Original Articles

Validated Protocol for FoxP3 Reveals Increased Expression in Type 1 Diabetes Patients

Jean Grant,[1] Katarzyna Bourcier,[1]* Stephen Wallace,[2] Dalin Pan,[2] Alexis Conway,[2] Vicki Seyfert-Margolis,[1] and Paul K. Wallace[2]

[1]Immune Tolerance Network, University of California San Francisco, Bethesda, Maryland

[2]Department of Flow and Image Cytometry, Roswell Park Cancer Institute, Buffalo, New York

Background: FoxP3 has become a key identifier of regulatory T cells. Investigators have used a variety of antibodies and methods for detecting FoxP3 by flow cytometry. To standardize FoxP3 antibody staining for use in clinical trial samples, we tested various antibodies from different vendors, cell preparation protocols and fix/perm reagents, and cell isolation procedures. Using this optimized staining protocol, we evaluated clinical specimens from patients with multiple sclerosis (MS) or type 1 diabetes.

Methods: FoxP3 antibodies from eBioscience (236A/E7 and PCH101) and BioLegend (206D) were evaluated along with their respective methods and fix/perm reagents for preparation and staining of FoxP3 for flow cytometry. Fresh washed blood and frozen or fresh PBMC were evaluated. Upon optimization of the protocol, clinical samples (frozen PBMC) from patients with MS or type 1 diabetes and healthy control donors were evaluated with the BioLegend antibody.

Results: Clone 206D from BioLegend yielded optimal staining and the fix/perm reagents from both eBioscience and BioLegend were comparable. Data were also comparable between cells separated by Ficoll (fresh or frozen) and washed blood samples, allowing this protocol to be applicable to different types of samples. We validated this protocol using clinical samples and saw a significant increase in FoxP3 expression in the patients with type 1 diabetes but not in the MS.

Conclusions: The results from this study will allow the assessment of FoxP3 by flow cytometry on samples from clinical sites that are analyzed in real time on fresh blood or frozen PBMC. © 2008 Clinical Cytometry Society

Key terms: FoxP3; Tregs; T1D; flow cytometry

How to cite this article: Grant J, Bourcier K, Wallace S, Pan D, Conway A, Seyfert-Margolis V, Wallace PK. Validated protocol for FoxP3 reveals increased expression in Type 1 diabetes patients. Cytometry Part B 2009; 76B: 69–78.

Regulatory T cells (Tregs) (also known as suppressor T cells) are a specialized subpopulation of T cells that act to suppress immune responses, thereby maintaining homeostasis and self-tolerance [reviewed in (1,2)]. Naturally occurring Tregs originate in the thymus and comprise 2–4% of mature peripheral $CD4^+$ T cells (3). It has been shown that Tregs are able to inhibit T cell proliferation and cytokine production and play critical roles in preventing autoimmunity, controlling tumor immunity, and establishing transplantation tolerance (4,5). Impairment of Treg development and/or function can precipitate a variety of autoimmune diseases, whereas a higher frequency of Tregs can render the immune system hyporesponsive to pathogens (6–8). Until recently, the primary method to detect Tregs by flow cytometry used gating on $CD4^+CD25^{high}$ cells (9). Other cell surface markers have been investigated for Treg identification in humans, such as GITR and CTLA-4 (10–12); however,

Jean Grant and Katarzyna Bourcier contributed equally to this work.

Grant sponsor: NIH; Grant number: NIAID-DAIT-07-33; Grant sponsor: National Cancer Institute; Grant number: 5 P30 CA016056-29.

*Correspondence to: Katarzyna Bourcier, Immune Tolerance Network, UCSF, 3 Bethesda Metro, Suite 400, Bethesda, MD 20814.

E-mail: kbourcier@immunetolerance.org

Received 18 January 2008; Accepted 18 May 2008

Published online 8 August 2008 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.b.20446

these molecules are expressed on other T cell subsets making them unreliable as a marker specific for Tregs. In recent years, Tregs have been further characterized by the expression of the transcription factor FoxP3 and a lack or decreased expression of IL-7R (CD127) (12–15). FoxP3, or forkhead box P3, is a transcriptional regulator that, when overexpressed in activated T cells, can downregulate cytokine production and upregulate GITR and CTLA-4 (14). Previous studies have shown FoxP3 to be required for the development and function of Tregs (13,16). Liu et al. demonstrated the combination of CD4, CD25, and CD127 to be an ideal biomarker set to use in combination with FoxP3 (15). They observed that CD127 is downregulated on T cells after activation and that $FoxP3^+$ cells remain $CD127^{low/-}$, whereas CD127 is reexpressed on the majority of memory and effector T cells.

Because FoxP3 has arisen as the prototype marker to measure Tregs (along with use of CD127), numerous antibodies have become commercially available. One of the first polyclonal antibodies (pAb) on the market was made by Abcam. Banham and colleagues evaluated several polyclonal and monoclonal FoxP3 antibodies, including Ab2481 (polyclonal, Abcam), hFoxy (eBioscience), and lab-derived mouse monoclonal antibodies (mAb) 236A/E7, 206D, and 259D (17). They found staining with hFoxy, 236A/E7, and 259D to result in nonspecific background staining. McCune and colleagues evaluated multiple surface markers for use in characterizing Tregs, including CD127, along with two FoxP3 antibody clones (206D and PCH101) (12). Although they found that the $CD4^+CD25^{high}CD127^-$ phenotype is characteristic of Tregs in mice, rhesus macaques, and human healthy controls, they did not identify which FoxP3 antibody clone or method of staining was optimal. Shevach and colleagues stained human peripheral blood with FoxP3 clones 236A/E7 and PCH101 (eBioscience) and 259D and 206D (BioLegend) and found that unlike the other clones, the PCH101 clone stained Treg cells and nonspecifically stained activated T cells (18). Bluestone and colleagues used the 206D BioLegend clone in a recent study and found this clone to work well at identifying $FoxP3^+$ cells (15). In addition to identifying the most suitable FoxP3 antibody clone, the optimal staining and fixation method have yet to be established. Furthermore, whether Tregs can be accurately identified in frozen samples as in fresh has yet to be determined.

This study was a two-part project. First, we evaluated several FoxP3 antibodies (clones 236A/E7, PCH101, and 206D) along with different staining procedures and cell preparation methods to detect FoxP3 expressing Tregs in fresh washed blood and frozen peripheral blood mononuclear cells (PBMC). Our findings show that the BioLegend clone (206D) and both the eBioscience and BioLegend fix/perm reagents provided optimal staining of $FoxP3^+$ positive cells. FoxP3 staining on cells separated by Ficoll was comparable to staining of fresh washed blood. Additionally, comparisons of our staining protocol on fresh and frozen cells yielded similar results. Second, when applying our optimized procedure (Biolegend 206D clone and fix/perm reagents) to clinical specimens from type 1 diabetes (T1DM) and multiple sclerosis (MS) patients, we observed significant differences in the number of T cells expressing higher levels of FoxP3 in T1DM patients compared to healthy controls.

MATERIALS AND METHODS

Clinical Specimens

Blood was collected from consenting donors (healthy donors, MS and T1DM patients) into 10 ml sodium heparin Vacutainer® tubes (BD Biosciences, San Jose, CA) (19–21) at Roswell Park Cancer Institute, Buffalo, NY, or blood collection bags (300 ml) at Brigham and Women's Hospital, Boston, MA, and at the Laboratories at Bonfils, blood collection bank, Denver, CO, according to the guidelines and recommendations of the each respective institution's IRBs. MS patients all had Relapsing Remitting type of disease, and T1DM patients had well-established disease with full metabolic control. PBMCs were isolated using a Ficoll isolation procedure as previously described (22) and frozen on liquid nitrogen in 10% DMSO plus human serum at 10×10^6 cells/ml at 1 ml aliquots. Clinical specimens were blinded and evenly distributed among numerous runs.

Antibodies and Reagents

Monoclonal antibodies (mAbs) were conjugated to Alexa Fluor 488 (Ax488), fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), phycoerythrin cyanine seven (PECY7), or allophycocyanin (APC). All surface-staining antibodies used in this study were from BD Biosciences. The FoxP3 antibodies and their respective isotype controls used in this study included FoxP3 clones 236A/E7 and PCH101 from eBioscience (San Diego, CA) and clone 206D from BioLegend (San Diego, CA). All mAbs were titered and used at saturating concentrations.

Two antibody panels were designed for preliminary work on eBioscience and BioLegend FoxP3 mAbs and respective isotype controls, referred to as the "evaluation panels": (1) FoxP3-Alexa Fluor 488 (either clone 236A/E7, PCH101 or 206D)/CD25-PE/CD3-PerCP/CD4-APC and (2) IgG1-Alexa Fluor 488/CD25-PE/CD3-PerCP/CD4-APC. Two antibody panels using the BioLegend 206D clone and isotype control were designed for the analysis of clinical samples from MS patients, T1DM patients, and healthy donors, referred to as the "clinical panels": (1) FoxP3-Alexa Fluor 488 (206D)/CD127-PE/CD4-PerCP/CD3-PECY7/CD25-APC and (2) IgG1-Alexa Fluor 488/CD127-PE/CD4-PerCP/CD3-PECY7/CD25-APC.

Cell Preparation and Staining with FoxP3 Antibody

Mononuclear cells (MNC) were isolated by density centrifugation on Ficoll Hypaque (22). Whole blood was diluted in an equal volume of Hanks Balanced Salt Solution (HBSS), then carefully overlaid on Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO), and centrifuged at 300*g*

for 30 min. The MNC rich interface was harvested, washed twice in FCM buffer (phosphate buffered saline pH 7.2 with 1% bovine serum albumin, 0.1% sodium azide, and 0.004% disodium ethylenediamine-tetraacetic acid; Sigma), counted on a cell counter (AcT10, Beckman Coulter, Miami, FL), and resuspended to 5–10 $\times$ 10^6 cells/ml in FCM buffer containing human IgG to block Fc receptors (2 mg/ml; IgG Cohn fraction II and III globulins, Sigma-Aldrich). For surface staining, 100 μl of cells was added to tubes containing cocktails of fluorochrome-labeled mAbs. The sample tubes were mixed and returned to the ice bath, shielded from light, and incubated for 60 min. Following incubation, 3.5 ml of lysing solution (0.155 M NH_4CI, 10 mM $KHCO_3$, and 0.089 mM EDTA) was added to the tubes, which were immediately inverted three times and held at 25°C for 5 min to promote lysis of residual erythrocytes. The tubes were centrifuged for 5 min at 400*g* and washed twice with 3 ml of FCM buffer. For intracellular FoxP3 staining, surface-stained cells were resuspended in 1 ml of FoxP3 fixation/permeabilization (Fix/Perm) Buffer (BioLegend or eBioscience) and incubated at room temperature in the dark for 30 min, followed by centrifugation at 400*g* for 5 min. Tubes were decanted, blotted, washed twice with FoxP3 Permeabilization (Perm) Buffer (BioLegend or eBioscience), and then incubated for 20 min in FoxP3 Perm Buffer. The cells were then centrifuged, decanted, and resuspended into ~50 μl of residual Perm Buffer left in each tube, to which human IgG was added 10 min before adding either the FoxP3 antibody or isotype control to the appropriate tubes. Cells were incubated with mAbs at room temperature for 1 hr in the dark, washed once in FoxP3 Perm buffer and once in FCM buffer before fixing in 2% formalin (methanol free formalin, Polysciences, Warrington, PA) [diluted in phosphate buffered saline (PBS)]. The tubes were capped, placed in the refrigerator, and analyzed within 3 days.

In one experiment, we compared Fix/Perm and Perm Buffers. In another experiment, alternate cell-processing procedures were evaluated. In these cases, either a washed blood lysis (23–27) or a dextran sedimentation technique was also used (28). For the washed blood lysis technique, 2–3 ml of blood was pipetted into a 15-ml conical centrifuge tube, along with ~12 ml PBS containing 10 U/ml heparin, centrifuged for 3 min at 1500*g*, and the clear supernatant aspirated to within 500 μl of the surface of the darker, cell-containing layer. After brief agitation of the cells by vortexing, the volume was brought up to 15 ml with PBS (without heparin). The cells were centrifuged a second time and the clear layer aspirated. The washed blood was resuspended in FCM buffer to a volume of 1.5 ml and human IgG to a final concentration of 2 mg/ml. In the dextran sedimentation method, WBCs were separated from RBCs by resuspending blood in an equal volume of 2% dextran (100,000 MW, Sigma-Aldrich) and incubating at 37°C for 15 min. WBCs were aspirated from the sedimented RBCs, centrifuged, and lysed to remove residual RBCs with ammonium chloride. The WBCs were then washed, counted, and resuspended at 2.5–5 $\times$ 10^6 cells/ml in FCM buffer containing Fc block.

In all experiments using frozen and thawed cells, Live/Dead Fixable Violet Dead Cell Stain (Invitrogen Molecular Probes, Carlsbad, CA) was added to the cocktail of surface mAbs to differentially stain live and dead cells.

Flow Cytometric Analysis

Cytofluorometric analysis was performed using either a FACSCanto or LSR II (BD BioSciences) flow cytometer. Both were equipped with 488-nm argon–ion and 633-nm He–Ne lasers. FITC, Alexa Fluor 488, PE, PerCP, and PECy7 fluorescence were detected off the 488-nm line using logarithmic amplification, and APC fluorescence was detected off the 635 nm line. The LSR II was also equipped with a 405-nm violet laser, which was used to excite the fixable violet live/dead reagent. A forward scatter (FSC) threshold was set to eliminate debris from list mode data and for each sample; a minimum of 200,000 events were collected using DiVA software (BD Bioscience). Data were analyzed using WinList (Verity Software House, Topsham, ME) and/or FlowJo software (Treestar, Ashland, OR). A region encircling lymphocytes was set using a forward versus side scatter (SSC) display, and all fluorescent parameters were gated on this population. Autofluorescent and FMO controls were used to establish the boundary between negative and positive fluorescent regions, and the results are expressed as the percentage of cells falling above the negative region for each mAb.

RESULTS

BioLegend Clone 206D Exhibits Optimal FoxP3 Staining

To determine the most favorable FoxP3 antibody to use for flow cytometry assessments, several antibodies were tested. Preliminary data showed that a rabbit polyclonal antibody from Abcam resulted in nonspecific and dim staining of FoxP3 and a monoclonal from eBioscience (hFoxy) resulted in minimal staining of FoxP3 (data not shown), similar to previously reported findings from other laboratories (17). Testing was expanded to include clones 236A/E7 (eBioscience), PCH101 (eBioscience), and 206D (BioLegend). Whole blood from two healthy controls was collected, PBMC separated by Ficoll, and cells fixed and permeabilized as required using either the eBioscience or BioLegend reagents with their respective antibody clones. Cells were also stained for CD3, CD4, and CD25 expression, using the evaluation panels (Fig. 1). Lymphocytes were gated on FSC versus SSC, followed by gating on $CD3^+$ and $CD4^+$. A region was set to determine the percentage of $CD25^+FoxP3^+$ cells (Fig. 1A). All three clones showed a similar frequency of $CD25^+FoxP3^+$ cells, ranging from ~7 to 11%. The PCH101 clone showed the greatest percent positive staining, and it exhibited a degree of nonspecific staining compared to its isotype control

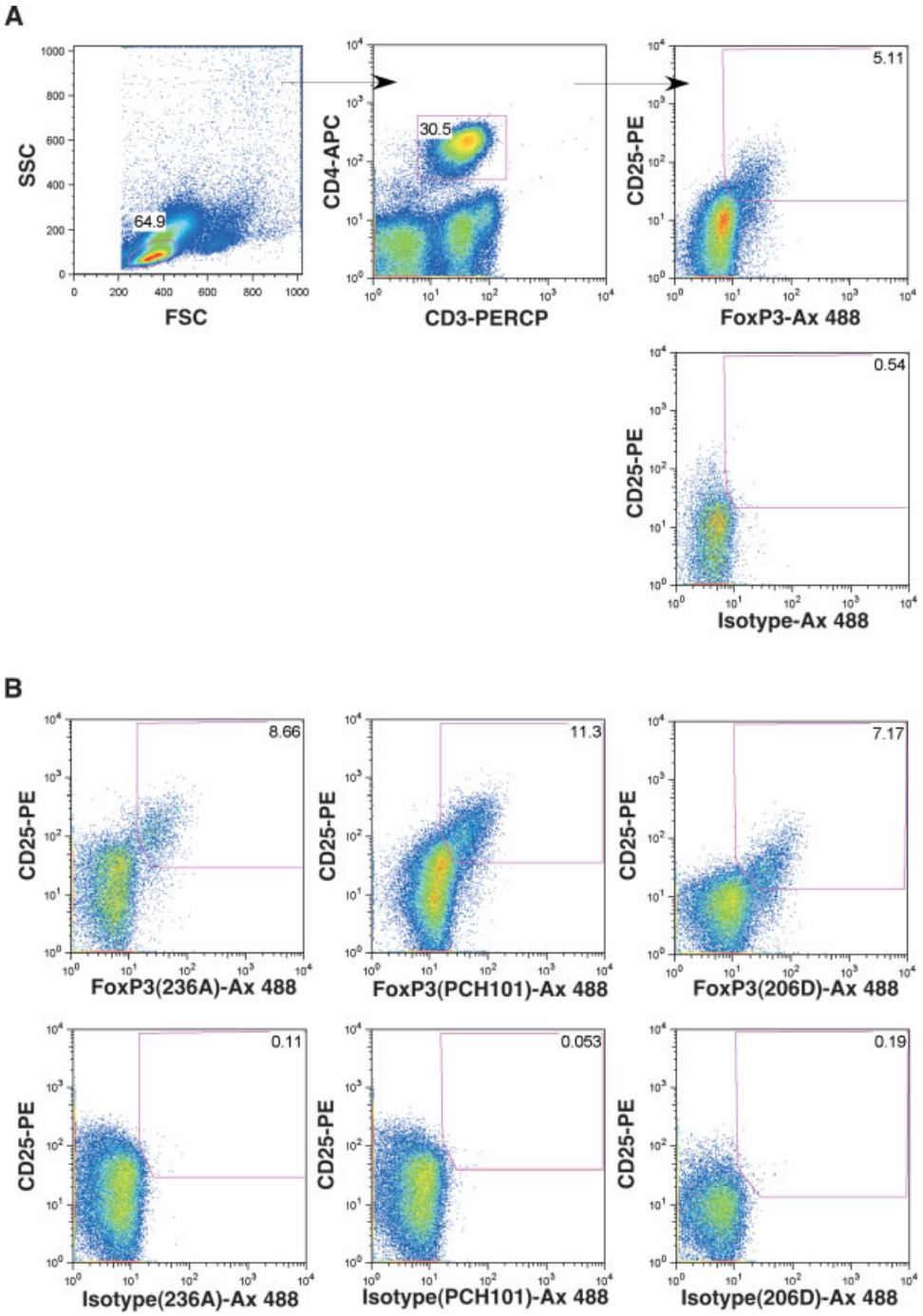

FIG. 1. Expression of FoxP3 after staining of blood with different FoxP3 antibodies. Blood from two healthy controls was drawn, cells separated by Ficoll, and stained with Foxp3 clones from eBioscience (236A or PCH101) or BioLegend (206D); the vendor's own fix/perm reagents were used with their respective clones. **A:** Gating strategy. Cells were gated on FSC versus SSC followed by the $CD3^{+}CD4^{+}$ population and then the percentage of $CD25^{high}FoxP3^{+}$ cells was determined. Data are represented as percent positive lymphocytes. **B:** Comparison of three antibody clones: 236A/E7 (eBioscience), PCH101 (eBioscience), and 206D (BioLegend). Antibody panel used: FoxP3-Alexa Fluor 488/CD25-PE/CD3-PerCP/CD4-APC with the manufacturer-suggested appropriate Alexa Fluor 488 isotype control. One donor is shown.

(Fig. 1B), realized by its shift in fluorescence by half a decade. This staining pattern was seen whether FoxP3 was conjugated to Alexa Fluor 488 (shown) or PE (not shown). Because other researchers have also found clones 236A and PCH101 to be less favorable and non-specific, we chose to exclude them from the remaining studies and proceeded with the rest of our studies using the BioLegend clone 206D (17,18).

Evaluating the FoxP3-Staining Protocol

After deciding to use the BioLegend antibody (clone 206D), the most suitable staining protocol for FoxP3 expression in flow cytometry samples was investigated. Cell fixation and permeabilization (fix/perm) reagents from eBioscience and BioLegend were tested. Data from healthy donor freshly isolated PBMC using either the eBioscience or the BioLegend fix/perm reagents demonstrated that the eBioscience and BioLegend fix/perm reagents resulted in comparable frequency of $FoxP3^+CD25^{high}$ cells (data not shown). In addition, lab-derived fix/perm reagents showed minimal staining (data not shown). Because we found that the staining intensity of CD25 to be dimmer when BioLegend fix/perm reagents were used (Fig. 1B), we decided to use the eBioscience fix/perm reagents for the remainder of the study.

Methods for cell preparation and isolation were then investigated. Healthy control whole blood was processed by Ficoll, dextran sedimentation, or a washed blood lysis technique, followed by staining with the evaluation antibody panels using the BioLegend FoxP3 clone and eBioscience fix/perm buffers. Washed blood and cells separated by Ficoll yielded the highest number of $FoxP3^+CD25^{high}$ cells (Fig. 2). Ficoll-separated cells gave a cleaner FSC versus SSC plot than washed blood-lysed cells (data not shown). Dextran sedimentation resulted in dimmer FoxP3 staining and was therefore eliminated from evaluation. Because cells separated by Ficoll did not impede detection of FoxP3 and gave a cleaner population than washed blood, the next objective was to determine whether frozen cells can be used for FoxP3 staining. Blood from five healthy controls was drawn and cells separated by Ficoll, half of the sample stained immediately and half frozen for 24 hr followed by staining. Cells were stained with the evaluation antibody panels using the BioLegend FoxP3 clone 206D and eBioscience fix/perm buffers. No difference could be evidenced between frozen and fresh cells (two-tailed P value of 0.8009) (Fig. 3), with about 2–4% of frozen cells staining $FoxP3^+CD25^{high}$, which is consistent with the results reported by other laboratories (3,12). Use of deoxyribonuclease I (DNAse I) during thawing was inconsequential (data not shown).

From these studies, we generated a FoxP3-staining protocol that used the BioLegend clone 206D and eBioscience fix/perm reagents, applicable to both whole/washed blood and frozen cells. Because previous studies demonstrated the combination of CD4, CD25, and CD127 to be an ideal biomarker set to use in combination with FoxP3, CD127 was added to the antibody panels (15). Furthermore, because CD127 is a dim marker that should be labeled with a fluorochrome having a high quantum yield, it was conjugated to PE. Therefore, the following panels were designed for use on clinical samples and are referred to as the "clinical panels": (1). FoxP3-Alexa Fluor 488 (206D)/CD127-PE/CD4-PerCP/CD3-PECY7/CD25-APC and (2). IgG1-Alexa Fluor 488/CD127-PE/CD4-PerCP/CD3-PECY7/CD25-APC

Staining of Clinical Samples Reveals Higher Expression of FoxP3 in Type 1 Diabetes Patients

After the protocol for staining of FoxP3 was optimized, the clinical antibody panels were used to assess FoxP3 expression on frozen PBMC obtained from patients with MS or T1DM and healthy controls. Because these panels differed from the evaluation panels and a live/dead stain was used, the gating strategy was expanded to include the $CD127^{low}$ population (Fig. 4). Cells were gated on FSC versus SSC followed by live cells gated for $CD3^+$ and then for $CD3^+CD4^+$ cells (Fig. 4A). Regions were then drawn on the $CD4^+CD25^+FoxP3^+$, $CD4^+CD25^{high}FoxP3^+$ (Fig. 4B), or $CD25^+CD127^{low}FoxP3^+$ (Fig. 4D) populations. Additionally, percentage of $CD3^+CD4^+CD25^+FoxP3^+$ cells of total lymphocytes was evaluated in a similar gating fashion as shown in Figure 1 (Fig. 4C).

Each sample was stained twice with the FoxP3 panel (and results averaged), and once with the isotype panel, which was used as a control and for setting FoxP3 regions. There was no significant difference between replicates, demonstrating a very high reproducibility of the staining method (data not shown). Samples were also stained with a sixth color live/dead dye, which was found critical when working with frozen cells, because the freezing/thawing process can result in a decreased viability (mean viability for these samples was 84 $\pm$ 12%) and nonspecific antibody uptake by dead cells. Percent positive and mean fluorescence intensity (MFI) of several subpopulations are shown in Figure 5. The purpose for performing MFI analysis in addition to determining percent positive cells was to evaluate the level of expression of FoxP3 in each cell, which provides information about the amount of FoxP3 expressed by the cells. Certain cell populations differ in their level of FoxP3 expression (such as anergic or activated cells), and this information cannot be concluded from percent positive evaluations alone. We found that the percentage of $CD3^+CD4^+$ of total $CD25^+$ cells that were also $FoxP3^+$ significantly increased in the T1DM samples. MFI values were also significantly higher in the patients with T1DM using this gating strategy. Additionally, the MFI of $CD4^+CD25^{high}FoxP3^+$ cells also increased significantly in the diabetes samples, but this was not reflected in the percent positive cells. We also saw a significant increase in the $CD127^{low}CD25^+FoxP3^+$ population in the diabetes samples by percent and MFI analysis. No significant increase in $CD127^{low}CD25^+FoxP3^+$ cells in the MS samples was seen although expression was higher than healthy controls.

DISCUSSION

Regulatory T (Tregs) cells play a critical role in the maintenance of peripheral tolerance and in the control of autoimmunity. Many previous studies used antibodies against CD4 and CD25 to determine frequencies of the $CD25^{high}$ population that was shown to contain a cell

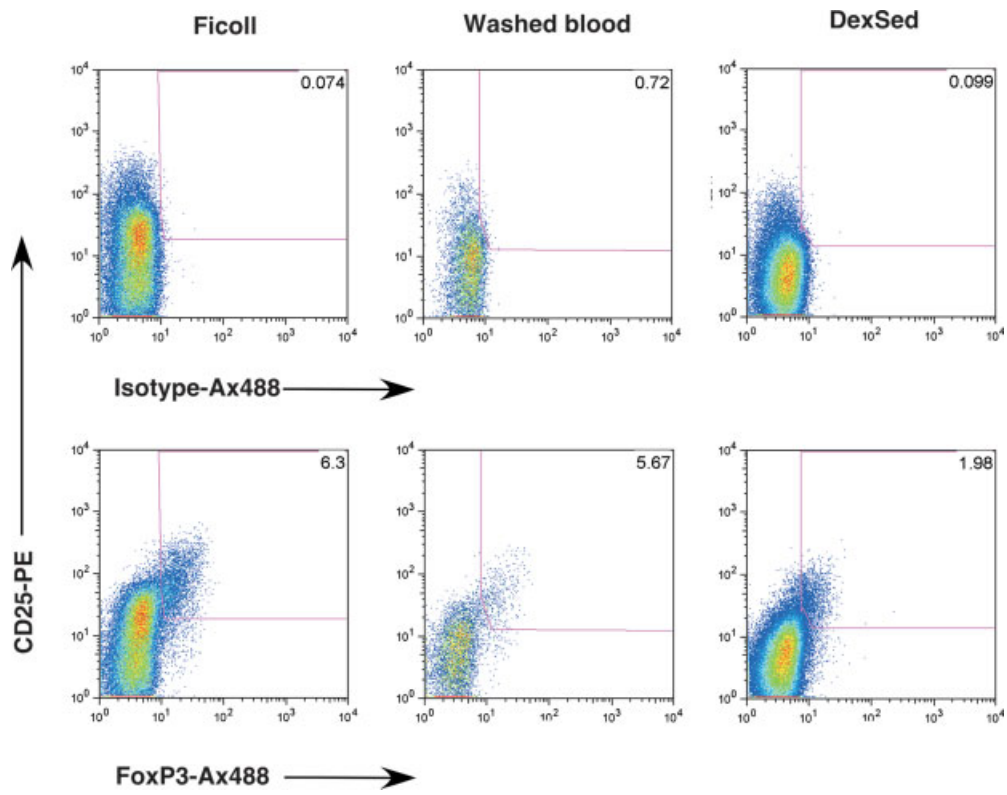

FIG. 2. The effects of different cell preparation methods on FoxP3 staining. Cells from fresh washed blood, cells separated by Ficoll, or blood processed by dextran sedimentation were stained with the following antibody panel: FoxP3-Alexa Fluor488 (clone 206D, BioLegend)/CD25-PE/CD3-PerCP/CD4-APC and its isotype control; eBioscience fix/perm reagents were used. Data are represented as percent positive of the lymphocyte gate. DexSed = dextran seditmentation.

population with regulatory potential (1,2,4,5,29). A transcription factor, FoxP3, has been shown as a key marker for identification of Tregs (13,14). In recent years, several antibodies to FoxP3 have become commercially available and can be used to characterize Tregs. However, there is still no clear consensus as to which antibody and method of staining is most suitable for determining frequencies of FoxP3 expressing cells in fresh

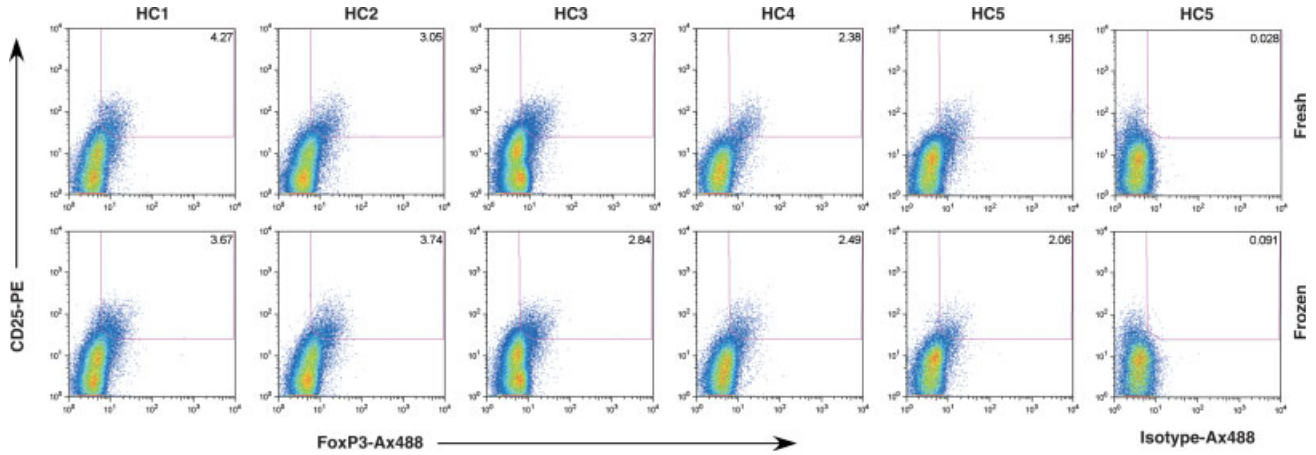

FIG. 3. Evaluation of freshly isolated PBMC and frozen PBMC. Blood from five healthy controls was drawn and cells separated by Ficoll, half of the samples stained immediately and half frozen for 24 hr. Frozen cells were thawed, viability assessed, and stained. Cells were stained with the following panel: FoxP3-Alexa Fluor 488 (clone 206D, BioLegend)/CD25-PE/CD3-PerCP/CD4-APC and isotype control using eBioscience fix/perm reagents. Data are represented as percent positive of the lymphocyte gate. Isotype staining from one sample (donor HC5) is shown. The two-tailed *P* value for the difference between frozen and fresh was 0.8009. HC, healthy control. Top row, fresh samples; bottom row, frozen samples.

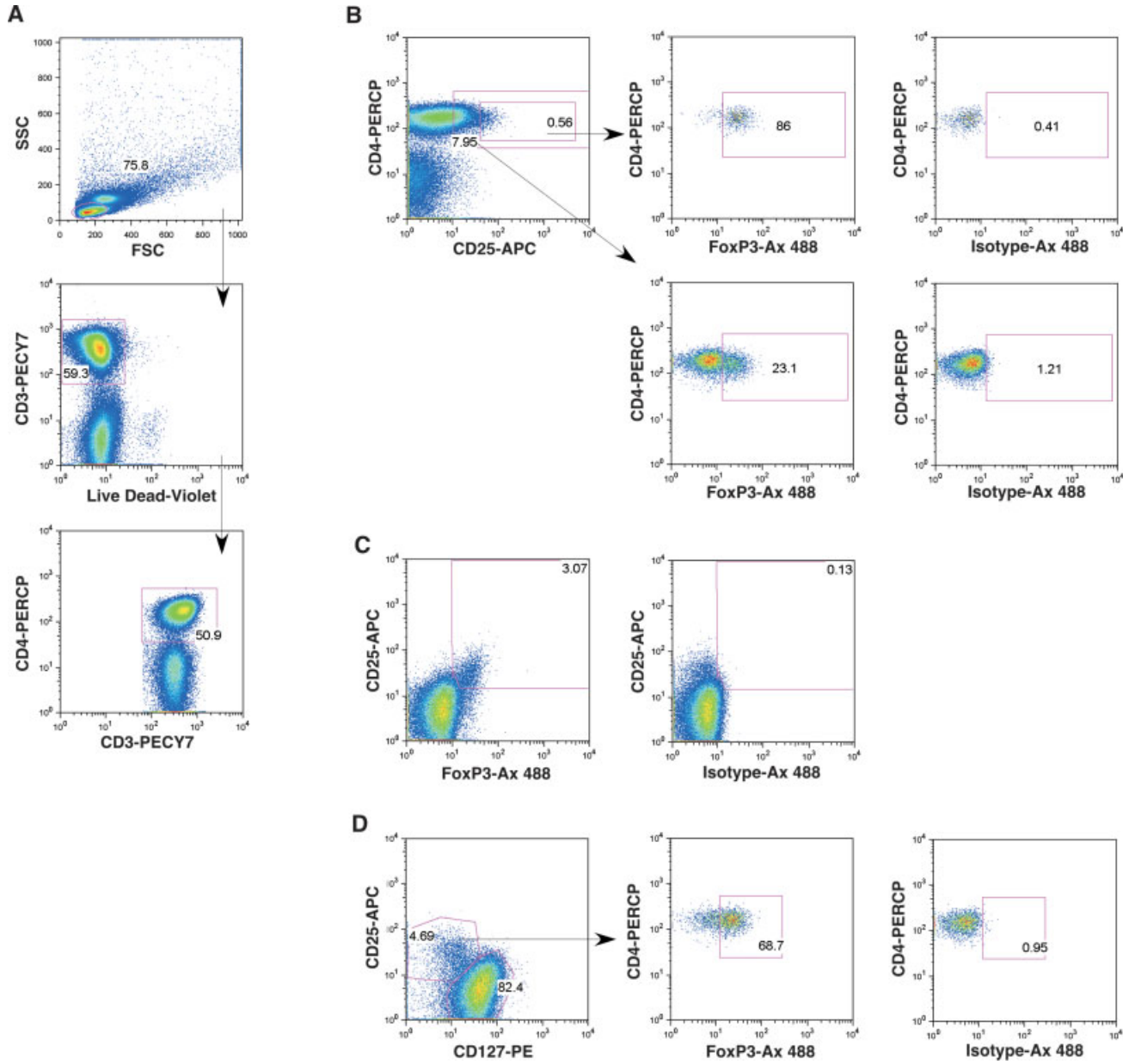

FIG. 4. Gating strategy for clinical specimens in Figure 5. One sample is represented, including isotype control. **A:** Cells were first gated on FSC versus SSC followed by live cells gated for $CD3^+$ and then $CD3^+CD4^+$. Parts B–D show subsequent regions drawn. **B:** $CD3^+CD4^+$ cells were gated on $CD4^+CD25^+$ (bottom row) or $CD4^+CD25^{high}$ (top row). Percent of $FoxP3^+$ cells within each of these populations is shown, with the respective isotype beside each. **C:** The percentage of $CD3^+CD4^+CD25^+FoxP3^+$ cells out of total lymphocytes with the respective isotype control. **D:** The percentage of $CD3^+CD4^+CD127^{low}FoxP3^+$ cells with the respective isotype control.

blood and frozen PBMC samples. Specifically, the widely used PCH101 clone was recently shown to be an unreliable indicator of FoxP3 expression in activated cells (18).

These observations prompted us to examine which of the available antibodies would be the most suitable to monitor frequencies of FoxP3 T regulatory cells with the goal to implement the resulting staining procedure in the clinical trial setting. In the first part of the study, we evaluated various conditions for the optimal FoxP3-staining protocol. These included testing of several antibodies as well as staining procedures. We found that the clone 206D antibody (BioLegend) gave the most favorable staining of Tregs, based on FoxP3 level of expression in the $CD25^{high}$ population. Additionally, we found that the cell fixation and permeablization reagents from eBioscience and BioLegend were comparable in detecting $FoxP3^+$ positive cells in the $CD25^{high}$ population.

When cell preparation methods were evaluated, we found that Ficoll-separated cells and washed blood

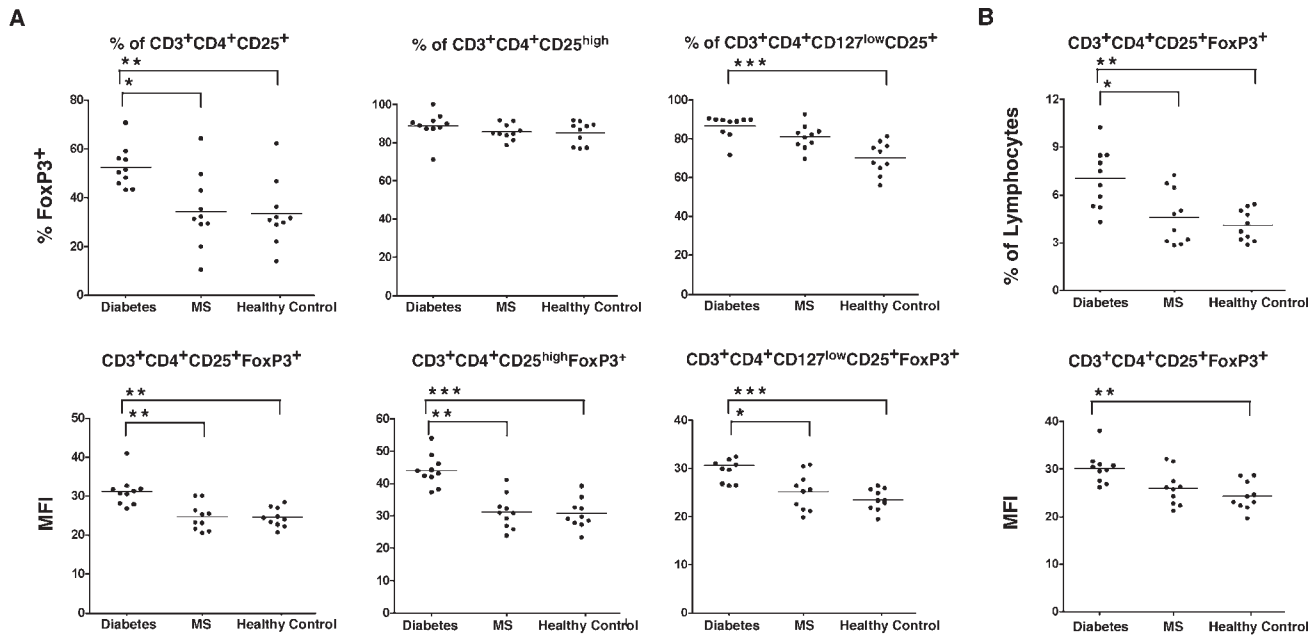

FIG. 5. Assessment of FoxP3 expression on frozen PBMC from MS patients, T1DM patients, and healthy controls. Frozen PBMC were thawed and stained with the following panel: FoxP3-Alexa Fluor 488 (clone 206D, BioLegend)/CD127-PE/CD4-PerCP/CD3-PECY7/CD25-APC and isotype control; eBioscience fix/perm reagents and Molecular Probes Live/Dead violet stain were used. Cells were stained in duplicate with the FoxP3 panel and once with the isotype panel. Isotype values were subtracted from percent positive values. Data are represented as percent positive (top row) or MFI (bottom row) of **(A)** $FoxP3^+$ cells within either $CD3^+CD4^+CD25^+$, $CD3^+CD4^+CD25^{high}$, or $CD3^+CD4^+CD127^{low}CD25^+$ populations, and **(B)** $CD3^+CD4^+CD25^+FoxP3^+$ cells within the lymphocyte gate. The average of the duplicate stains for FoxP3 is shown. T1DM, $n = 10$; MS, $n = 10$; healthy controls, $n = 10$. Significant values: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

yielded comparable results. Additionally, when fresh and frozen PBMCs were evaluated, FoxP3 staining results were comparable. This is a significant finding in that many clinical trials collect samples of blood at numerous sites nationally (or internationally), and the samples are either sent ambient to a central flow cytometry core facility (becasue flow cytometry staining at each site can be highly variable), processed on site into PBMC and frozen, or sent to a central cell processing core facility and processed into PBMC and frozen. Using frozen cells for staining would allow for maintaining effective quality assurance of samples and for more effective designing of experiments. Addition of DNAse I prevented frozen cells from clumping, yielding better thaw quality of the cells and DNAse I addition was inconsequential to FoxP3 staining. Interestingly, another group reported that the addition of DNAse I during the staining process on freshly purified $CD4^+CD25^+$ cells from the periphery might also enable better detection of endogenous FoxP3 (17).

In the second part of the study, we used the finalized staining protocol and antibody panel (BioLegend clone 206D and eBioscience fix/perm reagents) to evaluate FoxP3 expression in frozen PBMCs from patients with stable Relapsing Remitting MS or well-established T1DM. Banham and colleagues showed that roughly 50% of human $CD4^+CD25^+$ cells stained for $FoxP3^+$ and herein, we found ~35–45% of the CD25 population staining positive for FoxP3 (17).

Interestingly, we observed that, in T1DM patients, there was higher expression of FoxP3 in the $CD4^+CD25^+$ population and that finding correlated with increased expression of FoxP3 in individual cells (higher MFI). However, when we addressed the expression of FoxP3 in the $CD4^+CD25^{high}$ population, there was no difference in percent expression in T1DM patients and controls, but the differences remained higher in expression of FoxP3 protein at the cell level (MFI, Fig. 5). It is not known if the level of expression of FoxP3 per cell can be correlated with the level of suppressive activity and this finding needs to be further explored using a functional Treg assay. There was no difference in the level of expression of CD25 on CD4 cells between MS, T1DM, and healthy controls, indicating that activation state as measured by CD25 expression was comparable between subject groups (data not shown). However, the fact that the biggest difference between FoxP3 expression in T1DM patients occurred in the $CD4^+CD25^+$ population but not $CD4^+CD25^{high}$ population might indicate that increased expression could merely reflect a different type of activation state for this patient population. Previously reported findings indicated that there was no significant difference in FoxP3 expression in T1DM patients and healthy controls (15,30). One factor contributing to this discrepancy might be the use of different anti-FoxP3 antibodies. It must also be noted that there are no comprehensive studies comparing recent onset with well-established, longstanding disease in respect to these pop-

ulations and that can also contribute to the differences reported here and other studies. A study by Atkinson and colleagues on a large population of T1DM patients showed no alterations in the frequency of FoxP3 regulatory T-cells in T1DM (30). However, it was recently shown that the FoxP3 antibody used in this study (PCH101) is not reliable for FoxP3 evaluations in humans (18).

As shown in humans by Baecher-Allan et al., the Treg population is mainly confined to $CD4^+CD25^{high}$ expressing cells (3,9). In a study by Viglietta et al., it was shown that there was no difference in the frequency of the $CD4^+CD25^{high}$ population among MS patients and healthy subjects, which correlates with our findings (6). However, after sorting the $CD4^+CD25^{high}$ population and testing its regulatory potential, they found that the regulatory cells display impaired function in patients with MS. In our study, we did not show differences in FoxP3 expression between MS patients and healthy subjects. This stands in opposition to previous reports that FoxP3 expression, as measured by flow cytometry, PCR, and Western blot, actually decreases in MS patients (31,32). However, it is important to note that flow cytometry studies reported so far were done using the PCH101 antibody that as mentioned earlier was shown to have limited applicability to monitoring FoxP3 expression in humans (18). Also, indirect methods used to determine relative expression of FoxP3 such as using PCR amplification or Western blots might not reflect accurate expression levels and require cell sorting.

In summary, we show implementation of an optimized staining protocol for the evaluation of FoxP3 expression in washed blood or isolated PBMC. To our knowledge, it is the first report describing increased FoxP3 expression in T1DM patients. These findings may represent fluctuations in FoxP3 expression that may occur naturally during the course of disease. However, further studies need to be performed to address alteration of FoxP3 expression in a larger group of patients and in longitudinal clinical studies. Our analysis also demonstrates the importance of using percent positive and MFI values for data comparison. Overall, our results stress the necessity of careful assay development procedures that lead to a new method application for clinical trials. Interestingly, evaluation of the clinical samples illustrated the robust application of this panel and staining protocol to a large number of fresh or frozen samples for clinical trials and might be applicable for longitudinal patient assessment during treatment.

ACKNOWLEDGMENTS

We thank Christian Grant for his assistance with data analysis and review of the manuscript and Khadir Raddassi for assistance with blood collection. This research was performed as a project of the Immune Tolerance Network, a collaborative clinical research project headquartered at the University of California San Francisco and partially funded by the National Institute of Allergy and Infectious Diseases, with support from the National Institute of Diabetes, and Digestive and Kidney Disease and the Juvenile Diabetes Research Foundation. Flow cytometry was performed at Roswell Park Cancer Institute's Flow Cytometry Laboratory, which was established in part by equipment grants from the NIH Shared Instrument Program.

LITERATURE CITED

1. St Clair EW, Turka LA, Saxon A, Matthews JB, Sayegh MH, Eisenbarth GS, Bluestone J. New reagents on the horizon for immune tolerance. Annu Rev Med 2007;58:329–346.
2. Shevach EM. CD4+ CD25+ suppressor T cells: More questions than answers. Nat Rev Immunol 2002;2:389–400.
3. Baecher-Allan C, Viglietta V, Hafler DA. Human CD4+CD25+ regulatory T cells. Semin Immunol 2004;16:89–98.
4. Kang SM, Tang Q, Bluestone JA. CD4+CD25+ regulatory T cells in transplantation: Progress, challenges and prospects. Am J Transplant 2007;7:1457–1463.
5. Lee MKt, Moore DJ, Jarrett BP, Lian MM, Deng S, Huang X, Markmann JW, Chiaccio M, Barker CF, Caton AJ, Markmann JF. Promotion of allograft survival by CD4+CD25+ regulatory T cells: Evidence for in vivo inhibition of effector cell proliferation. J Immunol 2004;172:6539–6544.
6. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004;199:971–979.
7. Dejaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. Immunology 2006;117:289–300.
8. Bonelli M, von Dalwigk K, Savitskaya A, Smolen JS, Scheinecker C. Foxp3 expression in CD4+ T cells of patients with systemic lupus erythematosus (SLE): A comparative phenotypic analysis. Ann Rheum Dis 2008;67:664–671.
9. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol 2001;167:1245–1253.
10. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of $CD25^+CD4^+$ regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 2002;3:135–142.
11. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol 2001;19:225–252.
12. Hartigan-O' Connor DJ, Poon C, Sinclair E, McCune JM. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. J Immunol Methods 2007;319:41–52.
13. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330–336.
14. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057–1061.
15. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203:1701–1711.
16. Bacchetta R, Passerini L, Gambineri E, Dai M, Allan SE, Perroni L, Dagna-Bricarelli F, Sartirana C, Matthes-Martin S, Lawitschka A, Azzari C, Ziegler SF, Levings MK, Roncarolo MG. Defective regulatory and effector T cell functions in patients with FOXP3 mutations. J Clin Invest 2006;116:1713–1722.
17. Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, Pratap S, Toms C, Fox BC, Cerundolo V, Powrie F, Banham AH. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. Eur J Immunol 2005;35:1681–1691.
18. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-β dependent but does not confer a regulatory phenotype. Blood 2007;110:2983–2990.
19. Nicholson JK, Jones BM, Cross GD, McDougal JS. Comparison of T and B cell analyses on fresh and aged blood. J Immunol Methods 1984;73:29–40.

20. Hensleigh PA, Waters VB, Herzenberg LA. Human T lymphocyte differentiation antigens: Effects of blood sample storage on Leu antibody binding. Cytometry 1983;3:453–455.
21. Thornthwaite JT, Rosenthal PK, Vazquez DA, Seckinger D. The effects of anticoagulant and temperature on the measurements of helper and suppressor cells. Diagn Immunol 1984;2:167–174.
22. Muirhead KA, Wallace PK, Schmitt TC, Frescatore RL, Franco JA, Horan PK. Methodological considerations for implementation of lymphocyte subset analysis in a clinical reference laboratory. Ann NY Acad Sci 1986;468:113–127.
23. Stewart CC, Stewart SJ. Multiparameter data acquisition and analysis of leukocytes by flow cytometry. Methods Cell Biol 2001;64:289–312.
24. Stewart CC, Stewart SJ. Cell preparation for the identification of leukocytes. Methods Cell Biol 2001;63:217–251.
25. Wood BL, Levin GR. Interactions between mouse IgG2 antibodies are common and mediated by plasma C1q. Cytometry B Clin Cytom 2006;70:321–328.
26. Nicholson JK, Rao PE, Calvelli T, Stetler-Stevenson M, Browning SW, Yeung L, Marti GE. Artifactual staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. Cytometry 1994;18:140–146.
27. Ekong T, Gompels M, Clark C, Parkin J, Pinching A. Double-staining artefact observed in certain individuals during dual-colour immunophenotyping of lymphocytes by flow cytometry. Cytometry 1993; 14:679–684.
28. Fairhurst AM, Connolly JE, Hintz KA, Goulding NJ, Rassias AJ, Yeager MP, Rigby W, Wallace PK. Regulation and localization of endogenous human tristetraprolin. Arthritis Res Ther 2003;5:R214–R225.
29. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 2006;212:8–27.
30. Brusko T, Wasserfall C, McGrail K, Schatz R, Viener HL, Schatz D, Haller M, Rockell J, Gottlieb P, Clare-Salzler M, Atkinson M. No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes 2007;56:604–612.
31. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL, Medaer R, Hupperts R, Stinissen P. Compromised $CD4^+$ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. Immunology 2007;123:79–89.
32. Huan J, Culbertson N, Spencer L, Bartholomew R, Burrows GG, Chou YK, Bourdette D, Ziegler SF, Offner H, Vandenbark AA. Decreased FOXP3 levels in multiple sclerosis patients. J Neurosci Res 2005;81:45–52.