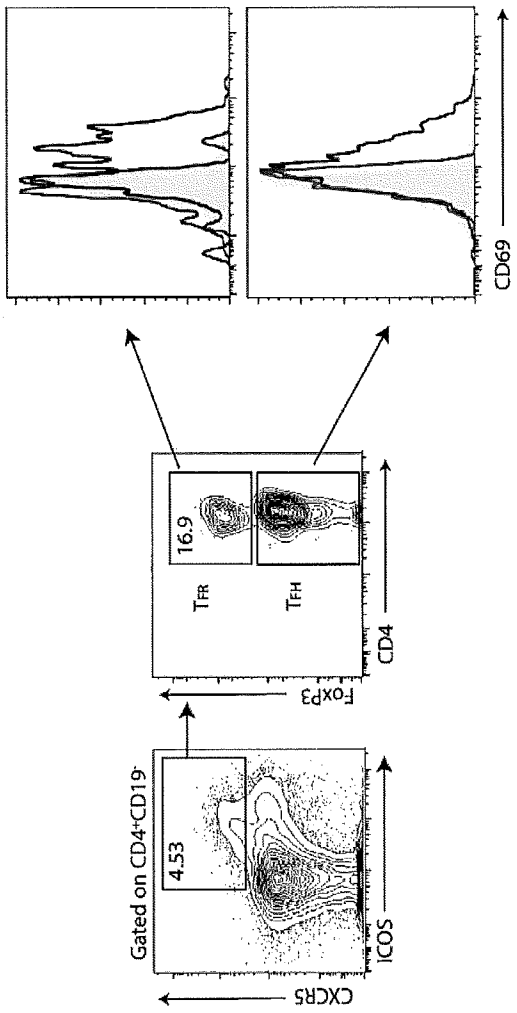


FIG. 16a

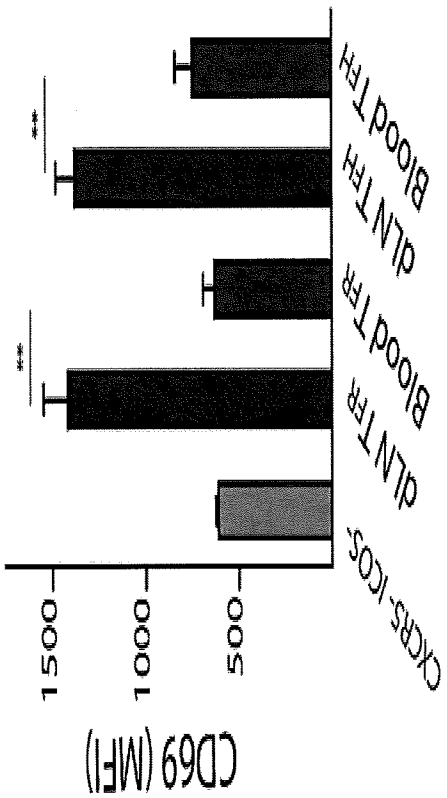

CD69 (MFI)
1500
1000
500
**
**
CXCR5-ICOS-
dLN TFR
Blood TFR
dLN TFH
Blood TFH

FIG. 16b

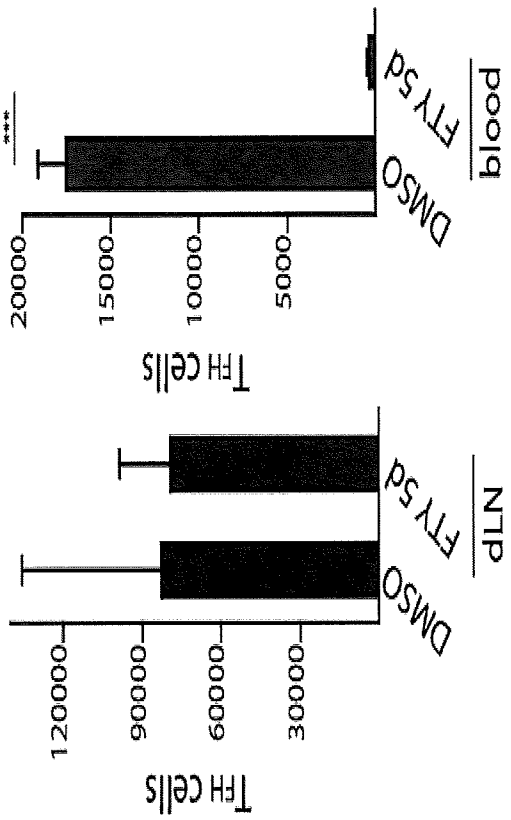

TFH cells
120000
90000
60000
30000
DMSO
FTY Sd
dLN
TFH cells
20000
15000
10000
5000
***
DMSO
FTY Sd
blood

FIG. 16c

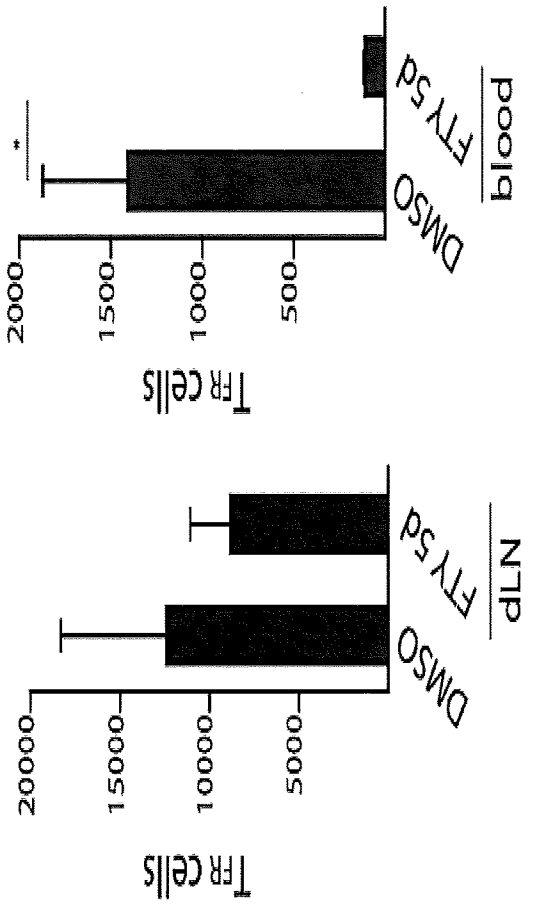

TFR cells
20000
15000
10000
5000
DMSO
FTY Sd
dLN
TFR cells
2000
1500
1000
500
*
DMSO
FTY Sd
blood

FIG. 16d

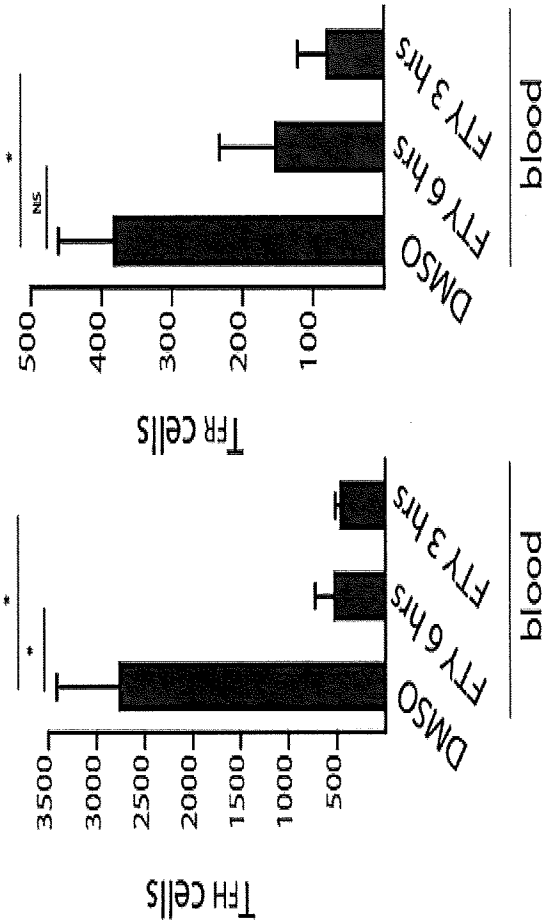

TFH cells
3500
3000
2500
2000
1500
1000
500
*
*
DMSO
FTY 6 hrs
FTY 3 hrs
blood
TFR cells
500
400
300
200
100
*
NS
DMSO
FTY 6 hrs
FTY 3 hrs
blood

FIG. 16e

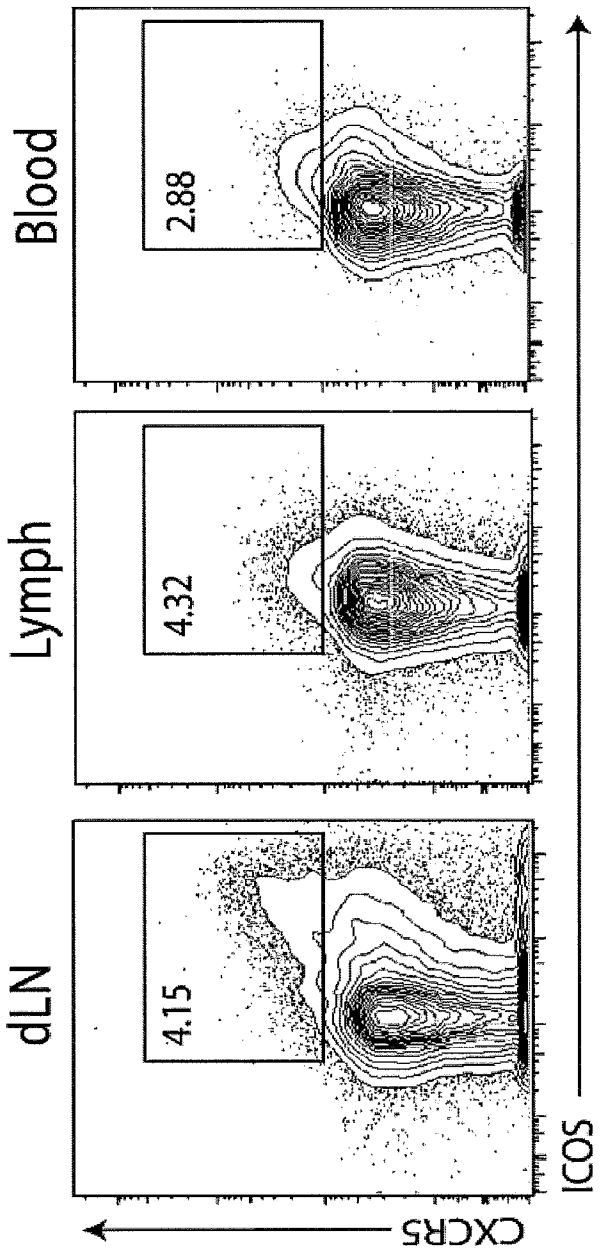


FIG. 16f

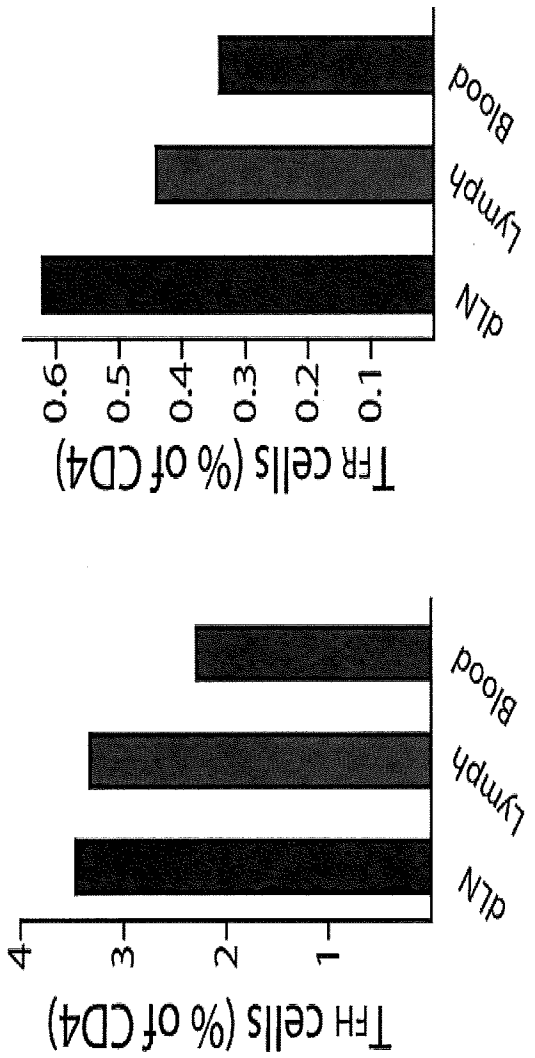

TFH cells (% of CD4)
4
3
2
1
dLN
Lymph
Blood
TFR cells (% of CD4)
0.6
0.5
0.4
0.3
0.2
0.1
dLN
Lymph
Blood

FIG. 16g

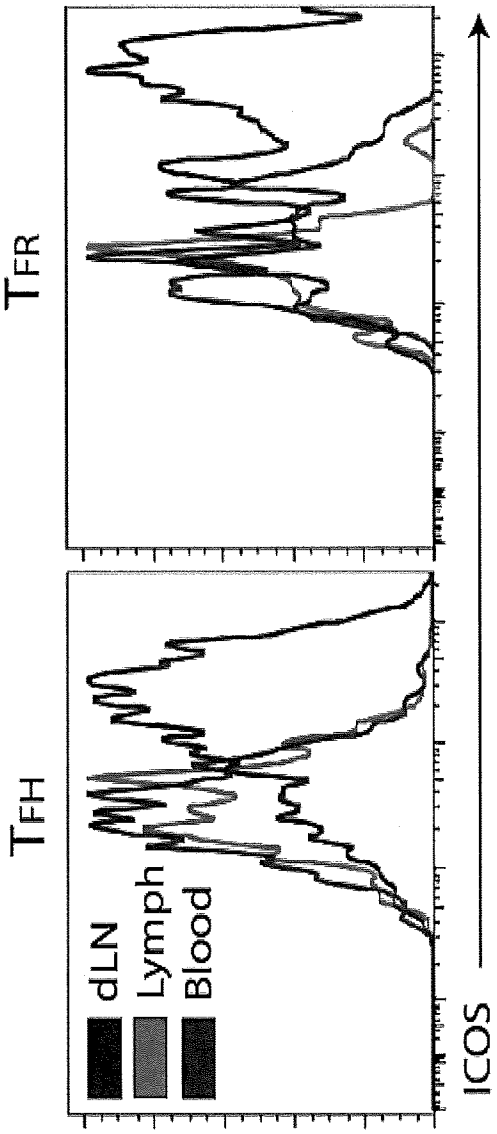


FIG. 16h

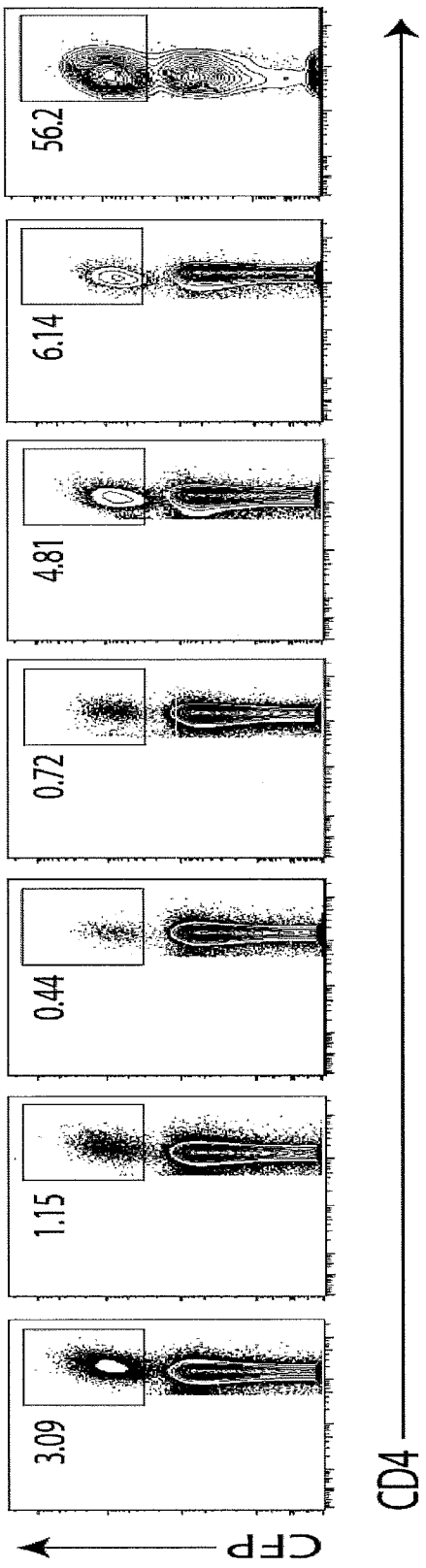

iLN
aLN
cLN
mLN
Spleen
Blood
Skin
3.09
1.15
0.44
0.72
4.81
6.14
56.2
CFP
CD4

FIG. 17a

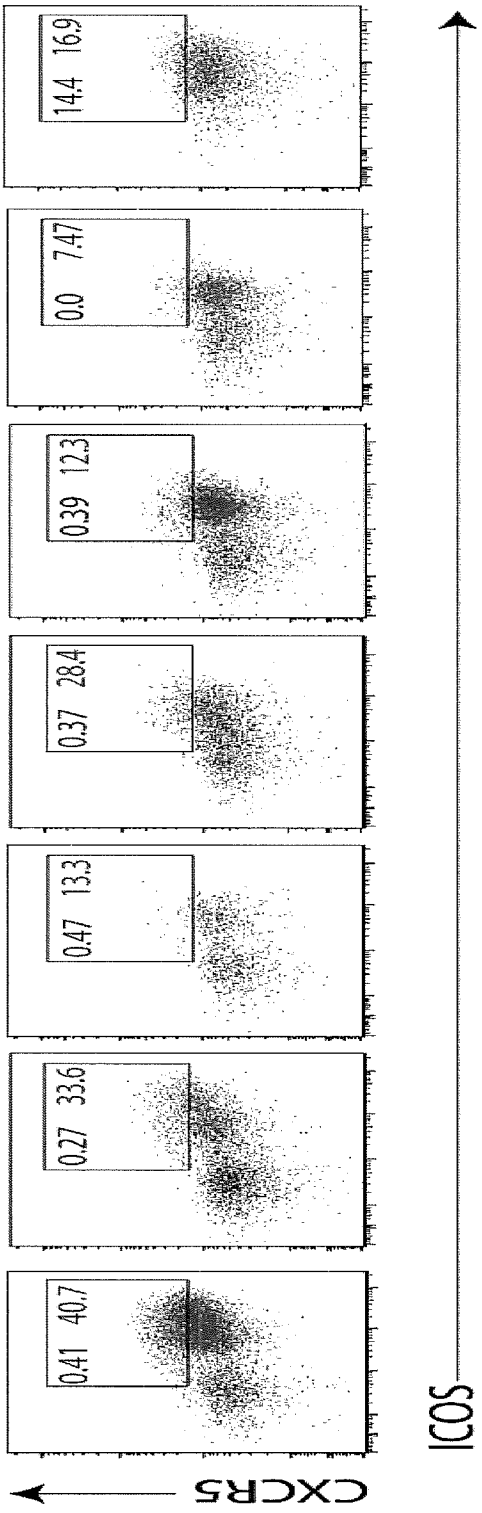

0.41 40.7
0.27 33.6
0.47 13.3
0.37 28.4
0.39 12.3
0.0 7.47
14.4 16.9
CXCR5
ICOS

FIG. 17b

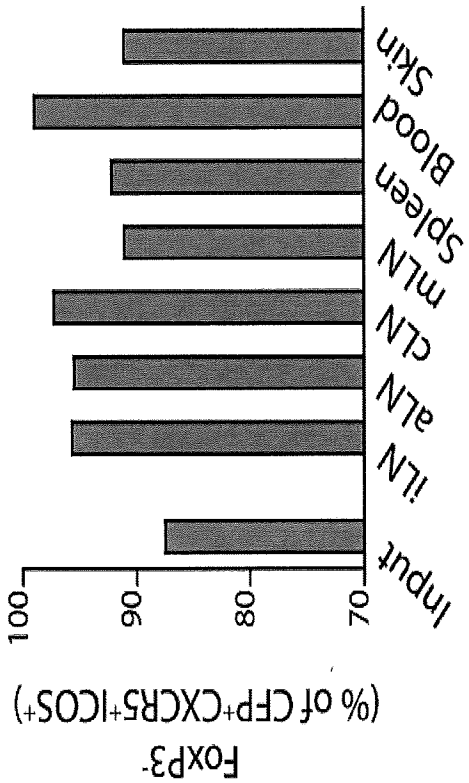

FoxP3-
(% of CFP+CXCR5+ICOS+)
100
90
80
70
Input
iLN
aLN
cLN
mLN
Spleen
Blood
Skin

FIG. 17c

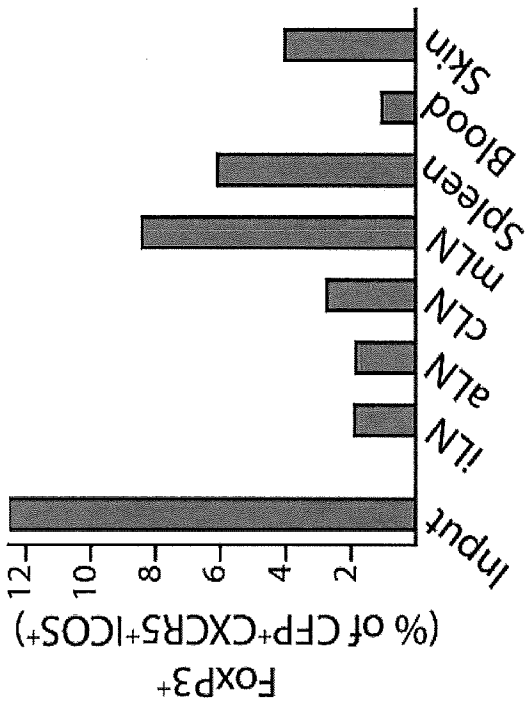

FoxP3+
(% of CFP+CXCR5+ICOS+)
12
10
8
6
4
2
Input
iLN
aLN
cLN
mLN
Spleen
Blood
Skin

FIG. 17d

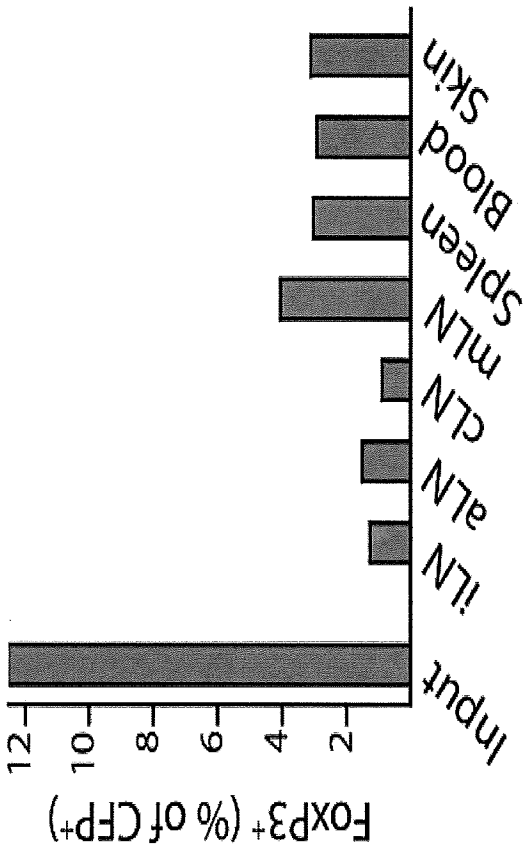

FoxP3+ (% of CFP+)
12
10
8
6
4
2
Input
iLN
aLN
cLN
mLN
Spleen
Blood
Skin

FIG. 17e

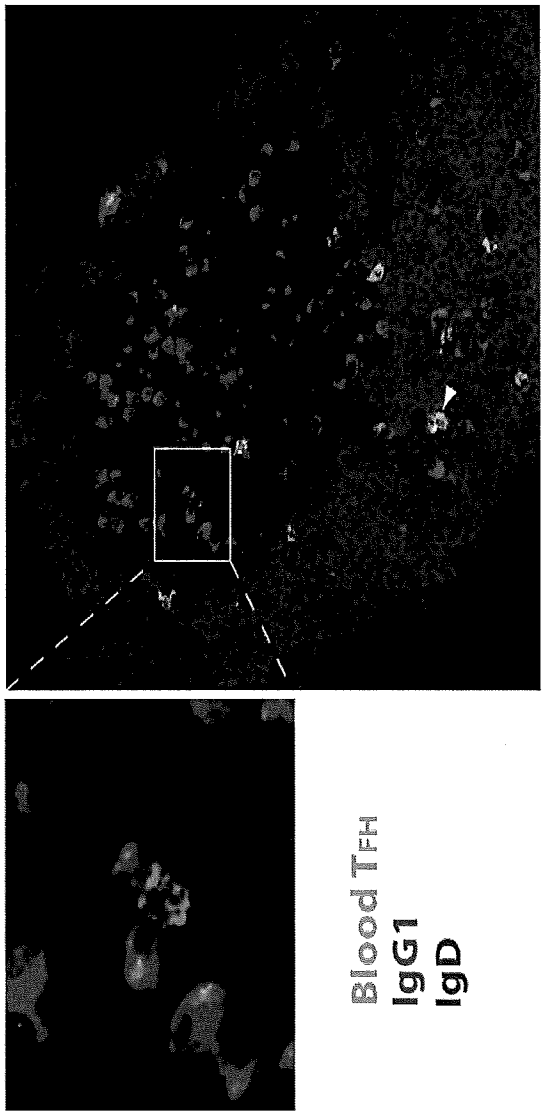


FIG. 17f

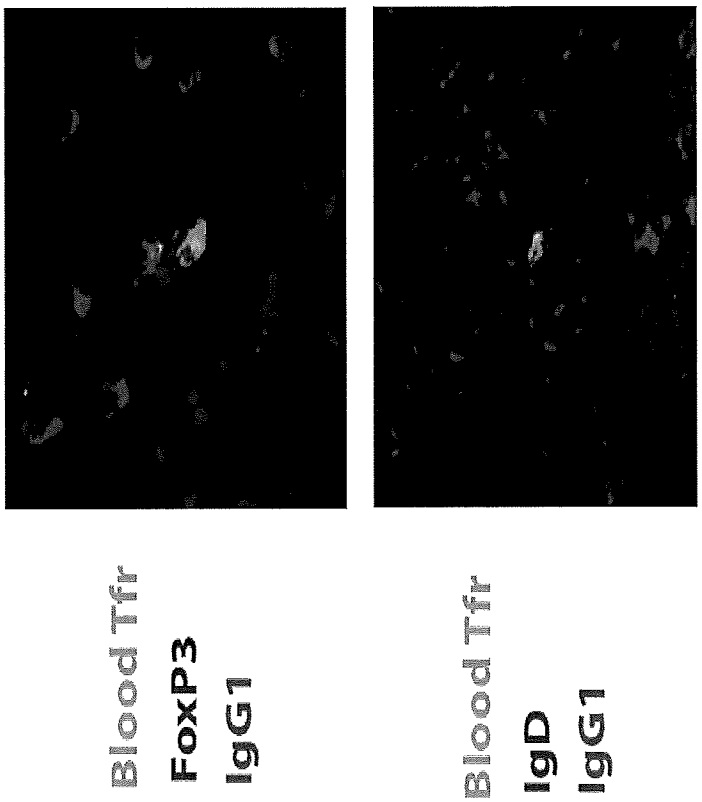


FIG. 17g

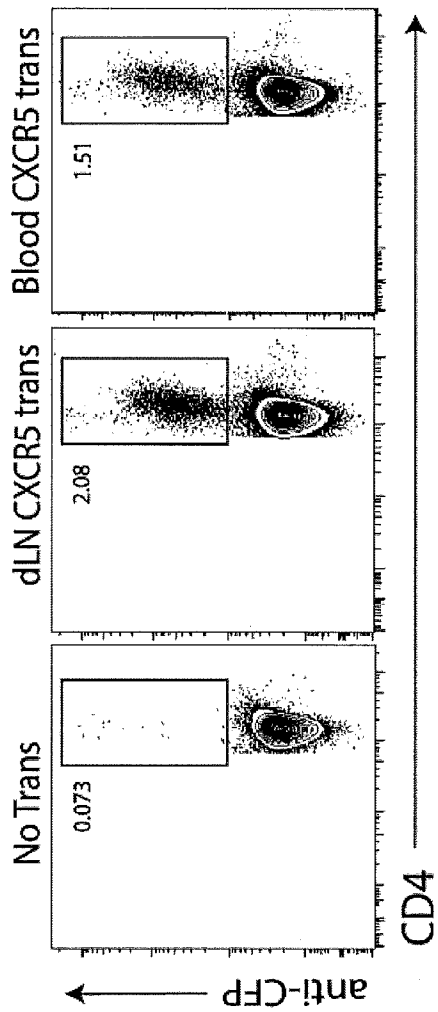

No Trans
0.073
dLN CXCR5 trans
2.08
Blood CXCR5 trans
1.51
anti-CFP
CD4

FIG. 18a

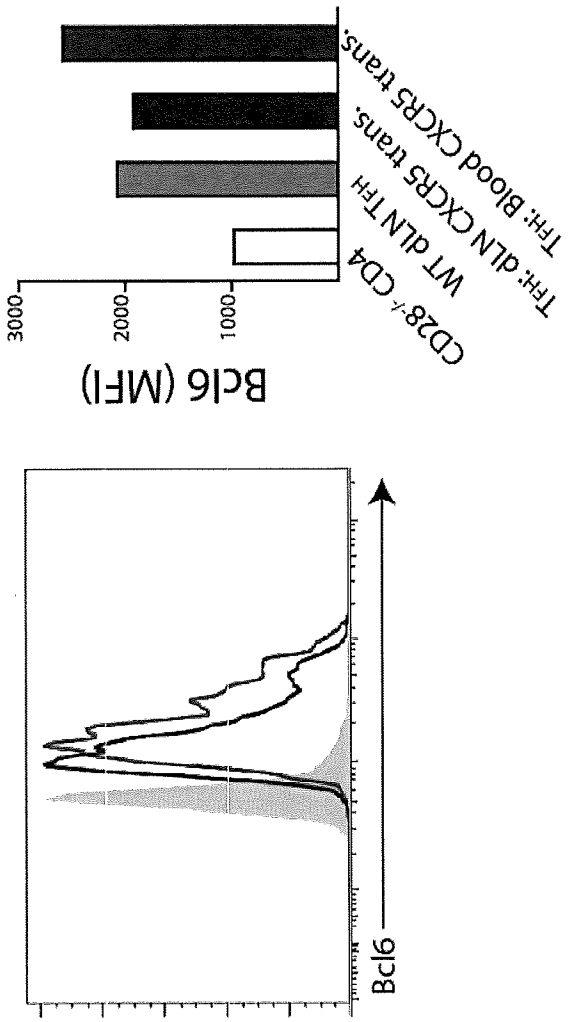

Bcl6 (MFI)
3000
2000
1000
CD28-/- CD4
WT dLN TFH
TFH: dLN CXCR5 trans.
TFH: Blood CXCR5 trans.
Bcl6

FIG. 18b

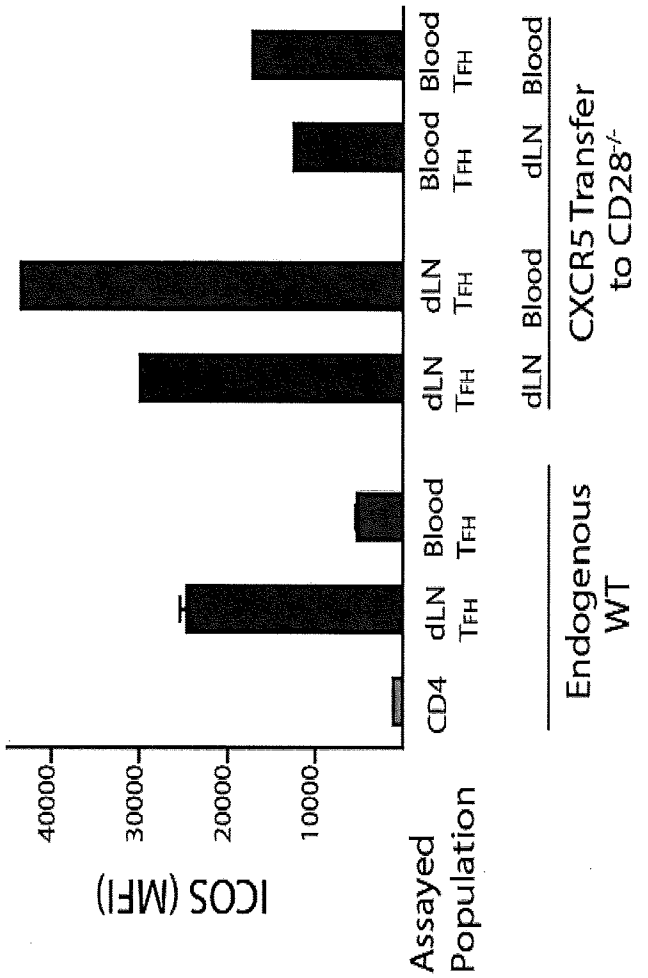

ICOS (MFI)
40000
30000
20000
10000
Assayed Population
CD4
dLN TFH
Blood TFH
dLN TFH
dLN TFH
Blood TFH
Blood TFH
Endogenous WT
dLN Blood
CXCR5 Transfer to CD28-/-
dLN Blood

FIG. 18c

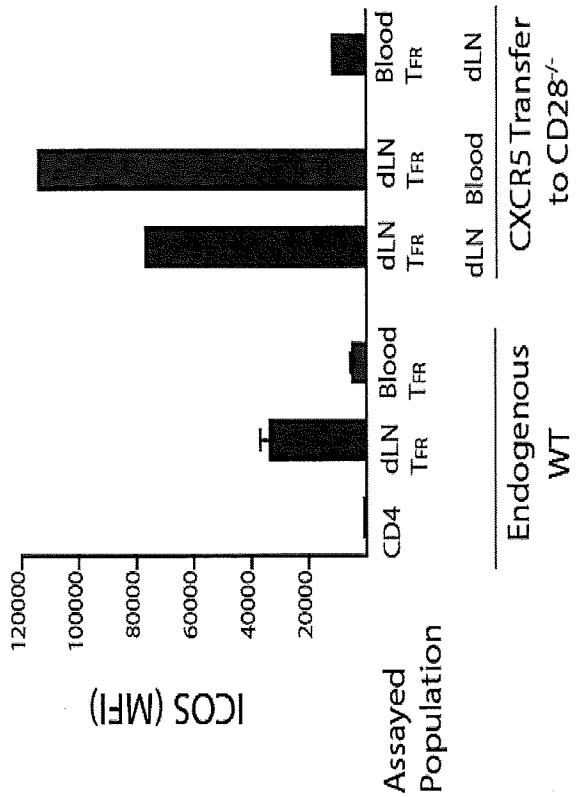

ICOS (MFI)
120000
100000
80000
60000
40000
20000
Assayed Population
CD4
dLN TFR
Blood TFR
dLN TFR
dLN TFR
Blood TFR
dLN TFR
Endogenous WT
dLN
Blood
dLN
CXCR5 Transfer to CD28-/-

FIG. 18d

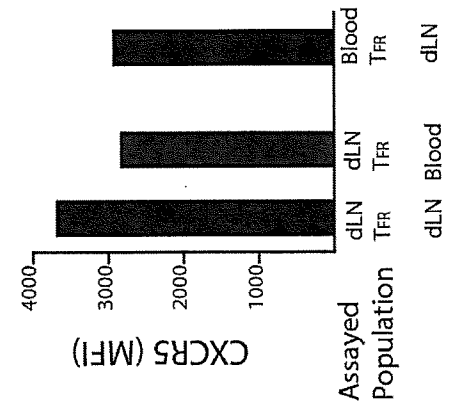


FIG. 18f

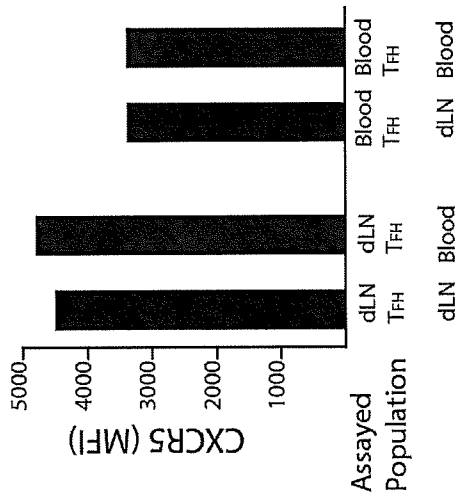


FIG. 18e

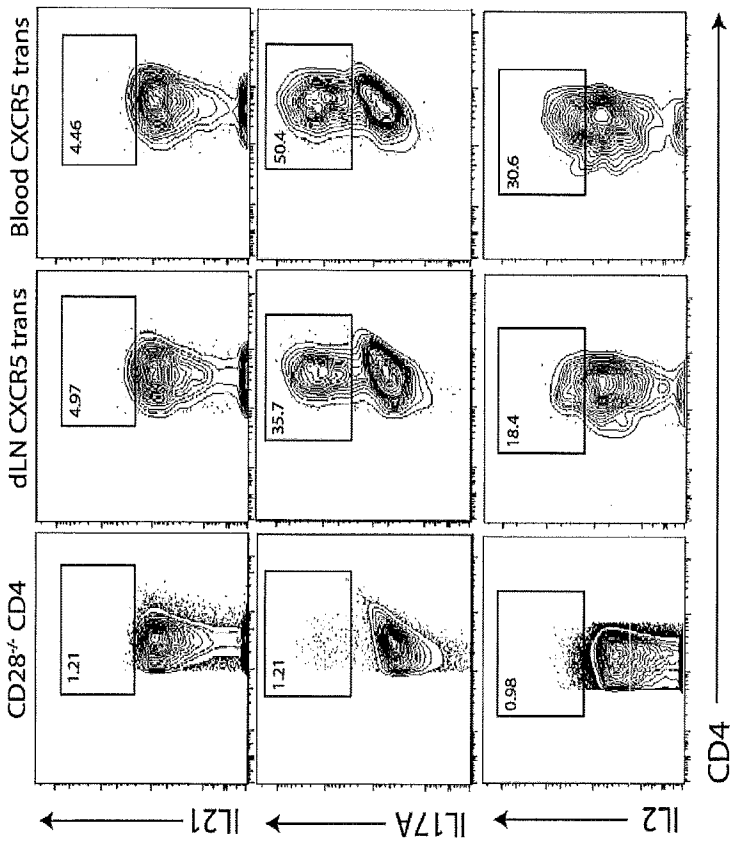

Blood CXCR5 trans
dLN CXCR5 trans
CD28-/- CD4
4.46
50.4
30.6
4.97
35.7
18.4
1.21
1.21
0.98
IL21
IL17A
IL2
CD4

FIG. 18g

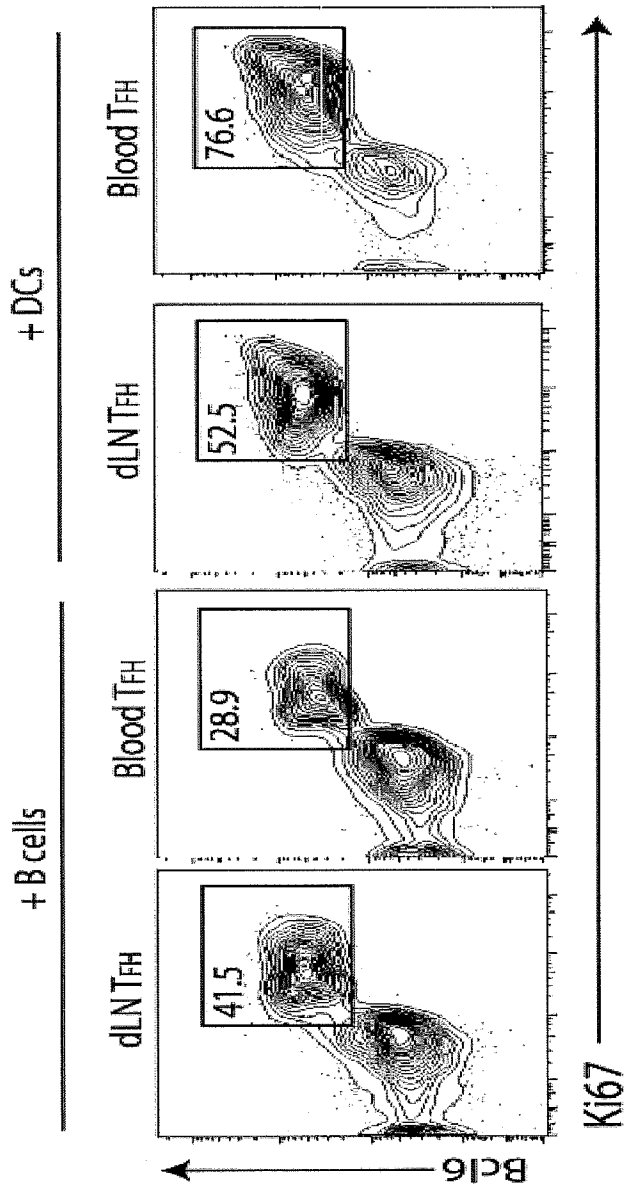

+ B cells
+ DCs
dLN TFH
Blood TFH
dLN TFH
Blood TFH
41.5
28.9
52.5
76.6
Bcl6
Ki67

FIG. 19a

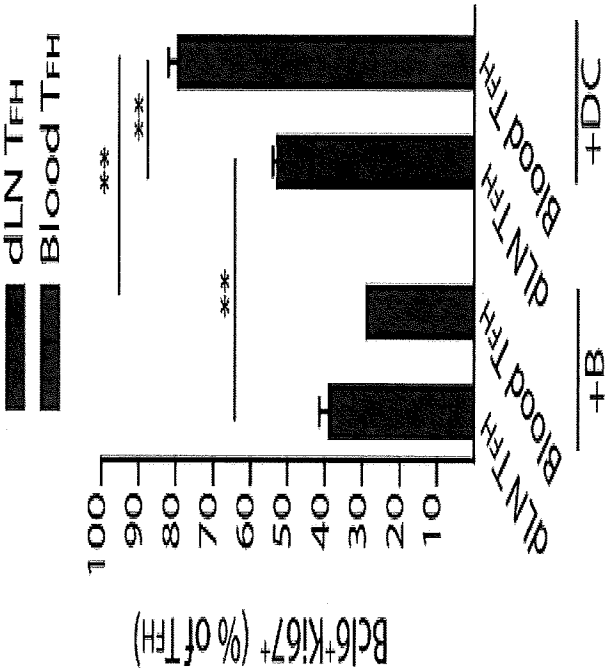

dLN TFH
Blood TFH
Bcl6+Ki67+ (% of TFH)
100
90
80
70
60
50
40
30
20
10
**
**
***
dLN TFH
Blood TFH
dLN TFH
Blood TFH
+B
+DC

FIG. 19b

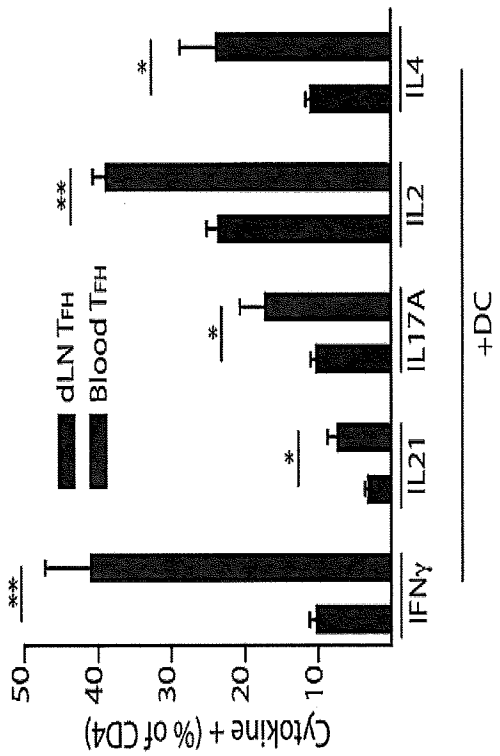

Cytokine + (% of CD4)
50
40
30
20
10
dLN TFH
Blood TFH
**
*
**
*
*
IFNγ
IL21
IL17A
IL2
IL4
+DC

FIG. 19c

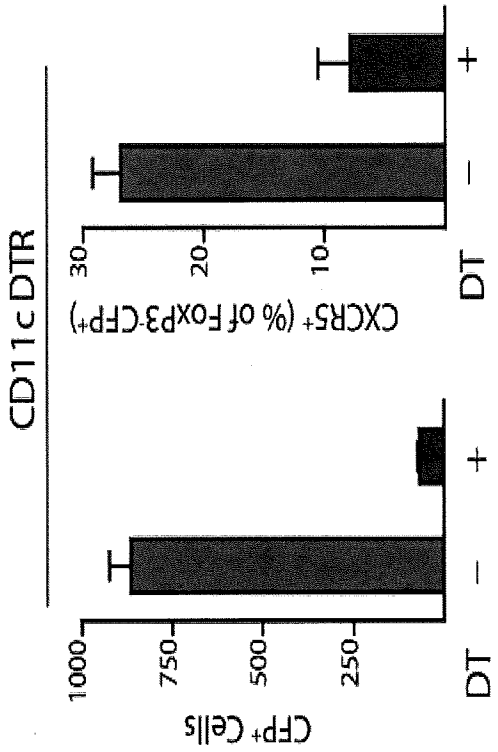

CD11c DTR
CXCR5+ (% of FoxP3-CFP+)
30
20
10
DT
–
+
CFP+ Cells
1000
750
500
250
DT
–
+

FIG. 19d

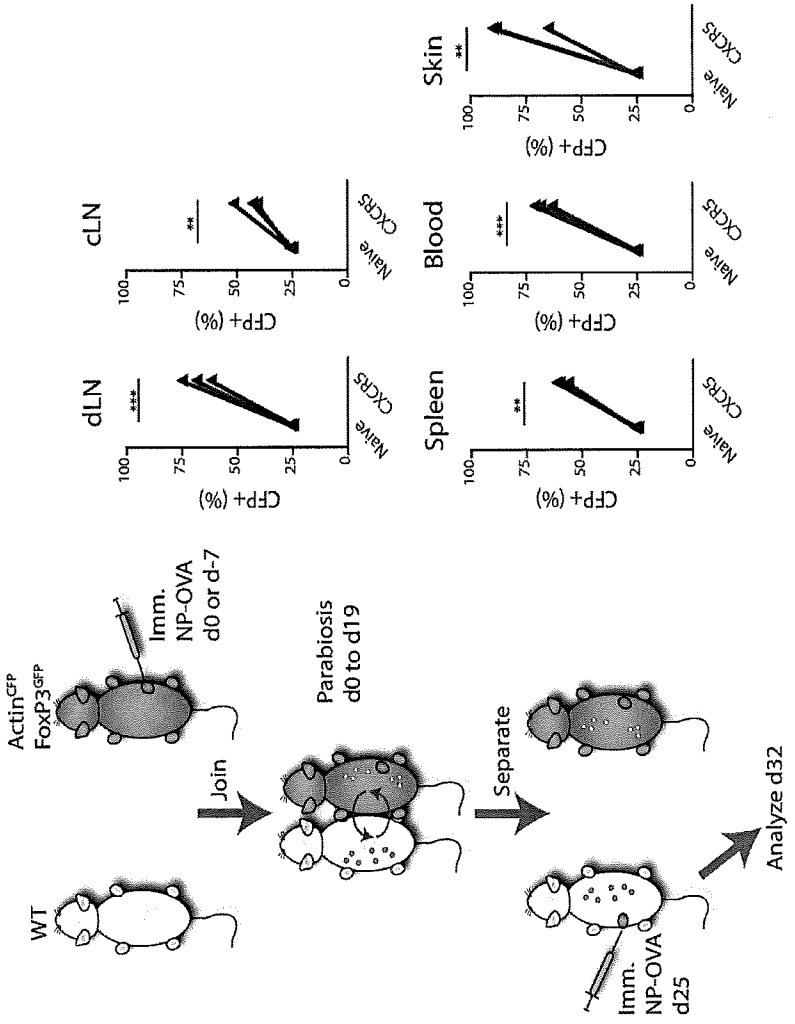

Actin^GFP
FoxP3^GFP
Imm.
NP-OVA
d0 or d-7
WT
Join
Parabiosis
d0 to d19
Separate
Imm.
NP-OVA
d25
Analyze d32
dLN
cLN
Spleen
Blood
Skin
CFP+ (%)
Naive
CXCR5

FIG. 19e

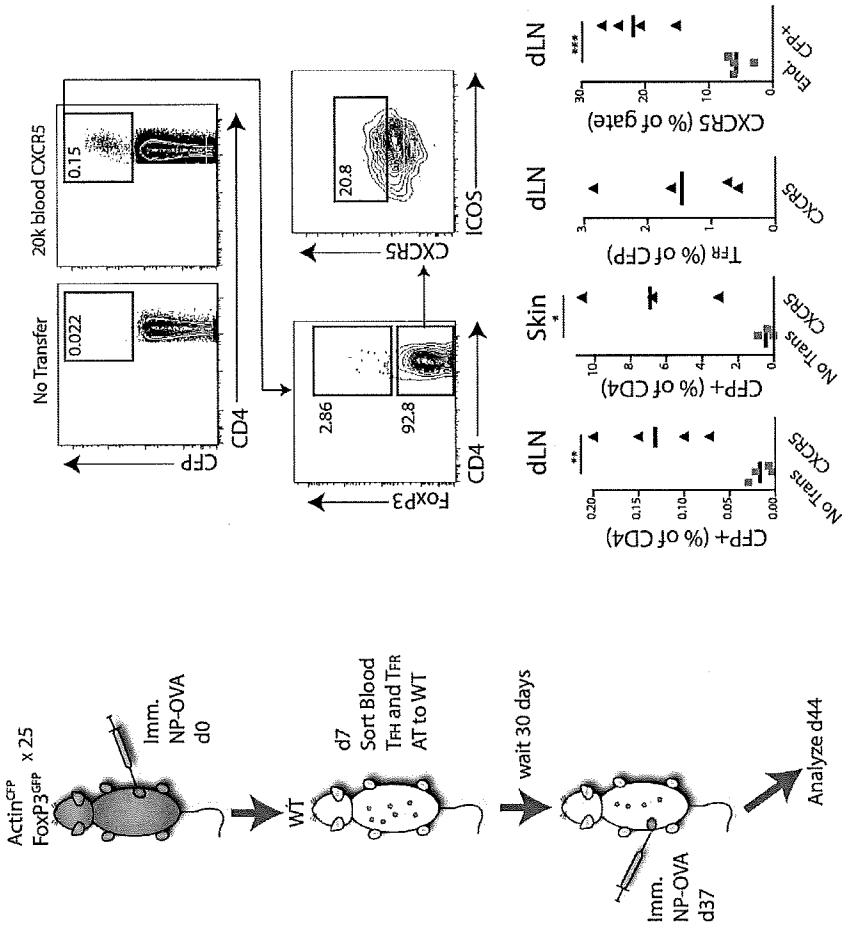

Actin^GFP FoxP3^GFP x 25
Imm. NP-OVA d0
WT
d7 Sort Blood TFH and TFR AT to WT
wait 30 days
Imm. NP-OVA d37
Analyze d44
No Transfer
20k blood CXCR5
0.022
0.15
CFP
CD4
2.86
92.8
FoxP3
20.8
CXCR5
ICOS
dLN
Skin
CFP+ (% of CD4)
No Trans
CXCR5
TFR (% of CFP)
End. CFP+
CXCR5 (% of gate)

FIG. 19f

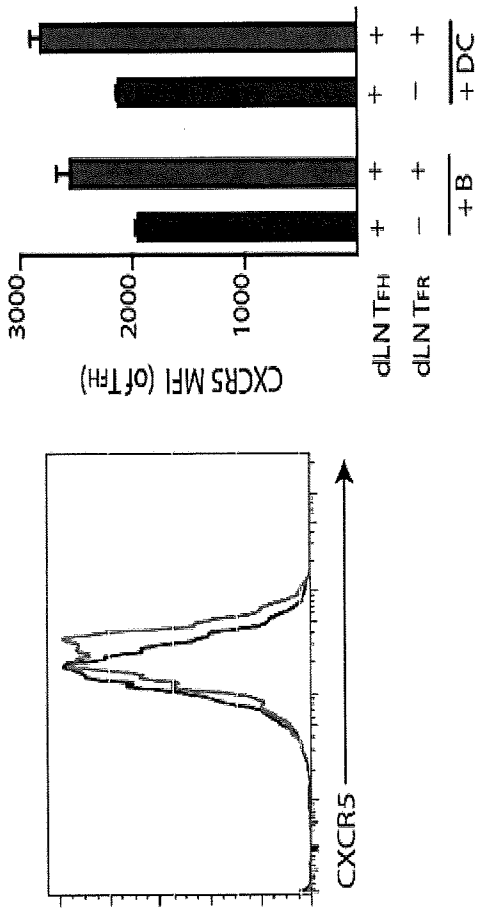


FIG. 20a

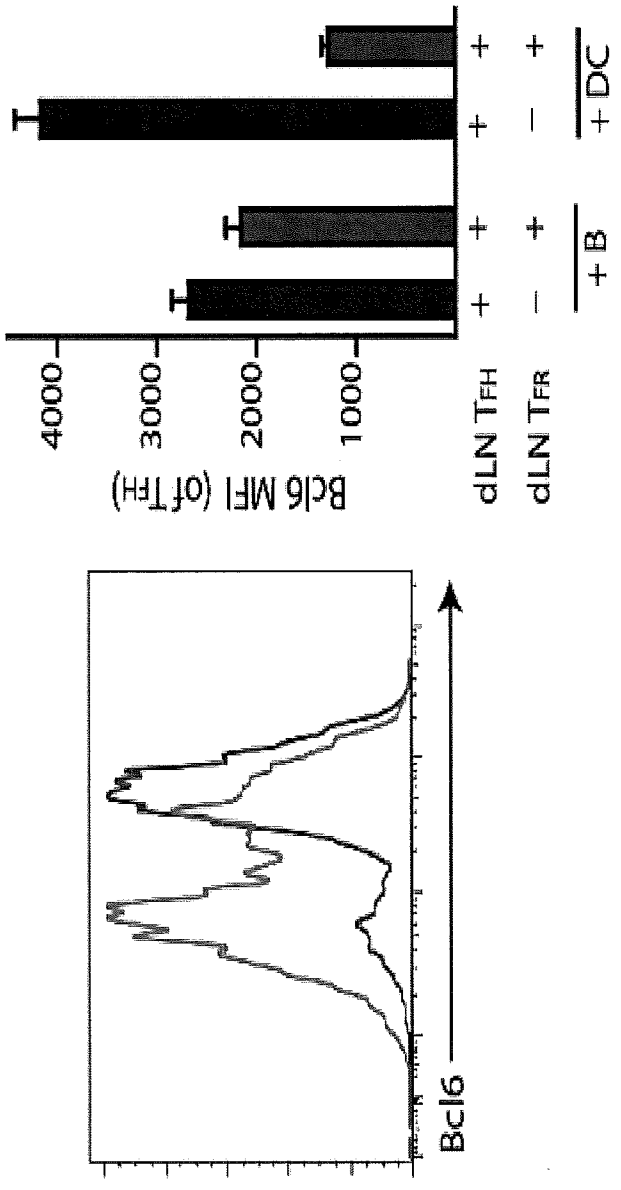


FIG. 20b

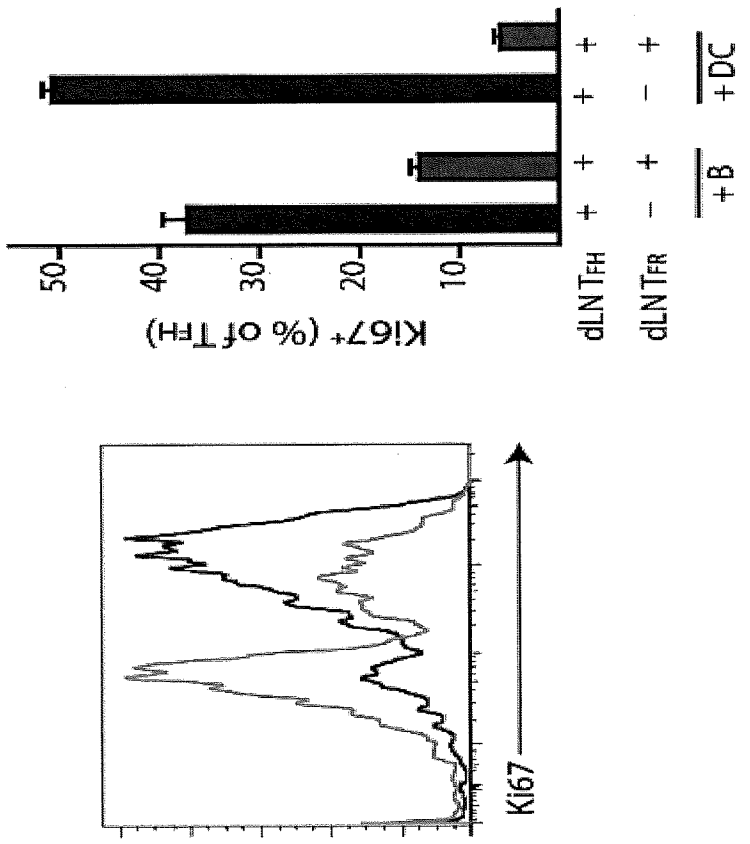


FIG. 20c

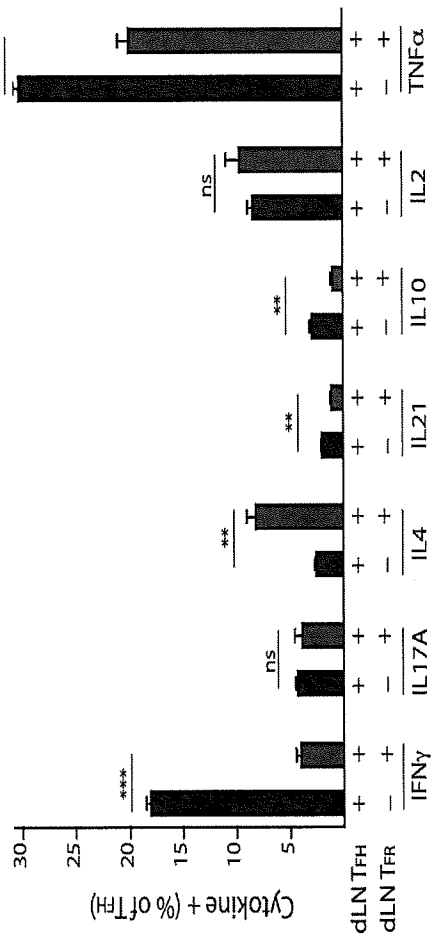

Cytokine + (% of TFH)
30
25
20
15
10
5
dLN TFH
dLN TFR
IFNγ
IL17A
IL4
IL21
IL10
IL2
TNFα
***
ns
**
**
**
ns
***

FIG. 20d

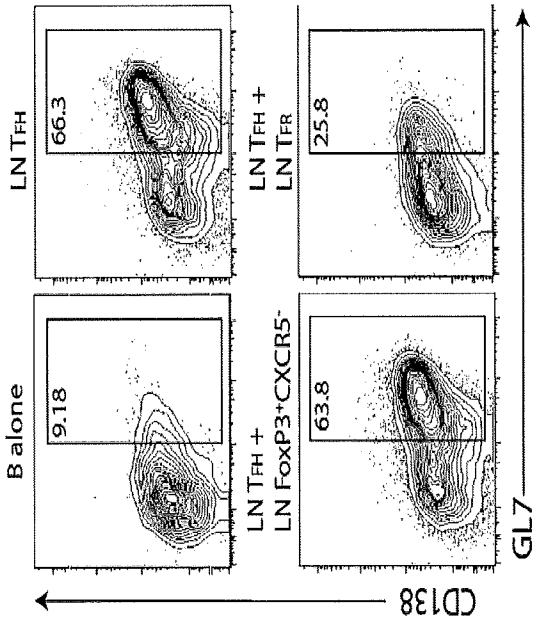


FIG. 20f

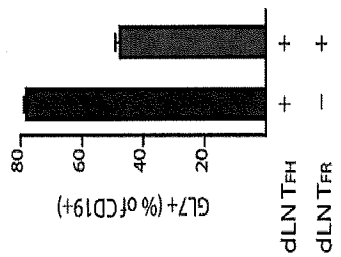


FIG. 20e

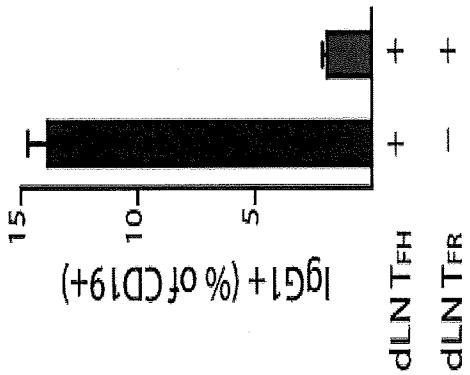


FIG. 20h

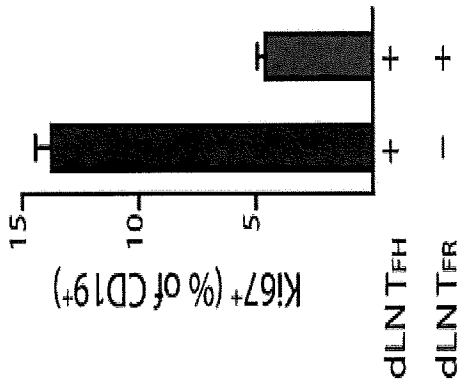


FIG.20g

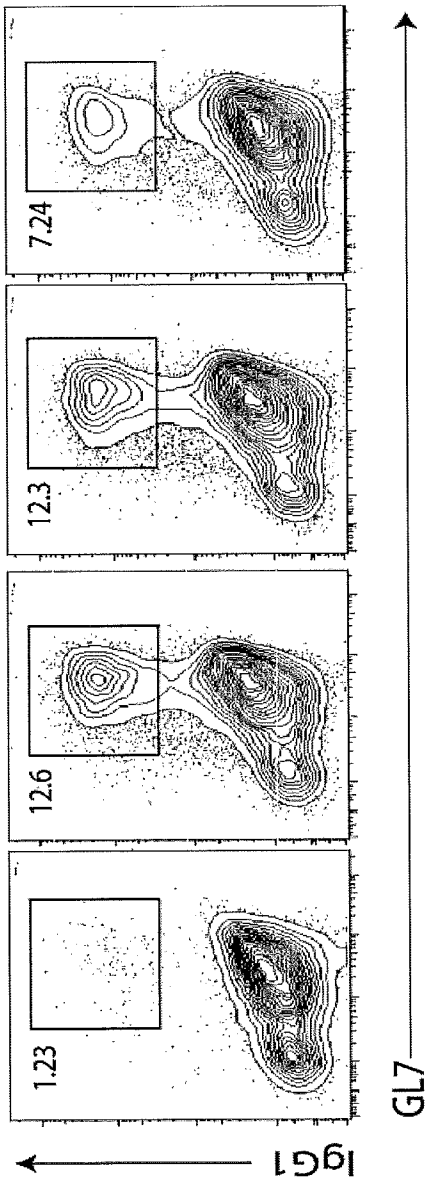

Naive CD4
LN TFH
LN TFH + LN FoxP3+CXCR5-
LN TFH + LN TFR
1.23
12.6
12.3
7.24
IgG1
GL7

FIG. 20i

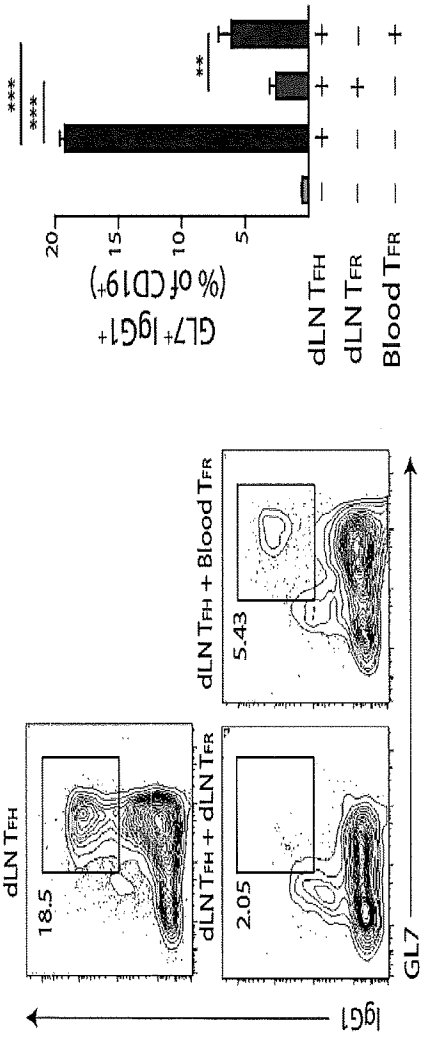

dLN TFH
18.5
dLN TFH + dLN TFR
2.05
dLN TFH + Blood TFR
5.43
IgG1
GL7
GL7+IgG1+ (% of CD19+)
20
15
10
5
***
***
**
dLN TFH
dLN TFR
Blood TFR

FIG. 21a

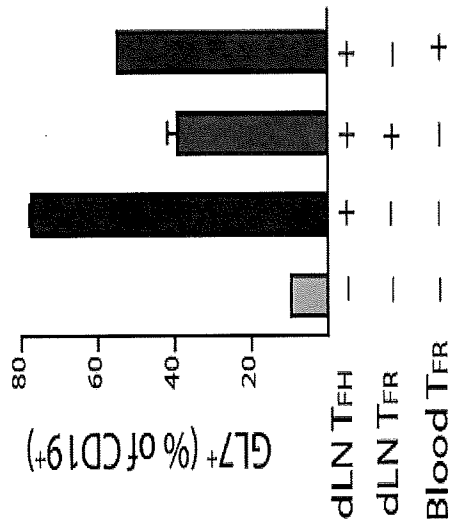


FIG. 21b

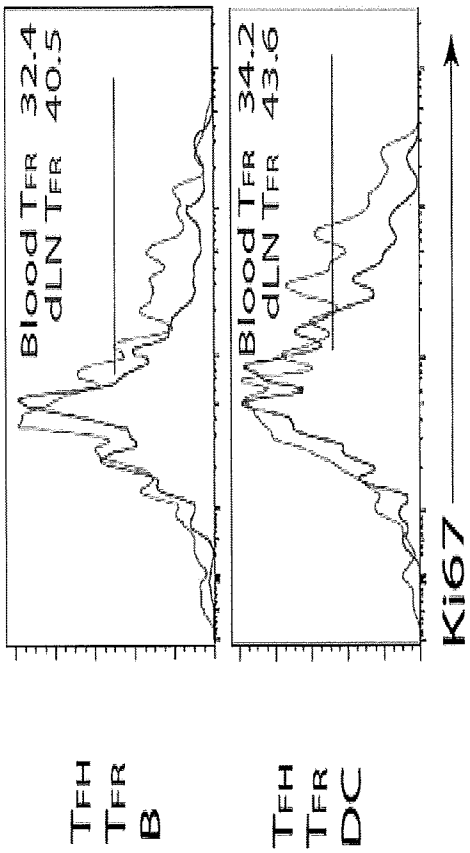

Blood TFR 32.4
dLN TFR 40.5
Blood TFR 34.2
dLN TFR 43.6
Ki67
TFH TFR B
TFH TFR DC

FIG. 21c

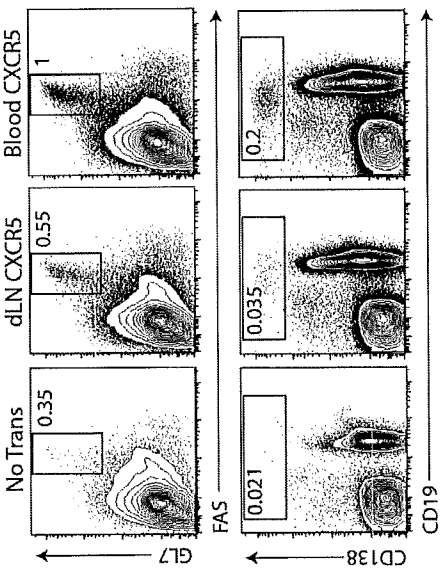


FIG. 21d

FIG. 21e

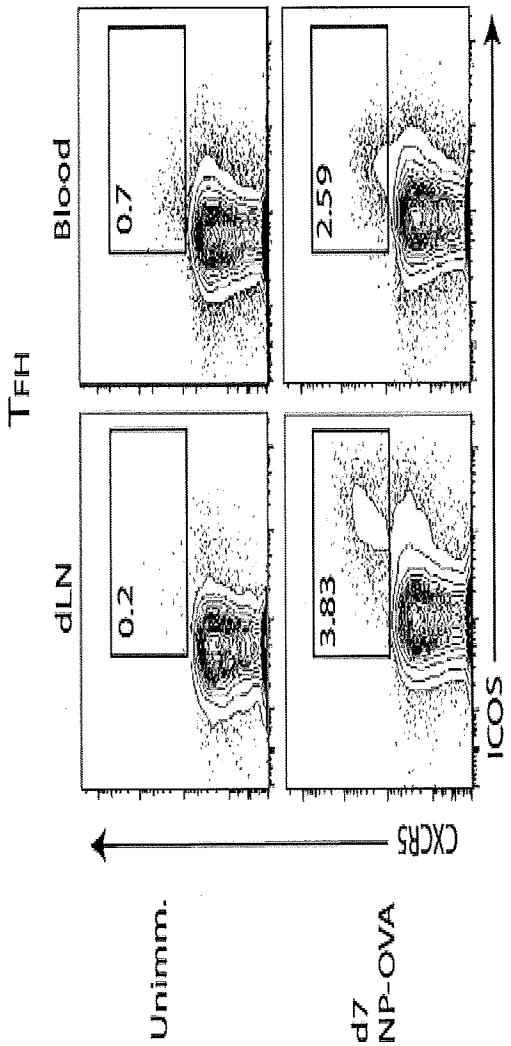


FIG. 22a

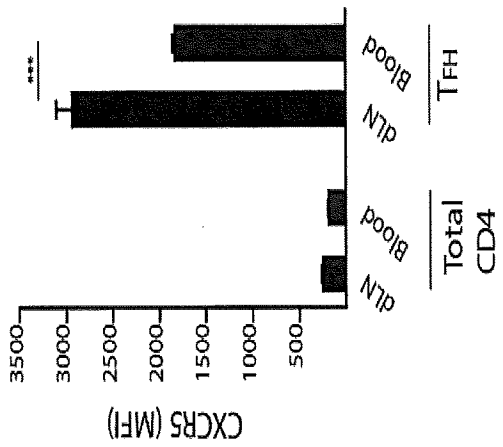


FIG. 22c

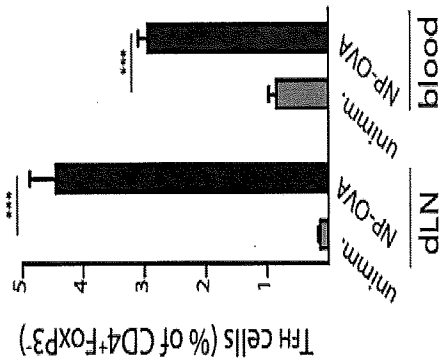


FIG. 22b

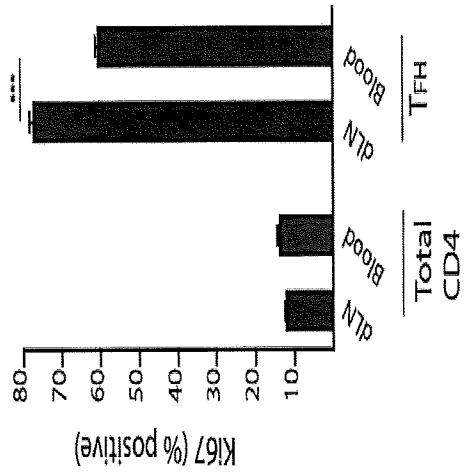


FIG. 22e

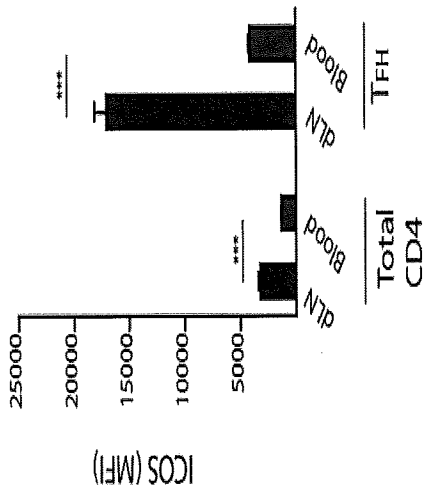


FIG. 22d

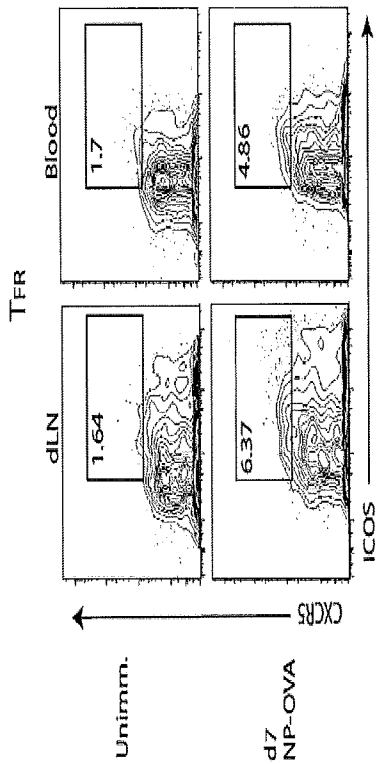


FIG. 22f

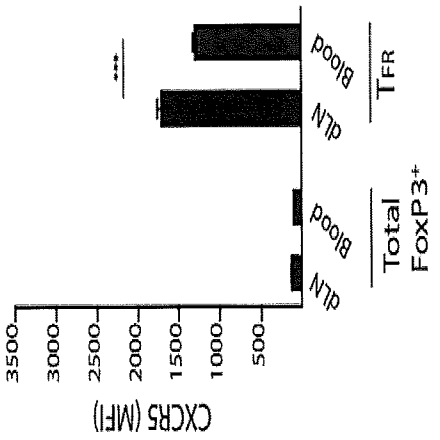


FIG. 22h

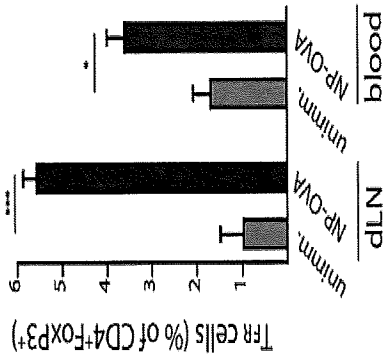


FIG. 22g

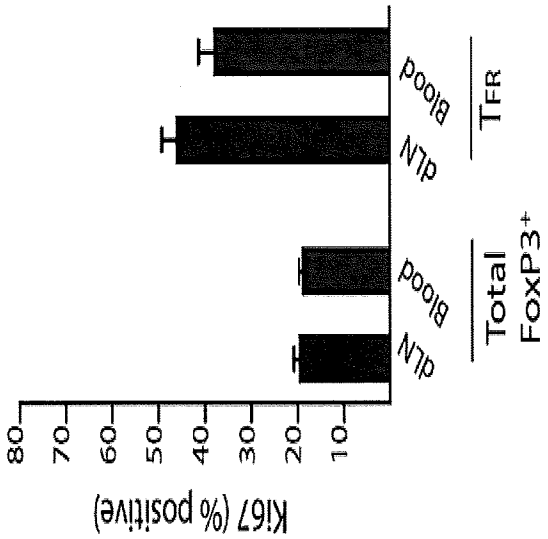


FIG. 22j

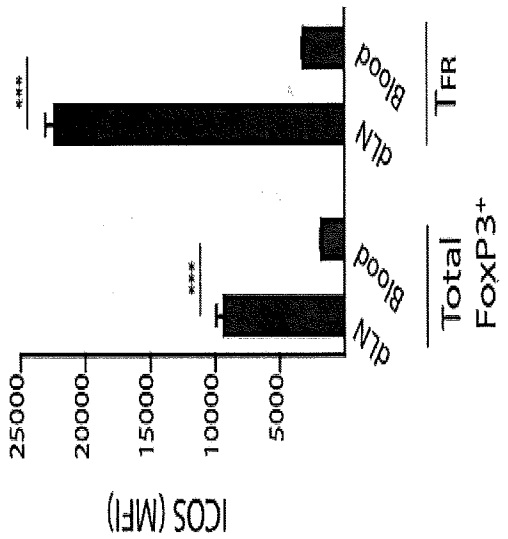


FIG. 22i

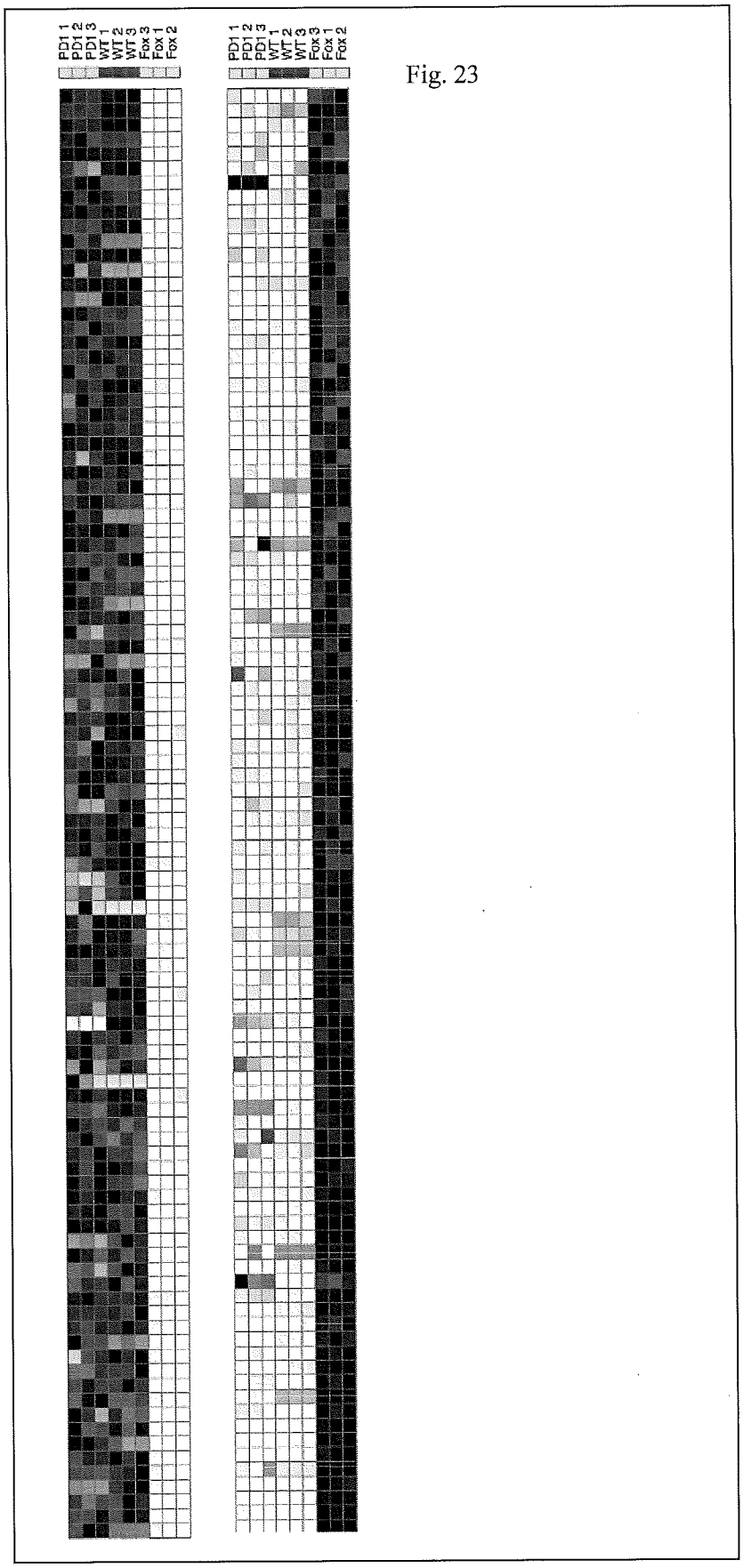
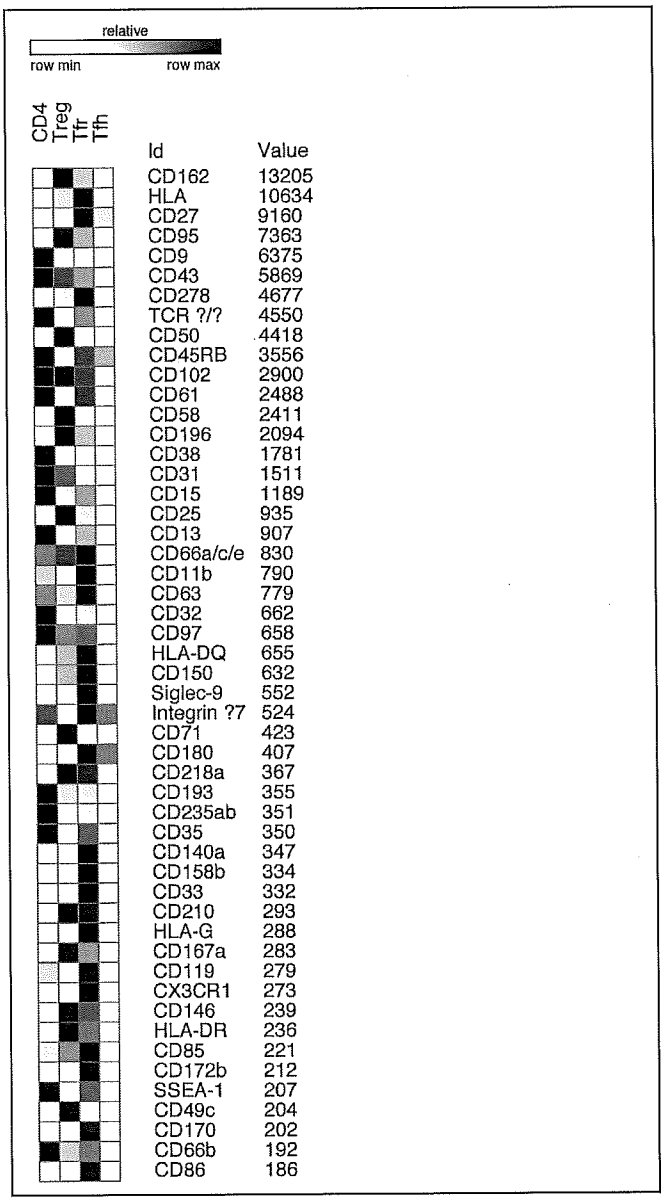
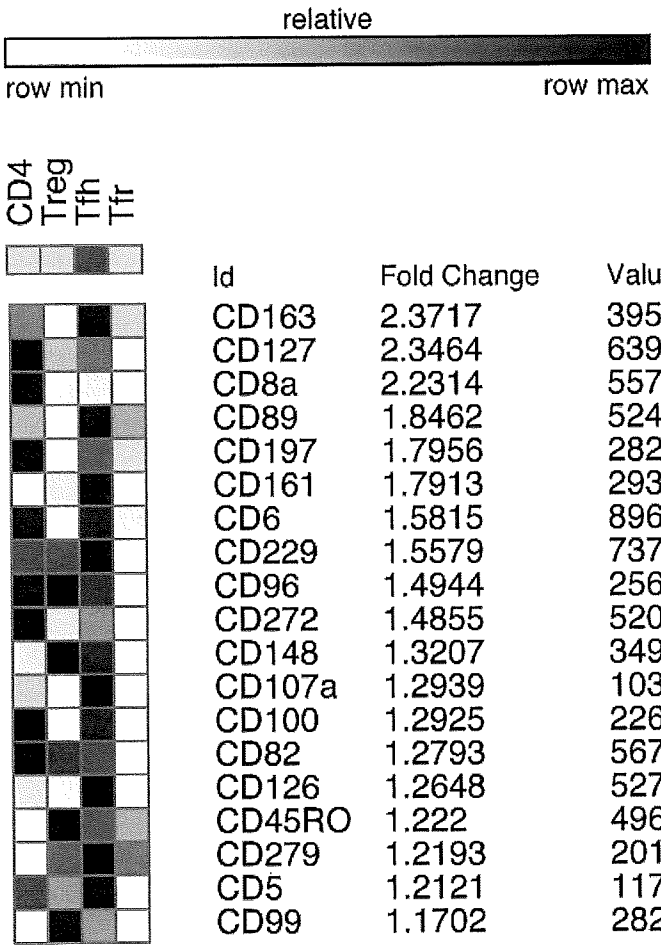

PD1 1
PD1 2
PD1 3
WT 1
WT 2
WT 3
Fox 3
Fox 1
Fox 2
PD1 1
PD1 2
PD1 3
WT 1
WT 2
WT 3
Fox 3
Fox 1
Fox 2

Fig. 23



Id	Value
CD162	13205
HLA	10634
CD27	9160
CD95	7363
CD9	6375
CD43	5869
CD278	4677
TCR ?/?	4550
CD50	4418
CD45RB	3556
CD102	2900
CD61	2488
CD58	2411
CD196	2094
CD38	1781
CD31	1511
CD15	1189
CD25	935
CD13	907
CD66a/c/e	830
CD11b	790
CD63	779
CD32	662
CD97	658
HLA-DQ	655
CD150	632
Siglec-9	552
Integrin ?7	524
CD71	423
CD180	407
CD218a	367
CD193	355
CD235ab	351
CD35	350
CD140a	347
CD158b	334
CD33	332
CD210	293
HLA-G	288
CD167a	283
CD119	279
CX3CR1	273
CD146	239
HLA-DR	236
CD85	221
CD172b	212
SSEA-1	207
CD49c	204
CD170	202
CD66b	192
CD86	186

FIG. 24



Id	Fold Change	Value
CD163	2.3717	395
CD127	2.3464	639
CD8a	2.2314	557
CD89	1.8462	524
CD197	1.7956	2821
CD161	1.7913	293
CD6	1.5815	8964
CD229	1.5579	737
CD96	1.4944	256
CD272	1.4855	520
CD148	1.3207	349
CD107a	1.2939	1033
CD100	1.2925	2262
CD82	1.2793	5676
CD126	1.2648	527
CD45RO	1.222	4964
CD279	1.2193	201
CD5	1.2121	11784
CD99	1.1702	28257

Figure X. Surface receptors differentially expressed by human blood TFH cells. Top 19 hits from a survey of surface receptor expression on human blood CD4+CXCR5+ ICOS+FoxP3+CD19- TFR, CD4+CXCR5+ ICOS+FoxP3-CD19- TFH, CXCR5- CD4 (CD4) and CXCR5- Tregs (Treg) are assessed at the protein level by flow cytometry. Hits are sorted based on receptors differentially expressed by TFH and TFR cells. Id indicates gene name. Fold change indicates MFI fold increase on TFH verus TFR cells. Value indicates mean fluorescence intensity on TFR cells. Heat map indicates relative high expression (black) or low expression (white).

FIG. 25

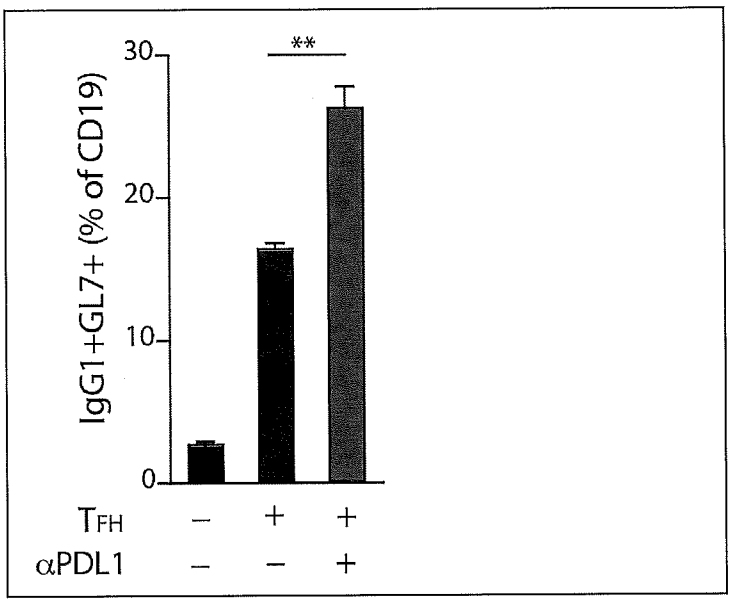


FIG. 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/069197

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i, A61P 37/00(2006.01)i

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)
A61K 39/395; C12Q 1/04; A61K 35/12; A61P 37/00; C12N 15/09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: follicular regulatory T cell, follicular helper T cell, immune, peripheral blood, PD-1, CD4, CXCR5, ICOS, GITR

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WOLLENBERG, I. et al., `Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells`, The Journal of Immunology, 2011, Vol. 187, No. 9, pages 4553-4560 See abstract, pages 4554, 4556, 4557, 4559.	89-90
A		1-6,25-30,36-38,43,53,97-98
X	WO 2012-063875 A1 (SYSMEX CORPORATION) 18 May 2012 See abstract, paragraphs [0002], [0003], [0009], [0102], tables 3 and 4.	36-38,91-92
A		1-6,25-30,43,53,89-90,97-98
A	WO 2010-003002 A2 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 07 January 2010 See abstract, claims 1-4.	1-6,25-30,36-38,43,53,89-92,97-98
A	WU, H. Y. et al., `Suppression of murine SLE by oral anti-CD3: inducible CD4+CD25-LAP+ regulatory T cells control the expansion of IL-17+ follicular helper T cells' , Lupus, 2009, Vol. 18, No. 7, pages 586-596 See abstract, page 587.	1-6,25-30,36-38,43,53,89-92,97-98

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2014 (20.02.2014)	**20 February 2014 (20.02.2014)**

Name and mailing address of the ISA/KR
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/069197

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.: 7-24, 31-35, 39-42, 44-52, 54-88, 93-96, 99-100
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 7-24, 31-35, 39-42, 44-52, 54-88, 93-96, 99-100 pertain to methods for treatment of the human body by surgery or therapy, as well as diagnostic methods practiced on the human body, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

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.:
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:

Group I. Claims 1-6, 25 (partially), 28-30, 53, 89, 90, and 97 directed to T follicular regulatory (TFR) cells.
Group II. Claims 25 (partially), 26, 27, 36-38, 43, 91, 92, and 98 directed to T follicular helper (TFH) cells.

1. ☐ As all required addtional 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 an additional fees, this Authority did not invite payment of any 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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/069197

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KING, C. et al., `T follicular helper (TFH) cells in normal and dysregulated immune responses`, Annual Review of Immunology, 2008, Vol 26, pages 741-766 See abstract.	1-6,25-30,36-38,43 ,53,89-92,97-98
PX	SAGE, P. T. et al., `The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood`, Nature Immunology, 16 December 2012 (E-pub.), Vol. 14, No. 2, pages 152-161 See abstract, pages 152, 160.	1-6,25-30,36-38,43 ,53,89-92,97-98

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/069197

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012-063875 A1	18/05/2012	EP 2639305 A1	18/09/2013
WO 2010-003002 A2	07/01/2010	WO 2010-003002 A3	22/04/2010
		WO 2010-003002 A9	20/05/2010