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(54) Title: NOVEL REGULATORY-CELLS, METHOD FOR THEIR ISOLATION AND USES

(57) Abstract: The invention pertains to methods of isolating a Treg from a subject, the method comprising the steps of analyzing a sample of cells obtained from the subject to determine the level of expression of polypeptides and/or polynucleotides corresponding to proteins CD 127, CD4, CD25, and CD226 and isolating, as the Treg, a cell which expresses CD4 and CD25, expresses low levels of CD 127 or does not express CD 127, and expresses low levels of CD226 or does not express CD226. The Treg isolated from a subject can be proliferated *in vitro*. Accordingly, the invention also provides compositions comprising Treg isolated/proliferated according to the methods of the invention and pharmaceutically acceptable carrier and/or excipient. The pharmaceutical compositions can be used to treat and/or prevent inflammation mediated disease, for example, autoimmune diseases, such as, systemic lupus erythematosus, rheumatoid arthritis, Multiple Sclerosis, type 1 diabetes, or inflammatory bowel disease.



DESCRIPTION

NOVEL REGULATORY T-CELLS, METHOD FOR THEIR ISOLATION AND USES

5 CROSS-REFERENCE TO A RELATED APPLICATION

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/156,506, filed May 4, 2015, which is incorporated herein by reference in its entirety.

GOVERNMENT SUPPORT

10 The subject invention was made with government support under a research project supported by the National Institutes of Health grant number 1-R01-DK-106191-01A1. The government has certain rights in the invention.

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BACKGROUND

The adaptive immune system provides a host with a vast receptor repertoire facilitating
20 protection from a wide array of pathogens. One consequence of this incredible diversity is the development of T and B cells specific for self-tissues. To counteract this autoreactivity, the immune system employs mechanisms to reinforce peripheral immune tolerance, including a dominant role for a small population of CD4⁺ regulatory T cells (Treg). The requirement for regulation is most apparent in individuals presenting with a mutation in the canonical transcription factor of the Treg lineage,
25 FOXP3, which results in an X-linked fatal autoimmune disease.

Treg exert their suppressive properties through a variety of mechanisms, including tolerogenic enzymatic pathways, production of immunoregulatory cytokines, and expression of cell-surface expressed negative regulators. Of these, CTLA-4 and PD-1 have been subject to extensive investigation for their ability to regulate T cell activation through interactions with antigen presenting
30 cells (APCs) and host tissues. Moreover, it is apparent that Treg, like their T_H cell counterparts, exhibit some level of lineage heterogeneity, as well as the potential for cellular plasticity in response to environmental cues.

Deficiencies in Treg cell frequency and/or function have been associated with the development of autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid
35 arthritis (RA), Multiple Sclerosis (MS), and type 1 diabetes (T1D). An increase in IFN γ ⁺Helios⁻ Treg

with reduced suppressive capacity was observed in patients with T1D. An analogous finding was also reported in patients with MS. This potential was also observed for the T_H17 lineage.

When analyzed at a single-cell level, IL-17-producing FOXP3⁺ Treg possessed some suppressive capacity, yet exhibited a transient loss of suppression in the presence of inflammatory cytokines. Collectively, these studies raise the intriguing potential that subsets of antigen-experienced Treg with reduced suppressor activity and effector-like properties may contribute to defective immune regulation in the context of persistent inflammation and autoimmune disease.

The ability to preserve and/or bolster the activity of Treg is useful for inhibiting autoimmune reactivity. Studies have generally focused on two subsets of Treg. Thymic-derived natural Treg (nTreg) express the transcription factors FOXP3 and Helios, are demethylated at the FOXP3-Treg Specific Demethylated Region (TSDR), and recognize primarily host antigens. In contrast, peripherally-induced CD4⁺FOXP3⁺Helios⁻ Treg (pTreg) develop from naïve T cells under tolerogenic conditions, are partially demethylated at the TSDR, and are generally directed to foreign antigens.

TIGIT has recently been associated with Treg biology through transcriptional profiling of Treg, and has also been identified as a locus epigenetically-marked as being highly demethylated in FOXP3⁺ T cells. As noted, TIGIT inhibits T cell activation intrinsically through its ITIM motif. Extrinsically, TIGIT ligation to CD155 on DCs leads to a reduction in IL-12 and a concomitant increase in IL-10 production. Importantly, TIGIT attenuates anti-tumor immunity by CD8⁺ T cells, and was recently demonstrated as a mechanism by which Treg exert their suppressive activity.

Protocols to generate human Treg for adoptive cell therapies are susceptible to contamination by non-Treg, heterogeneity from pTreg subsets, and the potential for lineage instability following extended periods of *in vitro* culture. Therefore, improved methods for the identification and isolation of Treg are needed.

BRIEF SUMMARY

The subject invention provides regulatory T cells (Treg), methods of identifying and isolating the Treg, and methods of using the Treg for treating and/or preventing diseases including, for example, inflammation-mediated diseases. In a specific embodiment, the inflammation-mediated disease is an autoimmune disease.

According to one embodiment of the invention, the method for isolating a Treg comprises the steps of:

a) analyzing a sample of cells obtained from a subject to determine the level of expression of polypeptides and/or polynucleotides corresponding to proteins CD4, CD25, CD226, and optionally, CD127, and

b) isolating, as the Treg, a cell which:

i) expresses CD4 and CD25, and

a) expresses low levels of CD127 or does not express CD127, and/or

b) expresses low levels of CD226 or does not express CD226.

The invention also provides a Treg isolated from a sample of cells from a subject, wherein the Treg expresses CD4 and CD25, expresses low levels of CD127 or does not express CD127, and expresses low levels of CD226 or does not express CD226. In a further embodiment, the Treg cell does not express CD226 protein.

In one embodiment, the Treg can be proliferated by culturing the Treg *in vitro*. Accordingly, the invention also provides a cultured Treg obtained by proliferating the Treg isolated from a subject.

The invention further provides pharmaceutical compositions comprising the Treg isolated from a subject, Treg proliferated from the Treg isolated from a subject, or a mixture thereof, and a pharmaceutically acceptable carrier and/or excipient.

Furthermore, the invention provides a method of treating and/or preventing an inflammation-mediated disease in a subject, the method comprising, administering to the subject a therapeutically effective amount of a composition comprising a Treg. In one embodiment, the Treg administered to the subject is autologous Treg, *i.e.*, Treg isolated from the subject and optionally, proliferated by culturing *in vitro*.

In further embodiments, the subject invention provides materials and methods for driving protective immune responses in the contexts of, for example, cancer treatment and vaccine development. In specific embodiments, the pathways identified according to the subject invention can be either inhibited or augmented to provide therapeutic benefit. This may be achieved using, for example, blocking monoclonal antibodies or through the use of Ig fusion proteins.

BRIEF DESCRIPTION OF THE FIGURES

To obtain a precise understanding of the invention, a more particular description of the invention described herein will be rendered by reference to specific embodiments thereof that are illustrated in the appended Figures. Thus, understanding that these Figures depict only certain embodiments of the invention and are not therefore limiting in scope, the invention will be described and explained with additional specificity and detail through the use of the accompanying Figures.

This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication, with color drawing(s), will be provided by the Office upon request and payment of the necessary fee.

Figures 1A-1C. TIGIT expression is enriched in FOXP3⁺Helios⁺ Tregs. CD4⁺ T cells were enriched from peripheral blood by negative selection and stained for CD4 and the intracellular transcription factors FOXP3 and Helios. (A) Cells were isolated by FACS to yield Tconv cells (*lower*

left gate), FOXP3⁺Helios⁻ T cells (lower right gate), and FOXP3⁺Helios⁺ Tregs (upper right gate), with representative populations shown. RNA was isolated from sorted populations and subjected to direct transcriptional profiling on the Human Immunology GX Panel on the NanoString platform. (B) TIGIT expression was 12.4-fold higher in FOXP3⁺Helios⁺ Treg compared to Tconv (N = 3, $p < 0.05$). (C) Representative TIGIT and Helios expression was shown in gated CD4⁺FOXP3⁺ T cells.

Figures 2A-2B. Differential expression of CD226 and TIGIT on CD4⁺ T cell subsets. PBMCs were stained for CD4, CD226, TIGIT and the transcription factors FOXP3 and Helios. (A) Representative plots show TIGIT and CD226 on total CD4⁺ T cells, Tconv, FOXP3⁺Helios⁻, and FOXP3⁺Helios⁺ Treg populations. (B) Graphs show summarized data demonstrating TIGIT is enriched in Treg populations (left graphs) analyzed by geometric Mean Fluorescence Intensity (gMFI) and percent positive, whereas CD226 is enriched in Tconv cells (right graphs). Shown are mean $\pm$ standard error with significance indicated (N = 5; n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Figures 3A-3G. High CD226 expression is associated with a memory CD4⁺ T cell phenotype. Cryopreserved PBMCs were thawed and stained for CD4, CCR7, CD45RA, CD45RO, CD226, and TIGIT or stimulated with PMA/ionomycin in the presence of monensin followed by intracellular staining of IFN γ . (A) A representative plot shows gated viable CD4⁺ naïve T cells (orange), central memory (dark blue), effector memory (green), and T effector memory CD45RA⁺ (TEMRA) (cyan) populations and their TIGIT/CD226 expression compared to total CD4⁺ T cells. A representative overlaid histogram shows CD226 expression on CD4⁺ T cells as a function of CD45RO expression. (B) Data from six subjects are summarized for frequency, CD226 gMFI, and percent TIGIT⁺ for each subset. (C) Cryopreserved PBMCs were thawed and stained for CD4, CD226, TIGIT, and the subset specific chemokine receptors CCR4 (T_H2), CCR6 (T_H17), and CXCR3 (T_H1). (D) Data from four subjects are summarized for Tregs (CD4⁺CD25⁺CD127^{-lo}) and Tconv (non-Tregs) and bifurcated by chemokine receptor expression. (E) Representative plots demonstrate that IFN γ ⁺ cells are predominantly CD226^{hi} (left plot) and are less frequent in TIGIT⁺ cells (right plot). (F) Summarized data of six subjects show CD226 and TIGIT expression on IFN γ ⁺ populations. (G) Cytokine capture assays allowed the isolation of Treg and Tconv subsets based on IFN γ production capacity. Expanded cells were restimulated with PMA/Ionomycin and analyzed by microarray (N = 5). IFN γ ⁺ and IFN γ ⁻ Tregs expanded into two distinct populations that could be distinguished from Tconv as shown by PCA analysis.

Figures 4A-4E. Direct ex vivo analysis of TIGIT and CD226 Treg. (A) CD4⁺CD25⁺CD127⁻ T cells were sorted by FACS and further subdivided based on TIGIT and CD226 expression. (B) Freshly sorted Treg subsets were analyzed for FOXP3-TSDR demethylation. (C) Treg subsets and autologous Tconv cells were stained with distinct proliferation dyes and cultured at

indicated ratios in the presence of Treg suppression Inspector beads for 4d. Percent suppression was calculated by division index of CD4⁺ Tconv cells in co-culture relative to Tconv cells alone. (D) The proliferation of each Treg subsets was shown in division index. (E) IL-10 production in suppression assay co-culture supernatants at indicated Treg:Tconv cell ratios.

Figures 5A-5I. *In vitro* Expansion of Tregs expressing TIGIT and/or CD226 yield subpopulations differing in proliferative ability, purity, and suppressive capacity. CD4⁺CD25⁺CD127^{lo} Tregs were isolated from fresh peripheral blood and further divided to yield four distinct populations based on CD226 and TIGIT expression (A) TIGIT⁺ Tregs were refractory to expansion as the number of cells yielded on day 0 (*left graph*) was inversely related to fold expansion following 14 d of culture (*right graph*). Following expansion, cells were reactivated with PMA/ionomycin for 4 h and assessed for FOXP3 and Helios (B, C), TIGIT and CD226 (D, E), and IFN γ and CD226 (F, G). (H) The *in vitro* suppressive capacity of expanded Tregs was assessed. Percent suppression was calculated by division index of the CD4⁺ or CD8⁺ gated responder T cells in co-culture relative to responders alone. (I) Graphs indicate the percent of cells demethylated at the FOXP3-TSDR (*left graph*) and the correlation between FOXP3-TSDR analysis and FOXP3 and Helios expression as analyzed by FACS following 14 d cultures (*right graph*; $R^2 = 0.94$, $p < 0.0001$). Data are represented as $\pm$ standard error of the mean (N = 4).

Figures 6A-6H. CD226⁺TIGIT⁺ Tregs display an intermediate effector gene expression profile. mRNA was isolated from 14 d *in vitro* expanded Treg subsets and Tconv cells and analyzed for gene expression on the Human Immunology GX Panel on the NanoString platform. (A) Treg and Tconv profiles shown by hierarchical clustering based on the Euclidian distance was used to create a heatmap and dendrogram of the genes in Partek Genomic Suite. Select genes of interest are shown and grouped based on (B) coinhibitory receptors, (C) cytokines, (D) cytokine receptors or subunits, and (E) lineage-associated transcription factors. Normalized gene counts are represented as $\pm$ standard error of the mean with grey horizontal lines indicating the background expression threshold (N = 3). (F) The similarity of each Treg subsets to Tconv cells was shown by PCA analysis. (G-H) Fresh Treg subsets sorted according to CD226 and TIGIT expression were activated by CD3/28 dynabeads for 24, 48, and 72 hrs. (G) Cytokine production was tested by multiplex assay and shown by bar graph and (H) heatmap for 72hrs. Data are represented as $\pm$ standard error of the mean (N = 4).

Figures 7A-7K. Exclusion of CD226⁺ Tregs reduces IFN γ production and augments the *in vitro* suppressive capacity of Tregs. (A) Total CD4⁺CD25⁺CD127^{lo/-} Tregs or CD4⁺CD127⁺ Tconv cells were FACS sorted and Tregs were further subdivided into CD226⁺ and CD226⁻ subsets (*right plot*). (B) Cells were expanded for 14 d, rested to 21 d, and then reactivated with PMA/ionomycin for 4 h. Treg purity was assessed by FACS for (C, D) FOXP3 and Helios, (E) CD226 expression by gMFI, (F, G) IFN γ and CD226 expression, and (H) TIGIT expression by gMFI.

(I) CD226⁺ and CD226⁻ Treg subsets were assessed for their ability to suppress autologous CD4⁺ or CD8⁺ responder T cells from PBMC. (J) The ratio of TIGIT and CD226 expression, as assessed by gMFI on gated Treg populations following the suppression assay (d4). (K) Graph indicates the percent of cells demethylated at the FOXP3-TSDR. Data are represented as $\pm$ standard error of the mean (N=4).

Figures 8A-8E. IL-12 augments T_H1-skewing of CD4⁺ T cells and attenuates TIGIT expression and proliferation by Tregs. Human PBMCs (2.5x10⁵ cells/well) were activated with anti-CD3 (2 μ g/mL) and anti-CD28 (1 μ g/mL) in media alone (*blue bars*) or with the addition of IL-12 (1 ng/mL; *red bars*). Production of IFN γ was assessed during the final 4 h of culture by addition of monensin prior to harvesting and intracellular cytokine analysis by FACS. (A) Representative plots show TIGIT and CD226 expression on FOXP3⁺ or FOXP3⁻CD4⁺ T cells at 24, 48, and 72 h of culture. Data are summarized as (B) gMFI of TIGIT on CD4⁺FOXP3⁺Helios⁺ and CD4⁺FOXP3⁺Helios⁻ cells, (C) CD226 gMFI on CD4⁺ T cells, (D) percent CD4⁺IFN γ ⁺ cells, and (E) frequency of TIGIT expression on FOXP3⁺Helios⁺ Tregs. Shown is the standard error of the mean (N = 6).

Figures 9A-9B. IFN γ ⁺ Treg express elevated CD226 and reduced TIGIT. Cytokine cell capture reagent was used to isolate Treg and Tconv subsets based on their capacity to produce IFN γ . (A) Shown are the overlapping differentially expressed genes between 4 hr PMA and ionomycin activated IFN γ ⁺ and IFN γ ⁻ Tregs immediately after FACS isolation (fresh) and following 14 d *ex vivo* expansion and 4 hr PMA and ionomycin reactivation (expanded). (B) Differentially expressed genes between all four populations (IFN γ ^{+/+} Treg and Tconv subsets) demonstrate that TIGIT is elevated in Treg and highly expressed in IFN γ ⁻ Treg subset (N = 5).

Figure 10. Model depicting CD226 and TIGIT signaling as key checkpoint impacting immune regulation in type 1 diabetes (T1D).

Figures 11A-11B. LV overexpression of CD226 and TIGIT. Human Treg were isolated by FACS and transduced at 48h post-activation to overexpress (A) GFP mock, CD226 (C, major wt allele), CD226 (T, mutant allele), wild-type TIGIT, or a dominant negative TIGIT (Y225A, Y231A). Six replicate wells were harvested at d14 for FACS analysis. (B) Dual dye labeling allows the simultaneous monitoring of Treg and Teff proliferation during *In vitro* suppression assays. Preliminary data suggests overexpression of full-length, but not a dominant neg. form of TIGIT, augments Treg-mediated suppression of CD4⁺ Tconv cell proliferation. CTV, cell trace violet.

Figure 12. LV gene transfer of the IGRP TCR generates MHC pentamer⁺ CD8⁺ T cells. Human CD8⁺ T cells were transduced with a LV vector encoding the IGPR-TCR (Table 4).

Figures 13A-13B. Cell-mediated lymphocytotoxicity (CML) assay for CD8⁺ T cells. A) Primary CD8⁺ T cells were stimulated with anti-CD3/28 beads and then transduced with LV encoding

the IGRP TCR. After 9 d, GFP⁺ cells were sorted and expanded for an additional 7 d. *B*) MART-1 (ctrl) or IGRP CTL were used in a CML against BL5 indicating effective lysis of β cells at a 1:5 E:T ratio.

Figures 14A-14D. IL-12 augments T_H1-skewing of CD4⁺ T cells and attenuates TIGIT expression and proliferation by Treg. Human PBMCs (2.5×10^5 cells/well) were activated with anti-CD3 (2 μ g/mL) and anti-CD28 (1 μ g/mL) in media alone (*blue bars*) or with the addition of IL-12 (1 ng/mL; *red bars*). IFN γ was assessed during the final 4 h of culture by FACS. Data are summarized as (*A*) gMFI of TIGIT on nTreg (CD4⁺FOXP3⁺Helios⁺), (*B*) CD226 gMFI on CD4⁺ T cells, (*C*) percent CD4⁺IFN γ ⁺ cells, and (*D*) frequency of nTreg. Shown is the mean + SE (N = 6).

Figure 15. CD226 and TIGIT expression in NOD mice. pDLNs were harvested from female NOD mice at 4 wks of age or at onset (Dbx). Single cell suspensions were stained for live/dead, CD4, FoxP3, CD226, and TIGIT. Shown are the ratio of CD226:TIGIT (as a ratio of gMFI) on gated Treg (CD4⁺FoxP3⁺) and Tconv (CD4⁺FoxP3⁻) cells (n=4, p=0.07).

Figure 16. IFN γ ⁺ Treg are enriched in T1D. PBMC from healthy controls (n = 10) or patients with T1D (n = 11, including 1 recent-onset case, *square*) were stimulated 4 h with PMA/ionomycin. Viable CD4⁺ lymphocytes were gated, and FOXP3, Helios, and IFN γ were analyzed by FACS. **P < 0.01.

Figures 17A-17C. IFN γ ⁺ Treg express elevated CD226 and reduced TIGIT. Human IFN γ ⁺ Treg and Tconv were isolated by FACS (9). (*A*) Shown is CD226 heat signature between 4 h PMA/ion activated IFN γ ⁺ and IFN γ ⁻ Treg immediately after FACS isolation (fresh) and following 14 d *ex vivo* expansion and reactivation (expanded). (*B*) Differentially expressed genes between all four populations (IFN γ ^{+/+} Treg and Tconv subsets, d14) demonstrate that TIGIT is elevated in Treg and highly expressed in IFN γ ⁻ Treg subset (N=5). (*C*) PCA analysis of d14 expanded populations.

Figures 18A-18C. Analysis of T cells from human spleen, pDLN, and islets in T1D. (*A*) A single cell suspension of lymphocytes was prepared from spleen and LN material and subjected to intracellular staining for Helios and FOXP3. Shown are representative plots from indicated tissues. Immunohistochemistry images depict insulin staining and T cell infiltration in pancreatic islets from a 1 yr old T1D (*B* and *C*).

Figure 19. Isolation of live viable human B cells, CD8⁺ T cells, CD4⁺ Treg and Tconv cell subsets. pDLN from a T1D donor (1 yr duration, insulinitis and triple auto-Ab⁺). Single cell suspensions were surface stained and FACS sorted with cell yields indicated.

Figure 20. Expansion of Treg expressing TIGIT and/or CD226 yield subpopulations differing in proliferative ability, purity, and suppressive capacity. CD4⁺CD25⁺CD127^{-lo} Treg (*upper left plot*) were isolated from 100 mL of human PB and further divided to yield four distinct

populations based on CD226 and TIGIT expression (*lower left, indicated sort quadrants*). Day 14 expanded Treg and Tconv were assessed for FOXP3 and Helios (N=4).

Figure 21A-21C. Elimination of CD226⁺ cells results in a highly pure population of Treg. CD4⁺CD25⁺CD127^{-lo} Treg were further divided based on initial CD226 expression and expanded for 14 d. (A) Shown are the frequency of CD226⁺IFN γ ⁺ cells post activation, (B) the ratio of TIGIT:CD226 on Treg following *in vitro* suppression culture, and C) the percent of cells demethylated at the TSDR.

Figures 22A-22C. Determination of the methylation status of the FOXP3 – Treg Specific Demethylated Region (TSDR). (A) A schematic view of the genomic exon-intron structure of the *foxp3* gene and TSDR position (CNS2). The enlarged TSDR fragment depicts the distribution and position of individual CpG motifs contained within the TSDR and the selected amplicon for real-time PCR assay (indicated by the black marked target region) covering the sequence: 5'-GGTCTGCATCTGGGCCCTGTTGTCACAGCCCCCGACTTGCCCAGATTTTTCCGCCATTGACGTCATGGCGGCCGGATGCGCCGGGCTTCATCGACACCACGGAGGAAGAGAAGAGGGCA
GATACCCACCCACAG-3' (SEQ ID NO: 1). (B) Graphical illustration of the FOXP3-TSDR methylation-specific real-time PCR assay. Sample DNA is bisulfite-converted and the target sequence is amplified. FAM and VIC dye-labeled internal MGB Taqman® probes are designed for binding specifically to either the unmethylated (VIC) or methylated (FAM) allele sequence, respectively. Ratios of methylated and unmethylated standards were serially titrated to generate a standard curve. (C) The difference of Ct values (Ct unmethylated probe – Ct methylated probe) is plotted against the standards to extrapolate the percent of cells demethylated at the TSDR.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: 1: Selected amplicon for real-time PCR assay for TSDR fragment of *foxp3* gene.

GGTCTGCATCTGGGCCCTGTTGTCACAGCCCCCGACTTGCCCAGATTTTTCCGCCATTGAC
GTCATGGCGGCCGGATGCGCCGGGCTTCATCGACACCACGGAGGAAGAGAAGAGGGCA
GATACCCACCCACAG

SEQ ID NO: 2: FOXP3_TSDRfwd primer for the standard curve
(ATATTTTATAGATAGGGATATGGAGATGATTTGTTTGG)

SEQ ID NO: 3: FOXP3_TSDRrev primer for the standard curve
(AATAAACATCACCTACCACATCCACCAACAC).

SEQ ID NO: 4: The probe used for PCR amplification, TSDR-Forward
GGTTTGTATTTGGGTTTGTGTTATAGT

SEQ ID NO: 5: The probe used for PCR amplification TSDR-Reverse
CTATAAAATAAAATATCTACCCTCTTCTCTTCCT

SEQ ID NO: 6: Sequence of the probe for methylated target sequence detection.
CGGTCGGATGCGTC

5 **SEQ ID NO: 7:** Sequence for the probe for unmethylated target sequence detection.
TGGTGGTTGGATGTGTTG

DETAILED DESCRIPTION

10 In one embodiment, the subject invention provides regulatory T cells (Treg), methods of identifying and isolating the Treg, and methods of using the Treg for treating and/or preventing diseases including, for example, inflammation-mediated diseases. In a specific embodiment, the inflammation-mediated disease is an autoimmune disease.

In specific embodiments, the subject invention provides markers to delineate functionally distinct Treg subsets that help direct cellular therapies and provide important phenotypic markers for
15 assessing the role of Treg in health and disease.

According to one embodiment of the invention, the method for isolating a Treg comprises the steps of:

- a) analyzing a sample of cells obtained from a subject to determine the level of expression of CD4, CD25, CD226, and optionally, CD127, and
- 20 b) isolating, as the Treg, a cell which:
 - i) expresses CD4 and CD25,
 - a) expresses low levels of CD127 or does not express CD127, and/or
 - b) expresses low levels of CD226 or does not express CD226.

25 In one embodiment of the invention, the level of expression of CD127, CD4, CD25, and CD226 is analyzed at the mRNA level. For example, oligonucleotides corresponding to the target mRNAs can be labeled and the sample of cells can be contacted with the labeled oligonucleotides. Oligonucleotides corresponding to different mRNAs can be labeled differently to facilitate identification of the presence of the target mRNAs within a cell.

30 In another embodiment of the invention, the level of expression of CD127, CD4, CD25, and CD226 is analyzed at the protein level. For example, the analysis of the expression of CD127, CD4, CD25, and CD226 proteins can be performed using flow cytometry using labeled antibodies specific for CD127, CD4, CD25, and CD226. Various techniques for labeling, identification, and isolation of cells by flow cytometry are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

The invention also provides a Treg isolated from a sample of cells from a subject, wherein the Treg expresses CD4 and CD25, expresses low levels of CD127 or does not express CD127, and expresses low levels of CD226 or does not express CD226. In a further embodiment, the Treg cell does not express CD226 protein.

5 In a further embodiment, Treg expresses CD4 and CD25, and expresses low levels of CD226 or does not express CD226. In certain embodiments, Treg cell is $CD4^+CD25^+CD226^-$.

In one embodiment, the Treg can be proliferated by culturing the Treg *in vitro*. Accordingly, the invention also provides a cultured Treg obtained from proliferating the Treg isolated from a subject.

10 The invention further provides pharmaceutical compositions comprising the Treg isolated from a subject, Treg proliferated from the Treg isolated from a subject, or a mixture thereof, and a pharmaceutically acceptable carrier and/or excipient.

Furthermore, the invention provides a method of treating and/or preventing an inflammation-mediated disease in a subject, the method comprising, administering to the subject a therapeutically effective amount of a composition comprising a Treg. In one embodiment, the Treg administered to the subject is autologous Treg, *i.e.*, Treg isolated from the subject and optionally, proliferated by culturing *in vitro*.

15 In one embodiment of the subject invention, immune responses associated with CD226 are enhanced in order to elicit an immune response useful for the treatment of cancer and/or to generate a protective immune response against a pathogen.

Definitions

As used herein the term “Treg” refers to regulatory T cells, either singular or plural.

25 As used herein, the terms “express,” “expresses,” or “expressing” at low levels (indicated by “^{lo}”), or any other synonymous terms, refer to a level of expression of a polypeptide or polynucleotide, such as CD127, CD4 or CD25, by a cell within a sample that is lower when compared to the level of expression of that polypeptide or polynucleotide by the population of cells comprising the whole of the sample being analyzed. For example, the term “CD127^{lo}” refers to a level of expression of CD127 by a particular cell within the sample that is lower compared to the level of expression of CD127 by the population of cells comprising the whole of the sample being analyzed.

30 Similarly, the terms “express,” “expresses,” or “expressing” (indicated by “⁺”), or any other synonymous terms, refer to a level of expression of a polypeptide or polynucleotide, such as CD127, CD4 or CD25, by a cell within a sample that is higher compared to the level of expression of that molecule or polynucleotide by the population of cells comprising the whole of the sample being analyzed. For example, the term “CD4⁺” refers to a level of expression of CD4 by a particular cell

within the sample that is higher compared to the level of expression of CD4 by the population of cells comprising the whole of the sample being analyzed. More particularly, the term “⁺” may refer to a distinct cell that expresses a particular molecule at a level that is higher than that expressed by one or more other distinct populations within a sample.

5 Further, the terms “express,” “expresses,” or “expressing” at high levels (indicated by “^{hi}”), or any other synonymous terms, refer to a level of expression of a particular polypeptide or polynucleotide, such as CD127, CD4 or CD25, by a particular cell within a sample that is substantially higher (for example, by more than 2 fold, 10 fold, 100 fold, 1000 fold or more) compared to the level of expression of that molecule or polynucleotide by the population of cells
10 comprising the whole of the sample being analyzed. For example, in one embodiment the term “CD25^{hi}” refers to a level of expression of CD25 by a particular cell within the sample that is about 10 times higher when compared to the level of expression of CD25 by the population of cells comprising the whole of the sample being analyzed.

Also as used herein, the terms “do not express,” “does not express,” or “not expressing”
15 (indicated by “⁻”), or any other synonymous terms, refer to a level of expression of a particular polypeptide or polynucleotide, such as CD127, CD4 or CD25, by a cell within a sample that cannot be detected by the standard molecular technique used to analyze the particular molecule. Therefore, a cell identified as “not expressing” a polypeptide or polynucleotide may be expressing some level of the polypeptide or polynucleotide; however, the level of expression is too low to be detected by the
20 technique used. For example, the term “CD127⁻” refers to a level of expression of CD127 protein by a particular cell within the sample which cannot be detected by the standard molecular biology technique used to detect CD127 protein expression.

Treg Subsets

25 To better elucidate human Treg subsets, a direct transcriptional profiling of CD4⁺FOXP3⁺Helios⁺ natural Treg (nTreg) and CD4⁺FOXP3⁺Helios⁻ peripheral Treg (pTreg) was conducted and was compared to CD4⁺FOXP3⁻Helios⁻ T cells, followed by comparison to CD4⁺FOXP3⁻Helios⁻ T conventional (Tconv) cells. This analysis revealed the coinhibitory receptor T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) to be
30 highly expressed on nTreg. Given that TIGIT competes with the costimulatory factor CD226 for binding to CD155, the cellular distribution and suppressive activity of isolated subsets of CD4⁺CD25⁺CD127^{lo/-} T cells expressing CD226 and/or TIGIT were analyzed. TIGIT expression on Treg is upregulated following activation and *in vitro* expansion, and correlates with lineage stability and suppressive capacity. Conversely, the CD226⁺TIGIT⁻ population was associated with reduced

Treg purity and suppressive capacity following expansion, along with a marked increase in IL-10 and effector cytokine production.

The subject invention provides isolated subsets of human CD4⁺CD25⁺CD127^{-/lo} Treg. These subsets were proliferated *ex vivo* to assess their phenotype and suppressive capacity. Production of the effector cytokine IFN γ within Tconv cells and the CD4⁺CD25⁺CD127^{-/lo} Treg pool was found to be tightly linked to co-expression of the costimulatory molecule CD226. Conversely, selection of CD226⁻ Treg, irrespective of initial TIGIT expression, leads to a highly enriched population of nTreg that are demethylated at the TSDR, express TIGIT and suppress T proliferation.

10 Methods for Isolating Treg

In one embodiment, the invention provides a method of isolating Treg, wherein the step of analyzing the level of expression of CD4, CD25, CD226, and optionally, CD127, proteins comprises:

- (a) obtaining a sample of cells from a subject;
- (b) contacting the cells with antibodies directed towards CD4, CD25, CD226, and optionally, CD127 proteins to allow binding of the antibodies to the corresponding proteins;
- (c) subjecting the sample of cells to flow cytometry;
- (d) examining the flow cytometry signal for the expression of CD4, CD25, CD226, and optionally, CD127 proteins; and
- (e) isolating, as the Treg, a cell which:
 - i) expresses CD4 and CD25,
 - a) expresses low levels of CD127 or does not express CD127, and/or
 - b) expresses low levels of CD226 or does not express CD226.

The analysis of the expression of CD127, CD4, CD25, and CD226 proteins can be performed simultaneously or sequentially.

For example, when the analysis of CD127, CD4, CD25, and CD226 proteins is performed sequentially, a sample of cells can be contacted with labeled antibodies against CD127, CD4, CD25, and CD226 proteins followed by the steps of:

- i) identifying and isolating CD4 expressing cells,
- ii) identifying and isolating CD25 expressing cells within the CD4 expressing cells,
- iii) identifying and isolating cells expressing low levels of CD127 and/or not expressing CD127 within the cells expressing both CD25 and CD4, and
- iv) identifying and isolating cells expressing low levels of CD226 and/or not expressing CD226 within the cells expressing CD24 and CD25 and expressing low levels of CD127 and/or not expressing CD127.

In a further example, when the analysis of CD4, CD25, and CD226 proteins is performed sequentially, a sample of cells can be contacted with labeled antibodies against CD4, CD25, and CD226 proteins followed by the steps of:

- i) identifying and isolating CD4 expressing cells,
- 5 ii) identifying and isolating CD25 expressing cells within the CD4 expressing cells,
- iii) identifying and isolating cells expressing low levels of CD226 and/or not expressing CD226 within the cells expressing CD24 and CD25.

A person of ordinary skill in the art can envision and design alternative methods of sequential separation of cells to obtain the Treg according to the claimed invention and such embodiments are
10 within the purview of the invention.

Similarly, when the analysis of CD127, CD4, CD25, and CD226 proteins is performed simultaneously, a sample of cells can be contacted with labeled antibodies against CD127, CD4, CD25, and CD226 proteins followed by the steps of:

- i) identifying and isolating the cells expressing both CD4 and CD25, and
- 15 ii) identifying and isolating the cells expressing low levels of CD226 and/or not expressing CD226 and expressing low levels of CD127 and/or not expressing CD127 within the cells expressing both CD24 and CD25.

Also, when the analysis of CD4, CD25, and CD226 proteins is performed simultaneously, a sample of cells can be contacted with labeled antibodies against CD4, CD25, and CD226 proteins
20 followed by the steps of:

- i) identifying and isolating the cells expressing both CD4 and CD25, and
- ii) identifying and isolating the cells expressing low levels of CD226 and/or not expressing CD226 within the cells expressing both CD24 and CD25.

A person of ordinary skill in the art can envision and design alternative methods of
25 simultaneous separation of cells to obtain the Treg according to the claimed invention and such embodiments are within the purview of the invention.

A person of ordinary skill in the art can determine the sequence of a particular protein or polynucleotide corresponding to a protein of interest (*e.g.*, CD4, CD25, CD127, and CD226) and corresponding antibodies or other techniques required to isolate Treg according the methods of the
30 invention.

The subject from which the Treg can be obtained according to the methods of the invention can be a mammal. Non-limiting examples of mammals include mice, rats, cats, dogs, bovine, porcine, non-human primates and humans.

The sample of cells can be obtained from a biological sample from the subject. The
35 biological sample may be, for example, bodily fluids or tissue. Non-limiting examples of bodily

fluids include whole blood, isolated peripheral blood mononuclear cells (PBMC) or leukophoresis products, urine, saliva, buccal mucosa, interstitial fluid, and lymph fluid. The bodily fluid can be appropriately treated before it is used pursuant to the methods of the invention.

Tissues from which Treg can be obtained according to the methods of the invention include, but are not limited to, tonsil, thymus, spleen, lymph node or a non-lymphoid tissue. Tissues can also be appropriately treated to obtain a sample of cells from the subject before implementing the methods of the invention.

Isolated Treg

The invention also provides a Treg isolated from a sample of cells obtained from a subject, wherein the Treg, at the protein or mRNA level, expresses CD4 and CD25, expresses low levels of CD127 or does not express CD127, and expresses low levels of CD226 or does not express CD226. In one embodiment, the Treg expresses CD4 and CD25 proteins, expresses low levels of CD127 protein or does not express CD127 protein, and expresses low levels of CD226 protein or does not express CD226 protein. In a further embodiment, the Treg cell does not express CD226 protein. In a further embodiment, the Treg expresses high levels of CD4 and CD25 proteins, does not express CD127 or expresses low levels of CD127 and does not express CD226. In an even further embodiment, the Treg expresses high levels of CD4 and CD25 proteins, does not express CD226 or expresses low levels of CD226.

The Treg can be proliferated by culturing the Treg *in vitro*. Accordingly, the invention also provides Treg obtained by proliferating the Treg isolated from a subject. Methods of proliferating Treg obtained from a subject in culture are well known to a person of ordinary skill in the art.

For the purposes of the invention, the term “proliferate” or any of the relevant terms or synonyms thereof (e.g. proliferating, proliferated, multiplied, amplified, *etc.*) refer to culturing the Treg under appropriate conditions that allows for an increase in the number of Treg.

Pharmaceutical Compositions

The invention further provides pharmaceutical compositions comprising Treg isolated from a subject; Treg proliferated from Treg isolated from a subject; or a mixture thereof and a pharmaceutically acceptable carrier and/or excipient.

Pharmaceutical compositions, as disclosed herein, can be formulated in accordance with standard pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York) known by a person skilled in the art. Pharmaceutical composition according to the invention may also be

formulated to release active agents (e.g., Treg as disclosed herein alone or in combination with a chemotherapeutic agent) substantially immediately upon administration or at any predetermined time or time period after administration.

Compositions for parenteral administration are generally physiologically compatible sterile solutions or suspensions that can optionally be prepared immediately before use from solid or lyophilized form. Adjuvants, local anesthetics, preservatives and/or buffering agents can be added to the vehicle and a surfactant or wetting agent can be included in the composition to facilitate uniform distribution of the active ingredient.

The composition can be formulated into conventional dosage forms, such as liquid preparations, syrups, and concentrated drops. Non-toxic solid carriers or diluents may be used, which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, magnesium, carbonate.

The pharmaceutical composition of the invention may be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as lyophilized preparations, liquids solutions or suspensions, injectable and infusible solutions, *etc.* The preferred form depends on the intended mode of administration and therapeutic application.

Administration of Treg

The Treg can be administered to a subject via conventional means. For example, Treg can be transferred to the desired tissue, either *in vitro* (e.g., as a graft prior to implantation or engrafting) or *in vivo*, to the subject's tissue directly.

Treg can be transferred to the desired tissue by any appropriate method depending on the tissue type. In the case of a graft tissue, Treg can be transferred to the graft by bathing the graft (or infusing it) with culture medium containing the cells or the Treg can be seeded onto the desired site within the tissue to establish a population. Devices such as catheters, trocars, cannulae, and stents seeded with the Treg can be used as appropriate.

In further embodiments, the composition of the invention can be administered to the subject via local or systemic route. Non-limiting examples of the systemic route include intravenous, intraperitoneal, intramuscular or subcutaneous. Additional methods and routes of administering pharmaceutical compositions of the claimed invention to a subject in need thereof are well known to a person of ordinary skill in the art and such embodiments are within the purview of the claimed invention.

Therapeutical Treatments

Furthermore, the invention provides a method of treating and/or preventing an inflammation-mediated disease in a subject, wherein the method comprises, administering to the subject a therapeutically effective amount of the pharmaceutical compositions comprising Treg and a pharmaceutically acceptable carrier and/or excipient.

5 The subject that can be treated according the methods of the invention can be a mammal. Non-limiting examples of mammals treatable according to the methods of the invention include a mouse, a rat, a cat, a dog, a bovine, a porcine, a non-human primate or a human.

For the purposes of this invention the term “inflammation-mediate disease” refers to a disease characterized by a dysregulation of the normal immune response. Inflammation mediated diseases
10 can cause organ damage, and are associated with increased morbidity and/or mortality. An example of immune dysregulation is the inappropriate activation of inflammatory cytokines, such as IL-12, IL-6 or TNF alpha, whose actions lead to pathological consequences.

For the purposes of this invention the terms “treatment, treating, treat” or equivalents of these terms refer to curing, healing, alleviating, relieving, altering, remedying, ameliorating, or improving
15 the condition or the symptoms of a subject suffering with a disease, for example, type 1 diabetes. Total absence of the disease symptoms is not required for “treating” a disease. The subject to be treated can be suffering from or at risk of developing the disorder, for example, type 1 diabetes.

For the purposes of this invention, the terms “preventing, preventive, prophylactic” or equivalents of these terms are indicate that the Treg is provided in advance of disease symptoms. The
20 prophylactic administration of the Treg serves to prevent or attenuate any subsequent symptoms or disease. Total avoidance of disease symptoms is not required for “preventing” the disease.

By “therapeutically effective dose,” “therapeutically effective amount”, or “effective amount” is intended to be an amount of the Treg that, when administered to a subject, decreases the inflammatory response, or prevents an inflammatory response from increasing. “Positive therapeutic
25 response” refers to, for example, improving the condition of at least one of the symptoms of an inflammatory disorder.

In one embodiment, the Treg administered to the subject is an autologous Treg, *i.e.*, Treg isolated from the subject and optionally, proliferated *in vitro*.

If the Treg administered to a subject is an autologous Treg, the method of treating and/or
30 preventing an inflammation mediated disease in the subject can comprise the steps of:

- a) isolating a Treg from a sample of cells obtained from the subject, wherein the Treg expresses CD4 and CD25, expresses low levels of CD127 or does not express CD127, and expresses low levels of CD226 or does not express CD226,
- b) optionally, proliferating the Treg isolated from the subject *in vitro*, and

c) administering to the subject, the Treg obtained from the subject or the Treg proliferated from the Treg obtained from the subject.

In one embodiment, the Treg administered to a subject is an autologous Treg, and the method of treating and/or preventing an inflammation mediated disease in the subject comprises the steps of:

5 a) isolating a Treg from a sample of cells obtained from the subject, wherein the Treg expresses CD4 and CD25, expresses low levels of CD226 or does not express CD226,

b) optionally, proliferating the Treg isolated from the subject *in vitro*, and

c) administering to the subject, the Treg obtained from the subject or the Treg proliferated from the Treg obtained from the subject.

10 Treg can be administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective. The quantity of Treg to be administered depends on the subject to be treated. The dose and route of administration will vary according to, for example, the type and weight of the subject, severity of the disease, *etc.* Precise number of cells administered to a subject depends on the judgment of the practitioner. However, suitable dosage ranges are of the order
15 of a few thousand cells to millions of cells. Suitable regimes for initial administration and subsequent administrations are also variable. Typically, an initial administration can be followed by subsequent administrations.

Various autoimmune diseases that can be treated according to the compositions and methods of the current invention include, but are not limited to, acute disseminated encephalomyelitis
20 (ADEM), Addison's disease, alopecia areata, amyloidosis, autoimmune retinopathy, autoimmune thyroid disease, axonal & neuronal neuropathies, chronic fatigue syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), Crohn's disease, Cocksackie myocarditis, dermatitis herpetiformis, experimental allergic encephalomyelitis, Evans syndrome, fibromyalgia, glomerulonephritis, granulomatosis with polyangiitis (GPA) (formerly called Wegener's
25 Granulomatosis), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, Hemolytic anemia, Kawasaki syndrome, Lupus (SLE), Lyme disease, Meniere's disease, multiple sclerosis, myasthenia gravis, myositis, neuromyelitis optica (Devic's), neutropenia, scleroderma, Sjogren's syndrome, stiff person syndrome.

Further embodiments of the invention provide uses of antagonists or agonists against CD226
30 or TIGIT for the treatment of cancer or an autoimmune disorder.

The antagonists or agonists of CD226 or TIGIT can be a small molecule compound or can be a biologic such as an antibody or fragment thereof. A person of ordinary skill in the art, having the benefit of the current disclosure, can obtain a small molecule antagonists or agonists of CD226 or TIGIT by, for example, screening small molecule compound libraries using assays routine in the art.

The antagonists or agonists of CD226 or TIGIT can also be a biologic such as, for example, an antibody, aptamer, fusion protean or other biomolecule capable of specifically binding to CD226 or TIGIT.

For the purposes of this invention, the binding between two molecules, which is based on specific interactions between sites present on the two molecules, is referred to as “specific binding”. The specific binding between two entities can be represented by, for example, their dissociation constant, which, according to the current invention can be, for example, less than about 10^{-6} , less than about 10^{-7} , or less than about 10^{-8} M. Examples of specific binding include, but are not limited to, binding between an antibody and a binding partner based on interactions between the binding sites present on the antibody and specific epitopes present on the binding partner, binding between an aptamer and its target biomolecule based on the interactions between the target binding sites present on the aptamer and the specific target sites present on the target biomolecule. Additional examples of specific binding between any two molecules and further aspects of specific binding are well known to a person of ordinary skill in the art.

Antibodies can be polyclonal antibodies, monoclonal antibodies, recombinant antibodies or hybrid antibodies. Non-limiting examples of recombinant or hybrid antibodies include chimeric antibodies, humanized monoclonal antibodies, single chain antibodies, single chain fragment variable (scFv) antibodies, and antigen-binding fragments (Fab fragment).

Fusion proteins capable of binding to CD226 or TIGIT can comprise, for example, a portion that specifically binds to CD226 or TIGIT fused to another portion. In one embodiment, a fusion protein comprises an antigen binding fragment of an antibody that specifically binds to CD226 or TIGIT and Fc portion of IgG.

In specific embodiments, biomolecules that specifically bind to CD226 or TIGIT are administered to a patient. Non-limiting examples of such biomolecules include antibodies, fragments of antibodies, aptamers, or fusion proteins. For example, a method of treating a disease, for example, cancer or an autoimmune disease, comprises administering polyclonal antibodies, monoclonal antibodies, recombinant antibodies or hybrid antibodies against CD226 or TIGIT. A method of treating a disease can also comprise administering to a patient in need thereof, a recombinant or hybrid antibody, for example, chimeric antibodies, humanized monoclonal antibodies, a single chain antibody, a single chain fragment variable (scFv) antibody, or a fragment antigen-binding (Fab fragment) capable of binding CD226 or TIGIT.

A further embodiment provides a method of treating a disease comprising administering to a patient in need thereof, fusion proteins capable of binding to CD226 or TIGIT for example, a fusion protein comprising an antigen binding fragment of an antibody which specifically binds to CD226 or TIGIT and Fc portion of IgG.

MATERIALS AND METHODS

Sample Procurement and processing

Peripheral blood was collected from healthy control donors (median age 28.8, range 22.5-46.7) or purchased from Life South Blood Centers (median age 23, range 20-26). Venous blood was collected in sodium-heparinized vacutainer tubes (BD Biosciences) or supplied in sodium citrate followed by PBMC isolation by density gradient centrifugation. For FACS experiments, whole blood was pre-enriched by negative selection with RosetteSep (Stemcell) prior to centrifugation.

Transcription factor sorting and expression analysis

CD4⁺ RosetteSep enriched T cells were stained with anti-CD4, fixed and permeabilized with the FOXP3 Fix/Perm (Biolegend) per manufacturer recommendations and stained for FOXP3 and Helios. tTreg (CD4⁺FOXP3⁺Helios⁺), CD4⁺FOXP3⁺Helios⁻ T cells and Tconv (CD4⁺FOXP3⁻Helios⁻) were sorted into RNeasy Lysis Buffer (Life Technologies). RNA was extracted with the RNeasy FFPE Kit (Qiagen) by proteinase K digestion followed by incubation at 80°C. RNA quality was verified on a Bioanalyzer (> 300 bp length for over 50% of transcripts) with the RNA Nano Chip (Agilent Technologies). RNA transcripts (100 ng) were directly quantified with the nCounter[®] and the Human Immunology GX Panel (NanoString Technologies, v1).

Flow cytometry and FACS

All samples were first stained with Fixable Live/Dead Yellow or Near IR (Invitrogen), and then stained for surface markers in stain buffer for 30 min at 4°C (PBS + 2% FBS + 0.05% NaN₃). For flow panels with only surface markers, cells were fixed with BD Cytofix™ - Fixation buffer (BD Bioscience) according to the manufacture's protocol. For intracellular staining, cells were fixed and permeabilized with the FOXP3 Fix/Perm buffer then stained for intracellular proteins in FOXP3 Perm buffer (BioLegend) according to manufacture's protocol. The antibodies used included CD4-Pacific Blue (RPA-T4), TIGIT-APC or -PerCP-eFluor710 (MBSA43), (eBioscience), CD226-PE (11A8) and CD8 (SK1), (BD Bioscience), CD25-APC or -AlexaFluor (AF)-488 (BC96) (BioLegend), IFN-γ-PE-Cy7 (4S.B3), Helios-PE or -Pacific Blue, -AF647 (22F6) (BioLegend), FOXP3-AF488 and -PE (206D) (BioLegend).

Flow cytometric analyses were performed on a LSR Fortessa (BD Bioscience). Data was collected using BD DIVA acquisition software as FCSv.3 files and imported into FlowJo V 9.7.5 (TreeStar Inc) for analysis. Cell proliferation data was determined by calculating the division index (DI) of responding populations gated as live with viability dye and lymphocyte gating. Percent marker positivity was determined by fluorescence minus one (FMO) method. Expression levels were calculated with geometric mean fluorescence (gMFI) intensity.

All cell sorting was conducted on a FACS Aria III (BD Bioscience) cell sorter. Treg (CD4⁺CD25⁺CD127⁻) and TConv (CD4⁺CD127⁺) were further enriched based on CD226 and/or TIGIT expression. Post-sort purities were typically greater than 93% (median 93%; range 90%-95%).

5 *In vitro* T cell expansions and activation cultures

Treg and Tconv cells were expanded as previously described by Putnam *et al.* (2009). After expansion, cells were analyzed for intracellular IFN γ by re-activation for 4 h with PMA (10 μ g/mL) and Ionomycin (500nM) in the presence of GolgiStop (4 μ l/6 mL culture; BD Biosciences). For multiplex cytokine detection, cells were activated with anti-CD3 and anti-CD28 coated dynabeads (Life Technologies) according to manufacturer recommendations and supernatants collected at 24, 48, and 72 h.

In vitro suppression assays

Expanded Treg subsets were tested for their ability to suppress autologous T cell proliferation, as described by Brusko *et al.* (2007), with the following modifications. Treg were labeled with CFSE (0.15 μ M), while responding cells were stained with Cell Trace Violet (2.5 μ M, Life Technologies) and activated with either autologous APCs or Treg Suppression Inspector beads (Miltenyi Biotec). Triplicate cultures were harvested and pooled following 96 h, stained with live/dead dye, CD4, CD8, CD226 and TIGIT, and proliferation was calculated by division index (DI) of gated live lymphocytes.

20

Analysis of the FOXP3-TSDR

TSDR demethylation is a hallmark of lineage-stable nTreg. This region coincides with the conserved non-coding sequence 2 (CNS2) within the first intron of the *foxp3* gene (Figure 22A). When DNA and RNA were required from the same samples, cells were processed with the AllPrep DNA/RNA Mini Kit (Qiagen). Otherwise, genomic DNA was isolated from purified T cell subsets using the DNeasy tissue kit (Qiagen). Bisulfite treatment of genomic DNA was performed with the EZ DNA Methylation Kit (Zymo Research). Briefly, 500 ng genomic DNA was sodium bisulfite treated overnight at 50°C, followed by 10 min on ice. After washing and desulphonation, bisulfite converted DNA was eluted with 16 μ l elution buffer.

To create a standard curve, fully methylated or unmethylated bisulfite-treated DNA was titrated at varying ratios. DNA was comprised of unmethylated bisulfite-converted human EpiTect control DNA (Qiagen) and universally methylated bisulfite-converted human control DNA (Zymo Research). The TSDR was PCR amplified using the following reaction: 50 μ L reaction volume containing 25 μ L of ZymoTaqTM PreMix buffer (Zymo Research) and 0.5 μ M each of the primers FOXP3_TSDRfwd (ATATTTT TAGATAGGGATATGGAGATGATTTGTTTGG) (SEQ ID NO: 2)

35

and FOXP3_TSDRrev (AATAAACATCACCTACCACATCCACCAACAC) (SEQ ID NO: 3). After incubation at 95°C for 10 min, amplification was performed as follows: 50 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Amplified PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen). The concentration of purified DNA was determined with a GE NanoVue spectrophotometer (GE Healthcare Life Sciences).

TSDR real-time PCR was performed in 384-well white trays with a Roche LightCycler 480 system (Roche Diagnostics) with real-time probes that target methylated or demethylated target sequences (Figure 22). Each reaction contained 10 μ L Lightcycler 480 Probes Master Mix (Roche), 1 μ L bisulfite converted DNA sample or standards, 1 μ M of each primer and 150 nM of each probe with a final reaction volume of 20 μ L. The probes used for amplification were TSDR-Forward GGGTTGTATTTGGGTTTTGTTGTTATAGT (SEQ ID NO: 4) and TSDR-Reverse CTATAAAATAAAATATCTACCCTCTTCTCTTCCT (SEQ ID NO: 5). The probes for target sequence detection were FAM-labeled methylated probe, FAM-CGGTCGGATGCGTC-MGB-NFQ (SEQ ID NO: 6), or VIC-labeled unmethylated probe, VIC-TGGTGGTTGGATGTGTTG-MGB-NFQ (SEQ ID NO: 7). All samples were tested in triplicate. The protocol for real-time amplification is as follows: after initial denaturation at 95°C for 10 min, the samples were subjected to 50 cycles at 95°C for 15 sec and at 61°C for 1 min. 14 different ratios of fully methylated and demethylated template were used as real-time standards (Figure 22C). The 6-order polynomial equation was used to extrapolate the percentage of cells demethylated at the TSDR for each sample.

T Cell Transcriptional Profiling

Using the nSolver Analysis Software (NanoString, Inc.), counts were first normalized to the geometric mean of the positive control spiked into the assay, then normalized to housekeeping genes built into the Human Immunology panel. Subsequent analyses were conducted with the Partek Genomic Suite (Partek Inc.). The signal-to-noise ratio was significantly higher in the expanded T cell counts compared to the fixed cell sorted T cell counts. Thus, two different statistical approaches were used.

For the count data from fixed cells, the average of the negative controls were subtracted from the counts and values less than 1 were converted to 1 prior to subsequent analysis. A paired ANOVA coupled with the Bonferroni multiple test correction (MTC) was used to determine differentially regulated genes with a significance value of $p < 0.05$. Genes were classified into T cell subsets and validated with the leave-one-out cross validation method using 100 permutations and a $p < 0.01$ considered significant.

When analyzing gene expression profiles from unfixed expanded cell subsets, genes that were below the background threshold (mean of negative controls count + 2 standard deviations) for both

Treg and Tconv were removed from the analysis. Because Tconv often have a variance different than Treg at both the RNA and proteins levels, A Welch's ANOVA with a Bonferroni MTC was used to determine significance ($p < 0.05$). Significantly regulated genes with a false discovery rate below 0.05 were normalized around zero and clustered using the average of the means.

5

Isolation of IFN γ producing Treg

Treg and Tconv were FACS isolated from five healthy subjects (median age 26, range 22-30) and sorted into two groups. Briefly, the first group was stimulated for 4 hours with PMA/ionomycin and labeled with the IFN γ cytokine cell-capture reagent (Miltenyi Biotech) followed by FACS
10 isolation of IFN γ^- and IFN γ^+ populations, as previously described (11). The second set was expanded to day 14 prior to reactivation and cytokine cell capture.

For each sample, 25 ng total RNA were amplified using the Ovation® Pico WTA System (NuGen) and labeled with Encore Biotin Module V2 (NuGen). GeneChip® Human Genome U133 Plus 2.0 arrays (Affymetrix) were hybridized to 5 μ g labeled, amplified cDNA, washed, stained, and
15 scanned according to the protocol described in the GeneChip Expression analysis manual (GEO accession number: GSE59786). Gene expression profiling data was extracted from the Affymetrix Microarray Suite 5.0 (MAS 5.0) software and used for subsequent statistical analyses.

Multiplex cytokine assay

20 Cytokine production was determined using the Human T_H17 Magnetic Bead Panel (HT17MG-14K-PX25, EMD Millipore) according to manufacturers instructions from culture supernatants collected and run in duplicate. Samples were processed on a Bio-Tek ELx405, detected with MAGPIX system (EMD Millipore), and analyzed with Milliplex Analyst software.

25 Data Analysis

An ANOVA with a posthoc Tukey multiple test correction was used for analysis of cytometric data utilizing Prism (GraphPad, v6). Geister-Greenhouse variance correction method was applied to the data to account for the difference in variance between Treg and Tconv, with values matched between each individual.

30

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. The examples and embodiments described herein are for illustrative purposes only and various modifications or changes in light thereof will be suggested to persons skilled in the art and are included within the spirit and purview of this application.

35

EXAMPLE 1 – TIGIT is enriched in the tTreg

Human Treg display a considerable degree of heterogeneity. To limit biases for putatively identified surface markers, Treg were FACS-sorted following intracellular staining for FOXP3 and Helios. Tconv (FOXP3⁻Helios⁻), FOXP3⁺Helios⁻, and tTreg subsets (FOXP3⁺Helios⁺) (Figure 1A) were sorted, which then underwent direct mRNA hybridization that facilitates the direct quantitation of mRNA transcripts without reverse-transcription and amplification. This approach is compatible with partially degraded RNA samples, such as those obtained from fixed samples.

Patient-to-patient variance accounted for 77% of the expression differences observed between the samples, however genes putatively associated with Treg were identified, including CTLA-4 and IL2RA (Table 1). Specifically, the mRNA levels of TIGIT were found to be 12.4-times higher in tTreg compared to Tconv ($p < 0.05$; Figure1B). In addition, strong co-expression of TIGIT and Helios in CD4⁺FOXP3⁺ T cells was observed (Figure 1C). Thus, TIGIT expression is maintained by FOXP3 and TIGIT may play an important role in immune regulation.

Given that TIGIT and CD226 compete for binding to the ligand CD155, their surface expression was analyzed on CD4⁺ T cells in combination with FOXP3 and Helios (Figure 2). Strikingly, tTreg had the highest percentage of TIGIT⁺ cells (83.4% $\pm$ 6.2%) and did not express CD226 in the absence of TIGIT (Figure 2A). In contrast, Tconv cells co-expressed TIGIT only with high CD226 expression, suggesting TIGIT may play an important role in Tconv after activation. TIGIT expression by tTreg was increased in frequency and gMFI compared to FOXP3⁺Helios⁻ T cells, and the lowest frequency of TIGIT⁺ cells and gMFI found in Tconv (Figure 2B, *left graphs*). In contrast, CD226 expression was higher in Tconv cells followed by FOXP3⁺Helios⁻, and then tTreg (Figure 2B, *right graphs*). FOXP3⁺Helios⁻ T cells demonstrated an intermediate phenotype for CD226 and TIGIT expression. CD96 (TACTILE; T cell activation, increased late expression), a ligand that also binds CD155 was also analyzed and CD96 expression positively correlated with CD226 but did not demonstrate the dynamic range observed for CD226 (data not shown). Hence, the downstream analyses were limited to TIGIT and CD226.

Table 1 - Differentially expressed genes between transcription factor-sorted CD4 ⁺ T cell subsets*				
Symbol	Gene Name	FOXP3 ⁺ Helios ⁻ vs Tconv	tTregs vs Tconv	FOXP3 ⁺ Helios ⁻ vs tTreg
ABU	c-abl oncogene 1, non-receptor tyrosine kinase	-	1.9	-
APP	amyloid beta (A4) precursor protein	-	-	-1.3
ATM	ataxia telangiectasia mutated	-	1.2	-
BCL2	B-cell CLL/lymphoma 2	-1.5	-	-
C8B	complement component 8, beta polypeptide	-	-	-1.4
CD6	CD6 molecule	-	-2.4	1.9
CD96	CD96 molecule	-1.6	-1.6	-
CHUK	conserved helix-loop-helix ubiquitous kinase	-2.5	-	-
CSF2RB	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	-	7.9	-10
CTLA4	cytotoxic T-lymphocyte-associated protein 4	5.2	4.9	-
CXCR3	chemokine (C-X-C motif) receptor 3	-	-	-1.7
EEF1G	eukaryotic translation elongation factor 1 gamma	-1.3	-	-
ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1	-	-	-1.3
FOXP3	forkhead box P3	21.8	48.7	-
HLA-DPB1	major histocompatibility complex, class II, DP beta 1	-	-	-5
IKZF2	IKAROS family zinc finger 2 (Helios)	-2.1	-	-
IL10RA	interleukin 10 receptor, alpha	2	2.2	-
IL11RA	interleukin 11 receptor, alpha	-1.4	-	-
IL13	interleukin 13	-	-	-1.4
IL1R2	interleukin 1 receptor, type II	1.5	-	-
IL23R	interleukin 23 receptor	-	-	-1.4
ILAR	interleukin 4 receptor	-2.6	-	-
IL6ST	interleukin 6 signal transducer	-2	-	-

Table 1 - Differentially expressed genes between transcription factor-sorted CD4 ⁺ T cell subsets*				
Symbol	Gene Name	FOXP3 ⁺ Helios ⁻ vs Tconv	tTregs vs Tconv	FOXP3 ⁺ Helios ⁻ vs tTreg
IL7R	interleukin 7 receptor	-	-2.3	-
IRF1	interferon regulatory factor 1	-	-	-1.3
IRF4	interferon regulatory factor 4	4.1	3.4	-
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-1.5	-	-
JAK3	Janus kinase 3	-	-1.4	-
MAP4K4	mitogen-activated protein kinase kinase kinase 4	-1.4	-	-
MARCO	macrophage receptor with collagenous structure	1.1	-	-
MYD88	myeloid differentiation primary response 88	-2	-	-
NCAM1	neural cell adhesion molecule I	-4	-	-
NCF4	neutrophil cytosolic factor 4, 40kDa	8.9	14	-
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	-	-1.4	-
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	-1.8	-	-
PRKCD	protein kinase C, delta	-	-	2.7
PSMB10	proteasome (prosome, macropain) subunit beta type 10	-	1.3	-
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8	2.2	-	-
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9	-1.4	-	-
PTGER4	prostaglandin E receptor 4 (subtype EP4)	-	-	1.5
PTK2	protein tyrosine kinase 2	-	-5	-
RUNX1	runt-related transcription factor 1	-1.5	-1.7	-
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	-1.9	-	-
STAT4	signal transducer and activator of transcription 4	-2.7	-4.2	-

Symbol	Gene Name	FOXP3 ⁺ Helios ⁻ vs Tconv	tTregs vs Tconv	FOXP3 ⁺ Helios ⁻ vs tTreg
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	-1.3	-	-
TAL1	T-cell acute lymphocytic leukemia 1	1.4	-	-
TGFB1	transforming growth factor, beta 1	-1.2	-	-
TIGIT	T cell immunoreceptor with Ig and ITIM domains	-	12.4	-
TLR1	toil-like receptor 1	-	-	10
TMEM173	transmembrane protein 173	-	-1.4	-
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	-1.7	-	-
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	11.4	13.4	-
TNFSF12	tumor necrosis factor (ligand) superfamily, member 12	-1.7	-	-
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	1.4	-	-
XCL1	chemokine (C motif) ligand 1	-	-	10

*The expression profile of CD4⁺ T cells FAC sorted by FXP3 and Helios was analyzed by the nCounter. A paired ANOVA coupled with the Bonferroni multiple test correction (MTC) was used to determine differentially regulated genes with a significance value of $p < 0.05$ ($n = 3$). The fold change was calculated by taking the geometric mean of the counts, then dividing one subset by another. Only the ratios of significant comparisons are shown.

Symbol	Description	p value	C-T- mean		C-T+ mean		C-T+ mean		TConv mean	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>CD40L</i> <i>G</i>	CD40 ligand	2.33E-02	1.33E+02	1.50E+01	5.94E+02	3.53E+01	1.79E+02	8.72E+01	1.62E+03	3.85E+01
<i>IL1RI</i>	interleukin 1 receptor, type I	2.36E-02	6.48E+02	1.75E+02	3.04E+02	5.86E+00	1.06E+03	8.40E+02	2.56E+01	2.08E+00
<i>TNFRSF1B</i>	tumor necrosis factor receptor superfamily, member 1B	2.44E-02	7.59E+03	6.16E+02	4.61E+03	1.53E+02	8.70E+03	8.13E+03	4.85E+02	9.47E+01

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*															
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		C+T+ mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>G6PD</i>	glucose-6-phosphate dehydrogenase	2.50E-02	7.82E-05	7.80E+02	6.08E+00	7.80E+02	3.65E+01	7.35E+02	1.31E+01	7.08E+02	3.87E+01	5.95E+02	4.26E+00	2	0
<i>FOX P3</i>	forkhead box P3	2.54E-02	1.54E-04	5.12E+03	4.15E+02	3.65E+03	3.08E+02	5.25E+03	4.83E+02	5.10E+03	2.55E+02	2.53E+03	1.05E+00	2	2
<i>FN1</i>	fibronectin 1	1.63E-04	1.63E-04	6.44E+01	5.66E+00	7.08E+01	1.74E+00	5.20E+01	9.66E+00	6.19E+01	1.04E+01	2.11E+01	1.58E+00	1	0
<i>TGFBR1</i>	transforming growth factor, beta receptor 1	1.64E-04	1.64E-04	1.04E+03	2.74E+01	6.40E+02	4.21E+01	9.63E+02	6.16E+01	9.79E+02	1.27E+02	2.90E+02	2.69E+00	2	1
<i>CD97</i>	CD97 molecule	2.56E-04	2.56E-04	1.73E+03	3.52E+01	1.56E+03	2.74E+01	1.89E+03	3.47E+01	1.75E+03	3.85E+01	1.33E+03	5.88E+00	3	0
<i>IL7</i>	interleukin 7	2.64E-04	2.64E-04	9.22E+01	1.51E+01	7.10E+01	3.48E+00	2.26E+02	1.19E+01	1.77E+02	9.03E+00	4.14E+01	4.12E+00	1	0
<i>POU2F2</i>	POU class 2 homeobox 2	2.67E-04	2.67E-04	7.77E+02	3.54E+01	5.68E+02	1.61E+01	9.21E+02	1.24E+02	9.34E+02	1.43E+02	2.27E+02	1.57E+00	2	1
<i>TIGIT</i>	T cell immunoreceptor with Ig and ITIM domains	3.17E-04	3.17E-04	1.31E+03	1.51E+02	4.52E+02	2.19E+01	1.80E+03	2.11E+02	2.34E+03	2.27E+02	5.18E+03	1.44E+00	1	0
<i>CTLA4</i>	cytotoxic T-lymphocyte-associated protein 4	3.43E-04	3.43E-04	5.46E+03	2.85E+02	5.85E+03	3.63E+02	8.53E+03	8.67E+02	7.44E+03	8.99E+02	1.15E+03	1.46E+00	3	2
<i>CD53</i>	CD53 molecule	3.83E-04	3.83E-04	7.03E+03	1.22E+02	6.02E+03	3.93E+02	7.64E+03	4.75E+02	7.20E+03	8.59E+02	3.87E+03	1.38E+00	3	2
<i>CD28</i>	CD28 molecule	4.10E-04	4.10E-04	3.10E+03	1.33E+02	3.32E+03	3.33E+02	4.55E+03	4.55E+02	3.99E+03	1.28E+02	1.73E+03	2.45E+00	3	1
<i>IL1R2</i>	interleukin 1 receptor, type II	4.38E-04	4.38E-04	6.42E+02	8.81E+01	5.80E+02	7.24E+01	1.11E+03	5.36E+01	1.05E+03	3.51E+02	6.07E+03	4.58E+00	1	0
<i>IL1RN</i>	interleukin 1 receptor antagonist	4.80E-04	4.80E-04	2.30E+02	2.84E+01	3.69E+02	9.46E+01	3.96E+02	1.44E+01	3.55E+02	1.97E+02	7.40E+03	1.29E+00	1	1
<i>IL7R</i>	interleukin 7 receptor	5.06E-04	5.06E-04	4.96E+01	3.40E+00	1.28E+02	8.14E+00	8.27E+01	1.86E+01	6.85E+01	1.01E+01	1.16E+03	7.06E+00	3	1
<i>IL2</i>	interleukin 2	6.18E-04	6.18E-04	1.99E+01	2.45E+00	2.27E+01	5.63E+00	2.39E+01	8.23E+00	3.81E+01	6.33E+00	9.38E+03	4.14E+00	1	0

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*													
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>CSF2R</i> <i>B</i>	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	6.66E-04	6.66E-04	5.05E+02	1.07E+02	1.65E+02	4.00E+01	7.84E+02	3.71E+01	8.62E+02	1.74E+02	1.06E+02	3.23E+01
<i>LCP2</i>	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	7.10E-04	7.10E-04	6.82E+03	2.18E+02	5.12E+03	1.33E+02	6.55E+03	4.53E+02	6.16E+03	5.98E+02	3.65E+03	9.98E+01
<i>ITGAM</i>	integrin, alpha M (complement component 3 receptor 3 subunit)	7.45E-04	7.45E-04	2.74E+03	1.22E+02	2.16E+03	2.65E+02	3.15E+03	4.56E+02	2.97E+03	5.27E+02	6.28E+02	7.83E+01
<i>XBP1</i>	X-box binding protein 1	8.26E-04	8.26E-04	2.45E+02	1.68E+01	5.37E+02	4.87E+01	2.57E+02	2.12E+01	2.43E+02	2.40E+01	1.16E+03	6.77E+01
<i>ICAM2</i>	intercellular adhesion molecule 2	8.38E-04	8.38E-04	1.74E+03	1.03E+02	1.71E+03	1.66E+02	2.20E+03	2.59E+02	2.01E+03	8.94E+01	7.88E+02	2.53E+01
<i>PTPN6</i>	protein tyrosine phosphatase, non-receptor type 6	8.98E-04	8.98E-04	2.57E+03	1.27E+02	2.62E+03	1.94E+02	3.17E+03	2.65E+02	3.08E+03	3.65E+01	2.03E+03	5.97E+01
<i>TMEM173</i>	transmembrane protein 173	9.26E-04	9.26E-04	7.90E+03	2.55E+02	9.61E+03	7.02E+02	1.06E+04	1.47E+02	1.02E+04	6.64E+02	5.06E+03	4.02E+02
<i>IL2RA</i>	interleukin 2 receptor, alpha	9.70E-04	9.70E-04	2.58E+04	3.38E+02	2.22E+04	1.34E+03	2.62E+04	1.23E+03	2.70E+04	2.36E+03	1.04E+04	9.43E+02
<i>CD59</i>	CD59 molecule, complement regulatory protein	1.01E-03	1.01E-03	3.31E+03	2.12E+02	3.99E+03	6.59E+02	6.71E+03	1.13E+03	4.82E+03	3.11E+02	1.01E+03	2.70E+01
<i>PDGFRB</i>	platelet-derived growth factor receptor, beta polypeptide	1.05E-03	1.05E-03	1.34E+02	1.30E+01	9.86E+01	2.29E+00	2.27E+02	2.84E+01	1.24E+02	3.25E+00	6.91E+01	2.91E+00
<i>NCF4</i>	neutrophil cytosolic factor 4, 40kDa	1.10E-03	1.10E-03	1.70E+03	4.47E+01	1.15E+03	4.98E+01	1.53E+03	3.95E+01	1.88E+03	1.33E+02	8.33E+02	6.35E+01

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*															
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		C-T- mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>CD46</i>	CD46 molecule, complement regulatory protein	1.11E-03	1.11E-03	1.43E+03	4.07E+01	1.39E+03	3.16E+01	1.77E+03	1.62E+01	1.77E+03	1.62E+01	1.64E+03	1.29E+02	1.24E+03	5.70E+01
<i>TNFSF13B</i>	tumor necrosis factor (ligand) superfamily, member 13b	1.12E-03	1.12E-03	6.66E+02	7.12E+01	1.77E+02	7.29E+00	6.22E+02	1.20E+02	6.44E+02	6.11E+01	1.03E+02	6.11E+01	1.03E+02	4.82E+00
<i>SMAD3</i>	SMAD family member 3	1.26E-03	1.26E-03	1.96E+02	1.14E+01	2.90E+02	2.31E+01	2.09E+02	2.75E+01	2.51E+02	2.07E+01	8.26E+02	2.07E+01	8.26E+02	4.66E+01
<i>CCR6</i>	chemokine (C-C motif) receptor 6	1.29E-03	1.29E-03	3.74E+01	3.98E+00	4.72E+01	7.31E+00	1.63E+02	1.20E+01	1.15E+02	1.31E+01	1.85E+02	1.31E+01	1.85E+02	1.92E+00
<i>IKZF2</i>	IKAROS family zinc finger 2 (Helios)	1.36E-03	1.36E-03	2.92E+03	2.39E+02	1.10E+03	1.53E+02	3.50E+03	9.31E+02	4.00E+03	4.87E+02	3.56E+03	4.87E+02	3.56E+03	1.53E-01
<i>TNFRSF13B</i>	tumor necrosis factor receptor superfamily, member 13B	1.41E-03	1.41E-03	3.83E+01	8.01E+00	3.15E+01	7.80E+00	8.65E+01	4.81E+00	5.55E+01	2.37E+00	2.47E+01	2.37E+00	2.47E+01	2.41E+00
<i>IL22</i>	interleukin 22	1.41E-03	1.41E-03	1.23E+01	6.97E-01	1.41E+01	3.54E+00	1.35E+01	4.14E+00	1.91E+01	2.49E+00	2.80E+01	2.49E+00	2.80E+01	1.80E+00
<i>BATF3</i>	basic leucine zipper transcription factor, ATF-like 3	1.45E-03	1.45E-03	3.49E+01	2.60E+00	1.15E+02	1.11E+01	6.54E+01	4.56E+00	5.58E+01	7.27E+00	3.57E+02	7.27E+00	3.57E+02	3.01E+00
<i>CD4</i>	CD4 molecule	1.48E-03	1.48E-03	1.51E+03	1.02E+02	1.42E+03	1.24E+02	1.87E+03	1.57E+02	2.01E+03	5.05E+01	7.83E+02	5.05E+01	7.83E+02	8.41E+00
<i>IL6R</i>	interleukin 6 receptor	1.52E-03	1.52E-03	3.59E+02	5.44E+01	3.91E+02	5.72E+01	1.00E+03	1.88E+02	6.40E+02	4.41E+01	7.65E+02	4.41E+01	7.65E+02	1.30E+00
<i>MAP4K2</i>	mitogen-activated protein kinase kinase 2	1.58E-03	1.58E-03	1.35E+03	1.53E+01	1.03E+03	3.13E+01	1.33E+03	5.32E+01	1.47E+03	6.27E+01	8.01E+02	6.27E+01	8.01E+02	5.23E+00
<i>NFIL3</i>	nuclear factor, interleukin 3 regulated	1.98E-03	1.98E-03	2.01E+02	3.57E+01	4.75E+02	1.55E+01	3.50E+02	2.80E+01	1.95E+02	2.16E+01	9.10E+02	2.16E+01	9.10E+02	1.71E+00
<i>HLA-B</i>	major histocompatibility	2.02E-	2.02E-	3.18E+	1.75E	2.83E+	1.62E+	3.67E+	4.11E	3.59E+	6.67E+	2.03E+	6.67E+	2.03E+	9.95E+0

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*													
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	complex, class I, B	03	-03	04	+03	04	02	04	+03	04	02	4	2
<i>SPP1</i>	secreted phosphoprotein 1	2.08E-03	2.08E-03	4.08E+02	2.81E+01	4.49E+02	1.33E+02	3.33E+02	1.17E+02	2.34E+02	5.00E+01	2.75E+01	4.12E+00
<i>CD99</i>	CD99 molecule	2.15E-03	2.15E-03	3.97E+03	8.16E+01	6.06E+03	5.97E+02	5.41E+03	3.40E+02	4.66E+03	1.80E+01	6.52E+03	1.71E+00
<i>BCL6</i>	B-cell CLL/lymphoma 6	2.16E-03	2.16E-03	1.93E+02	6.77E+00	1.25E+02	2.86E+01	1.39E+02	1.85E+01	2.02E+02	5.80E+01	7.12E+01	7.54E+00
<i>CD40</i>	CD40 molecule, TNF receptor superfamily member 5	2.31E-03	2.31E-03	6.12E+01	4.52E+00	4.49E+01	2.99E+00	9.80E+01	2.31E+01	8.36E+01	1.25E+01	1.89E+01	1.04E+00
<i>IRF8</i>	interferon regulatory factor 8	2.44E-03	2.44E-03	4.99E+01	6.73E+00	4.77E+01	6.50E+00	5.63E+01	8.71E+00	3.48E+01	3.97E+00	9.88E+01	4.40E00
<i>PDCD2</i>	programmed cell death 2	2.46E-03	2.46E-03	3.79E+02	1.76E+01	3.76E+02	2.86E+01	3.72E+02	8.39E+00	3.87E+02	5.00E+01	2.72E+02	1.45E+00
<i>CD79A</i>	CD79a molecule, immunoglobulin-associated alpha	2.71E-03	2.71E-03	2.41E+02	3.35E+01	1.19E+02	1.35E+01	2.27E+02	6.15E+01	3.02E+02	2.54E+01	3.78E+01	1.36E+00
<i>MAF</i>	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog	2.80E-03	2.80E-03	2.24E+03	3.65E+02	2.49E+03	2.48E+02	4.25E+03	2.54E+02	3.39E+03	4.48E+02	6.75E+02	2.29E+00
<i>GFI1</i>	growth factor independent 1 transcription repressor	2.92E-03	2.92E-03	3.71E+02	3.21E+01	6.04E+02	4.82E+01	2.76E+02	5.63E+01	3.36E+02	3.33E+01	1.03E+03	6.57E+00
<i>BID</i>	BH3 interacting domain death agonist	2.98E-03	2.98E-03	6.12E+01	4.52E+00	4.15E+01	5.89E+00	5.50E+01	2.92E+00	6.77E+01	1.61E+00	2.78E+01	3.42E+00
<i>MX1</i>	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	3.23E-03	3.23E-03	1.24E+04	8.49E+02	2.47E+03	6.31E+02	1.14E+04	2.15E+03	1.52E+04	3.71E+03	3.08E+03	7.56E+00
<i>CD86</i>	CD86 molecule	3.24E-	3.24E-	1.58E+	2.75E	2.95E+	4.51E+	3.35E+	2.32E	1.57E+	1.26E+	3.62E+	1.85E+0

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*													
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C-T+ mean		C+T+ mean		C-T+ mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
		03	-03	02	+01	02	01	02	+01	02	01	02	01
<i>SIGIRR</i>	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	3.38E-03	3.38E-03	9.28E+02	5.27E+01	6.36E+02	9.82E+01	9.44E+02	9.18E+01	1.16E+03	2.18E+02	3.73E+02	2.04E+01
<i>SELPLG</i>	selectin P ligand	3.45E-03	3.45E-03	4.09E+02	2.71E+01	3.17E+02	2.82E+01	5.20E+02	7.55E+01	5.09E+02	3.89E+01	1.93E+02	3.14E+00
<i>FKBP5</i>	FK506 binding protein 5	3.72E-03	3.72E-03	2.49E+03	2.15E+02	1.33E+03	1.99E+02	1.59E+03	3.21E+02	2.13E+03	1.81E+02	6.59E+02	4.94E+01
<i>SKI</i>	v-ski avian sarcoma viral oncogene homolog	4.07E-03	4.07E-03	1.03E+03	5.16E+01	8.42E+02	6.15E+01	1.16E+03	8.27E+01	1.19E+03	1.92E+02	5.81E+02	1.69E+01
<i>IGF2R</i>	insulin-like growth factor 2 receptor	4.43E-03	4.43E-03	5.49E+02	3.28E+01	5.43E+02	4.16E+01	7.77E+02	7.75E+01	6.54E+02	6.29E+01	2.84E+02	2.10E+01
<i>TCF7</i>	transcription factor 7 (T-cell specific, HMG-box)	4.47E-03	4.47E-03	1.77E+02	8.23E+00	7.01E+01	4.94E+00	2.67E+02	8.44E+01	2.89E+02	1.03E+02	1.08E+02	1.23E+00
<i>CEACAM1</i>	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	4.75E-03	4.75E-03	6.93E+02	1.07E+02	5.91E+02	4.51E+01	7.64E+02	7.57E+01	6.35E+02	9.50E+01	2.43E+02	1.16E+01
<i>C1QB</i>	complement component 1, q subcomponent binding protein	4.87E-03	4.87E-03	3.38E+03	3.60E+02	3.16E+03	2.69E+02	2.55E+03	4.34E+02	2.55E+03	2.16E+02	4.57E+03	8.97E+01
<i>CASP8</i>	caspase 8, apoptosis-related cysteine peptidase	4.91E-03	4.91E-03	1.84E+03	8.66E+01	1.60E+03	8.06E+01	3.38E+03	4.84E+02	2.80E+03	3.32E+02	1.11E+03	5.18E+01
<i>FAS</i>	Fas cell surface death receptor	5.10E-03	5.10E-03	2.92E+02	6.81E+01	2.18E+02	2.54E+01	5.83E+02	1.24E+02	3.41E+02	2.15E+01	1.17E+02	1.15E+01
<i>BCAP31</i>	B-cell receptor-associated protein 31	5.15E-03	5.15E-03	1.42E+03	6.57E+01	1.32E+03	4.73E+01	1.37E+03	4.99E+01	1.32E+03	6.87E+01	1.03E+03	1.51E+01
<i>CD7</i>	CD7 molecule	5.19E-03	5.19E-03	1.61E+03	1.15E+02	1.05E+03	6.71E+01	1.14E+03	6.09E+01	1.63E+03	7.40E+01	6.05E+02	1.00E+02
<i>IL11RA</i>	interleukin 11 receptor,	5.34E-03	5.34E-03	1.70E+03	6.63E+01	1.40E+03	1.21E+01	2.02E+03	3.99E+01	2.39E+03	5.98E+01	8.43E+03	6.46E+01

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*														
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean		
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
	alpha	03	-03	02	+00	02	01	02	+01	02	01	1	0	
CD80	CD80 molecule	5.40E-03	5.40E-03	1.37E+03	1.29E+02	8.40E+02	1.15E+02	1.35E+03	1.43E+02	1.35E+03	2.66E+02	3.41E+02	5.56E+01	
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	5.41E-03	5.41E-03	8.22E+02	4.59E+01	6.15E+02	6.42E+01	1.01E+03	1.18E+02	8.99E+02	1.31E+01	5.04E+02	3.88E+01	
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	5.42E-03	5.42E-03	1.80E+02	1.86E+01	1.46E+02	1.80E+01	2.05E+02	1.12E+01	2.15E+02	2.26E+01	9.24E+01	7.21E+00	
CD44	CD44 molecule (Indian blood group)	5.70E-03	5.70E-03	1.46E+03	2.52E+01	1.33E+03	5.84E+01	1.50E+03	1.10E+02	1.61E+03	1.99E+02	8.06E+02	6.24E+01	
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	5.87E-03	5.87E-03	3.27E+03	8.28E+01	3.34E+03	3.52E+02	3.45E+03	2.11E+02	3.55E+03	4.93E+02	2.22E+03	8.74E+01	
BCL3	B-cell CLL/lymphoma 3	6.30E-03	6.30E-03	5.29E+02	4.63E+01	5.85E+02	1.48E+01	6.76E+02	4.39E+01	5.63E+02	4.75E+01	7.68E+02	1.54E+01	
CCR2	chemokine (C-C motif) receptor-like 2	6.39E-03	6.39E-03	2.24E+02	9.69E+00	1.48E+02	1.64E+01	2.39E+02	3.80E+01	2.21E+02	2.91E+01	1.00E+02	1.04E+01	
HPRT1	hypoxanthine phosphoribosyltransferase 1	6.58E-03	6.58E-03	2.29E+03	8.38E+01	2.80E+03	8.12E+01	2.18E+03	1.33E+02	1.95E+03	1.28E+02	2.97E+03	7.68E+01	
CISH	cytokine inducible SH2-containing protein	7.18E-03	7.18E-03	2.80E+03	1.81E+02	2.00E+03	1.62E+02	2.65E+03	3.45E+02	3.03E+03	2.71E+02	1.45E+03	4.18E+01	
IRAK1	interleukin-1 receptor-associated kinase 1	7.22E-03	7.22E-03	7.38E+02	4.19E+01	9.42E+02	5.99E+01	9.09E+02	6.31E+01	7.44E+02	3.61E+01	1.10E+03	3.10E+01	
LTA	lymphotoxin alpha	7.23E-03	7.23E-03	8.76E+03	6.07E+02	5.32E+03	1.80E+03	4.93E+03	7.88E+02	5.99E+03	1.54E+03	1.88E+03	4.86E+02	
CTSC	cathepsin C	9.02E-03	9.02E-03	6.42E+03	1.19E+03	6.43E+03	9.78E+02	7.01E+03	1.13E+03	6.43E+03	4.74E+02	2.69E+03	7.04E+01	
ETS1	v-ets avian	9.28E-03	9.28E-03	1.88E+03	1.65E+02	1.50E+03	8.24E+02	1.89E+03	2.40E+02	1.89E+03	1.16E+02	1.11E+03	3.14E+01	

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*														
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C-T+ mean		C+T+ mean		TConv mean		
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
	erythroblastosis virus E26 oncogene homolog 1	03	-03	04	+03	04	02	04	+03	04	03	4	2	
CD3D	CD3d molecule, delta (CD3-TCR complex)	9.32E-03	9.32E-03	9.16E+03	3.74E+02	8.26E+03	5.04E+02	1.05E+04	1.32E+03	9.45E+03	8.88E+02	6.04E+03	2.05E+03	2
TNFSF12	tumor necrosis factor (ligand) superfamily, member 12	9.38E-03	9.38E-03	1.48E+03	1.61E+02	9.37E+02	3.34E+01	1.33E+03	1.39E+02	1.52E+03	1.87E+02	5.63E+02	5.20E+03	1
IL16	interleukin 16	9.83E-03	-	3.63E+03	4.05E+02	1.65E+03	2.48E+01	2.87E+03	2.36E+02	3.88E+03	6.11E+02	1.56E+03	2.45E+03	1
CXCL2	chemokine (C-X-C motif) ligand 2	1.02E-02	-	2.81E+01	5.60E+00	3.24E+01	5.05E+00	3.78E+01	1.42E+00	4.18E+01	5.05E+00	2.29E+01	1.19E+01	0
TGFB1	transforming growth factor, beta 1	1.04E-02	-	2.82E+03	2.48E+02	2.57E+03	3.21E+02	3.06E+03	4.02E+02	3.03E+03	1.62E+02	1.77E+03	2.51E+03	1
HLA-A	major histocompatibility complex, class I, A	1.07E-02	-	3.18E+04	4.28E+03	2.71E+04	1.82E+03	3.97E+04	7.80E+03	3.75E+04	2.27E+03	1.95E+04	7.43E+03	2
IL18RAP	interleukin 18 receptor accessory protein	1.10E-02	-	5.94E+01	9.69E+00	4.11E+01	7.20E+00	3.77E+01	1.34E+00	6.14E+01	7.87E+00	2.09E+01	2.02E+01	1
TLR1	toll-like receptor 1	1.13E-02	-	5.85E+02	8.02E+01	1.64E+02	2.41E+01	4.28E+02	1.29E+02	4.57E+02	3.93E+01	1.75E+02	8.54E+00	0
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1.14E-02	-	4.92E+02	3.73E+00	7.32E+02	1.15E+02	7.06E+02	3.99E+01	6.08E+02	6.29E+01	1.50E+02	1.38E+02	2
LAI1	leukocyte-associated immunoglobulin-like receptor 1	1.15E-02	-	1.02E+03	3.67E+01	8.86E+02	4.07E+01	9.29E+02	7.48E+01	1.07E+03	1.29E+02	5.49E+02	4.96E+03	1
CD247	CD247 molecule	1.18E-02	-	5.33E+03	3.99E+02	4.05E+03	3.67E+02	7.26E+03	1.13E+03	6.40E+03	1.24E+03	2.62E+03	7.86E+03	1
MYD88	myeloid differentiation primary response 88	1.19E-02	-	1.79E+03	1.46E+02	1.50E+03	1.09E+02	2.05E+03	2.63E+02	2.06E+03	1.20E+02	1.07E+03	7.64E+03	1
SELL	selectin L	1.20E-02	-	9.20E+03	1.12E+03	7.86E+03	1.09E+03	1.28E+04	2.47E+03	1.25E+04	1.28E+03	3.63E+03	5.18E+03	2

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*														
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean		
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
<i>TLR5</i>	toll-like receptor 5	1.25E-02	—	3.22E+01	1.90E+00	2.84E+01	4.74E+00	4.76E+01	5.96E+00	3.10E+01	4.24E+00	1.74E+01	1.27E+00	
<i>NFKB1Z</i>	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	1.28E-02	—	2.23E+03	5.43E+02	1.61E+03	1.48E+02	3.14E+03	4.11E+02	2.48E+03	3.25E+02	7.77E+02	1.10E+02	
<i>CD27</i>	CD27 molecule	1.33E-02	—	3.65E+03	6.26E+02	2.59E+03	4.28E+02	4.28E+03	9.77E+02	4.13E+03	6.92E+02	6.59E+02	4.01E+02	
<i>CXCR4</i>	chemokine (C-X-C motif) receptor 4	1.41E-02	—	8.15E+03	6.12E+02	6.08E+03	4.18E+02	1.07E+04	1.88E+03	9.62E+03	4.59E+02	4.88E+03	5.88E+02	
<i>BATF</i>	basic leucine zipper transcription factor, ATF-like	1.41E-02	—	1.02E+03	7.83E+01	1.14E+03	1.14E+02	1.39E+03	1.16E+02	1.16E+03	2.60E+02	6.33E+02	3.89E+02	
<i>STAT2</i>	signal transducer and activator of transcription 2, 113kDa	1.44E-02	—	2.70E+03	4.34E+02	1.40E+03	6.65E+01	3.68E+03	8.40E+02	3.34E+03	6.23E+02	9.16E+02	6.31E+02	
<i>CCR5</i>	chemokine (C-C motif) receptor 5 (gene/pseudogene)	1.57E-02	—	7.72E+02	8.47E+01	4.70E+02	1.10E+02	6.71E+02	6.80E+01	8.85E+02	1.11E+02	3.41E+02	5.53E+02	
<i>HLA-DMB</i>	major histocompatibility complex, class II, DM beta	1.59E-02	—	5.66E+02	5.90E+01	4.98E+02	1.98E+01	6.44E+02	6.31E+01	6.27E+02	3.54E+01	3.36E+02	2.72E+02	
<i>PDCD1</i>	programmed cell death 1	1.59E-02	—	4.21E+01	1.25E+00	5.21E+01	1.19E+01	9.39E+01	6.60E+00	5.57E+01	6.49E+00	9.31E+01	1.94E+01	
<i>ITGB1</i>	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.60E-02	—	5.53E+02	7.96E+00	1.52E+03	2.78E+02	1.86E+03	1.78E+02	9.18E+02	1.37E+02	5.78E+02	6.54E+02	
<i>POLR2</i>	polymerase (RNA) II	1.65E-	—	1.75E+	2.22E	1.63E+	3.38E+	1.82E+	2.76E	1.71E+	6.81E+	1.51E+	3.68E+	

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*														
Symbol	Description	p value	FDR	C-T - mean		C+T - mean		C+T+ mean		C-T+ mean		TConv mean		
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
A	(DNA directed) polypeptide A, 220kDa	02		03	+01	03	01	03	03	+01	03	01	3	1
PRDM1	PR domain containing 1, with ZNF domain	1.71E-02	-	2.76E+03	6.01E+01	3.23E+03	2.59E+02	5.03E+03	3.24E+02	3.71E+03	4.64E+02	2.04E+03	2.48E+02	
IRF7	interferon regulatory factor 7	1.73E-02	-	8.30E+02	6.92E+01	3.72E+02	1.53E+01	1.06E+03	2.21E+02	1.19E+03	2.18E+02	4.31E+02	5.79E+01	
ICAM3	intercellular adhesion molecule 3	1.81E-02	-	4.24E+03	3.07E+02	3.67E+03	4.46E+02	4.63E+03	4.88E+02	4.57E+03	6.10E+02	2.49E+03	7.15E+01	
PSMB7	proteasome (prosome, macropain) subunit, beta type, 7	1.85E-02	-	3.48E+03	1.20E+02	3.74E+03	6.65E+01	3.23E+03	5.05E+01	3.26E+03	5.01E+01	3.74E+03	1.75E+02	
IL10RA	interleukin 10 receptor, alpha	1.92E-02	-	4.25E+03	6.50E+02	2.84E+03	3.96E+02	6.08E+03	1.38E+03	5.27E+03	7.65E+02	1.44E+03	1.24E+02	
CCR7	chemokine (C-C motif) receptor 7	1.94E-02	-	1.49E+03	1.61E+02	7.00E+02	7.93E+01	2.00E+03	3.36E+02	1.65E+03	1.90E+02	7.17E+02	2.95E+01	
TNFRSF11A	tumor necrosis factor receptor superfamily, member 11a, NFKB activator	1.95E-02	-	1.07E+03	1.19E+02	8.10E+02	1.03E+02	1.19E+03	1.71E+02	1.06E+03	1.69E+02	4.35E+02	4.30E+01	
APP	amyloid beta (A4) precursor protein	2.02E-02	-	3.64E+02	3.78E+01	2.88E+02	1.15E+01	2.89E+02	4.54E+01	3.36E+02	2.04E+01	5.72E+02	3.92E+01	
FCER1G	Fc fragment of IgE, high affinity 1, receptor for; gamma polypeptide	2.05E-02	-	1.33E+03	1.98E+02	2.89E+02	7.86E+01	5.45E+02	2.23E+02	1.06E+03	3.08E+02	6.41E+01	1.10E+01	
IRF1	interferon regulatory factor 1	2.12E-02	-	1.73E+03	2.42E+02	1.56E+03	6.91E+01	2.70E+03	3.35E+02	1.93E+03	5.40E+01	1.04E+03	1.47E+02	
B2M	beta-2-microglobulin	2.16E-02	-	2.59E+05	3.47E+04	2.33E+05	2.23E+04	3.18E+05	6.29E+04	2.81E+05	2.73E+04	1.41E+05	8.94E+02	
NOD2	nucleotide-binding oligomerization domain	2.22E-02	-	2.32E+01	3.14E+00	2.56E+01	7.71E+00	4.93E+01	2.55E+00	3.56E+01	2.47E+00	3.49E+01	5.05E+00	

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Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	containing 2												
<i>ITGA4</i>	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	2.28E-02	-	1.89E+03	1.68E+02	1.75E+03	1.13E+02	1.52E+03	2.24E+02	1.62E+03	1.40E+02	2.62E+03	1.20E+02
<i>MAP4K4</i>	mitogen-activated protein kinase kinase 4	2.33E-02	-	2.76E+03	3.83E+02	2.30E+03	3.32E+02	3.21E+03	6.34E+02	2.76E+03	2.23E+02	1.27E+03	1.38E+02
<i>TNF</i>	tumor necrosis factor	2.36E-02	-	1.12E+02	7.83E+00	8.96E+01	1.00E+01	1.17E+02	2.53E+00	1.13E+02	6.05E+00	6.92E+01	6.88E+00
<i>TAPBP</i>	TAP binding protein (tapasin)	2.44E-02	-	2.72E+03	1.14E+02	2.58E+03	1.10E+02	3.49E+03	3.24E+02	2.75E+03	1.72E+02	2.02E+03	9.10E+01
<i>TNFRSF9</i>	tumor necrosis factor receptor superfamily, member 9	2.50E-02	-	6.06E+02	8.20E+01	5.38E+02	1.58E+02	8.74E+02	4.65E+01	8.21E+02	1.50E+02	3.21E+02	7.29E+01
<i>LILRB4</i>	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	2.54E-02	-	1.04E+01	1.42E+00	1.36E+01	2.62E+00	1.12E+01	5.32E+00	2.12E+01	9.93E-01	4.60E+01	1.63E+01
<i>PPARG</i>	peroxisome proliferator-activated receptor gamma	2.54E-02	-	2.30E+01	5.41E+00	8.07E+01	1.61E+01	5.17E+01	4.33E+00	2.38E+01	3.47E+00	4.32E+01	3.94E+00
<i>RARRES3</i>	retinoic acid receptor responder (tazarotene induced) 3	2.56E-02	-	1.02E+03	7.55E+01	6.40E+02	5.38E+01	9.41E+02	1.53E+02	1.15E+03	1.03E+02	6.25E+02	3.98E+01
<i>CXCR3</i>	chemokine (C-X-C motif) receptor 3	2.57E-02	-	2.00E+03	4.30E+02	1.29E+03	2.62E+02	2.27E+03	4.13E+02	2.54E+03	5.20E+02	4.59E+02	7.45E+01
<i>IL2RB</i>	interleukin 2 receptor, beta	2.60E-02	-	4.50E+03	1.36E+02	4.01E+03	3.46E+02	4.19E+03	2.51E+02	4.60E+03	8.86E+01	2.52E+03	3.09E+02
<i>GATA3</i>	GATA binding protein 3	2.69E-02	-	1.06E+02	2.36E+00	1.56E+02	6.83E+00	1.46E+02	2.24E+01	1.26E+02	2.01E+01	1.12E+02	1.56E+01

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*															
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean			
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM		
CFP	complement factor properdin	2.80E-02	-	1.30E+02	1.25E+01	8.02E+01	9.38E+00	1.40E+02	4.61E+01	1.55E+02	2.20E+01	5.16E+01	8.28E+00		
LY96	lymphocyte antigen 96	2.84E-02	-	5.88E+02	5.28E+01	4.63E+02	4.55E+01	9.00E+02	1.70E+02	6.76E+02	8.16E+01	3.35E+02	2.17E+01		
CD58	CD58 molecule	2.89E-02	-	1.06E+03	5.45E+01	1.42E+03	4.91E+01	1.73E+03	1.00E+02	1.26E+03	1.27E+01	1.21E+03	6.13E+01		
LCK	lymphocyte-specific protein tyrosine kinase	2.93E-02	-	5.45E+03	4.84E+02	4.77E+03	5.82E+02	5.50E+03	7.25E+02	5.55E+03	3.76E+02	3.50E+03	1.41E+02		
CD5	CD5 molecule	3.04E-02	-	3.70E+03	1.49E+02	3.45E+03	1.62E+02	4.47E+03	2.53E+02	4.31E+03	3.91E+02	2.49E+03	2.36E+02		
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	3.08E-02	-	7.08E+01	5.02E+00	2.40E+03	5.60E+02	1.09E+02	9.04E+00	6.84E+01	9.51E+00	6.58E+03	2.11E+03		
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	3.08E-02	-	1.85E+02	1.28E+01	2.17E+02	1.54E+01	2.25E+02	2.30E+01	2.25E+02	1.57E+01	3.38E+02	2.13E+01		
FYN	FYN oncogene related to SRC, FGR, YES	3.12E-02	-	2.42E+03	2.74E+02	1.64E+03	1.07E+02	2.83E+03	3.38E+02	2.90E+03	2.69E+02	1.55E+03	9.68E+01		
ATM	ataxia telangiectasia mutated	3.17E-02	-	6.64E+01	8.65E+00	8.54E+01	6.24E+00	1.08E+02	8.19E+00	1.12E+02	1.14E+01	6.64E+01	1.83E+00		
FCGR2C	Fc fragment of IgG, low affinity IIc, receptor for (CD32) (gene/pseudogene)	3.24E-02	-	5.26E+01	4.32E+00	4.64E+01	3.83E+00	5.16E+01	2.51E+00	5.15E+01	6.52E+00	3.14E+01	2.64E+00		
FCGRT	Fc fragment of IgG, receptor, transporter, alpha	3.25E-02	-	6.48E+01	2.27E+00	8.42E+01	1.19E+01	8.96E+01	1.24E+01	9.65E+01	1.27E+01	1.33E+02	1.12E+01		
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase	3.32E-02	-	3.74E+02	4.45E+01	2.65E+03	8.18E+02	3.49E+02	3.87E+01	3.33E+02	1.83E+02	1.85E+04	3.20E+03		

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*													
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C+T+ mean		C-T+ mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	1)												
<i>CD3E</i>	CD3e molecule, epsilon (CD3-TCR complex)	3.40E-02	-	5.76E+03	4.81E+02	5.60E+03	6.12E+02	6.33E+03	7.99E+02	6.28E+03	5.27E+02	3.95E+03	9.19E+01
<i>STAT6</i>	signal transducer and activator of transcription 6, interleukin-4 induced	3.50E-02	-	2.05E+03	1.81E+02	1.30E+03	7.91E+01	2.37E+03	3.75E+02	2.34E+03	3.24E+02	1.15E+03	8.34E+01
<i>MRI</i>	major histocompatibility complex, class I-related	3.51E-02	-	1.70E+02	3.42E+01	1.17E+02	1.26E+01	2.18E+02	5.30E+01	2.18E+02	6.75E+01	5.47E+01	6.10E+00
<i>TNFRSF8</i>	tumor necrosis factor receptor superfamily, member 8	3.59E-02	-	3.75E+02	1.32E+02	5.61E+02	1.32E+02	5.06E+02	1.13E+02	3.46E+02	7.13E+01	7.91E+02	3.87E+01
<i>MAPK1</i>	mitogen-activated protein kinase 1	3.61E-02	-	9.32E+01	3.31E+00	7.57E+01	3.11E+00	1.11E+02	1.49E+01	1.11E+02	8.40E+00	9.70E+01	3.05E+00
<i>TLR3</i>	toll-like receptor 3	3.62E-02	-	5.39E+01	6.64E+00	4.52E+01	7.74E+00	6.64E+01	9.66E+00	6.36E+01	6.65E+00	3.20E+01	1.71E+00
<i>JAK1</i>	Janus kinase 1	3.63E-02	-	2.91E+03	3.48E+02	2.65E+03	3.25E+02	3.43E+03	5.05E+02	3.22E+03	2.73E+02	1.90E+03	1.87E+01
<i>IFIT2</i>	interferon-induced protein with tetratricopeptide repeats 2	3.63E-02	-	1.68E+02	2.08E+01	6.16E+01	5.57E+00	1.96E+02	5.39E+01	2.40E+02	6.61E+01	1.57E+02	3.29E+01
<i>CASP2</i>	caspase 2, apoptosis-related cysteine peptidase	3.82E-02	-	9.68E+02	9.84E+01	1.17E+03	1.01E+02	1.06E+03	5.13E+01	1.01E+03	5.98E+01	1.44E+03	6.24E+01
<i>TIRAP</i>	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	3.88E-02	-	9.93E+01	7.28E+00	8.81E+01	8.80E+00	1.09E+02	1.66E+01	1.14E+02	9.15E+00	6.65E+01	4.06E+00
<i>RORC</i>	RAR-related orphan receptor C	4.02E-02	-	3.79E+01	5.21E+00	6.23E+01	1.38E+01	8.03E+01	7.88E+00	6.09E+01	8.82E+00	3.62E+01	2.01E+00
<i>REL4</i>	v-rel avian reticuloendotheliosis viral oncogene homolog A	4.04E-02	-	6.49E+02	4.10E+01	6.25E+02	1.63E+01	6.77E+02	2.24E+01	6.65E+02	1.07E+01	5.28E+02	2.25E+01

Table 2 - Differentially expressed genes between expanded Tconv and Treg subsets expressing CD226 and TIGIT.*													
Symbol	Description	p value	FDR	C-T- mean		C+T- mean		C-T+ mean		C+T+ mean		TConv mean	
				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>PSMB8</i>	proteasome (prosome, macropain) subunit, beta type, 8	4.14E-02	-	1.10E+04	1.97E+02	1.12E+04	1.16E+02	9.74E+03	1.32E+02	1.13E+04	3.38E+02	1.02E+04	2.55E+02
<i>CD83</i>	CD83 molecule	4.16E-02	-	3.66E+02	7.11E+01	1.97E+02	2.47E+01	3.43E+02	3.83E+01	3.92E+02	2.34E+01	2.07E+02	3.51E+01
<i>FCGR3A</i>	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	4.35E-02	-	8.28E+01	8.73E+00	7.40E+01	7.62E+00	9.85E+01	2.15E+01	1.00E+02	6.17E+00	5.83E+01	4.12E+00
<i>TNFSF11</i>	tumor necrosis factor (ligand) superfamily, member 11	4.53E-02	-	4.46E+01	2.64E+00	1.52E+02	3.22E+01	4.76E+01	1.05E+01	5.20E+01	2.74E+00	1.30E+02	1.77E+01
<i>HLA-DRB3</i>	major histocompatibility complex, class II, DR beta 3	4.57E-02	-	2.90E+03	5.02E+02	3.07E+03	2.94E+02	2.96E+03	5.10E+02	3.17E+03	4.46E+02	1.75E+03	2.03E+01
<i>ENTPD1</i>	ectonucleoside triphosphate diphosphohydrolase 1	4.68E-02	-	1.37E+01	4.20E+00	1.38E+01	3.60E+00	3.03E+01	5.00E+00	4.30E+01	7.49E+00	1.46E+01	1.26E+00
<i>TNFSF15</i>	tumor necrosis factor (ligand) superfamily, member 15	4.69E-02	-	5.93E+01	7.26E+00	7.05E+01	7.55E+00	7.82E+01	4.27E+00	7.24E+01	8.19E+00	5.18E+01	9.04E-01
<i>IFNAR2</i>	interferon (alpha, beta and omega) receptor 2	4.73E-02	-	2.15E+03	3.67E+02	1.84E+03	2.99E+02	2.37E+03	5.90E+02	2.78E+03	3.23E+02	1.06E+03	4.90E+01

*Tconv (CD4⁺CD127⁺) and Tregs (CD4⁺CD25⁺CD127⁻) subdivided by CD226 and TIGIT population were FAC sorted then expanded for 14 days in vitro. The expression profile of the expansion product was analyzed by the nCounter.

EXAMPLE 2 – Differentiated T cells concomitantly express high CD226 and chemokine receptors

Signaling through CD226 suppresses T_H2 differentiation and promotes T_H1 responses and IFN γ secretion. The cellular distribution and expression profiles of CD226 and TIGIT on human naïve, central and effector memory, and effector CD45RA⁺ (TEMRA) subsets (Figure 3A) were studied. While naïve T cells were the most abundant population (mean $\pm$ SEM, 44.8% $\pm$ 14.6), they expressed the lowest surface levels of CD226 (495.4 $\pm$ 73.5 gMFI), when compared to effector memory (T_{EM}) and central memory (T_{CM}) subsets (1785 $\pm$ 357.2 and 3185 $\pm$ 492.6, respectively)(Figure 3B). Furthermore, the cellular distribution of CD226 in the bulk CD4⁺ T cell population demarcates naïve (CD226^{low}) versus CD226^{hi} memory subsets. Few naïve cells express TIGIT (5.3% $\pm$ 2.6) compared to the memory subsets (29.0% $\pm$ 3.5 for T_{EM} and 32.7% $\pm$ 8.2 for T_{CM}). One subject with a prominent TEMRA population was CD226^{hi}TIGIT⁺ (Figure 3A), which coincides with their role as a pro-inflammatory T cell subset.

In addition to robust CD226 expression in T_{EM} and T_{CM}, we also noted that chemokine receptors expressed by T_H2, T_H17, and T_H1 cells were co-expressed with high levels of CD226 (Figure 3C; *upper plots*). TIGIT⁺ populations also expressed chemokine receptors, albeit to a lesser extent than with CD226 (Figure 3C; *lower plots*). Increased CD226 and TIGIT on differentiated subsets were observed for both CD4⁺CD127⁺ Tconv and CD4⁺CD25⁺CD127^{lo} Treg (Figure 3D).

EXAMPLE 3 - CD226 expression is associated with effector cytokine production

CD226 and TIGIT have opposing roles in the regulation of IFN γ . The influence of CD226 and TIGIT on IFN γ production by PBMC following activation was studied (Figure 3E). IFN γ tracked primarily to the CD226^{hi}TIGIT⁺ fraction, with the CD226^{hi}TIGIT⁺ population consistently containing a minor population of IFN γ ⁺ T cells (64.7% $\pm$ 8.7 vs. 24.9 $\pm$ 8.3; p=0.011). IFN γ was co-expressed with CD226, as CD226^{int}TIGIT⁺ and CD226^{int}TIGIT⁺ populations contained significantly lower percentages of IFN γ ⁺ cells than their CD226^{hi} counterparts (Figure 3F; 7.03% $\pm$ 3.29 and 3.42 $\pm$ 1.03%, respectively). The CD226⁺ subset was largely devoid of IFN γ ⁺ T cells (0.183% $\pm$ 0.053). Overall, these results demonstrate a close association between CD226 and the production of IFN γ by antigen-experienced T cells.

To determine if this association was influenced by T_H1-skewing conditions, T cells were activated in the presence of IL-12 (Figure 8). While both CD226 and TIGIT increase upon T cell activation, the change in TIGIT expression by tTreg has not been characterized following culture with IL-12. CD226 expression increased over the 72 hr time course (Figure 8A). Likewise, TIGIT increases following TCR activation. Notably, TIGIT upregulation was attenuated by IL-12 in

FOXP3⁺Helios⁺ Treg (Figure 8B). IL-12 upregulated CD226 and IFN γ as expected (Figure 8C-D), however, the proportion of tTreg recovered from the culture decreased in IL-12 (Figure 1E). As such, IL-12 exposure may potentiate Teff cytokine production concomitant with a reduction in both tTreg proliferation and TIGIT expression.

5 IFN γ ⁺ Treg are elevated in patients with T1D. To further characterize this subset, IFN γ ⁺ or IFN γ ⁻ Treg and Tconv subsets were isolated and a transcriptional profile of the FACS isolated subsets was conducted. Principal component analysis (PCA) indicated divergence of Treg and Tconv populations, with further discordance in IFN γ ⁺ and IFN γ ⁻ Treg (Figure 3G). Importantly, both freshly isolated and expanded IFN γ ⁺ Treg express significantly more CD226 than the IFN γ ⁻ Treg subset
10 (Figure 9A). Moreover, TIGIT expression was increased in IFN γ ⁻ Tregs compared to IFN γ ⁺ Tregs, while the inverse is true for Tconv (Figure 9B).

EXAMPLE 4 – CD226 and TIGIT identify functionally distinct subpopulations of Treg

Human CD4⁺CD25^{hi}CD127⁻ Tregs contain a significant degree of heterogeneity in terms of
15 lineage diversity and antigen exposure. CD4⁺CD25^{hi}CD127⁻ Tregs were subdivided based on CD226 and TIGIT expression (Figure 4A, *right plot*). FOXP3-TSDR demethylation at the conserved non-coding sequence 2 (CNS2) has been previously associated with Treg stability. Hence, we measured the percentage of cells demethylated at the TSDR. When analyzed *ex vivo*, CD226⁺TIGIT⁻ T cells were reduced in TSDR demethylation (30.0% $\pm$ 8.3), in comparison to the other Treg subsets and
20 Tconv cells (Figure 4B). Despite this reduction in TSDR-demethylation, the resulting population suppressed to comparable levels when compared to the other freshly isolated CD4⁺CD25^{hi}CD127⁻ Treg populations (Figure 4C). Dye dilution analysis facilitated further analysis of Treg proliferation and viability. Increased proliferation of both the CD226⁻TIGIT⁻ (naïve) and CD226⁺TIGIT⁻ populations was observed. Interestingly, increased IL-10 in the suppression assay co-culture with
25 CD226⁺TIGIT⁻ Treg was also noted. A trend toward more CD226⁺TIGIT⁺ cell death was observed; however, this did not reach statistical significance for any Treg subset. Thus, freshly isolated CD226⁺TIGIT⁻ T cells exhibit comparable *ex vivo* suppression when freshly isolated, yet differ in FOXP3-TSDR demethylation and cytokine production.

Protocols to generate expanded human Treg are susceptible to outgrowth of non-Treg and the
30 potential for lineage instability. Therefore, the purity and suppressive activity of Tregs was analyzed post *in vitro* expansion based on CD226 and TIGIT expression (Figure 5A). While the TIGIT⁺ fractions constituted the majority of Treg isolated from PBMC, these cells were highly refractory to expansion, limiting the overall Treg yield (Figure 5A). This decrease in proliferative capacity of the sorted TIGIT⁺ Treg supports the role of TIGIT as an intrinsic negative regulator, and also reflects an

enrichment of antigen-experienced (CD45RO⁺) cells with limited expansion capacity. In contrast, the TIGIT⁻ Treg fraction expanded robustly from a naïve state.

To further assess the purity and functional capacity of these subsets, FOXP3 and Helios were analyzed following expansion (Figure 5B-C). The highest purity of post-expansion Treg originated from cells lacking CD226 expression at the time of the initial sort (*i.e.*, prior to *in vitro* expansion). The highest percentage of FOXP3⁺Helios⁺ cells were observed in the CD226⁻TIGIT⁺ population, followed by CD226⁻TIGIT⁻ and CD226⁺TIGIT⁺ cultures (90.73% ± 3.7, 83.69 ± 8.69, and 74.30 ± 10.13, respectively). In contrast, the CD226⁺TIGIT⁻ population contained the least FOXP3⁺Helios⁺ cells post expansion (38.18 ± 11.70). Tconv expanded with very little FOXP3 and Helios co-expression (Figure 5B). In terms of surface CD226 and TIGIT expression, TIGIT was maintained or upregulated on TIGIT⁺ and TIGIT⁻CD226⁻ cells following expansion, but remained low on TIGIT⁻CD226⁺ Treg and Tconv populations (Figure 5D-E).

Expanded Treg from T1D subjects were enriched in FOXP3⁺Helios⁻ cells with the capacity to produce IFN γ . Accordingly, CD226⁺ Treg have increased capacity to produce IFN γ upon stimulation, while CD226⁻ Treg were almost completely devoid of IFN γ (Figure 5F-G). CD226⁻ Treg (TIGIT⁺ or TIGIT⁻) were both able to potently suppress autologous CD4 and CD8 T cell proliferation, with some diminution observed in the CD226⁺ expanded subset (Figure 5H). The CD226⁻TIGIT⁻ and CD226⁻TIGIT⁺ subsets were almost completely demethylated at the TSDR (92.0% ± 3.13% and 94.5% ± 3.71, respectively, Figure 5I). The degree of demethylation at the TSDR correlated strongly with FOXP3 and Helios co-expression determined by FACS (Figure 5I; $R^2 = 0.94$, $p < 0.0001$).

EXAMPLE 5 – CD226⁺TIGIT⁻ T cells express an activated effector gene expression profile and produce IL-10 and effector cytokines

Gene expression profiles provide a powerful signature of the regulatory and effector mechanisms employed by T cells. Given that most Treg therapies will require some form of expansion, a gene profile was conducted on *in vitro* expanded Tconv (CD4⁺CD25⁻CD127⁺) or Treg (CD4⁺CD25⁺CD127^{-/lo}) that were further sub-divided based on initial TIGIT and CD226 expression (Figure 6A). From this analysis, 159 genes were found differentially expressed between the groups ($p < 0.05$). These data are summarized in the heatmap and dendrogram (Figure 6A and Table 2). The normalized transcript counts were used to cluster the genes. 18.6% of the variance observed was attributable to individual subject variance, while the remainder segregated based on the initial sorted populations. Tconv showed a clear demarcation from Treg, with the CD226⁺TIGIT⁻ population demonstrating an intermediate expression profile between Treg and Tconv (Figure 6A, *green bars*). Interestingly, the CD226⁺TIGIT⁺ population has a Treg signature highly enriched in negative

regulators and immunoregulatory pathways (Figure 6B-E). Despite this regulatory signature, it did not completely correlate with their suppressive capacity following *in vitro* expansion (Figure 5H). This may imply that CD226 negatively impacts suppression, or may reflect a preferential outgrowth of non-Treg by d14, as indicated by the TSDR results (Figure 5I). PCA analysis of Treg subsets demonstrated the CD226⁺TIGIT⁻ population shared some common features with Tconv cells (Figure 6F). An extensive multiplex cytokine profile of freshly isolated Tregs further confirmed the capacity of CD226⁺TIGIT⁻ T cells to produce a broad array of cytokines (Figure 6G-H).

EXAMPLE 6 – Exclusion of CD226⁺ eliminates unstable Treg following *in vitro* expansion

Improving Treg stability and limiting contamination non-tTreg may be critical for future Treg therapies. Prior efforts demonstrated that CD226 expression, irrespective of initial TIGIT expression, resulted in a sizable fraction of cells that were reduced in suppressive activity and methylated at the TSDR (Figure 5). In addition, the TIGIT⁺ population was highly refractory to expansion. Thus, eliminating CD226⁺ cells during the initial sort would increase purity without significantly constraining the final cell yield (Figure 7A). Total Treg and CD226⁻ Treg expanded with comparable kinetics (Figure 7B). Importantly, the purity of the Treg subsets (FOXP3⁺Helios⁺) was consistently higher in the CD226⁻ Treg subset compared to the CD226⁺ Treg fraction (89.33 ± 3.45 vs. 57.2 ± 5.96 , respectively) (Figure 7C-D). CD226 expression correlated with IFN γ (Figure 7E-F). This observation was inversely related to TIGIT expression following culture (Figure 7G,H). In terms of suppressive capacity, CD226⁻ Treg were consistently more able to suppress CD4⁺ and CD8⁺ responder T cells than their CD226⁺ Treg counterparts (Figure 7I). Indeed, an elevated ratio of TIGIT to CD226 expression on the tTreg population following an *in vitro* suppression assay was associated with increased suppressive activity and TSDR demethylation (Figure 7I-K). These results suggest that elimination of cells expressing CD226 provides an effective means to further enrich a stable population of human Treg.

EXAMPLE 7 – Identification of Treg based on TIGIT and CD226 expression

Prior efforts to define the transcriptional profile of human Treg have relied primarily upon the use of surrogate surface markers for isolation. This methodology is subject to alterations in surface marker expression following antigen exposure and cellular activation (particularly for CD45RA, CD25, and CD127). A direct transcriptional profile of Treg by FACS sorting cells based on the transcription factors FOXP3 and Helios was conducted. This analysis identified TIGIT, an important negative regulator, as highly expressed on tTreg relative to Tconv or FOXP3⁺Helios⁻ T cells.

TIGIT expression on Treg was characterized in the context of the competing costimulatory molecule CD226. This analysis identified four distinct subpopulations of cells based on their surface expression of these receptors. CD226 expression marks both T_{CM} and T_{EM} and Treg subsets capable of producing IFN γ and IL-10 for Treg. TIGIT expression was stable or upregulated on Treg following *in vitro* expansion. An incipient concept in Treg biology relates to the ability of Treg to co-opt the transcription programs of the T_H cells they are posed with suppressing (*e.g.*, Tbet⁺ Treg suppress T_H1 immunity, T_H2-Treg suppress humoral responses, *etc.*). This may also be the case for antigen-experienced Treg that are CD226⁺TIGIT⁺. Lineage associated chemokine receptor expression on both CD226 and TIGIT expressing Treg is noted.

TIGIT⁺CD226⁻ Treg expressed high levels of FOXP3 and Helios and were demethylated at the TSDR. Moreover, data from *in vitro* suppression assays indicated TIGIT expression on Treg was associated with robust suppressive activity. The relative ratio of these receptors might provide an informative biomarker. These findings are particularly timely given the genetic associations of CD226 in autoimmune diseases and multiple reports of Treg functional defects and effector cytokine production by Treg (*e.g.*, IFN γ and IL-17). Interestingly, analysis of IL-10 producing T regulatory-type 1 (T_R1) cells also reported high CD226 expression, in addition to CD49b and LAG3.

These findings for TIGIT⁺ Treg draw some distinctions from those recently reported by Joller *et al.*, who suggested TIGIT⁺ Treg share features with T cells of a proinflammatory lineage. This phenotype may be more representative of cells co-expressing CD226 and TIGIT, as few cytokines or effector genes were upregulated in TIGIT single positive populations. In fact, IL-10 expression and the IL-10-associated transactivator PRDM1 were only discernible in the CD226⁺TIGIT⁺ Treg population. Moreover, the shift toward an effector-like lineage was most prominent in the CD226⁺TIGIT⁻ population.

EXAMPLE 8 – Immunotherapies using CD226⁻ Treg

Immunotherapeutics targeting coinhibitory molecules such as CTLA-4 and PD-1 have garnered increasing interest following notable clinical successes. This disclosure provides important implications for future therapies that may seek to target the CD226/TIGIT axis. CD226 is associated with proinflammatory Teff and this disclosure demonstrate CD226 is also expressed at low to intermediate levels on naïve T cells and may play a key role in IL-10 producing Treg. Moreover, CD226 is upregulated on the majority of tTreg following activation. Thus, therapies seeking to block CD226 to attenuate Teff activity must be carefully dosed to target CD226^{hi} expressing Teff, while preserving naïve T cells and IL-10 producing T_R1. The CD226/TIGIT axis may be susceptible to control by innate inflammatory cytokines, as demonstrated for IL-12. One potential benefit of anti-

IL-12 antibody therapy may be the preservation of TIGIT expression on tTreg. Finally, the disclosure provides implications for Treg adoptive cell therapies that are currently progressing in clinical trials for a number of autoimmune conditions. Overall, the disclosure supports that $CD4^+CD25^+CD127^{-/lo}$ Tregs maintain a high degree of purity following expansion over a period of 14 d. Extending these findings, the selection of the TIGIT⁺ Treg population is shown to result in a highly enriched population, but this came at the cost of initial Treg recovery and resulted in a highly refractory population limiting the overall yield. However, the isolation of CD226⁻ Treg, irrespective of initial TIGIT expression, results in a highly pure and potent population of TIGIT⁺ Treg for use in cell therapies. Therefore, these data provide a biological context in which the autoimmune candidate gene *CD226* may modulate T cell biology. Moreover, this disclosure provides markers to identify highly suppressive Treg for use in cell therapies.

EXAMPLE 9 – Immunotherapy of T1D using CD226⁻ Treg

T1D is a disorder of failed immune regulation. The immune system has evolved multiple mechanisms to elicit protective immunity to foreign agents while preserving tolerance to self. Treg play a central role in limiting self-reactive T cells that escape negative selection, thereby maintaining dominant peripheral tolerance to self-antigens. This regulatory pathway fails to protect against autoimmune tissue destruction in T1D and several other autoimmune diseases. An imbalance in Treg and effector T cell (Teff) cell activity may represent a central defect leading to T1D. Moreover, an imbalance may occur at the cellular level and also in controlling the activity of co-stimulation, whereby an activating co-stimulatory signal is favored over negative checkpoint regulators resulting in uncontrolled activation. Genome-Wide Association Studies (GWAS) in humans with T1D suggest concomitant defects occur in innate and adaptive immunity, predisposing T1D individuals to favor inflammatory signaling and a functional imbalance in adaptive immune regulation (Figure 10). Genetic variations result in TCR and BCR signaling, alterations in the balance of costimulatory factors, increased innate inflammation, type 1 interferon production, and IL-2R defects.

T1D is treatable through administration of exogenous insulin, but no intervention allows prevention of T1D onset or reverses autoimmunity. Several immunotherapies have strived to induce tolerance to T1D self-antigens by inhibiting Teff or augmenting Treg functions, however incomplete knowledge of how Teff/Treg develop and function in T1D has hindered success. This embodiment of the claimed invention provides novel therapies for T1D.

Lentiviral (LV) modulation of CD226 and TIGIT

A library of overexpression and shRNA knockdown constructs to modulate CD226 and TIGIT expression was constructed to determine its impact on human T cell subsets (Table 3). Preliminary studies have validated constructs, as demonstrated for the overexpression constructs shown in (Figure 11). In addition, our LV CD226 constructs demonstrated increased IFN γ in response to activation with anti-CD3 and anti-CD28.

Table 3	
Overexpression	Predicted Phenotype
*CD226C(major allele).eGFP	↑ Treg IFN γ ; ↑ Th1 cell
*D226T(minor allele).eGFP	↑↑Treg IFN γ ; ↑↑ Th1 cell
*TIGIT(wt). eGFP	↑ Treg cell; ↓ Th1 cell
**DN TIGIT(mut).eGFP	Trans effect on APC ↑ IL-10; ↓ IL-12
*eGFP alone	Transduction control
shRNA Gene Knockdown	
***CD226-shRNA	↑ Treg cell; ↓ Th1 cell
***Scramble shRNA	Transduction control
*TIGIT-shRNA.eGFP	↓ Treg cell activity; ↑ Th1 cell
*Scramble shRNA.eGFP	Transduction control
* Gene of interest driven by CMV promoter. Enhanced GFP reporter. ** Dominant negative (DN) TIGIT mutated intracellular signaling domain (Y225A, Y231A). ***shRNA driven by U6 promoter. Transduced cells are selected by puromycin.	

LV gene transfer facilitates the study of human autoreactive TCRs. A robust LV gene transfer platform to interrogate Ag-specific T cell activity was developed. This system allows for multicistronic expression of TCR α and TCR β chains in addition to reporter genes. These constructs include the ability to alter the specificity of both CD4⁺ and CD8⁺ T cells recognizing MHC class I and II-restricted peptides presented in the context of HLA-DR*03-01, DR*04-01, and HLA-A*02-01 (an example is shown in Figure 13). This provides an unprecedented ability to assess the function of T1D Ag-specific Treg, Tconv, and CD8⁺ T cells, as demonstrated in (Figure 14).

IL-12 negatively impacts TIGIT expression

While it has been previously shown that both CD226 and TIGIT increase upon T cell activation, the change in TIGIT expression by human nTreg has not been characterized following culture with IL-12. As shown previously, CD226 expression increased over the 72 h. Likewise, TIGIT increases following TCR activation. TIGIT upregulation was attenuated in IL-12 conditions in FOXP3⁺Helios⁺ Treg (Figure 14A). IL-12 upregulated IFN γ as expected (Figure 14C), however, the proportion of nTreg recovered from the culture decreased with IL-12 (Figure 14D). These data suggest a mechanism whereby IL-12 may augment T_H1 production of IFN γ concomitant with a

reduction in both nTreg proliferation and TIGIT expression. These findings provide an important biomarker of anti-IL-12 antibody (ustekinumab) therapy in trials in Chron's Disease, psoriasis, and proposed in T1D through the Immune Tolerance Network (ITN).

5 Development of TCR expression constructs

To express functional *de novo* TCRs in CD4⁺ and CD8⁺ T cells, LVs were generated using a high-affinity tumor-reactive TCR recognizing the melanoma antigen, Melan A, in the context of HLA-A*0201. Likewise, T cell clones recognizing β cell autoantigens were isolated from T1D patients. Full TCR α - and β -chain genes encoding TCRs recognizing peptides from pre-proinsulin (PPI), glutamic acid decarboxylase (GAD), and insulin-related glucose-6-phosphatase catalytic subunit 2 related protein (IGRP), were cloned into a pFUGW expression vector (Table 4). These TCR LV constructs facilitate equimolar expression of TCR α - and β -chains via multicistronic T2A and P2A elements. Virus production and transduction can be conducted as described previously (Ventura *et al.* (2004)). Cells can be cultured with IL-2 until d 9. On d 9 cells can be sorted using fluorescent reporter and transgene positive cells re-stimulated with Dynabeads and expanded for an additional 7 d. Preliminary studies validate expression of these TCRs following transduction of primary human CD4⁺ T cells and CD8⁺ T cells through MHC-multimer staining (Figure 12) and activation (CD69 upregulation and IL-2 production) following incubation with K562 HLA expressing cells with cognate peptides.

Table 4. TCR constructs to be used for the generation of antigenic specific CD4 and CD8 T cells					
Clone	Antigen Target	TCR α	TCR β	HLA Restriction	Ref/Source
1E6	PPI ₁₈₋₂₄	13-2/13-2	8	HLA-A*0201	(1-3)
32	IGRP ₂₅₅₋₂₇₃	12.1	20	HLA-A*0201	(4,5)
MART1	Melan-A ₂₇₋₃₅	35	10.3	HLA-A*0201	(6)
*R164	GAD ₅₅₅₋₅₆₇	12.1	5.1	HLA-DR*0401	(7,8)
*4.13	GAD ₅₅₅₋₅₆₇	12.1	5.1	HLA-DR*0401	(7,8)
PM1#11	GAD ₅₅₅₋₅₆₇	35.02	5.1	HLA-DR*0401	(11)

TRC-LV will encode an expressed far-red fluorescent tag for sorting.

*R164 and 4.13 recognize GAD with high and intermediate affinity respectively.

To determine the effectiveness of these cells cell-mediated lymphocytotoxicity (CML) assays were performed with the Class I HLA-A*0201⁺ BL5 cells as targets and HLA-A*0201 restricted, IGRP-reactive CTL as the effector cells. Numerous CML assays were performed with this system to study required effector to target (E:T) ratios, mechanisms of CTL-mediated lysis, as well as changes

in lysis due to priming of BL5 cells with IFNs. IGRP-CTL lyse BL5 cells while MART1-CTL only lyse MART-1 peptide pulsed BL5 cells (Figure 13). Lysis can be titrated by decreasing the E:T ratio. As BL5 cells express IGRP, BL5 cells do not require pulsing with IGRP peptide. IFN γ increased lysis of BL5 cells by IGRP-CTL. Lysis is partially dependent on Fas and perforin as an anti-Fas antagonistic antibody and concanamycin (CMA) respectively both partially block lysis. Killing is further reduced by the combination of CMA and anti-Fas. Lysis is dependent on HLA, as antibody blockade of HLA eliminates lysis. For these experiments, CTL can be co-transduced with the IGRP TCR and the vectors in Table 3 to assess the impact of these costimulatory molecules on CTL activity. Co-reporter expression and TCR (far red reporter) and CD226 or TIGIT directed vectors (eGFP) can be used to ensure uniform stable expression on FACS purified populations. For MHC class II restricted TCRs, proliferation, effector cytokine production, and *in vitro* suppression using peptide stimulations and autologous APC or HLA-expressing artificial APCs for stimulation (K562.HLA-DR*0301 or HLA-DR*0401) can be assessed. This system can expand antigen-specific T cells (CD4 and CD8), utilizing starting clinically feasible material (~10-20 ml blood draw), ~100-fold at d9, and 1000-fold at d14 following FACS sorting and restimulation to enrich transduced populations. Furthermore, these expanded T cells remain Ag-reactive and are effective at lysing β cells facilitating experiments with gene modified CTL.

Impact of innate inflammatory cytokines

In recent onset T1D where insulinitis is present, infiltrates (containing CTL, macrophages, B cells, and CD4⁺ T cells) associate with insulin positive islets as opposed to insulin deficient endocrine clusters. Persisting insulinitis is associated with decreases of only β cells (not α cells) and increases in MHC I and FAS. Thus, specific immune reactivity to β cell antigens is central to T1D progression, and CTL responses are likely key effectors eliminating β cells. However, several unknowns exist with regards to additional requirements, including the immune checkpoint regulators involved in controlling effector molecules used by autoreactive CTL to kill β cells. The maintenance of immune regulation is susceptible to environmental control (Figure 10). Localized innate inflammatory cytokines may attenuate TIGIT, allowing for the activation and expansion of pathogenic autoreactive T cells.

Impact on skewing, cytokine production, and *in vitro* suppression

The impact of type 1 IFN α , IFN β , IL-1 β , IL-18, IL-12, IL-23, and IL-6 on the ratio of CD226 and TIGIT can be tested. Innate cytokines may cause increased T cell skewing and CD226 relative to TIGIT on Treg and Teff/CTL-ultimately rendering Treg less suppressive. Treg may adopt a Teff-like

phenotype (loss of FOXP3, acquisition of IFN γ and IL-17) or Teff cells may become refractory to suppression, as has been described in patients with T1D.

Determining the impact of innate cytokines on CTL activity and killing of BL5

5 BL5 or monolayered islets can be treated with or IFN γ (100U/mL) for 24 h. The cells can then be washed and CTL generated and genetically manipulated as described above with the addition of treatment during the *in vitro* expansion protocol with titrating amounts of cytokine (3-fold serial dilutions from 20 ng/ml each). CML can be collected, washed and subjected to flow cytometry (CD8, IFN γ , CD107a, perforin, granzymes, CD226, TIGIT). Supernatants can be collected for multiplexed
10 cytokine analysis (e.g., by Miliplex). Optimal concentrations can be determined and moved into CML assays. CML can be performed by using β cells or monolayered islets as targets. CTL can be used at a 5:1 E:T ratio as this provides for observations of increased as well as decreased lysis. The specific impact of CD226 and TIGIT can be further tested with agents (agonists/antagonists).

 TIGIT is likely to be enriched on Treg with potent suppressor activity. Thus, overexpression
15 constructs can augment the suppressive capacity of Treg, and potentially, alter APC and Tconv into a more tolerogenic state (e.g., IL-10 producing). Conversely, shRNA constructs designed to downregulate TIGIT can reduce the suppressive activity of Treg. Increased expression and/or signaling via CD226 can be seen in individuals carrying the T1D-associated risk allele (T) at rs763361. In addition, overexpression of CD226 can augment the capacity of autoreactive T cells to
20 produce effector cytokines (e.g., IL-17 and IFN γ), and likewise, increase the cytotoxic activity of CTL.

 Biologics now play an increasingly important role in controlling autoimmune reactivity by modulating cytokine signaling (e.g., ustekinumab and tocilizumab). Some of this beneficial effect is due to blocking inflammation that attenuates suppressive activity and negative regulators normally
25 expressed by Treg. Specifically, inflammatory cytokines can attenuate FOXP3 and TIGIT expression by Treg, and conversely augment CD226 expression and differentiation into T_H1 and T_H17 cell lineages.

**EXAMPLE 10 – Assessing the expression of CD226 and TIGIT throughout the natural history
30 of T1D in the NOD mouse and in humans with T1D**

 Destruction of islet β cells may occur when a functional imbalance results favoring activation and expansion of autoreactive effector CD4⁺ and CD8⁺ T cells overwhelms the capacity of Treg to maintain immune tolerance. CD226 on tissue- and islet resident T cells may increase and TIGIT may reduce during the pathogenesis of disease in the NOD and in humans with T1D.

The NOD mouse develops spontaneous disease with many similarities to human T1D, albeit with a much more pronounced islet infiltration and presentation. Importantly, this model affords the ability to directly assess the impact of specific genes and pathways in an immune replete host. The data indicates a higher ratio of CD226:TIGIT at disease onset relative to 4 wk old mice (Figure 15).

5

Investigating the role of CD226 and TIGIT in the NOD mouse model of T1D

Female NOD mice, Non-Obese Resistant (NOR), and C57BL/6 control mice (Jackson Laboratories) can be followed longitudinally at defined checkpoints in the disease process (4, 8, 12, 16 wk, and at disease onset). Groups of mice (N=10/group) can be sacrificed at each time point for flow cytometric and histological analysis of CD226 and TIGIT expression on T cells and NK cell populations. CD112 and CD155 can also be measured on islet resident APC populations. Incidence studies can be conducted in female NOD (N=20/group) and can be defined as BG>250 mg/dL on 2 successive days. 80% of female NOD mice developed disease at 21 wks of age. In addition to standard NOD mice, the specific impact of these receptors can be assessed on disease development through ZFN and CRISPR targeted knockouts (NOD.TIGIT^{-/-} and NOD.CD226^{-/-}). Pancreata can be processed for immunohistochemistry and stained for Ki67, insulin, glucagon, CD3, CD4, CD8, CD226, TIGIT, FoxP3, B220, F4/80, CD112, CD155 and CD11c to assess the expression of the markers of interest on resident and infiltrating lymphocytes. PB, splenocytes, pDLN, axillary LN, and BM cells can be taken for immunological assays and FACS.

20 Immune profiling

Complete blood count (CBC) by coulter counter can be followed by immunophenotyping via flow cytometry. Antibodies can be used for the detection of CD3, CD4, CD8, CD226, TIGIT, CD25, FoxP3, Helios, IFN γ , IL-10, CD56, CD16, CD11b, CD11c, CD112, and CD155. Single cell suspensions prepared from islets, spleens, pancreatic and mesenteric lymph nodes, as well as BM and thymus can be subjected to flow cytometry using a BD LSR Fortessa. These single cell suspensions can be probed for CD4⁺ and CD8⁺ T cells with BDC2.5 and IGRP tetramers (NIH Tetramer Core) and markers for naïve, activated, memory, and regulatory phenotypes, as well as CD226 and TIGIT. DCs can be assessed for markers to distinguish pDC and mDC, maturation status, co-stimulatory and co-inhibitory molecules as well as monocyte expression of CD112 and CD155 ligands and production of IL-12 and IL-10 in response to TLR ligands (LPS, Poly I:C, CpG).

30

Adoptive transfer (AT) and mixed bone marrow chimera experiments

The impact can be ascertained of Treg and Teff cell expression of CD226 and TIGIT through AT, and adoptive co-transfer experiments in NOD.RAG^{-/-} recipients alone, or following co-AT with

diabetogenic splenocytes (2×10^7 cells injected into NOD.RAG^{-/-} recipient mice). Recipient mice can be followed for T1D development via blood glucose as indicated.

Mixed bone marrow chimeric mice consisting of TIGIT or CD226 deficient and WT immune cells can be generated. In these mice, WT cells can provide the necessary diabetogenic environment with cytokines, chemokines, and surface ligands during T1D. An equal amount of NOD.Thy1.2 WT and either NOD.Thy1.1 TIGIT^{-/-}, Thy1.1 CD226^{-/-}, or Thy1.1 WT bone marrow as control can be transferred into lethally irradiated NOD Rag1^{-/-} mice to exclude the interference of endogenous T cells. Antibiotics can be added into drinking water for 6 weeks to prevent infection. Six weeks after transplantation, the reconstitution of CD4⁺, CD8⁺ T cells, B cells, DC, macrophage, and neutrophils in PBMC can be confirmed by flow cytometry. Incidence can be followed at indicated above. To understand if TIGIT and CD226 play roles in CD4 T cell activation, proliferation, and migration, diabetic mice can be sacrificed and pancreata, spleens, draining, and non-draining lymph nodes can be collected. The proportion of allelic marked donor cells and expression of activation markers and Ki-67 can be assessed by FACS and histology.

CD226 and TIGIT are reported to regulate lineage differentiation and cytokine production. The expression of various transcriptional factors (Tbet, ROR γ t, GATA3, FoxP3) and cytokines including IFN γ , IL-17, IL-4/IL-5, Foxp3, and IL-10 can be determined.

Analysis of *in vitro* suppressor function

Freshly isolated Treg and T responder splenocytes from NOD mice or NOD.TIGIT^{-/-}, CD226^{-/-} mice can be assessed for their ability to suppress, or be suppressed in cross-over *in vitro* suppression assays. Tresp (labeled with Cell Tracker Violet) were plated in triplicate with purified Treg (labeled with APC channel dye) added at varying ratios from 1:1 to 1:64 Treg to Tresp cells.

Vaccine studies

Female NOD, NOD.CD226^{-/-}, and NOD.TIGIT^{-/-} mice at 8 wk of age can be used to explore the effects of costimulation on recall responses to exogenous antigen challenge. This provides insight into whether immune sufficiency is maintained and whether effector, memory, or regulatory mechanisms are involved with CD226:TIGIT signaling. Group i) consists of a control, no injections, Group ii) HEL antigen given in prime and 2 wk later boost with alum adjuvant. Groups can be bled weekly for serum assessment of HEL specific IgM and IgG titers by ELISA. 8 wk post-boost of HEL, the animals can be euthanized and spleens removed. Cells can be placed into culture and stimulated with HEL antigen to test recall responses assessing differentiation and proliferation by FACS, and cytokine secretion in supernatants (Milliplex).

Human data suggests that CD226 is tightly associated with memory and effector T cell responses. Thus, NOD mice, when compared to NOR and C57BL/6, can exhibit a progressive increase in CD226 relative to TIGIT within the islets and in the pDLNs. Given that TIGIT^{-/-} mice exhibit exacerbated Experimental autoimmune encephalomyelitis (EAE), the NOD background can exhibit a higher incidence and a potentially more rapid progression of T1D. Given the costimulatory nature of CD226, CD226 deficient mice may have attenuated disease; however, the opposite was observed in CD28 deficient mice, suggesting costimulation may also be necessary for the maintenance of Treg activity. While the use of gene targeting in the NOD represents the most rapid means to assess these questions, it lacks the cell specificity normally afforded by conditional knockout systems (e.g., FoxP3-Cre). Hence, CD226 or TIGIT-LoxP sites for lineage specific deletion can be produced.

PBMC cross-sectional studies of CD226 and TIGIT

Studies can be conducted with cross-sectional samples with cohorts comprising normal healthy controls, at risk relatives (single and multiple autoantibody positive), new onset subjects (defined as ≤ 3 months post diagnosis), established T1D (> 3 months), and subjects with T2D (to address questions of immune versus metabolic effects). Analysis of clinical samples from fresh PB and cryopreserved PBMC can be performed.

nPOD *in situ* analysis of CD226 and TIGIT

In addition to studies in PB, samples from a unique and innovative resource (*i.e.* nPOD) that obtains cells and tissues of pancreatic or lymphoid origin (spleen, LN – both pancreatic and peripheral, PB) from groups with or at various risks for T1D can be studied. This can permit directly addressing pertinent and long-standing questions in human T1D as to whether PBMC reflect activities at the pancreatic LN and insulinitis lesion. The pancreata, spleens, pancreatic LN, peripheral LN, anticoagulated whole blood and serum can be collected, processed, and stored. Samples from individuals with T1D, T2D, and non-autoimmune controls (from 0 to 80 yr) can be collected. In addition, tissues from “pre-diabetic” individuals through screening organ donors to identify those with one or more autoantibodies can be obtained. Post-acquisition analyses can include high resolution HLA typing, ImmunoChip (SNP) analysis, C-peptide analysis, as well as standard H&E histology and immunohistochemistry.

IFN γ producing Treg are enriched in PB of patients with T1D

Defective suppression by Treg and/or Teff cell resistance is observed in patients with T1D. An increase of IFN γ ⁺ Treg that were Helios⁻, significantly reduced in suppressive activity, and partially methylated at the TSDR (Figure 16). IFN γ ⁺ or IFN γ ⁻ Treg and Tconv subsets were isolated for microarray profiles. Notably, both freshly isolated and 14d expanded IFN γ ⁺ Treg expressed significantly more CD226 than the IFN γ ⁻ Treg subset (Figure 17A), and TIGIT expression was higher in the IFN γ ⁻ Treg compared to IFN γ ⁺ Treg (Figure 17B). Principal component analysis (PCA) of these populations indicated clear divergence between Treg and Tconv populations, with further variance in IFN γ ⁺ and IFN γ ⁻ Treg (Figure 17C).

10 Isolation of immune subsets from nPOD material

A four-way sort (FACS Aria III) is performed to isolate snap frozen cell pellets and live cell aliquots of B cells, Tconv, Treg, and CD8⁺ T cells. Figure 19 illustrates feasibility of isolating live viable cells and their subsets from an nPOD donor.

15 Characterization of the islet and pDLN resident expression of CD226 and TIGIT

Tissues obtained from nPOD (pDLN, irrelevant DLN, spleen, PB) can all be collected immediately and processed into single cell suspensions (Miltenyi Gentle MACS Dissociator). Samples can then be stained in parallel for surface and intracellular flow cytometry or FACS isolation. Pancreatic immunohistochemistry (antibody) or *in situ* hybridization (ISH) can be used, as necessary, to delineate the cells compromising the T1D lesion.

The following tests can be performed:

Immunohistochemistry - Pancreatic tissue sections can be stained for CD4, CD226, TIGIT, and FOXP3. Fluorescent images can be scanned (Zeiss LSM510 Meta confocal microscope/Aperio Scanscope) and data can be analyzed and quantitative measures assessed using Zeiss LSM510, Metamorph, or Aperio software.

Transcriptional Analysis - Tissue samples, and the potential for laser capture of islets or infiltrates, can be analyzed with a Nanostring nCounter as noted for fixed tissues (Figure 1).

The milieu at the draining lymph node and islet of patients with T1D may be enriched in autoreactive T cells expressing high levels of CD226. A relative reduction in the negative regulatory TIGIT may be observed. Moreover, activated lymphocytes within the lesion and draining lymph node may be observed in higher proportion than PB and also in higher proportion of those with T1D or multiple autoantibodies compared with control lymph node tissue.

EXAMPLE 11 - Testing the therapeutic potential of modulating CD226 and TIGIT activity

One embodiment of the invention provides therapeutics that block CD226 costimulation, or alternatively augment the regulatory profile of Treg through bolstering TIGIT to avert autoimmunity.

Implications for Treg cell therapy - CD226 and TIGIT identify functionally distinct subpopulations of Treg.

Treg may lead to the induction of long-term tolerance and preservation of endogenous or transplanted β -cell mass. Current protocols to generate human Treg are susceptible to contamination by non-Treg and the potential for lineage instability following *in vitro* culture (31, 69-71). This embodiment of the invention provides FACS-isolated Treg ($CD4^+CD25^{hi}CD127^-$ T cells) that are subdivided into quadrants based on CD226 and TIGIT expression (Figure 20). TIGIT⁺ Treg constitute the majority of the initial population but are highly refractory to expansion. CD226⁺TIGIT⁻ Treg adopt a transcriptional program (Nanostring) similar to Tconv cells, are reduced in suppressive capacity, produce IFN γ , and are methylated at the FOXP3-TSDR. CD226⁻ Treg, irrespective of initial TIGIT expression, upregulate TIGIT post-expansion and are highly suppressive and pure. Elimination of CD226-expressing Treg alone could improve Treg purity post-expansion (Figure 21). While Treg were largely suppressive, elimination of CD226 improved the suppressive capacity of the final product, indicating this ratio may emerge as an important surrogate measure of Treg suppressive activity.

Identification of drugs, tolerogenic cytokines, and modified Treg cell therapies to bolster TIGIT and Treg cell activity

TIGIT expression may augment the suppressive capacity of Treg, while simultaneously limiting Teff cell activity. Various immunomodulatory drugs and tolerogenic cytokines can augment TIGIT expression. Drugs and cytokines known to increase Treg may also upregulate TIGIT (e.g., rapamycin, All-trans Retinoic Acid (ATRA), IL-10, TGF β , low-dose IL-2, PI3K inhibitors). Additional agents can be screened and identified which modulate TIGIT activity and/or expression in Treg.

Determining if antibodies, Somamers, and Ig-fusion proteins can modulate Treg and Tconv cell activity *in vitro*

The identification of CD226 and TIGIT has generated a new axis susceptible to immune modulation for cancer, immunizations, and autoimmunity. CD226 and TIGIT can be targeted through the use of activating and neutralizing antibodies, CD112 and CD155-Ig fusion proteins, and CD226 and TIGIT somamer reagents to impact Treg and Tconv cell activity.

CD226 expression clearly demarcates a population of human Treg that are unstable and produce effector cytokines, including IFN γ . Isolation of CD226⁺ Treg will increase Treg suppressive activity and stability following *in vitro* expansion. This is important during acute activation, as CD127 is downregulated following activation, whereas, CD226 is upregulated. Therefore, addition of CD226 may increase the ability to discriminate bona-fide Treg from Teff populations during periods of ongoing T cell activation.

CD226 and TIGIT inhibitors can be screened in a high-throughput binding assay with CD155 expressing cell lines (K562) and fluorescently-labeled CD226 and TIGIT Ig fusion proteins. These studies can then be validated during *in vitro* suppression assays. In terms of CD226 and TIGIT inhibitors, the targeted inhibitors and agonists can recapitulate the results from LV overexpression and knockdown experiments and gene targeted mouse models. Larger drug libraries (e.g., those available through the NIH – National Cancer Institute) can also be screened to identify molecules modulating CD226 or TIGIT activity and/or expression.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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CLAIMS

We claim:

1. A method for isolating a regulatory T cell (Treg) from a subject, the method comprising the steps of:

a) analyzing a sample of cells obtained from the subject to determine the level of expression of polypeptides and/or polynucleotides corresponding to proteins CD4, CD25, CD226, and optionally, CD127, and

b) isolating, as the Treg, a cell which:

i) expresses CD4 and CD25, and

a) expresses low levels of CD127 or does not express CD127, and/or

b) expresses low levels of CD226 or does not express CD226.

2. The method of claim 1, wherein the method comprises analyzing the level of expression proteins CD127, CD4, CD25, and CD226.

3. The method of claim 2, wherein the step of isolating comprises isolating the cell which:

i) expresses CD4 and CD25,

ii) expresses low levels of CD127 or does not express CD127, and

iii) expresses low levels of CD226 or does not express CD226.

4. The method of claim 2, wherein the step of isolating comprises isolating the cell which:

i) expresses CD4 and CD25,

ii) expresses low levels of CD127 or does not express CD127, and

iii) does not express CD226.

5. The method of claim 2, wherein the step of analyzing the level of expression proteins CD127, CD4, CD25, and CD226 is performed using flow cytometry.

6. The method according to claim 5, wherein the step of analyzing the level of expression proteins comprises the steps of:

(a) obtaining the sample of cells from the subject;

- (b) contacting the sample of cells with antibodies directed towards CD127, CD4, CD25, and CD226 to allow binding of the antibodies to the corresponding proteins;
- (c) subjecting the sample of cells to flow cytometry;
- (d) examining the flow cytometry signal for the expression of CD127, CD4, CD25, and CD226, and
- (e) isolating, as the Treg, the cell which:
 - i) expresses CD4 and CD25,
 - ii) expresses low levels of CD127 or does not express CD127, and
 - iii) expresses low levels of CD226 or does not express CD226.

7. The method of claim 1, wherein the method comprises analyzing the level of expression proteins CD4, CD25, and CD226.

8. The method of claim 7, wherein the step of isolating comprises isolating the cell which:

- i) expresses CD4 and CD25, and
- iii) expresses low levels of CD226 or does not express CD226.

9. The method of claim 2, wherein the step of analyzing the level of expression proteins CD4, CD25, and CD226 is performed using flow cytometry.

10. The method according to claim 7, wherein the step of analyzing the level of expression proteins comprises the steps of:

- (a) obtaining the sample of cells from the subject;
- (b) contacting the sample of cells with antibodies directed towards CD4, CD25, and CD226 to allow binding of the antibodies to the corresponding proteins;
- (c) subjecting the sample of cells to flow cytometry;
- (d) examining the flow cytometry signal for the expression of CD4, CD25, and CD226, and
- (e) isolating, as the Treg, the cell which:
 - i) expresses CD4 and CD25, and
 - iii) expresses low levels of CD226 or does not express CD226.

11. The method of claim 1, the method further comprises proliferating the Treg *in vitro*.

12. The method according to claim 1, wherein the sample of cells is obtained from a biological sample selected from a cell line, a bodily fluid, or a tissue.

13. The method according to claim 8, wherein the bodily fluid comprises blood or lymph.

14. The method according to claim 8, wherein the tissue is thymus, lymph node, spleen, tonsil.

15. A Treg isolated from a sample of cells obtained from a subject, wherein the Treg expresses CD4 and CD25, expresses low levels of CD127 or does not express CD127, and expresses low levels of CD226 or does not express CD226.

16. The Treg of claim 11, wherein the Treg express high levels of CD4 and CD25.

17. The Treg of claim 11, wherein the cell does not express CD226.

18. A cultured Treg obtained from proliferating the Treg of claim 11 *in vitro*.

19. A composition comprising the Treg of claim 11, the cultured Treg of claim 14, or the combination thereof and a pharmaceutically acceptable carrier and/or excipient.

20. A Treg isolated from a sample of cells obtained from a subject, wherein the Treg expresses CD4 and CD25, and expresses low levels of CD226 or does not express CD226.

21. The Treg of claim 11, wherein the Treg express high levels of CD4 and CD25.

22. The Treg of claim 11, wherein the cell does not express CD226.

23. A cultured Treg obtained from proliferating the Treg of claim 20 *in vitro*.

24. A composition comprising the Treg of claim 20, the cultured Treg of claim 23, or the combination thereof and a pharmaceutically acceptable carrier and/or excipient.

25. A method of treating and/or preventing an inflammation mediated disease in a patient, the method comprising, administering to the patient the composition of claim 19 or 24.

26. The method of claim 25, wherein the composition is administered to the subject via local or systemic route.

27. The method of claim 26, wherein the systemic route is selected from intravenous, intraperitoneal, intramuscular, or subcutaneous.

28. The method of claim 27, wherein the inflammation mediated disease is an autoimmune disease.

29. The method of claim 28, wherein the autoimmune disease is systemic lupus erythematosus, rheumatoid arthritis, Multiple Sclerosis, type 1 diabetes, or inflammatory bowel disease.

30. A method of treating and/or preventing an inflammation mediated disease in a subject, the method comprising the steps of:

a) isolating a Treg from a sample of cells obtained from the subject, wherein the Treg expresses CD4 and CD25, expresses low levels of CD127 or does not express CD127, and expresses low levels of CD226 or does not express CD226,

b) optionally, proliferating the Treg isolated from the subject by culturing the Treg *in vitro*, and

c) administering to the subject, the Treg obtained from the subject and/or the Treg proliferated from the Treg isolated from the subject.

31. The method of claim 30, wherein the Treg isolated from the subject and/or the proliferated Treg is administered to the subject via local or systemic route.

32. The method of claim 31, wherein the systemic route is selected from intravenous, intraperitoneal, intramuscular, or subcutaneous.

33. The method of claim 30, wherein the inflammation mediated disease is an autoimmune disease.

34. The method of claim 33, wherein the autoimmune disease is systemic lupus erythmatosus, rheumatoid arthritis, Multiple Sclerosis, type 1 diabetes, or inflammatory bowel disease.

35. A method of treating and/or preventing an inflammation mediated disease in a subject, the method comprising the steps of:

- a) isolating a Treg from a sample of cells obtained from the subject, wherein the Treg expresses CD4 and CD25, and expresses low levels of CD226 or does not express CD226,
- b) optionally, proliferating the Treg isolated from the subject by culturing the Treg *in vitro*, and
- c) administering to the subject, the Treg obtained from the subject and/or the Treg proliferated from the Treg isolated from the subject.

36. The method of claim 35, wherein the Treg isolated from the subject and/or the proliferated Treg is administered to the subject via local or systemic route.

37. The method of claim 36, wherein the systemic route is selected from intravenous, intraperitoneal, intramuscular, or subcutaneous.

38. The method of claim 35, wherein the inflammation mediated disease is an autoimmune disease.

39. The method of claim 38, wherein the autoimmune disease is systemic lupus erythmatosus, rheumatoid arthritis, Multiple Sclerosis, type 1 diabetes, or inflammatory bowel disease.

40. An antagonist or agonist against CD226 or TIGIT

41. A method of treating cancer or an autoimmune disease wherein said method comprises administering, to a subject in need of such treatment, an antagonist or agonist of claim 40, wherein the antagonist or agonist is a small molecule or a biologic.

42. The method of claim 41, wherein the biologic is an antibody, fragment of an antibody, aptamer, fusion protein or biomolecule capable of specifically binding to CD226 or TIGIT.

43. The method of claim 42, wherein the antibody is a polyclonal antibody, monoclonal antibody, recombinant antibody or hybrid antibody.

44. The method of claim 42, wherein the fusion protein comprises a binding fragment of an antibody that specifically binds to CD226 or TIGIT, and an Fc portion of IgG.

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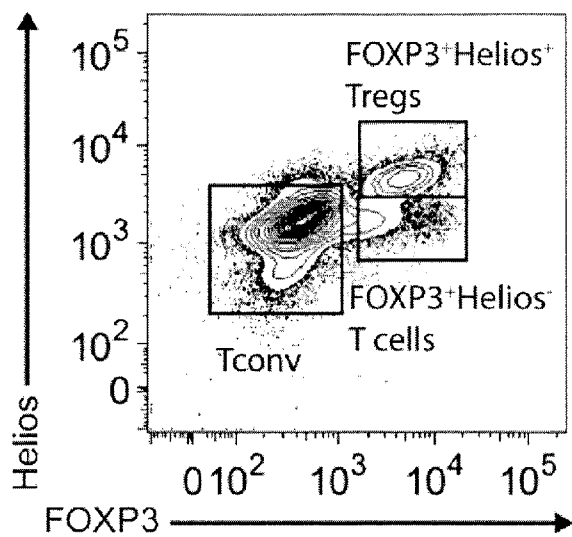


FIG. 1A

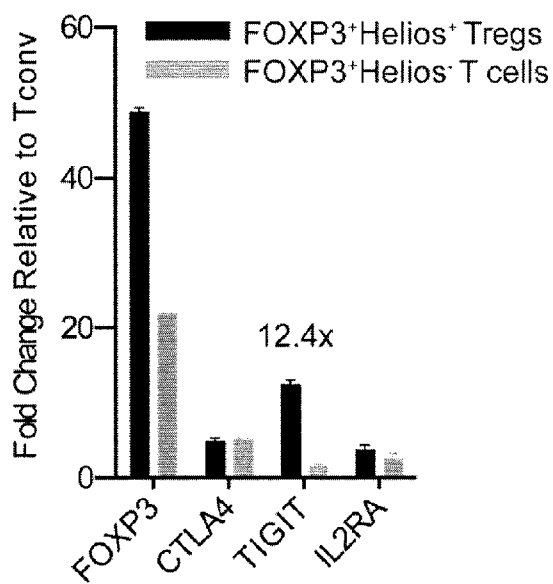


FIG. 1B

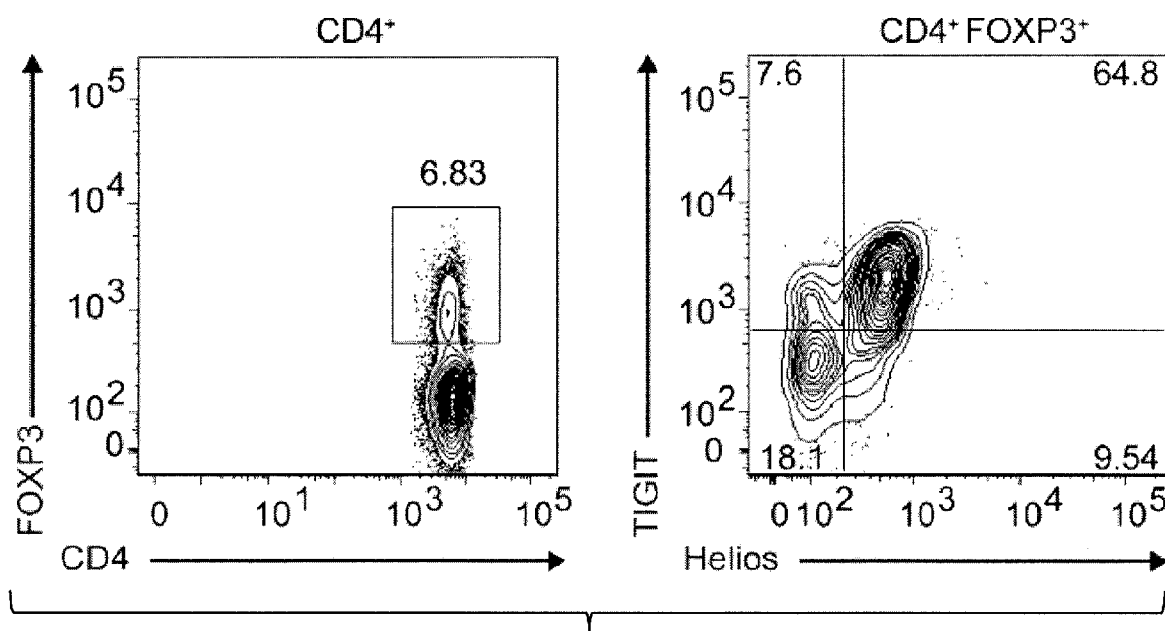


FIG. 1C

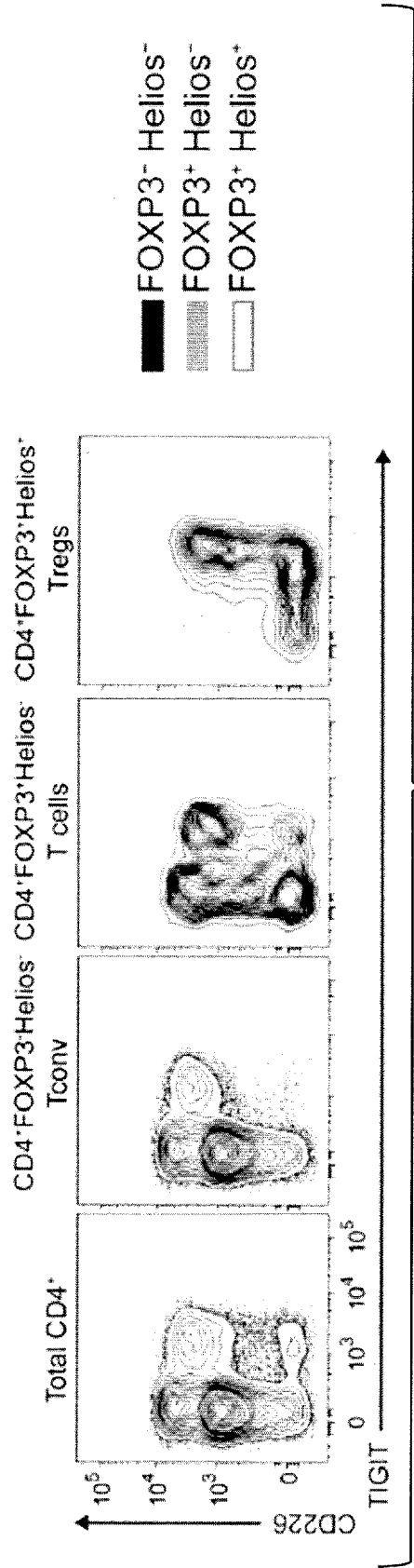


FIG. 2A

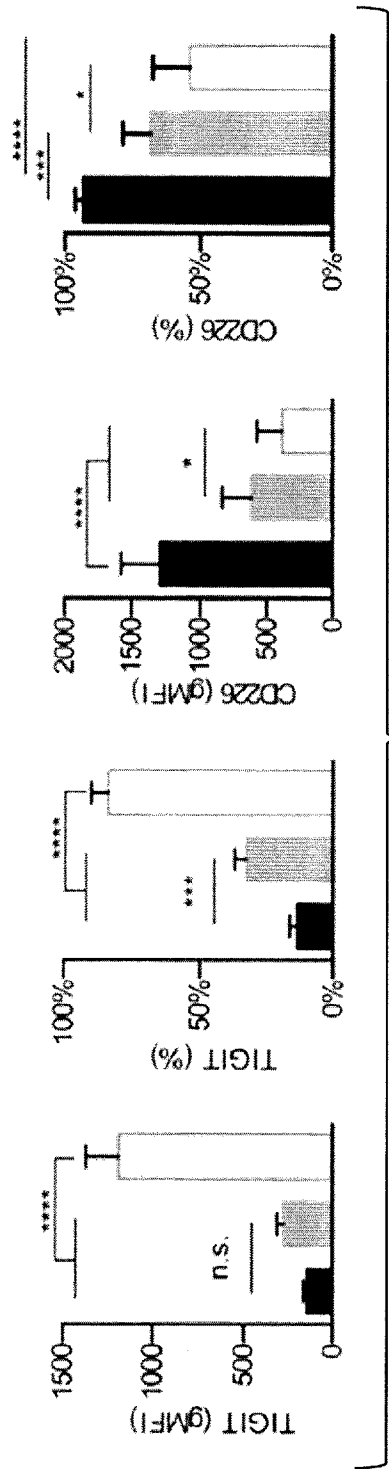


FIG. 2B

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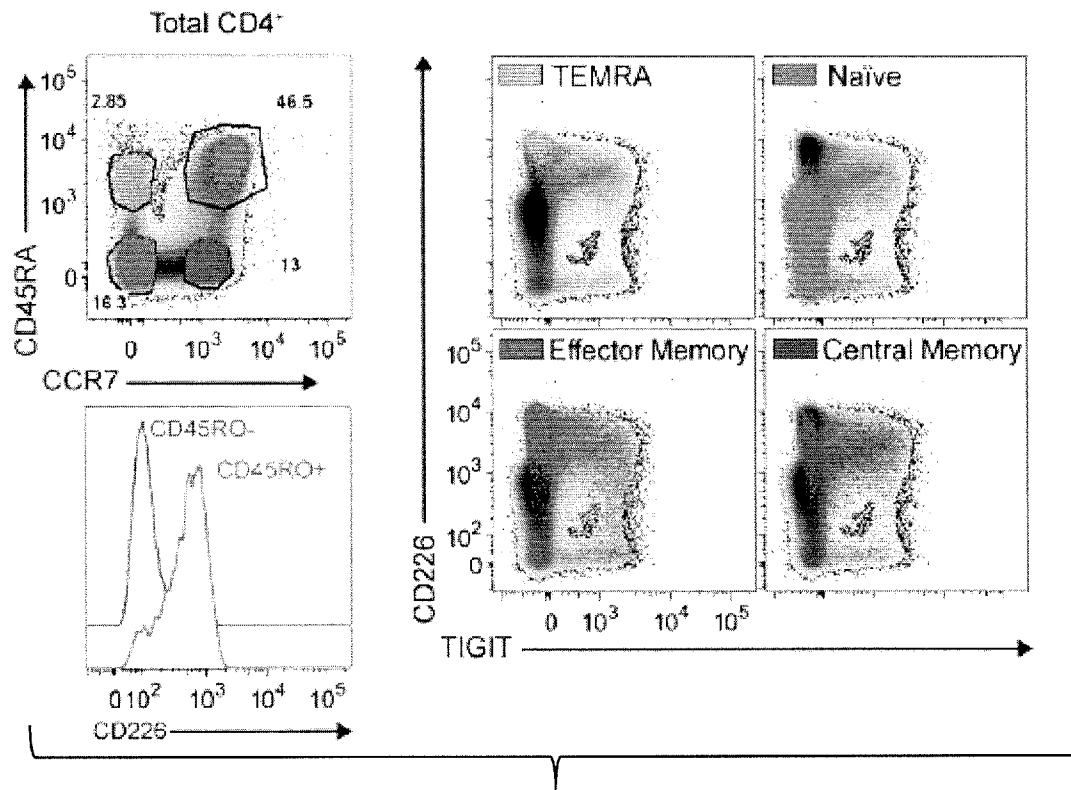


FIG. 3A

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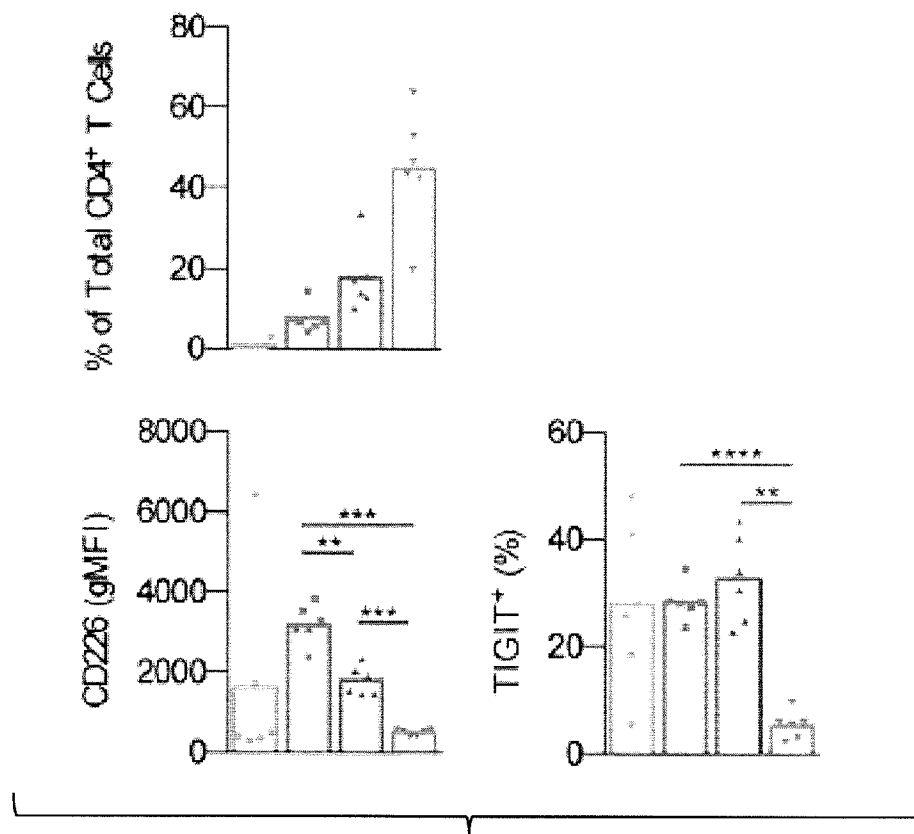


FIG. 3B

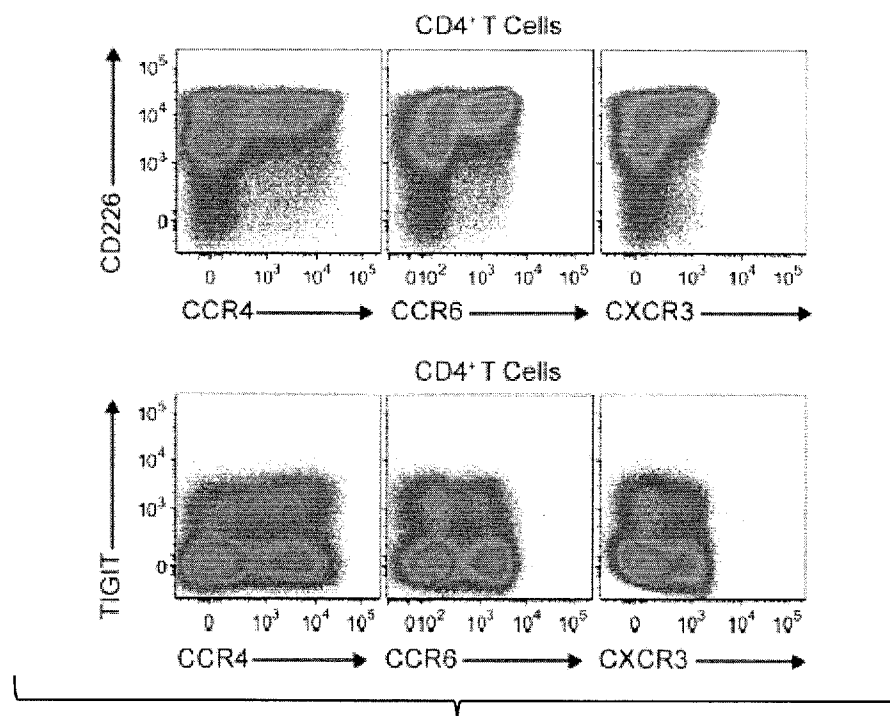


FIG. 3C

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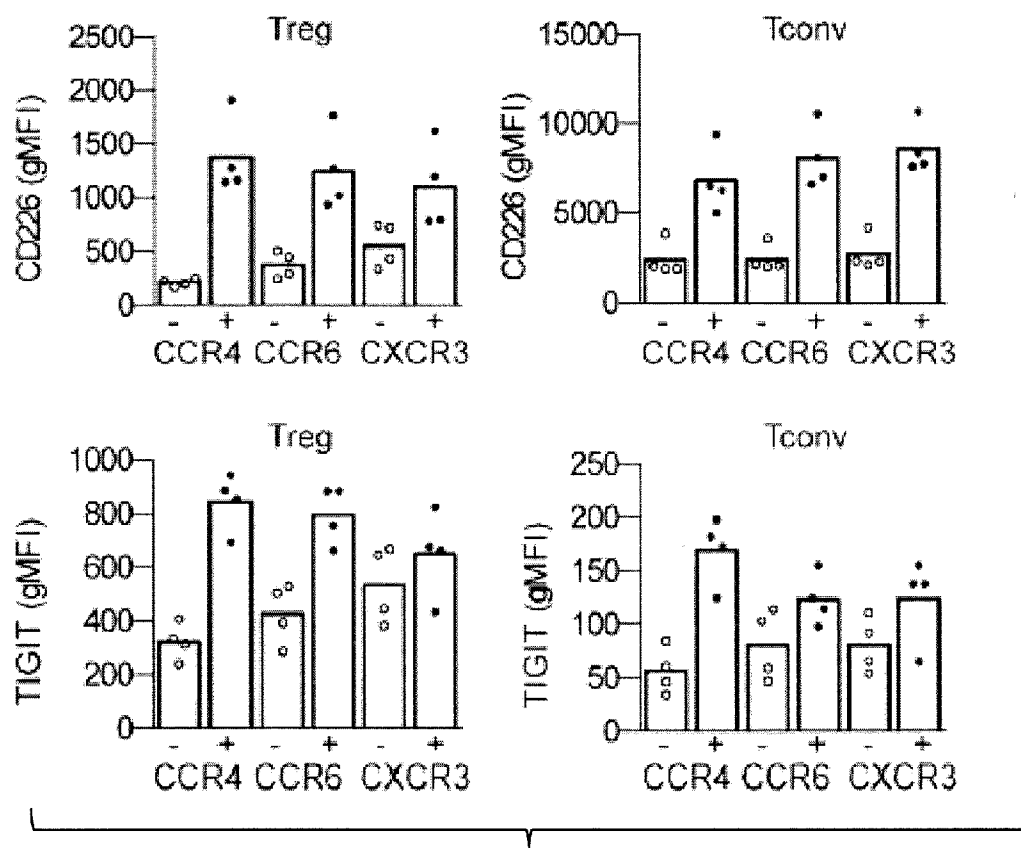


FIG. 3D

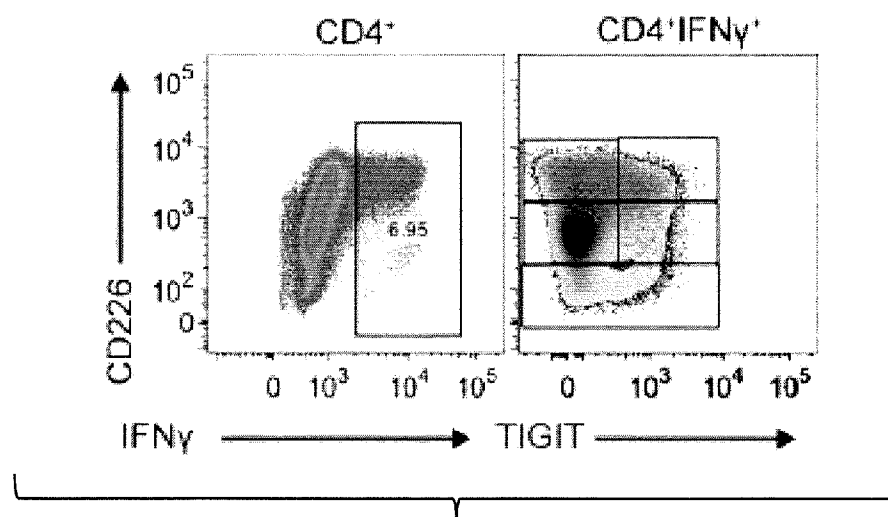


FIG. 3E

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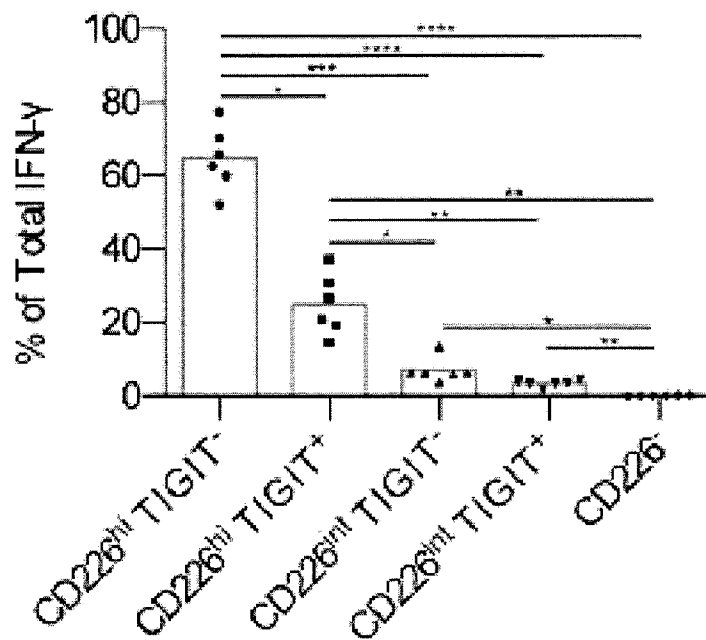


FIG. 3F

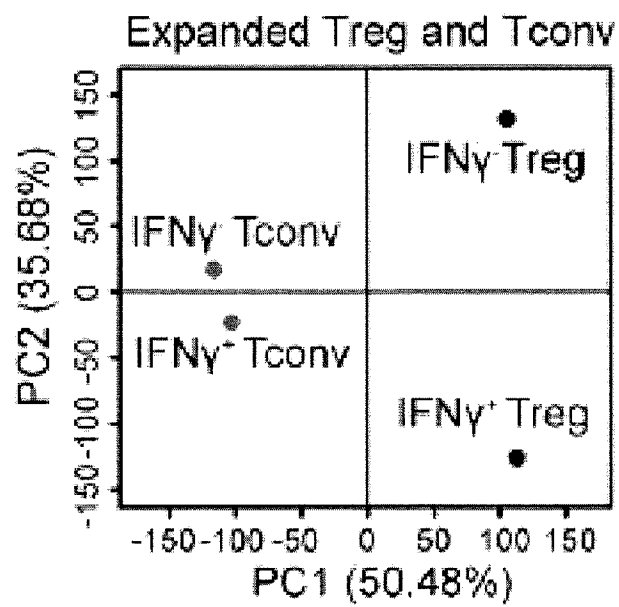


FIG. 3G

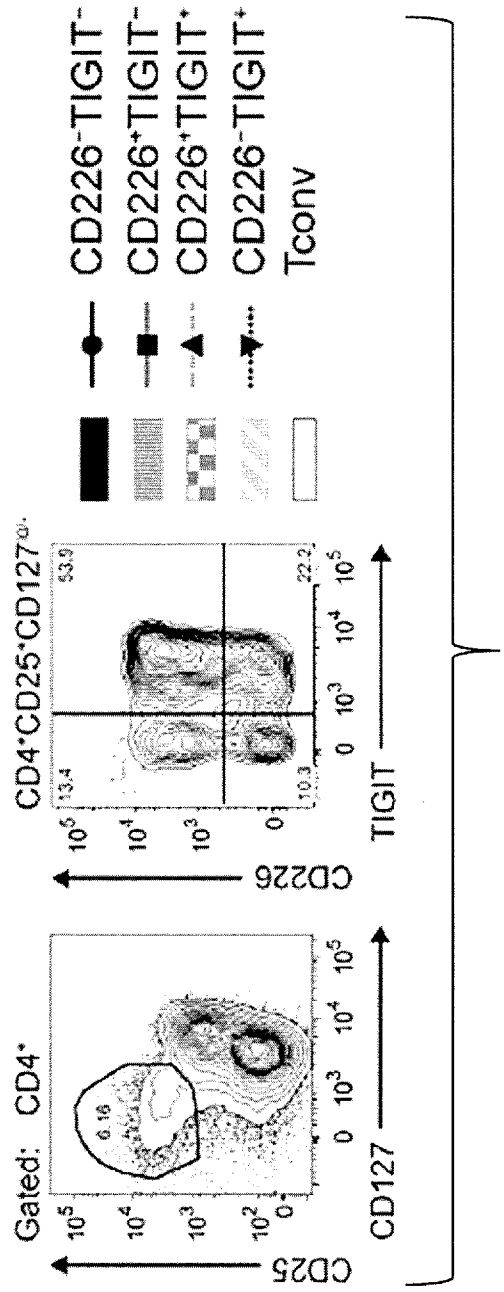


FIG. 4A

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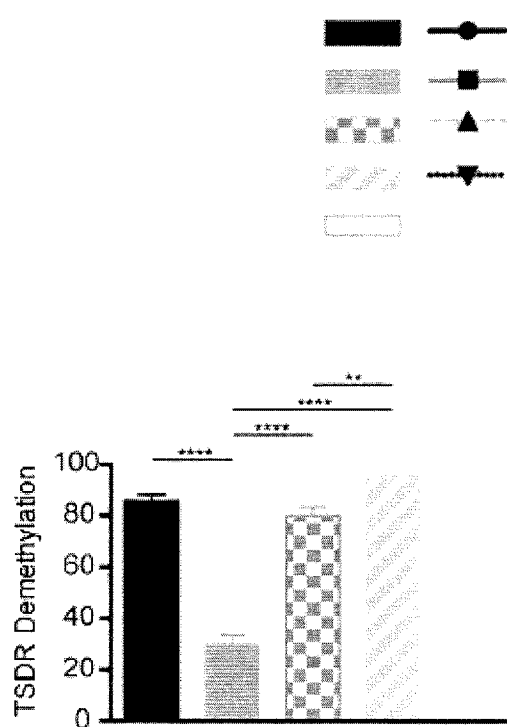


FIG. 4B

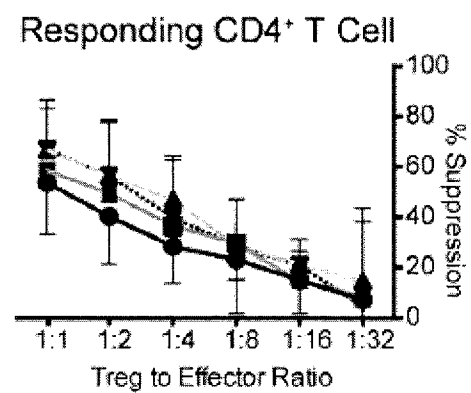


FIG. 4C

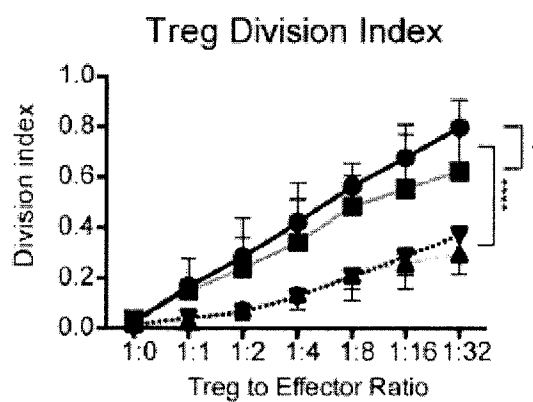


FIG. 4D

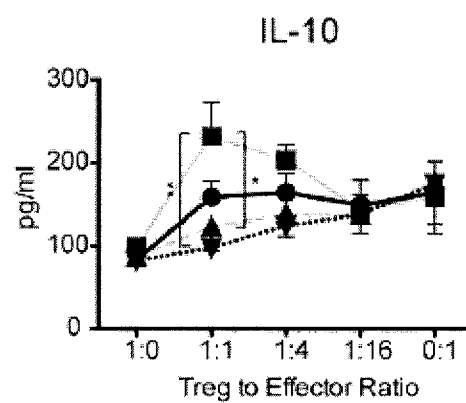


FIG. 4E

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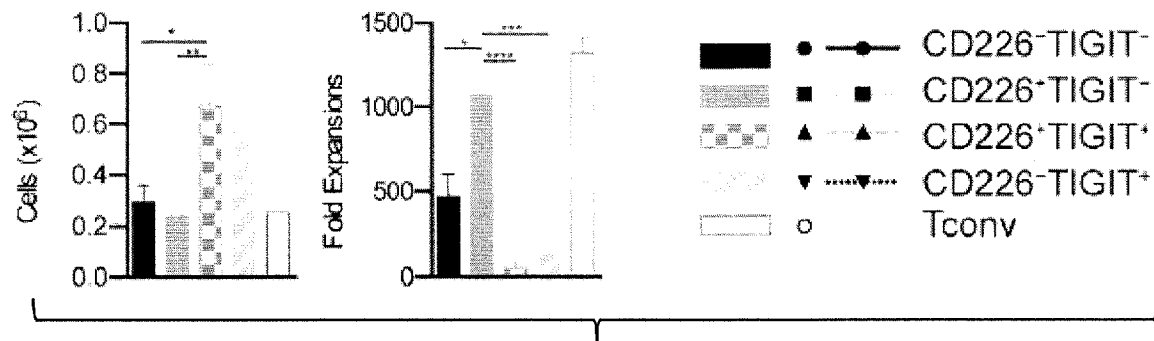


FIG. 5A

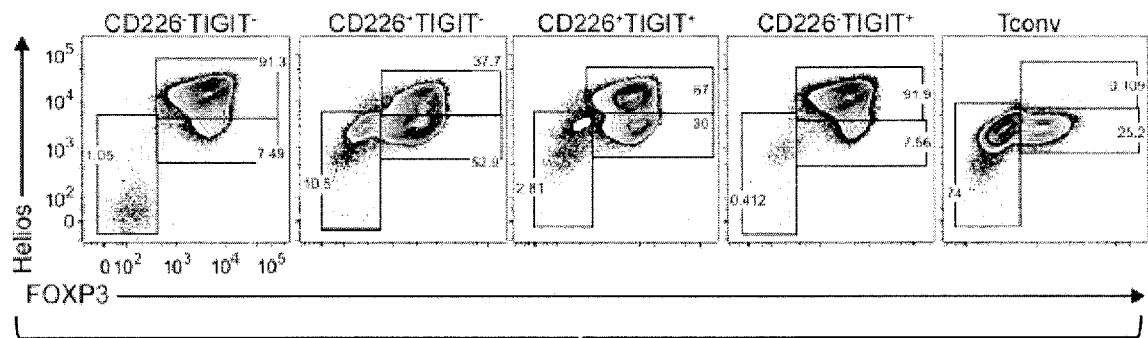


FIG. 5B

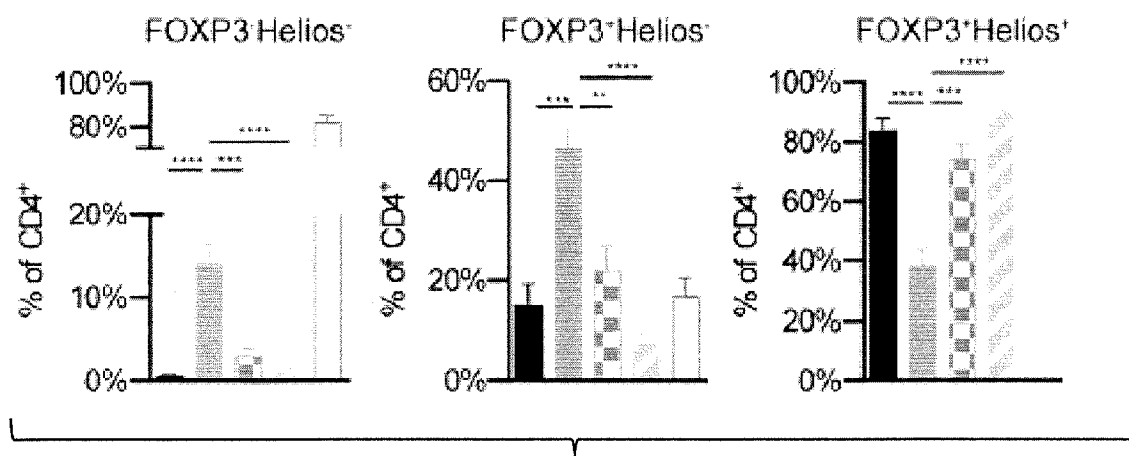


FIG. 5C

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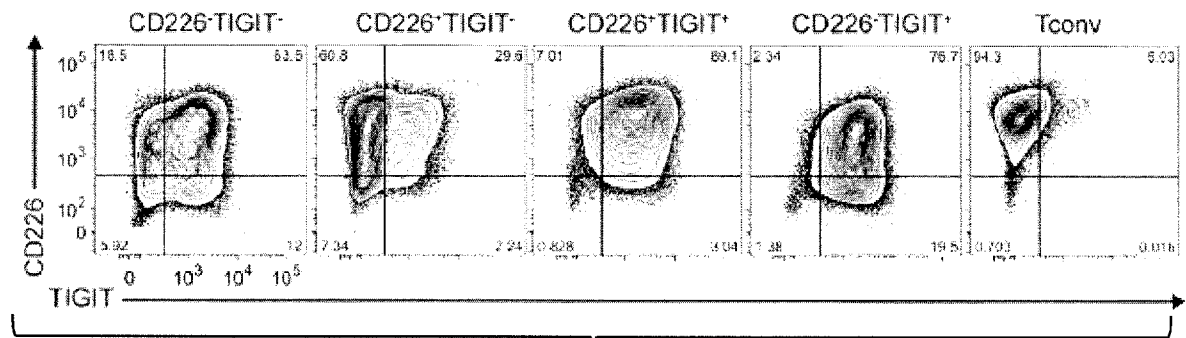


FIG. 5D

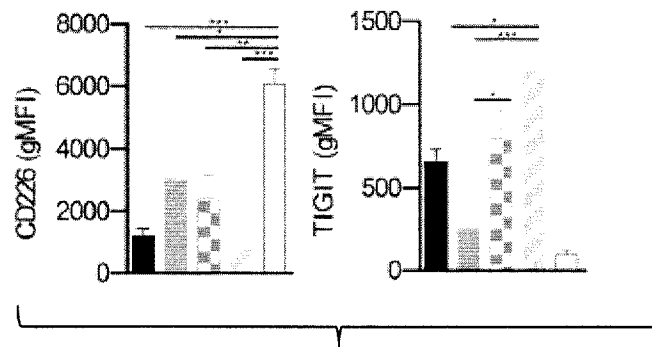


FIG. 5E

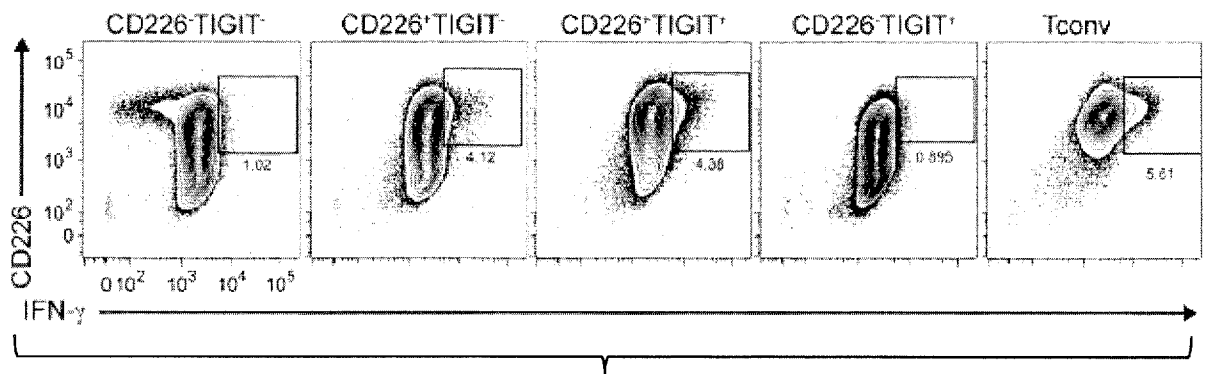


FIG. 5F

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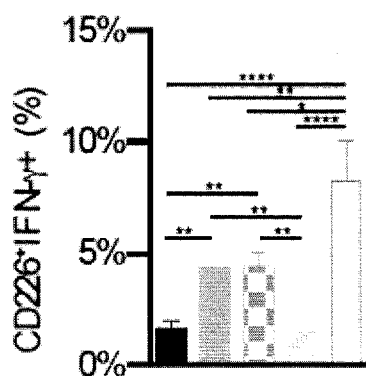


FIG. 5G

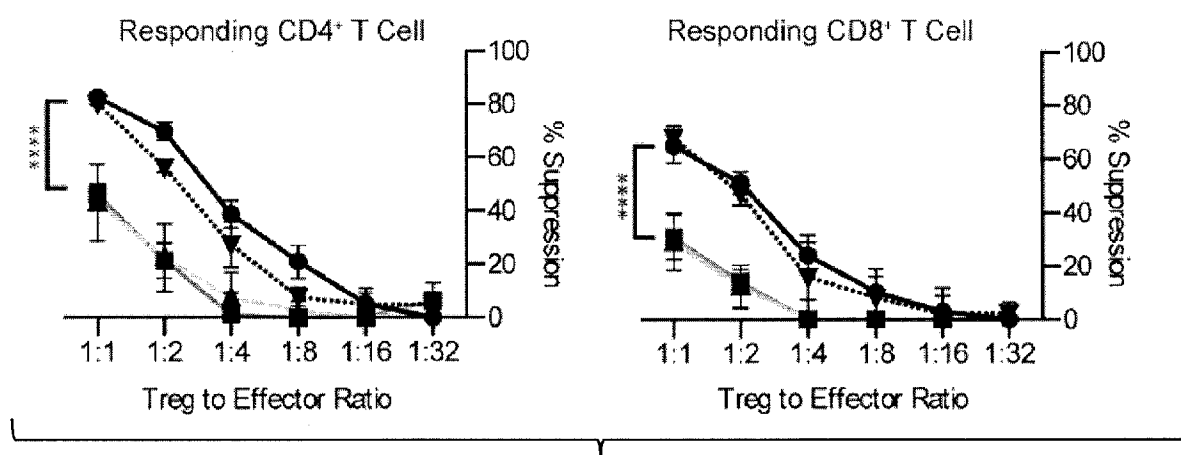


FIG. 5H

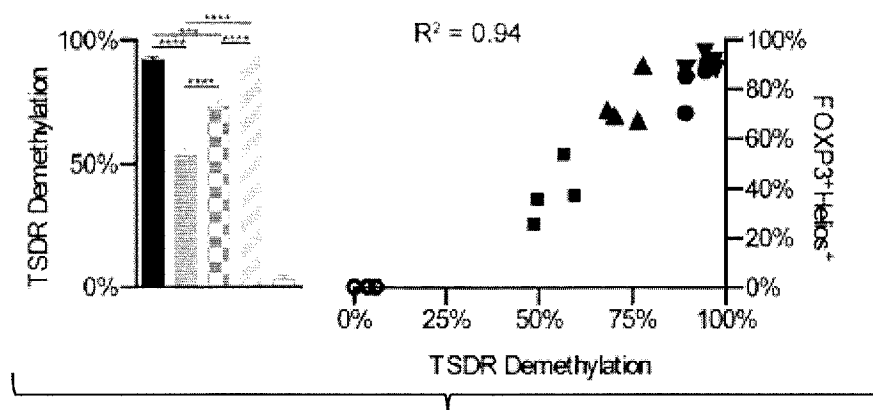


FIG. 5I

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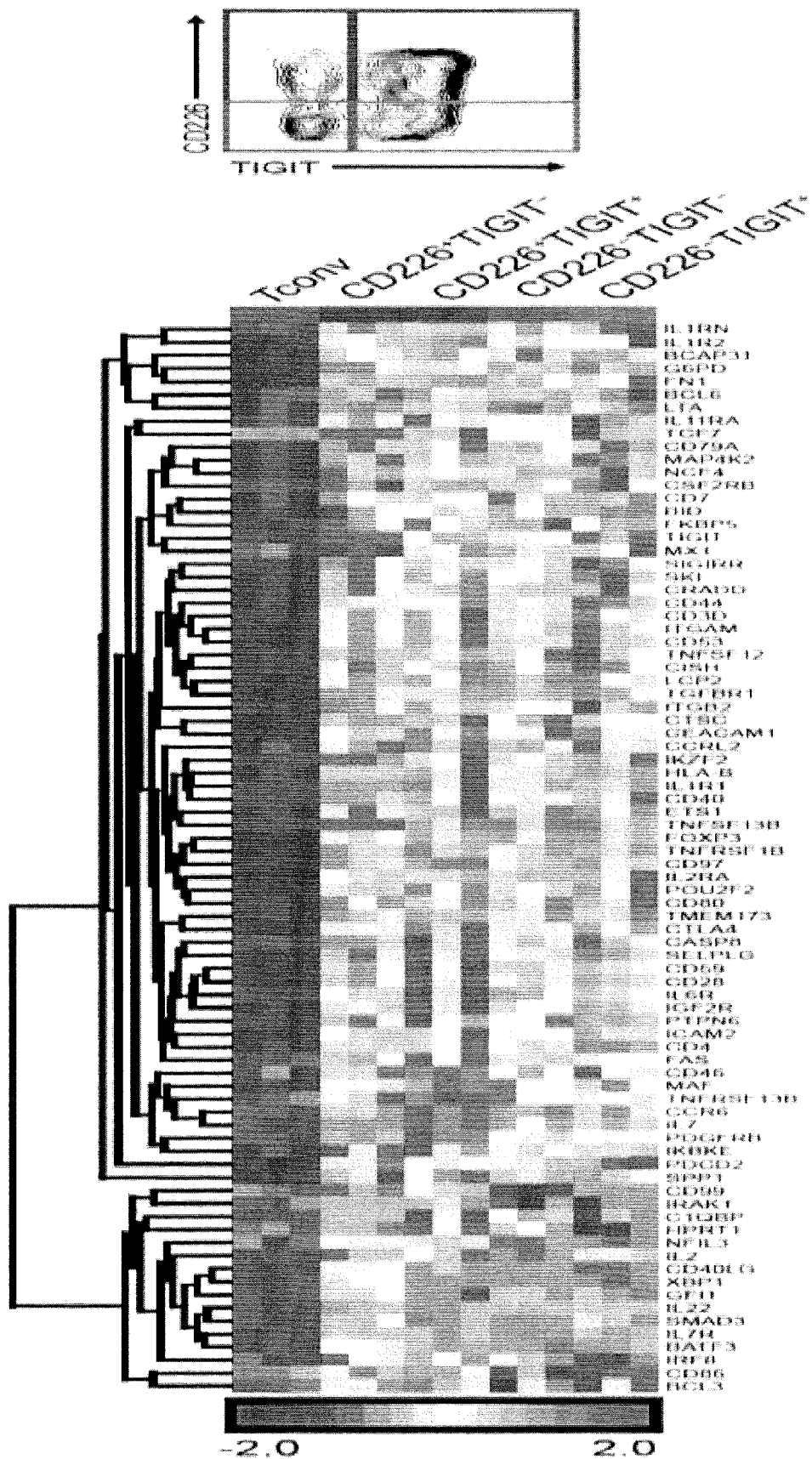


FIG. 6A

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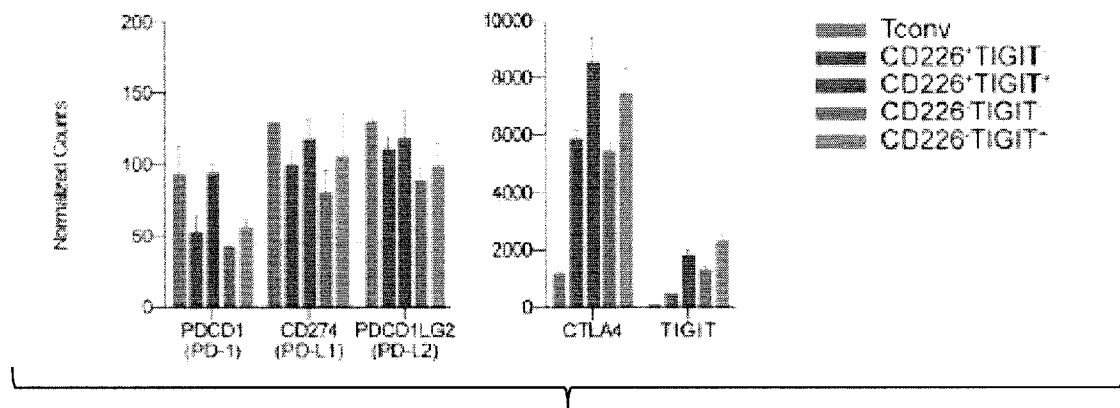


FIG. 6B

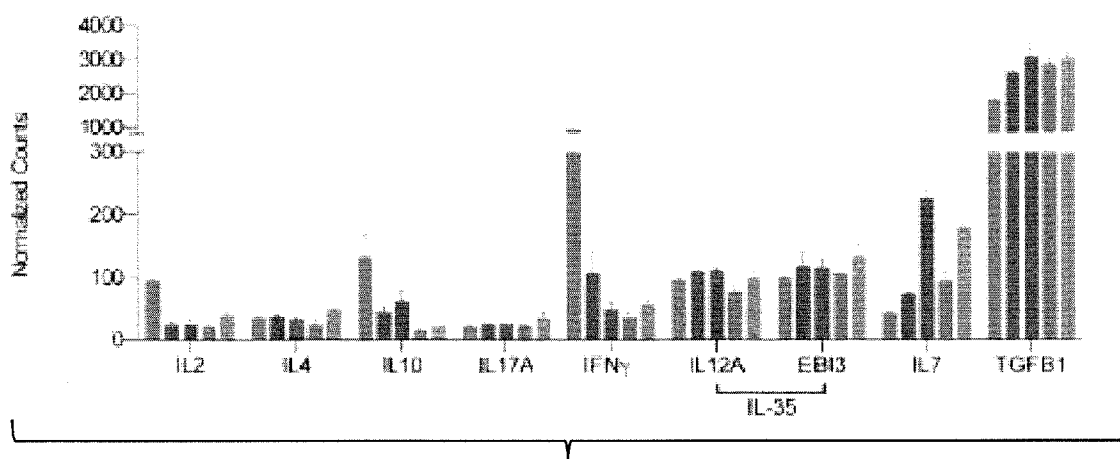


FIG. 6C

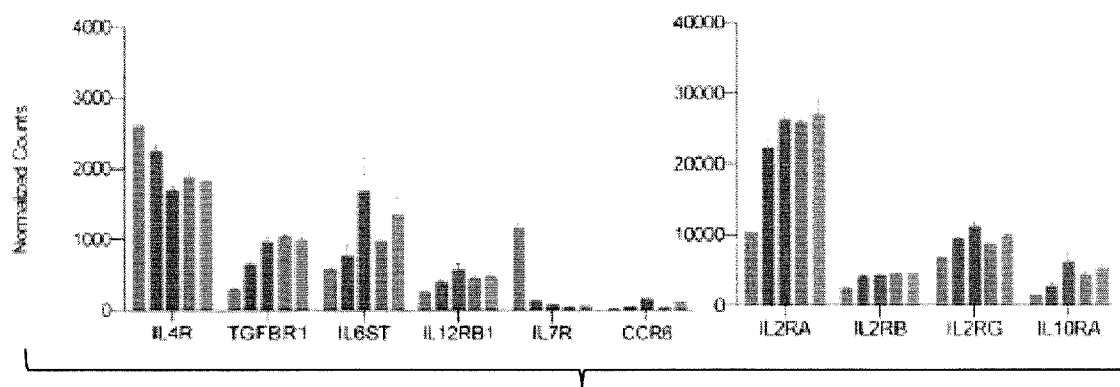


FIG. 6D

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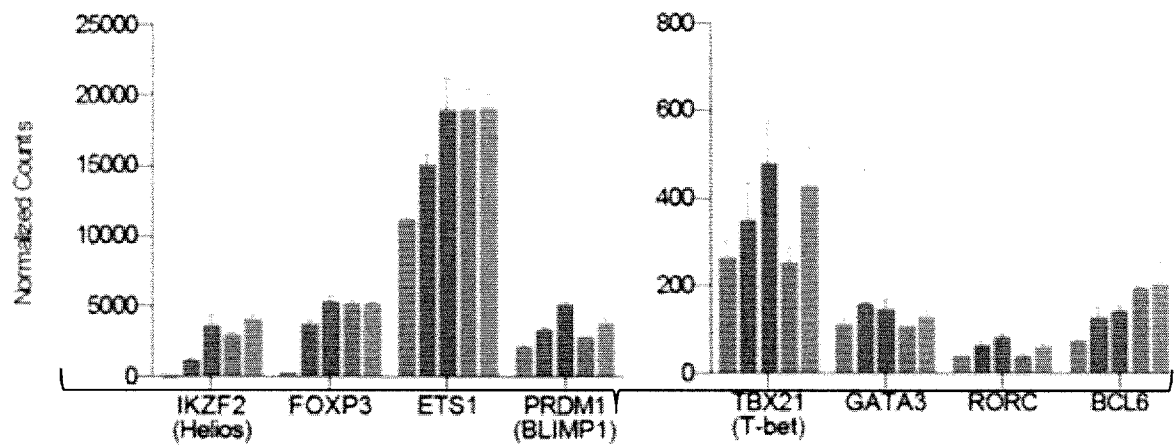


FIG. 6E

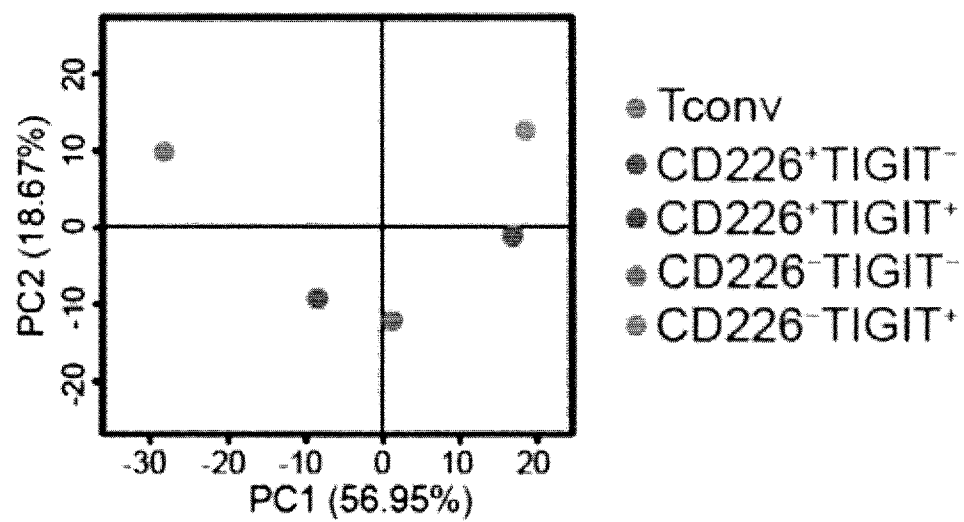


FIG. 6F

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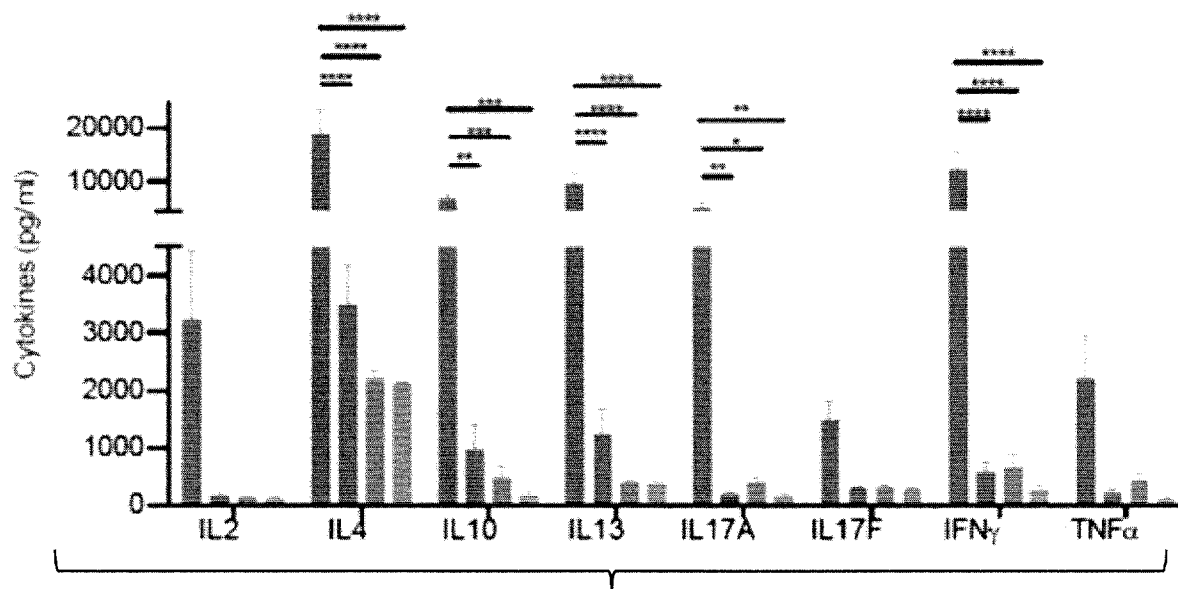


FIG. 6G

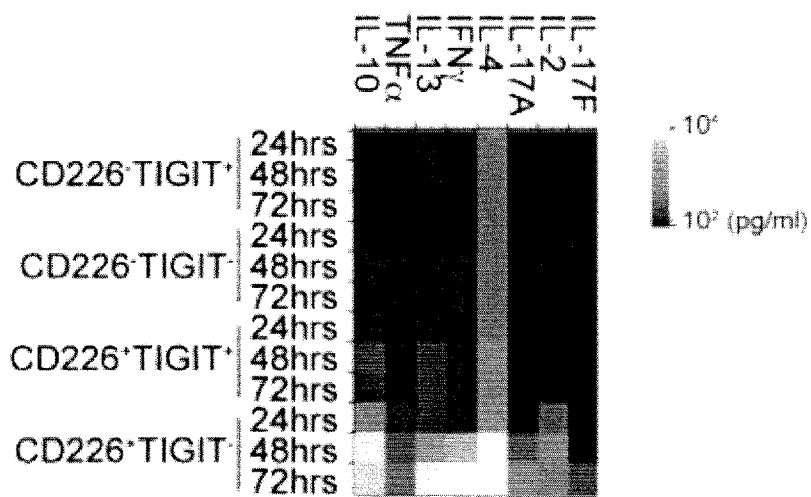


FIG. 6H

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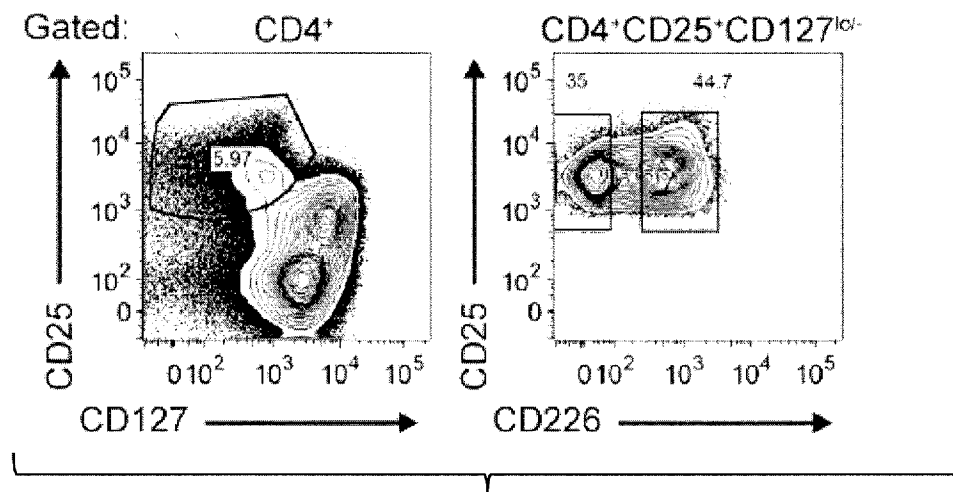


FIG. 7A

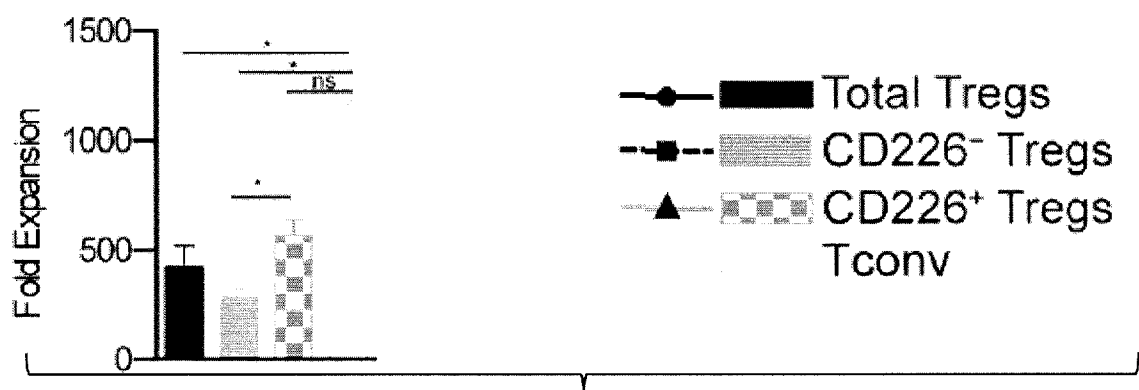


FIG. 7B

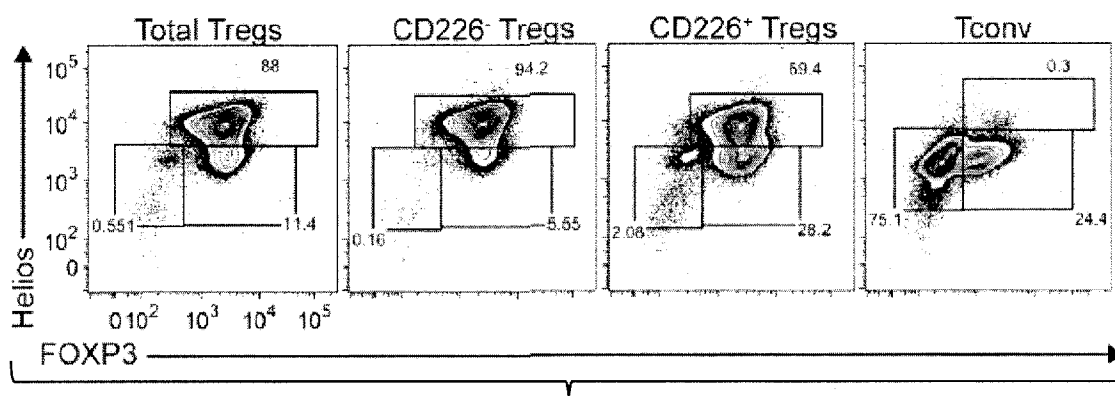


FIG. 7C

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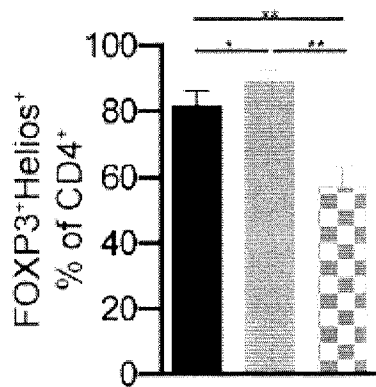


FIG. 7D

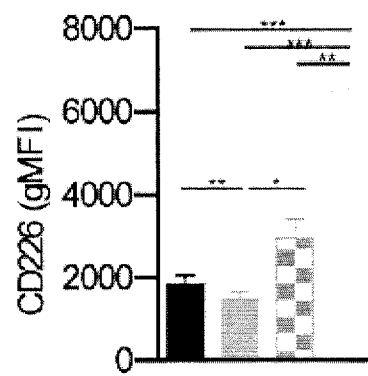


FIG. 7E

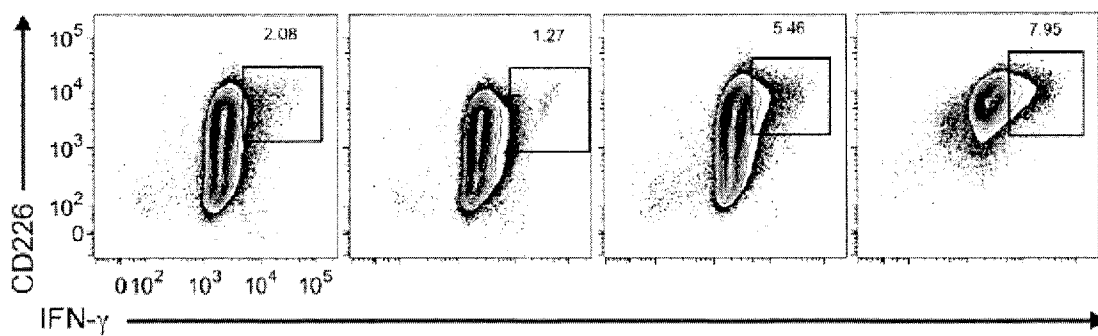


FIG. 7F

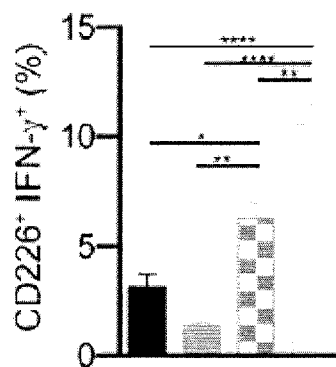


FIG. 7G

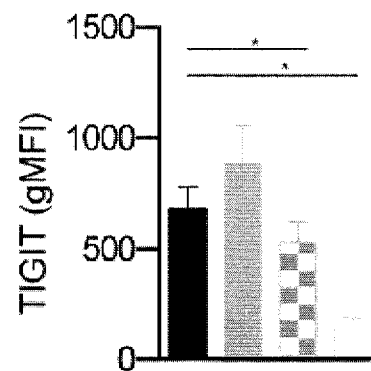


FIG. 7H

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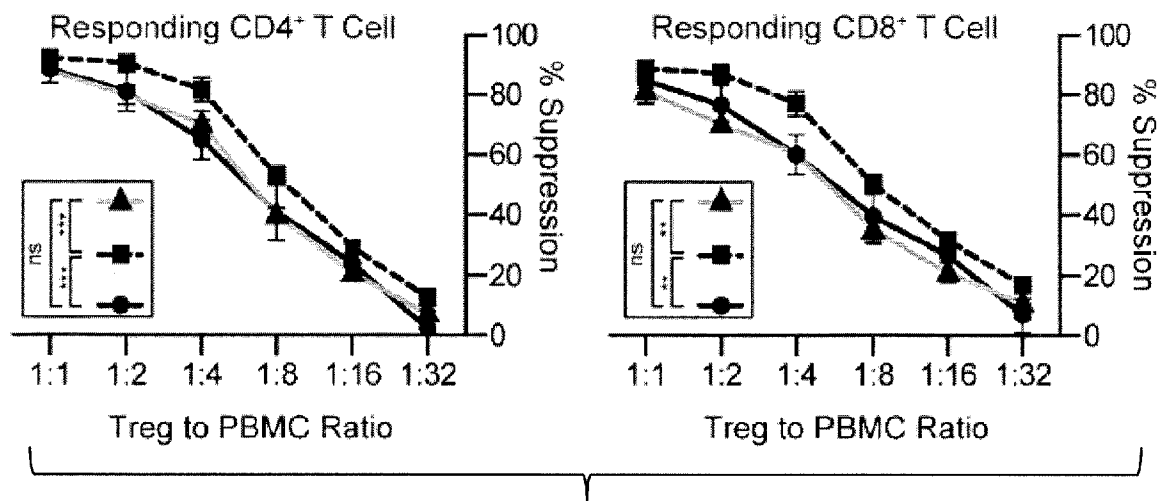


FIG. 7I

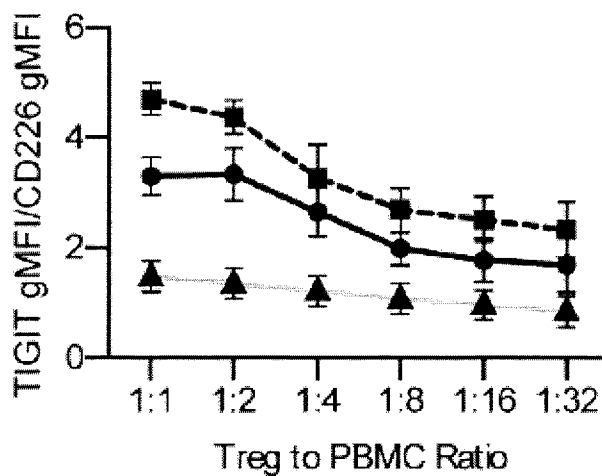


FIG. 7J

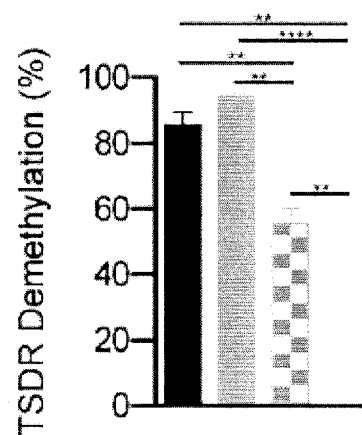


FIG. 7K

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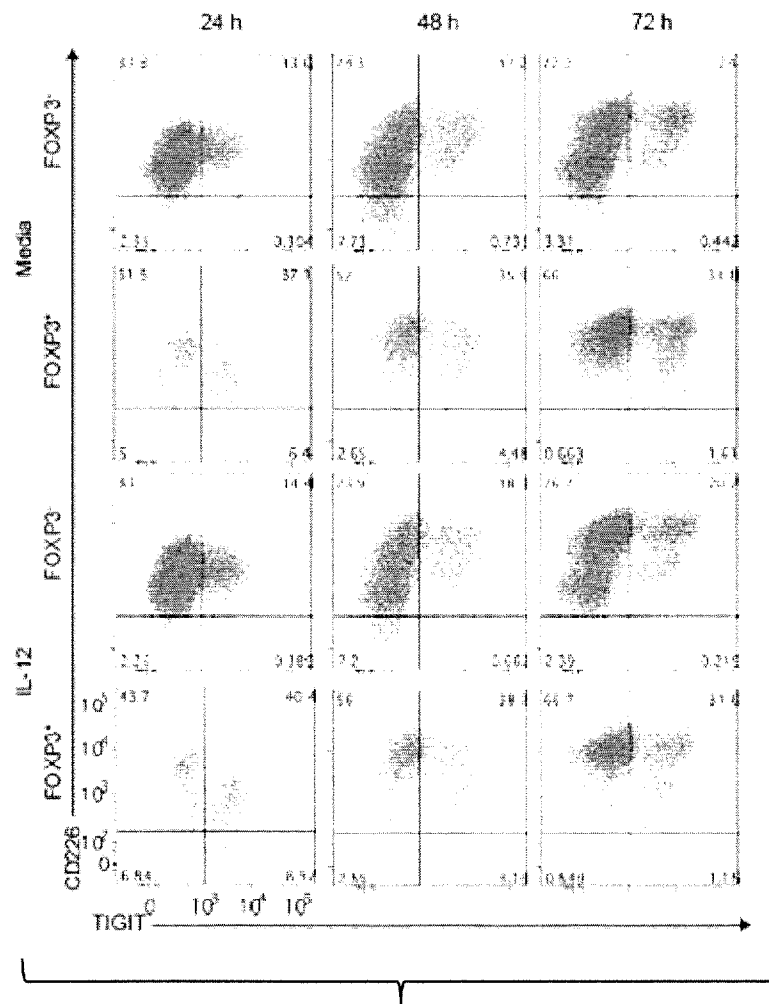


FIG. 8A

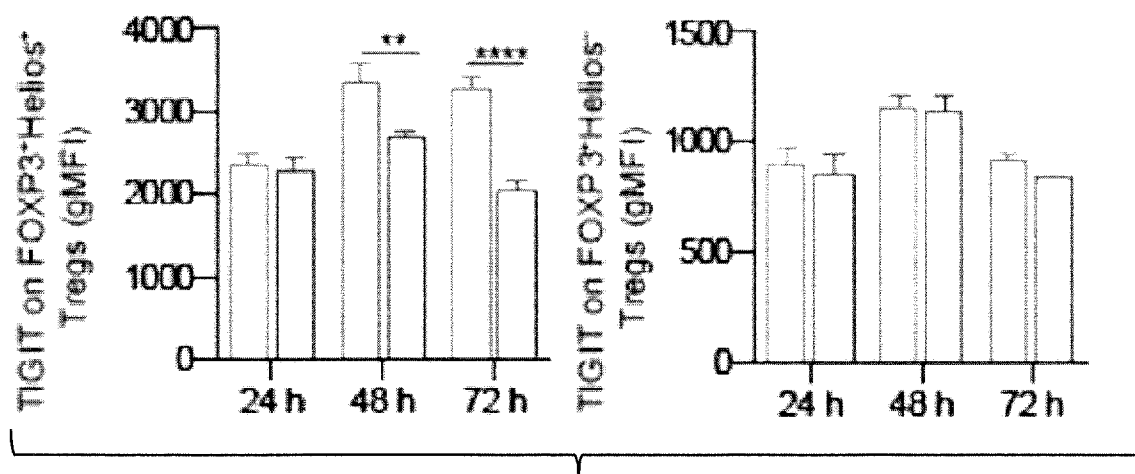


FIG. 8B

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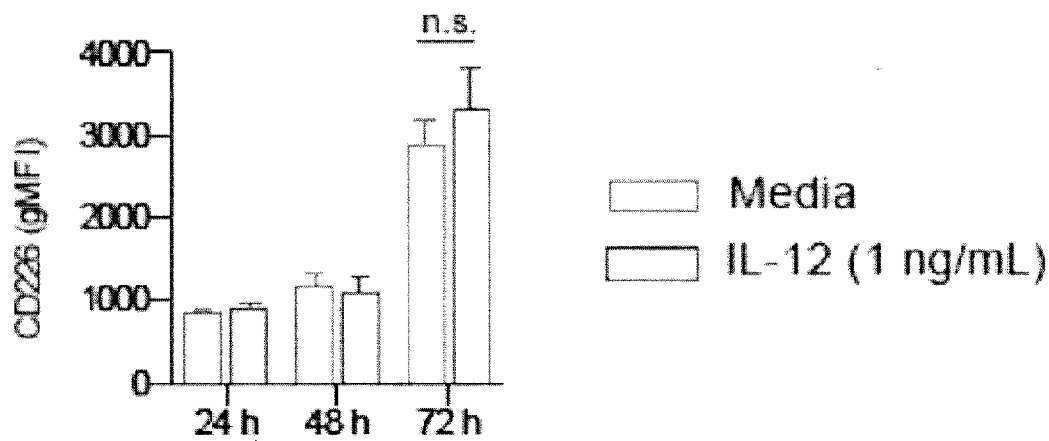


FIG. 8C

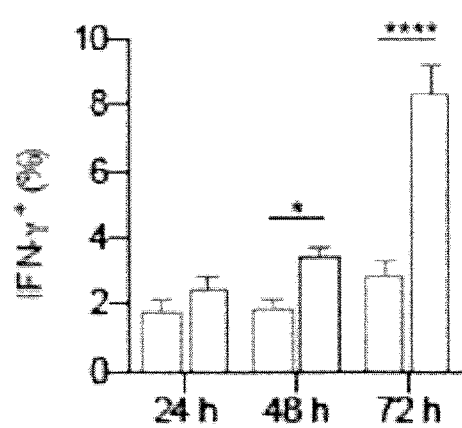


FIG. 8D

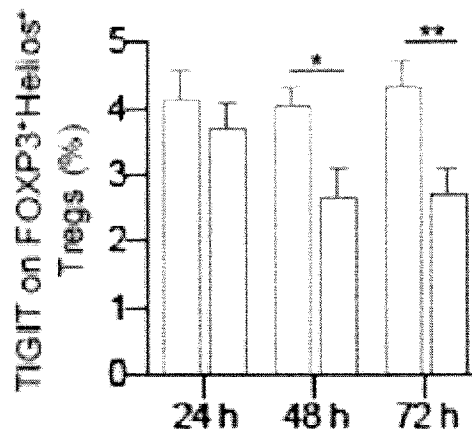


FIG. 8E

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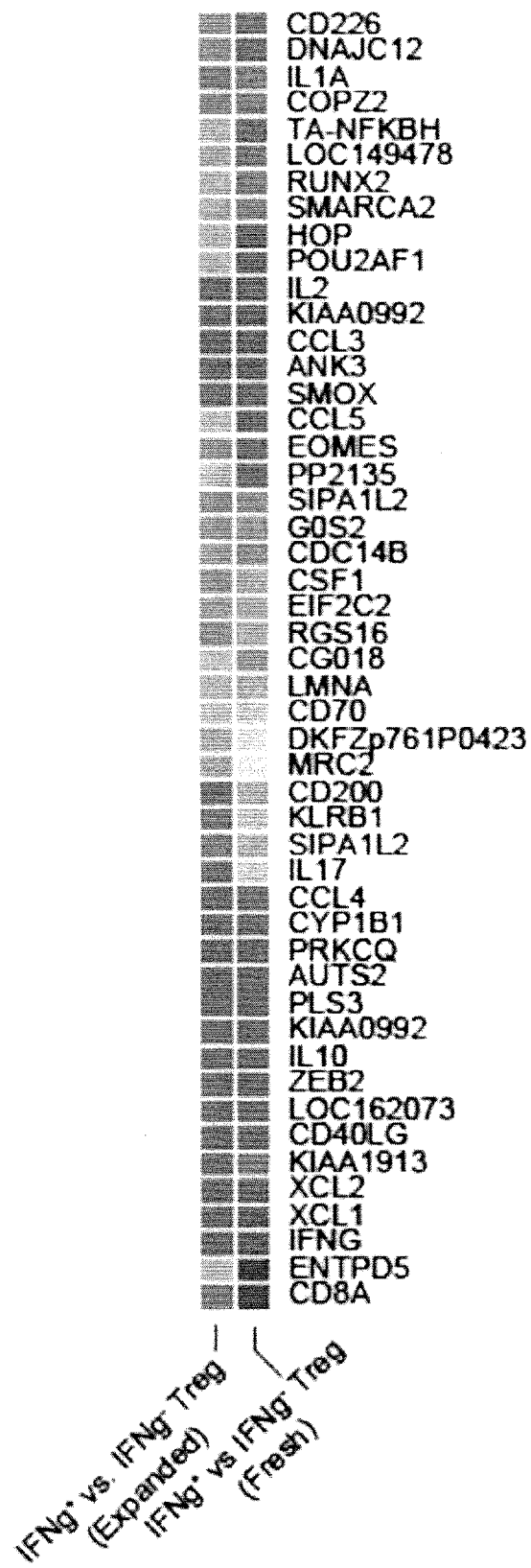


FIG. 9A

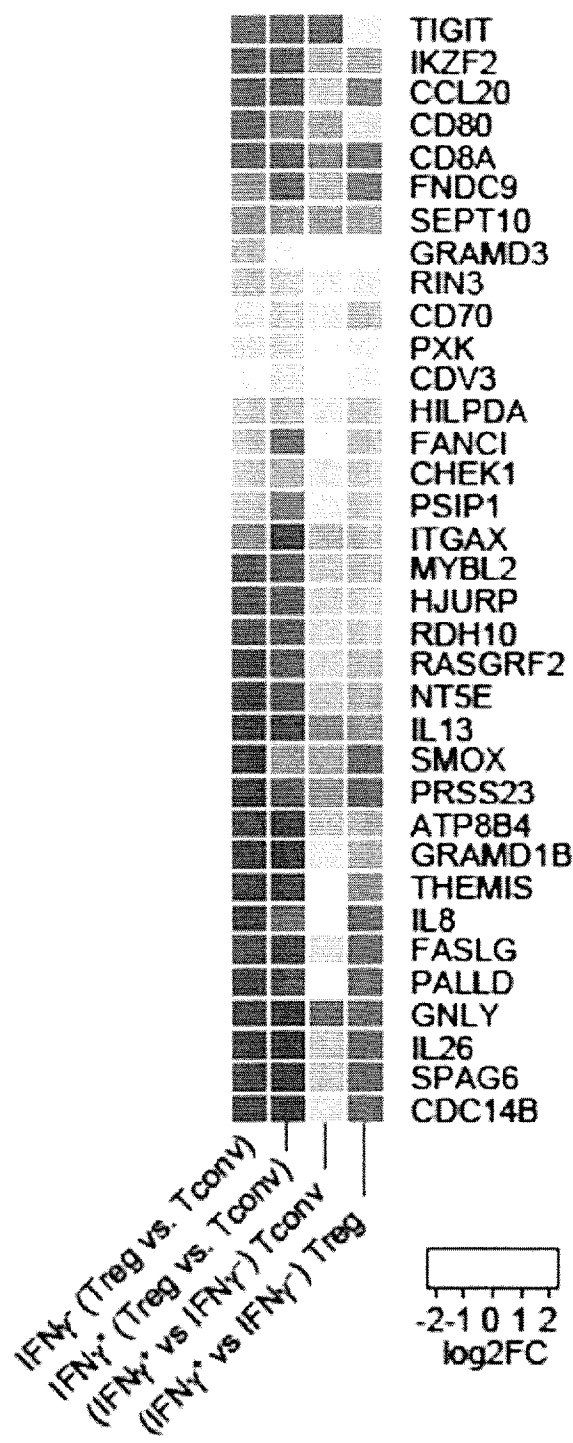


FIG. 9B

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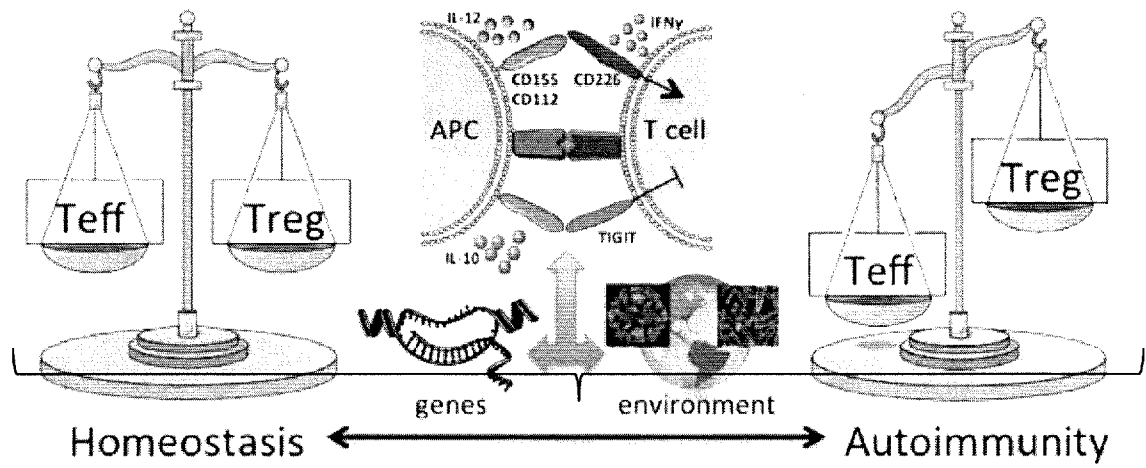


FIG. 10

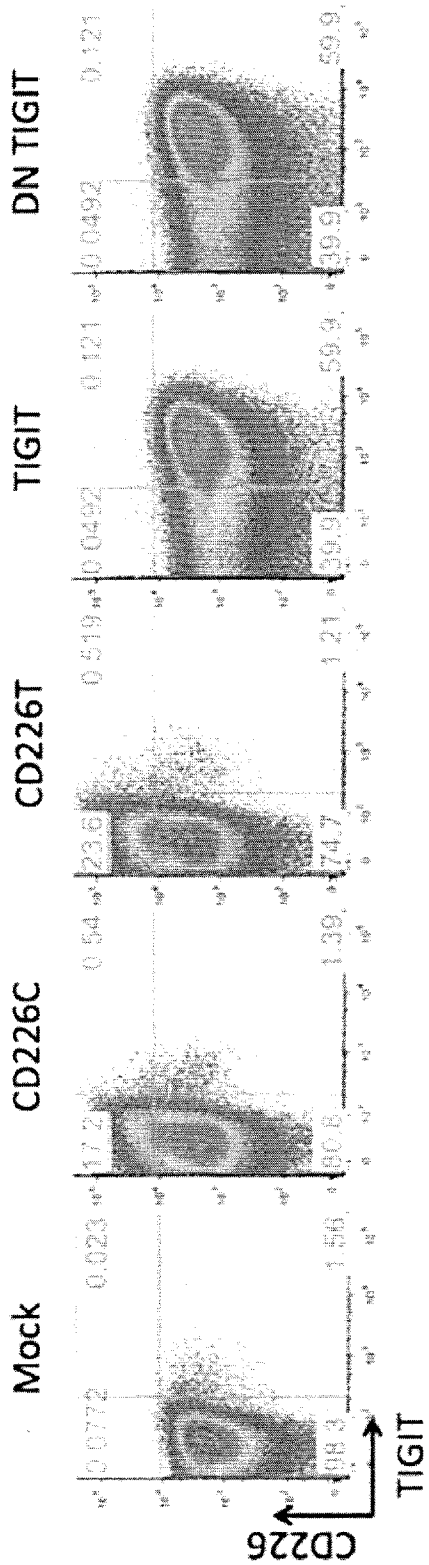


FIG. 11A

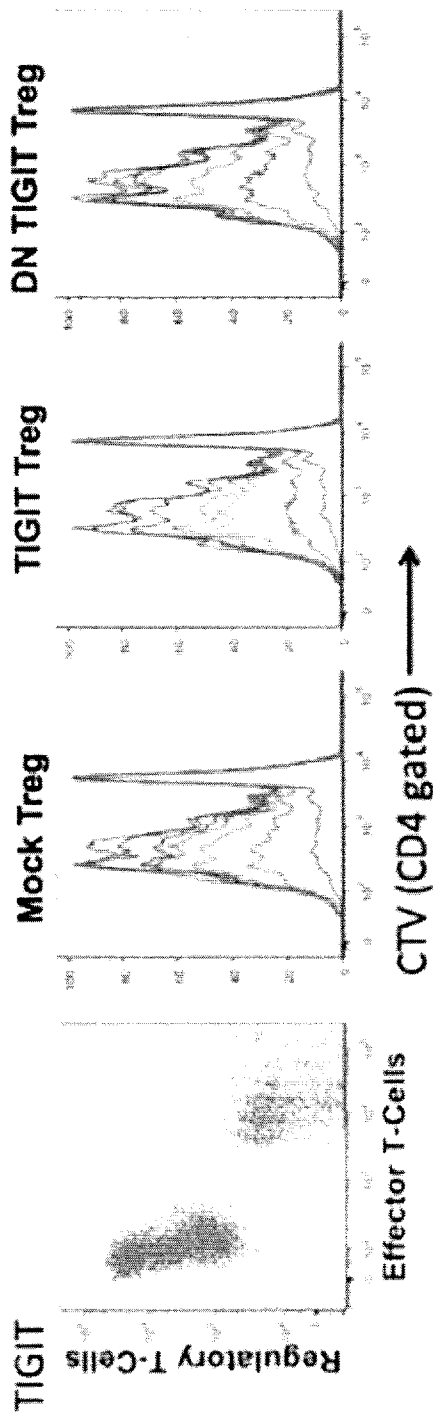


FIG. 11B

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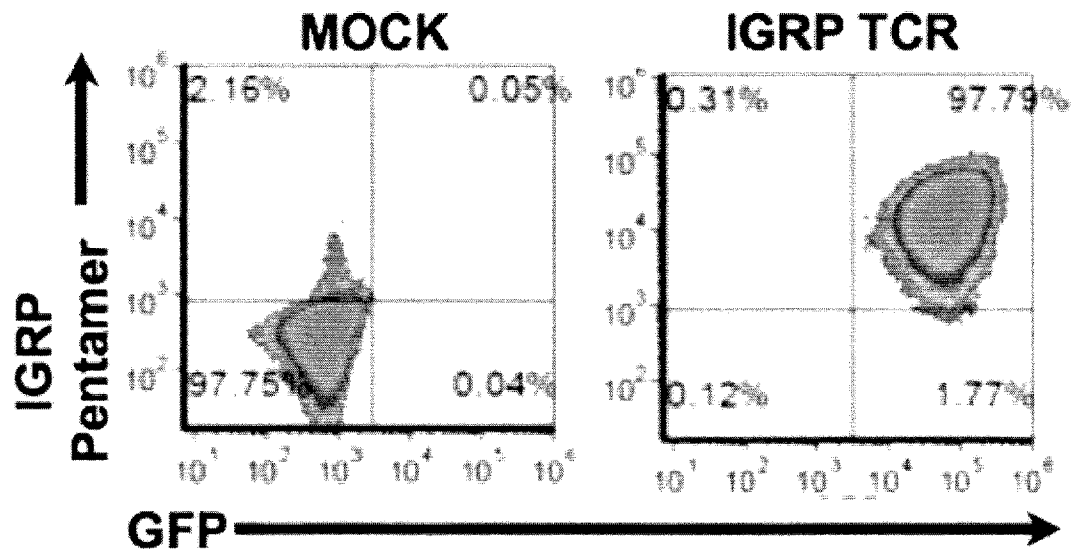


FIG. 12

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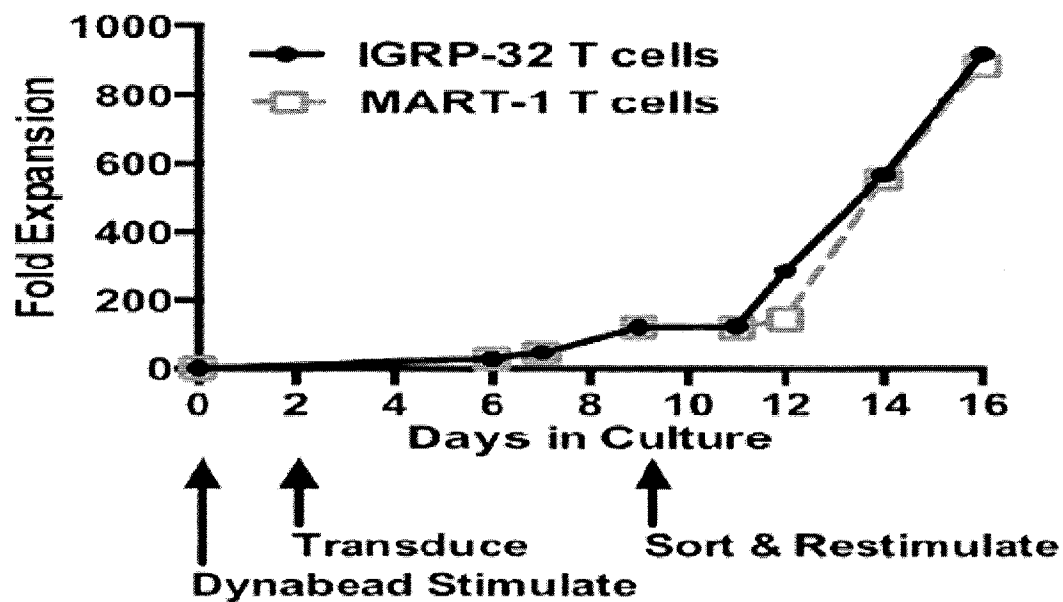


FIG. 13A

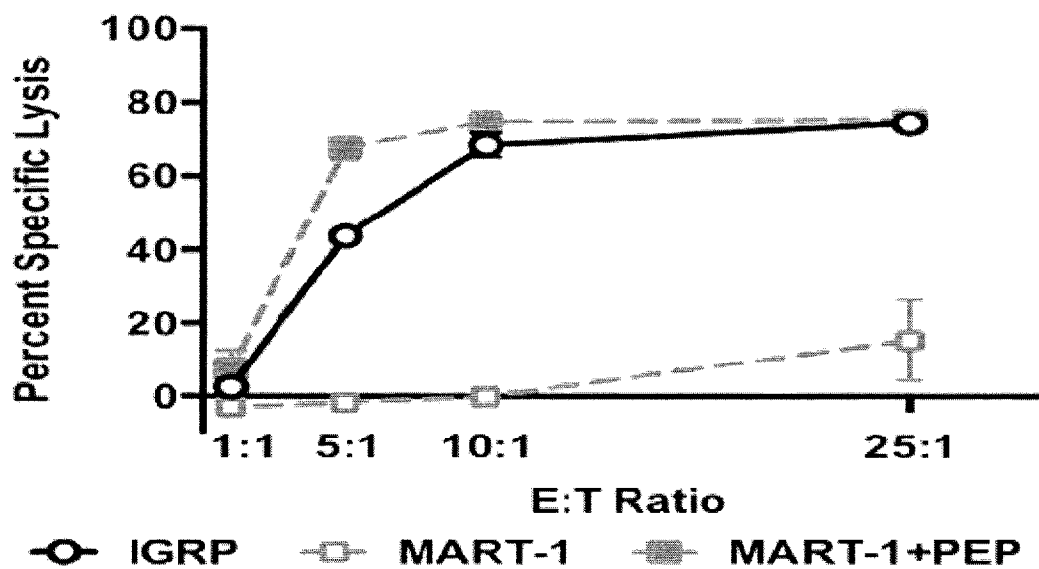


FIG. 13B

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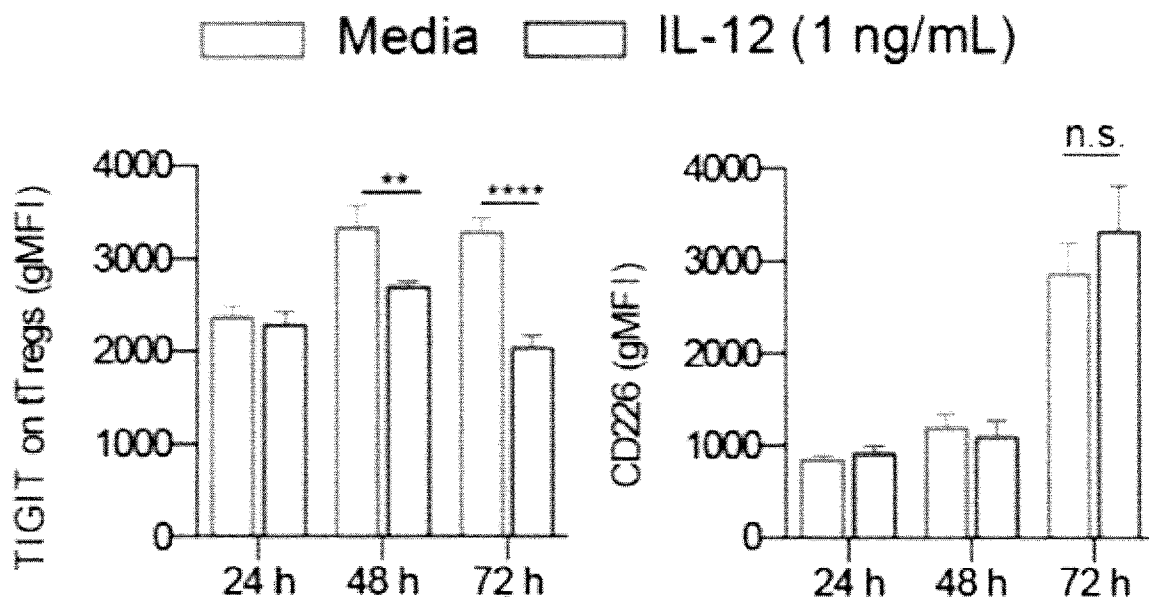


FIG. 14A

FIG. 14B

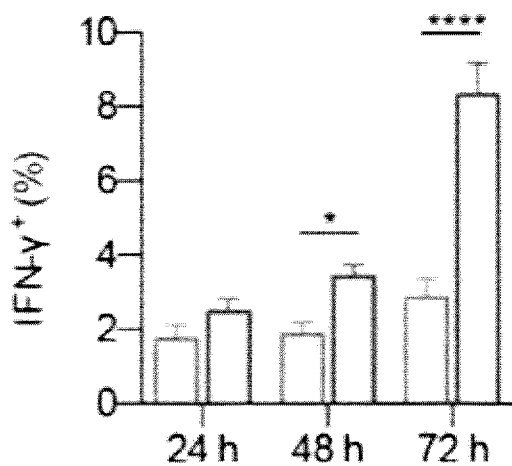


FIG. 14C

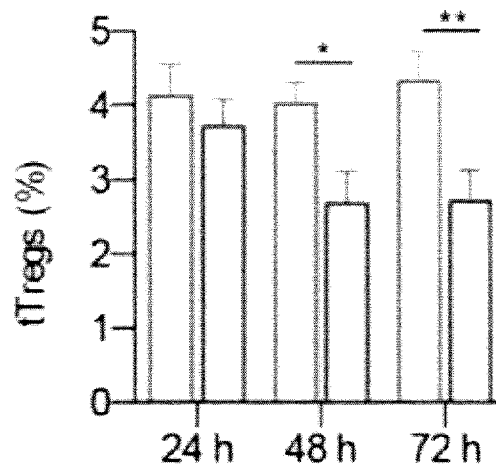


FIG. 14D

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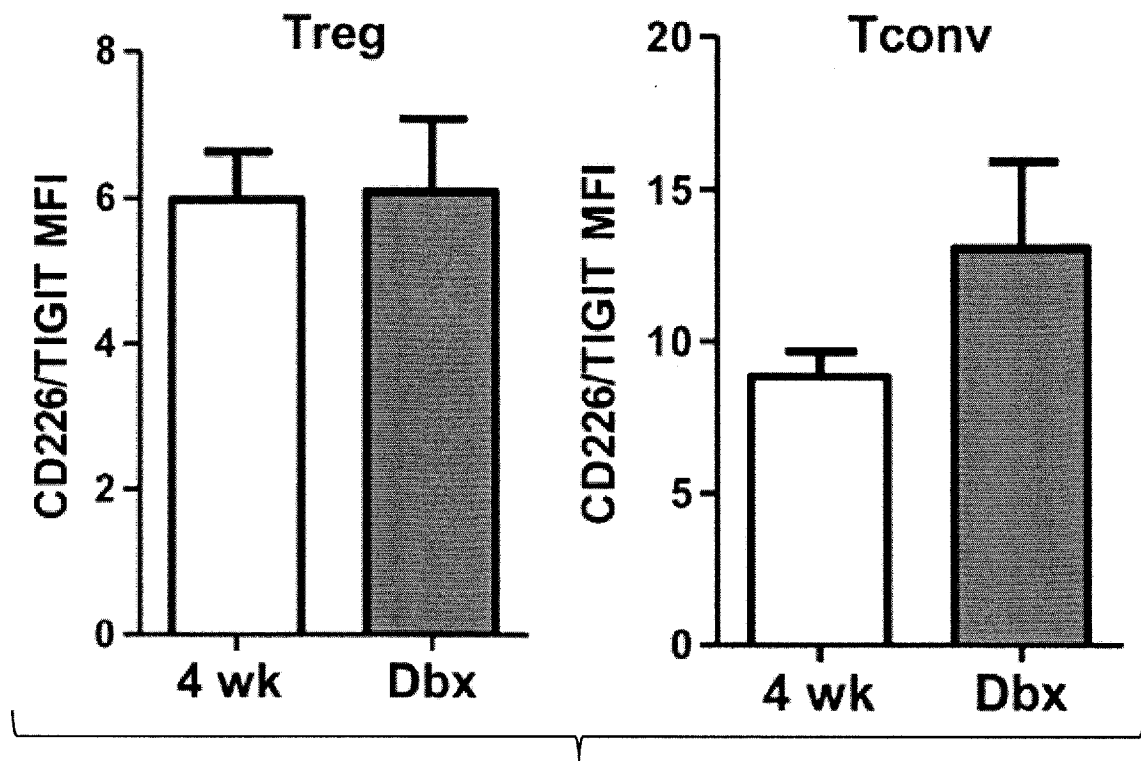


FIG. 15

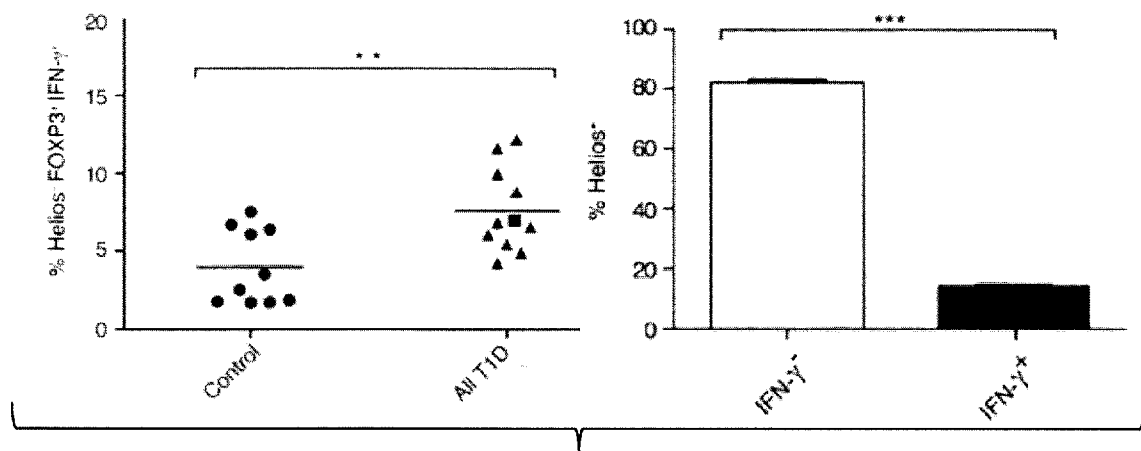


FIG. 16

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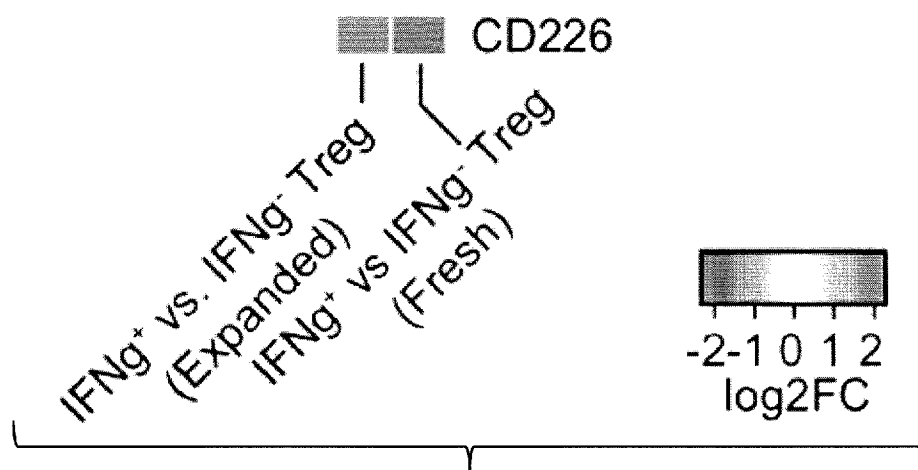


FIG. 17A

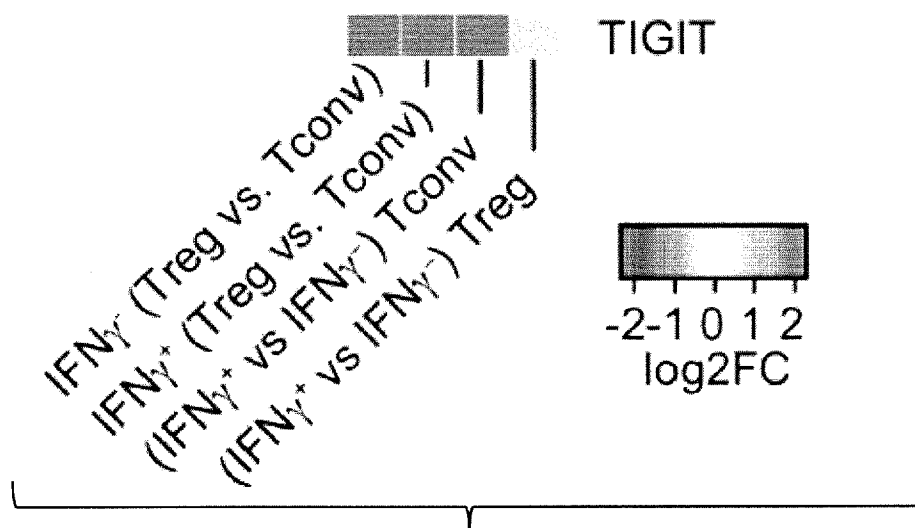


FIG. 17B

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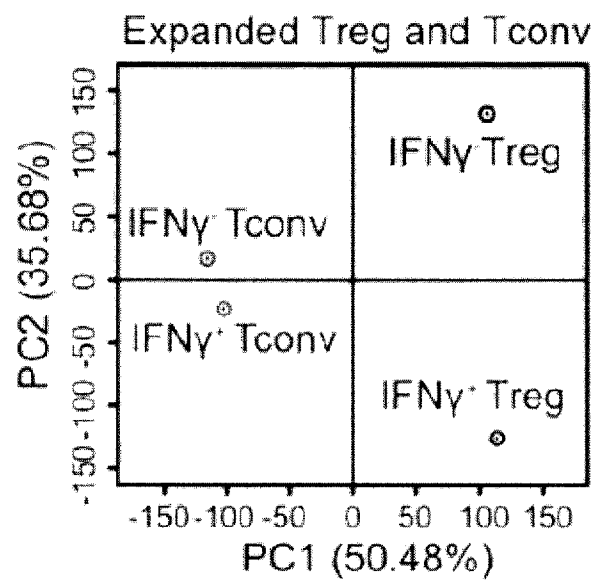


FIG. 17C

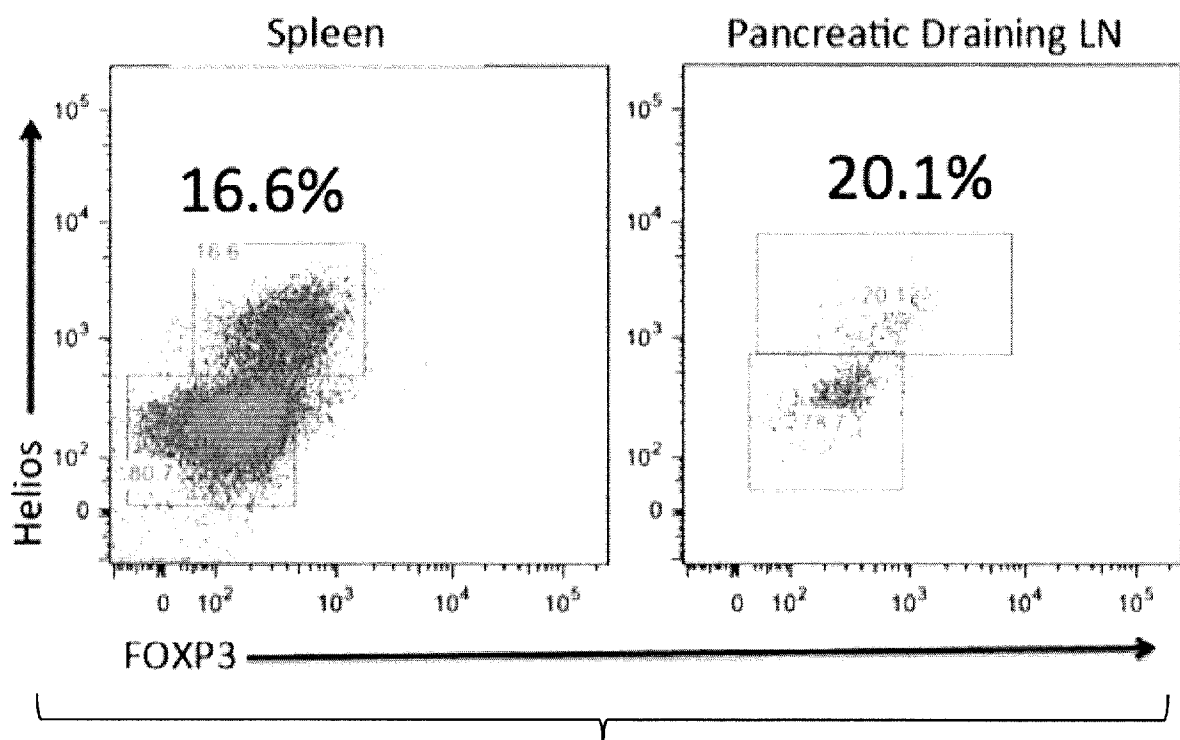


FIG. 18A

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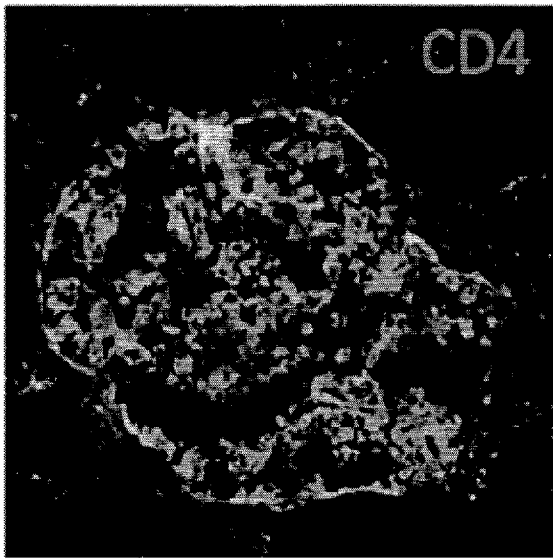


FIG. 18B

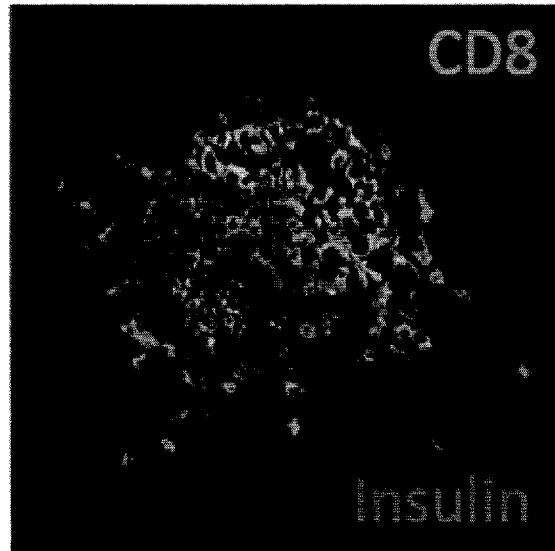


FIG. 18C

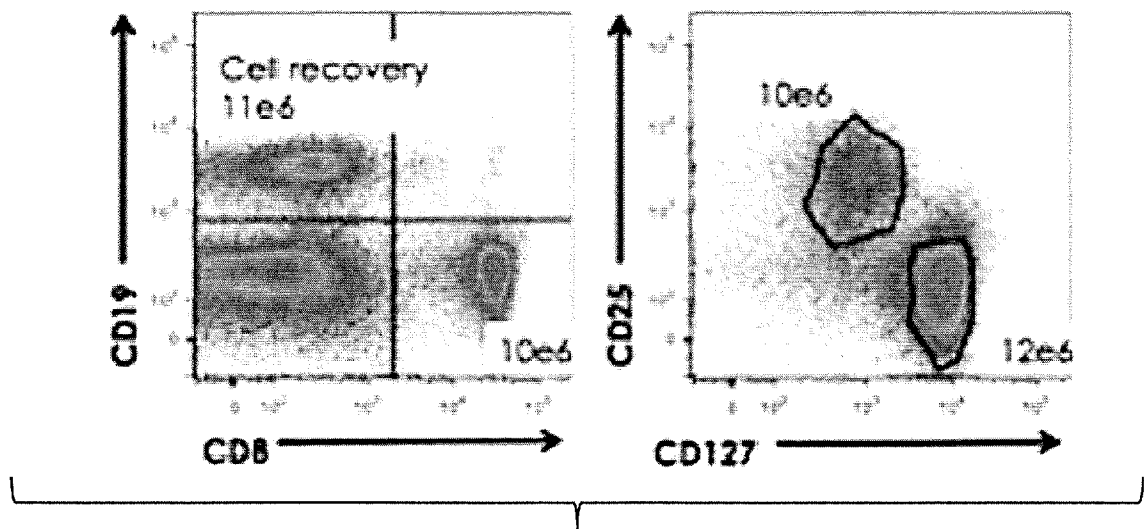


FIG. 19

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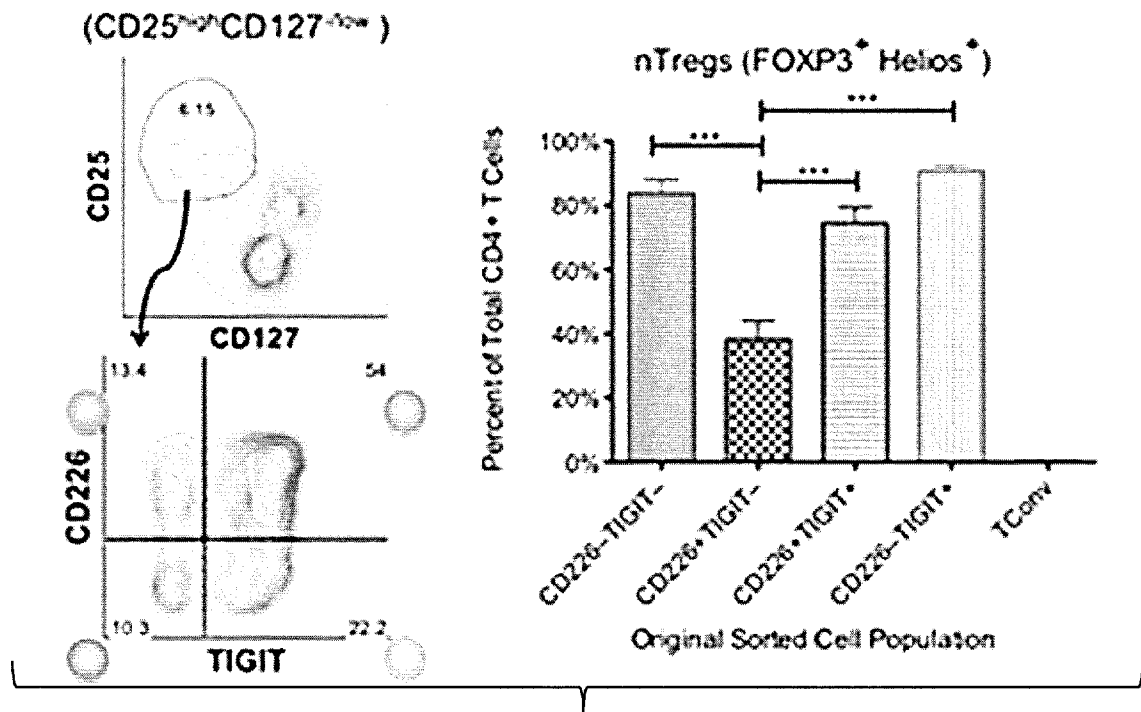


FIG. 20

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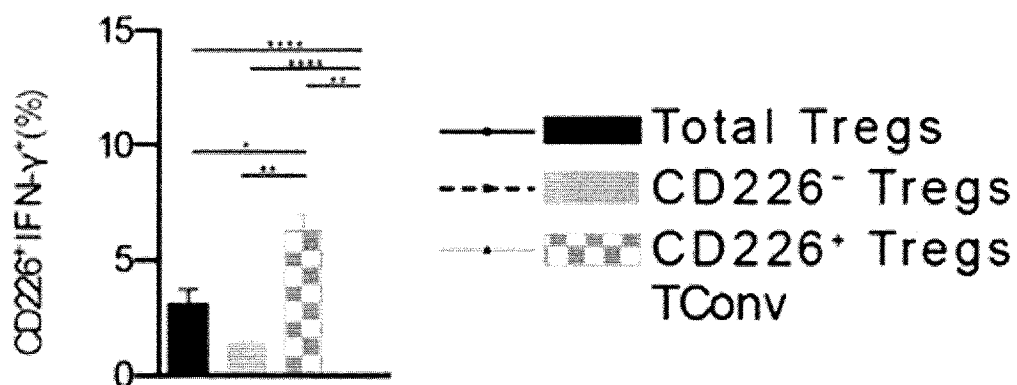


FIG. 21A

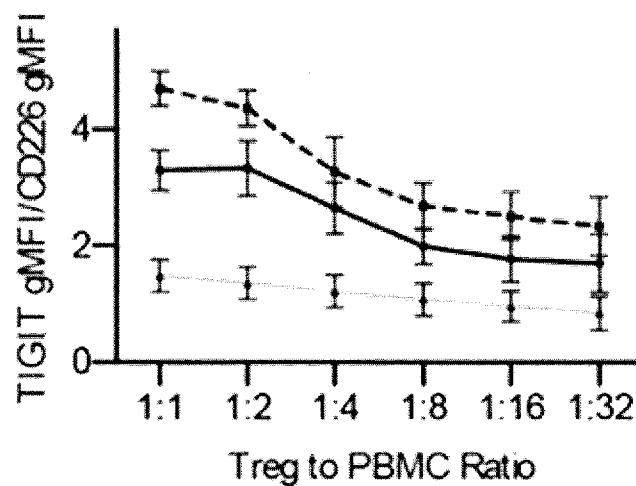


FIG. 21B

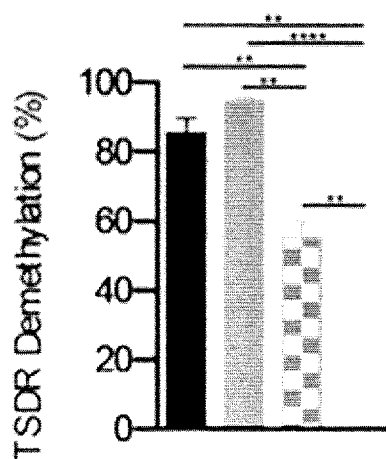


FIG. 21C

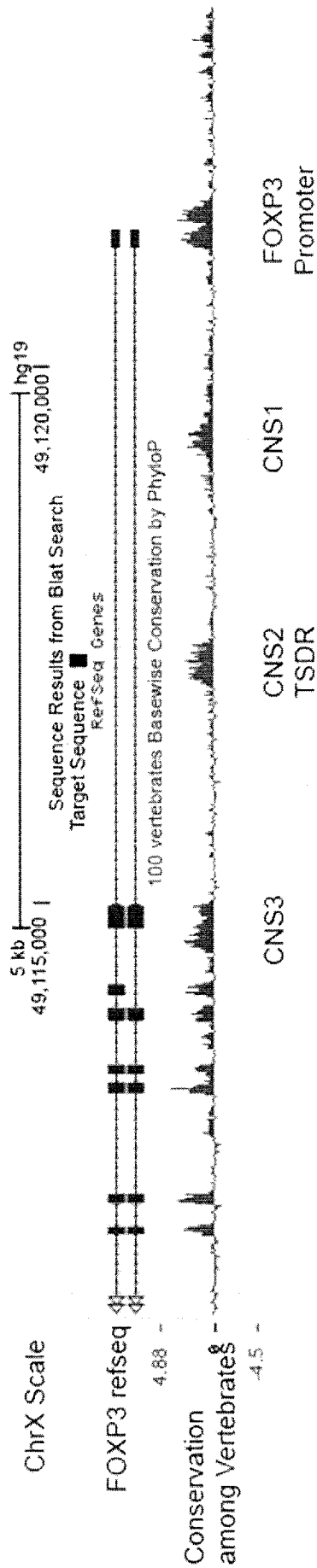


FIG. 22A

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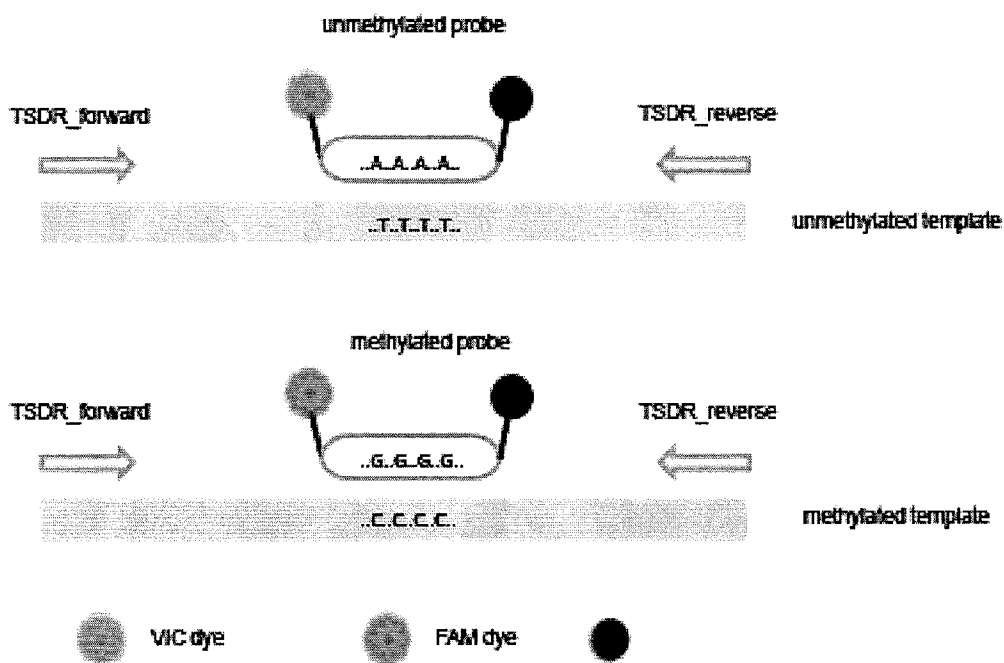


FIG. 22B

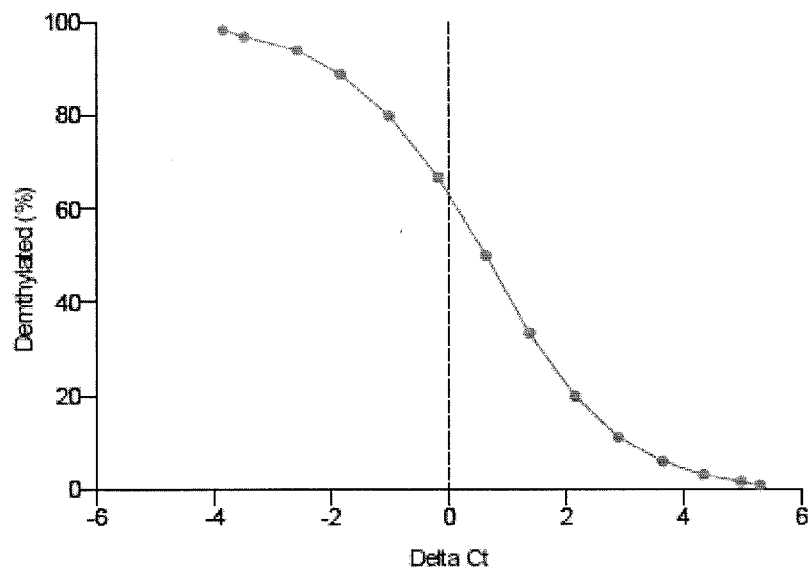


FIG. 22C

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0783(2010.01)i, G01N 15/10(2006.01)i, G01N 33/68(2006.01)i, A61K 31/7088(2006.01)i, A61K 35/17(2014.01)i, A61K 38/00(2006.01)i, 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)

C12N 5/0783; A61K 35/17; A61K 35/14; G01N 15/10; G01N 33/68; A61K 31/7088; A61K 38/00; A61K 39/395; A61P 37/00

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: regulatory T cell, cd226, cd127, autoimmune, flow cytometry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PUTNAM, AMY L. et al., `Expansion of human regulatory T-cells from patients with type 1 diabetes`, Diabetes, 2009, Vol.58, No.3, pp.652-662 See abstract; pages 652-654; and figure 1.	1-24
A		40
X	JOLLER, NICOLE et al., `TIGIT has T cell-intrinsic inhibitory functions`, The Journal of Immunology, 2011, Vol.186, No.3, pp.1338-1342 See abstract; page 1338, left column, lines 30-31; page 1340, left column, lines 24-26; page 1341, right column, paragraph 4; page 1342; left column, paragraphs 1 and 2; and figure 1.	40
Y		1-24
A	LOZANO, ESTER et al., `The TIGIT/CD226 axis regulates human T cell function`, The Journal of Immunology, 2012, Vol.188, pp.3869-3875 See the whole document.	1-24, 40
A	LIU, WEIHONG et al., `CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells`, The Journal of Experimental Medicine, 2006, Vol.203, NO.7, pp.1701-1711 See the whole document.	1-24, 40

☒ 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

11 August 2016 (11.08.2016)

Date of mailing of the international search report

12 August 2016 (12.08.2016)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

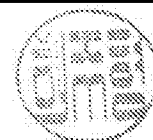
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/030789

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TRENADO, AURELIE et al., `Recipient-type specific CD4+ CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia`, The Journal of Clinical Investigation, 2003, Vol.112, No.11, pp.1688-1696 See the whole document.	1-24, 40
PX	WO 2015-158855 A1 (GENOVIE AB) 22 October 2015 See abstract; claims 1-33.	1-24, 40

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.: 25-39, 41-44
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 25-39 and 41-44 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☒ Claims Nos.: 26-29
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:
Claims 26-29 are considered to be unclear since they directly or indirectly refer to one of the multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying 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

Information on patent family members

International application No.

PCT/US2016/030789Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

WO 2015-158855 A1

22/10/2015

None