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The importance of Foxp3 antibody and fixation/permeabilization buffer combinations in identifying CD4+CD25+Foxp3+ regulatory T cells

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Abstract

Background—Foxp3 is a key marker for $CD4^+$ regulatory T cells (T_{regs}) and was utilized in developing a multiparameter flow cytometric panel to identify T_{regs}. Achieving reproducible staining and analysis first required optimization of Foxp3 staining.

Methods—We present a comparative study of PCH101, 236A/E7, 3G3, 206D, 150D, and 259D/C7 clones of anti-human-Foxp3 antibodies, used in combination with five different fixation/permeabilization buffers. Staining for CD25, CD152, and CD127 was also compared between fixation/permeabilization treatments. Promising antibody/buffer combinations were tested in a panel of PBMCs from 10 individuals, then on fresh versus frozen cells from four individuals. Finally, different fluorochromes coupled to two representative antibodies were compared to optimize separation of $Foxp3^+$ from $Foxp3^-$ events. Foxp3 gates were set using two gating strategies, based on $CD127^+CD25^-$ "non-T_{regs}" or based on isotype controls.

Results—For Foxp3 staining the best conditions for fixation/permeabilization were obtained using the eBioscience Foxp3, Imgenex, BioLegend, and BD Foxp3 buffers. Comparing results from 10 subjects, 259D/C7, PCH101, 236A/E7, and 206D antibodies yielded statistically higher levels of Foxp3 cells than 150D and 3G3 antibodies (mean=6.9, 5.1, 4.7, and 3.7% compared to 1.7, and 0.3% of $CD25^+Foxp3^+$ events within $CD4^+$ cells, respectively). Importantly, the "non-specificity" of some antibodies observed with a Foxp3 gate based on isotype controls could be eliminated by setting the Foxp3 gate on "non-T_{regs}". Better separation of $Foxp3^+$ and $Foxp3^-$ populations was observed using the PCH101 clone coupled to Alexa647 compared to FITC, or the 259D/C7 clone coupled to PE compared to Alexa488 fluorochrome.

Conclusions—Foxp3 staining can be highly variable and depends on the choice of antibody/buffer pair and the fluorochrome used. Selecting the correct population for setting the Foxp3 gate is critical to avoid including non-T_{regs} in the $Foxp3^+$ gate. The experiments presented here will aid in optimization of flow cytometry staining panels to quantify T_{reg} frequencies in humans.

Keywords

Foxp3 staining; Anti-human Foxp3 antibodies; PCH101; 259D/C7; 236A/E7; 3G3; 206D; 150D; Regulatory CD4+ T cells

INTRODUCTION

$CD4^+$ regulatory T cells (T_{reg} cells) suppress effector T cells and prevent or limit reactivity to self-antigens (1) and pathogens (2), blunt inflammation, and maintain antigen-specific T cell homeostasis (3). Multiple T_{reg} cell populations have been reported (3), with natural T_{reg} cells the best characterized (4). Natural T_{reg} cells constitutively express CD25 (IL-2-receptor α-chain) (5), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) (6), and the forkhead/winged helix transcription factor Foxp3, which is a key control gene in their development and function (7). While no single marker uniquely identifies T_{reg} cell populations, panels of markers allow identification of $CD4^+$ T cells with suppressive activity. T_{reg} cells are often defined as $CD3^+CD4^+CD25^+Foxp3^+$ cells (5-9). The expression of intracellular CTLA-4 and Foxp3 is positively correlated with surface CD25 expression (10). Higher levels of intracellular Foxp3 and CTLA-4 and lower levels of surface CD127 consistently distinguish $CD25^{hi}$ cells from $CD25^{int/low}$ and $CD25^-$ cells (11). More recent studies demonstrated that T_{reg} cells also express low levels of CD127 marker (IL-7 receptor) (11,12).

Our laboratory focuses on the role of T cell responses in controlling viral infections (13-15) and the ability of the immune system to reject donor white blood cells during blood transfusion (16). Regulatory T cells likely play an important role in both these processes. The materials used in many immunology studies reside in repositories of frozen peripheral blood mononuclear cells (PBMCs) derived from study subjects, so we aimed to optimize T_{reg} cell identification in frozen blood samples. After blood collection, PBMCs were isolated on Ficoll-Paque and frozen to be further used in batches for flow cytometry studies. We present here the results of optimization studies on Foxp3 staining that were performed on healthy normal controls to ensure the reproducibility of the staining, acquisition, and analysis before quantifying T_{reg} cells in frozen PBMCs from infected study subjects. In early studies, few clones of anti-human Foxp3 antibodies were available, with the PCH101 clone from eBioscience (San Diego, CA) the most frequently used in published studies (17-20). Prior publications noted concerns about the specificity of Foxp3 staining using clones such as the PCH101 antibody from eBioscience (21,22), and other antibodies have been recommended (23). In our system, we noted several levels of variability in the results obtained using different clones of Foxp3 antibodies coupled to different fluorochromes and even using antibodies paired with different fixation/permeabilization buffers to stain PBMC aliquots isolated from the same donor. To determine the optimal Foxp3 staining conditions, we adopted a three-step process. First, we compared commercially available kits.. Second, we quantified the variability in the staining attributable to each of the commercially available buffer sets. Third, we observed the quality of the separation of the $Foxp3^+$ population from the $Foxp3^-$ population after staining of fresh versus frozen PBMCs, or when using different Foxp3 antibody-fluorochrome combinations.

To optimize Foxp3 staining in our system, six different anti-human Foxp3 clones (PCH101, 236A/E7, 3G3, 206D, 150D, 259D/C7) used with five different buffer sets from five vendors were compared in a matrix format. Our systematic review of commercially available antibody/buffer pairs showed that the variability of the staining depended not only on the clone of anti-Foxp3 antibody, but also on the staining buffer composition and fluorochrome used.

METHODS

Human Cells

PBMCs were isolated from peripheral blood on a Ficoll-Paque PLUS density gradient (GE Healthcare Bio-Sciences, Piscataway, NJ). Aliquots of 10×10^6 cells were frozen in media that contained 90% fetal bovine serum (HyClone, Logan, UT) and 10% dimethyl sulfoxide (DMSO, Fisher Bio-Reagents, Pittsburg, PA) and stored in liquid nitrogen vapor.

Multiparameter Flow Cytometric Studies

Antibodies—Six multiparameter panels of monoclonal antibodies were used to stain PBMCs for T_{reg} cells. The antibodies to surface markers consisted of the following antibodies: anti-CD4-Alexa Fluor 700 (Alexa700) (clone RPA-T4), anti-CD25-PE-Cy7 (clone M-A251 or clone BC69 for staining using the BioLegend buffer set), and anti-CD127-PE (clone hIL-7R-M21) (all from BD Pharmingen, San Diego, CA). The antibodies to intracellular markers consisted of the following: anti-CD3-Pacific Blue (PB) (clone UCHT1), anti-CD152-APC (clone BNI3) (both from BD Pharmingen), and anti-Foxp3-FITC or anti-Foxp3-Alexa Fluor 488 (Alexa488). Each of the six panels had one of the following anti-Foxp3 antibodies: FITC-conjugated clones included PCH101 and 236A/E7 (eBioscience), 3G3 (Imgenex, San Diego, CA), and 206D (BioLegend, San Diego, CA). The Alexa488-conjugated Foxp3 clones were 259D/C7 (BD Pharmingen) and 150D (BioLegend). Each antibody was titrated using serial dilutions starting with the concentration recommended by the vendor using the corresponding buffer system, and the optimal concentration (or volume) determined by each titration matched the vendor's recommended concentration. Additional antibody-fluorochrome combinations were tested at the end of the study as the PCH101 clone coupled to Alexa488, PE and Alexa647 fluorochromes and the 259D/C7 clone coupled to PE and Alexa647 became commercially available. Each of these antibodies was used at the recommended concentration (or volume). Fluorescence minus one (FMO) controls were stained in parallel using the panel of antibodies with sequential omission of one antibody, with the exception of the anti-Foxp3 antibody, which was replaced by an isotype control rather than simply omitted. One million cells were used for each test. The LIVE/DEAD® aqua amine-reactive dye (1 μl of a 1/10 dilution, Invitrogen, Carlsbad, CA) was used to stain dead cells in a 10-minute incubation protected from light, followed by addition of antibodies to surface markers and incubation for 20 minutes at 4°C. Cells were washed twice in PBS before fixation and permeabilization.

Fixation and permeabilization protocols

Foxp3 Staining Buffer Sets from eBioscience (eBio-Foxp3) and Imgenex: Following viability and surface staining, 1 ml of freshly prepared fixation/permeabilization working solution was added to each sample, pulse vortexed and incubated at 4°C for 60 minutes in the dark. Cells were washed twice with 2 ml of permeabilization buffer. Antibodies to intracellular markers were added in 100 μl of permeabilization buffer, incubated at 4°C for 45 minutes in the dark, and then followed by two washes with 2 ml of permeabilization buffer. Cells were fixed in 1% paraformaldehyde (PFA) prior to flow acquisition.

FOXP3 Fix/Perm Buffer Set from BioLegend (BioLegend): Following viability and surface staining, 1 ml of BioLegend's FOXP3 Fix/Perm solution was added to each tube, vortexed and incubated at room temperature in the dark for 20 minutes. Cells were washed once with PBS followed by a second wash with 1 ml of BioLegend's FOXP3 permeabilization buffer. Cells were resuspended in 1 ml of BioLegend's FOXP3 permeabilization buffer, incubated at room temperature in the dark for 15 minutes then centrifuged and the supernatant was discarded. Antibodies to intracellular markers were added to the 100 μl remaining in the tube and incubated for 30 minutes at room temperature protected from light. After two washes in PBS, cells were fixed in 1% PFA prior to flow acquisition.

Human Foxp3 Buffer set from BD Pharmingen (BD-Foxp3): Following viability and surface staining, 2 ml of freshly prepared human Foxp3 buffer A was added to each sample, pulse vortexed and incubated at room temperature for 10 minutes. Cells were washed twice with 2 ml of PBS. Cells were permeabilized during 30 minutes of incubation at room temperature in 500 μl of freshly prepared human Foxp3 buffer C, protected from light. Then cells were washed twice with 2 ml of PBS and antibodies to intracellular markers were added

to 100 μl of PBS. A 30-minute incubation at room temperature in the dark was followed by two washes with 2 ml of PBS. Cells were re-suspended in 1% PFA prior to flow acquisition.

Caltag Fix and Perm cell permeabilization kit (Invitrogen): Following viability and surface staining, 100 μl of Fix/Perm medium A was added to each sample, pulse vortexed and incubated at room temperature for 15 minutes in the dark. Cells were washed twice with 2 ml of PBS. Antibodies to intracellular markers were added to cells in 100 μl of permeabilization medium B, incubated for 20 minutes at room temperature protected from light. Cells were washed twice with 2 ml of PBS. Cells were fixed in 1% PFA prior to flow acquisition.

Data collection—List mode data files of at least 50,000 and usually 100,000 gated viable lymphocytes were collected for each sample. A 3-laser (20 mW Coherent Sapphire 488nm blue, 25 mW Coherent Vioflame 405nm violet, and 17 mW JDS Uniphase HeNe 633nm red) LSR II benchtop flow cytometer equipped with BD FACS Diva v5.0.2 software with the filter configuration noted in Table 1 was used for acquisition.

Calibration—Instrument set-up was standardized to reduce batch-to-batch (or experiment-to-experiment) variation. Optimal cytometer photomultiplier tube (PMT) voltages were initially determined for each detector and filter set using dimly fluorescing Rainbow Fluorescent Particles (peak 2 of Rainbow Calibration Particles, Spherotech, Inc.), after which target channels were determined using mid range FL1 Rainbow Fluorescent Particles (BD Biosciences) (24,25). Prior to each experiment, mid-range FL1 Rainbow Fluorescent Particles were run at the pre-optimized detector PMT voltages to ensure that the pre-determined baseline PMT voltages were appropriately set, that the predetermined target channels were met. Minor adjustments were made to PMT voltages prior to each experiment's data acquisition to ensure that the target channels were maintained, and that no instrument drift was observed.

Compensation—Compensation controls were created for each fluorochrome in the T_{reg} panels. Unstained cells that were fixed and permeabilized with the same protocol as respective test samples were used as unstained compensation controls. Fixed and permeabilized heat-killed cells and viable cells were stained as compensation controls for the aqua amine-reactive dye. BD CompBeads (BD Bioscience) were stained as compensation controls for Pacific Blue, A700, A488, A647, and PE-Cy7. CaliBrite beads (BD Bioscience) were used for PE, APC and FITC controls. Single-stained compensation tubes were checked to ensure each stain was the brightest in its own channel prior to acquisition of 10,000 events per compensation tube. Compensation controls were run and compensation values were determined by FACSDiva v5.0.2 Software.

Flow cytometric Data Analysis

FCS (Flow Cytometry Standard format) 3.0 files were exported and data were evaluated using FlowJo (Treestar US, Mac-Version 8.7.3, Ashland, OR). Lymphocytes were gated based on SSC/FSC characteristics. Dead cells were excluded if aqua amine-reactive dye$^+$. $CD3^+CD4^+$ cells were gated within the viable lymphocyte gate. $CD25^+$, $CD152^+$ and $CD127^-$ gates were set using FMO control tubes. The $Foxp3^+$ gate was set using the isotype control or based on "non-T_{reg}" $CD127^+CD25^-CD4^+$ T cells. T_{reg} cells were gated as $CD25^+Foxp3^+$ events as a percentage of $CD3^+CD4^+$ cells, and the expression of CD152 and CD127 markers were quantified within $CD3^+CD4^+CD25^+$ cells.

Statistical Analysis

The Kruskal-Wallis test followed by the Dunn's multiple comparison test were used to evaluate differences in the mean percentage of $CD25^+Foxp3^+$ cells stained with the six Foxp3 antibody clones across 10 individuals. The Student t-test for paired comparisons was used to compare

the mean percentages of $CD25^{-}Foxp3^{+}$ or $CD25^{+}Foxp3^{+}$events within fresh versus frozen PBMCs from four donors stained by each of the six Foxp3 antibody clones. Statistical significance was defined as $p < 0.05$.

RESULTS

The present study first compared different Foxp3 antibody/buffer pairs using frozen PBMC aliquots from a single donor. Results were confirmed using frozen PBMCs from a second donor to ensure the consistency of the results. Once antibody/buffer combinations were determined, the six Foxp3 antibodies were compared using frozen cells from 10 normal control donors. Then the six antibodies were compared using fresh versus frozen cells from four normal control donors. Finally, different fluorochromes coupled to the two antibodies providing the highest staining were compared for clarity of the separation between $Foxp3^{+}$ and $Foxp3^{-}$ populations. The first series of experiments compared seven pairs of anti-Foxp3 antibodies and fixation/permeabilization.

Comparison of Foxp3 staining kits

The PCH101 and 236A/E7 clones of anti-Foxp3 from eBioscience were used with the Foxp3 Staining Buffer Set from eBioscience. The 3G3 clone from Imgenex was used with the Foxp3 Flow Intracellular Staining Buffer Set from Imgenex. The 206D and 150D clones from BioLegend were used with the FOXP3 Fix/Perm Buffer Set from BioLegend. The 259D/C7 clone from BD Pharmingen was used with two different buffers: the BD Pharmingen Human Foxp3 Buffer set and the Caltag Fix and Perm cell permeabilization kit from Invitrogen.

We first noted that the fixation and permeabilization treatment could affect the SSC/FSC scatter characteristics of the cells, particularly the eBioscience buffer set (Fig. 1, row 1). Dead cells were excluded if stained with the aqua amine-reactive dye, and viable $CD3^{+}$ events were included in the gate (Fig. 1, row 2). The CD3 staining was consistent, with 74 to 78% of $CD3^{+}$ events in the viable lymphocyte gate (Fig. 1, row 2). The CD4 staining was also consistent with 63 to 73% of $CD4^{+}$ events in the $CD3^{+}$ gate (data not shown). On the contrary, the staining for CD25, CD127 and CD152 was highly variable and dependent on the combination anti-CD25 antibody-buffer set used, with 4 to 35% of $CD4^{+}$ cells staining positive for CD25 (Fig. 1, row 3). The highest proportion of $CD152^{+}CD127^{-}$ events within the $CD25^{+}$ gate was found when the CD25 staining was the lowest (Fig. 1, rows 3 and 4) using the BioLegend buffer, likely due to the fact that only the brightest $CD25^{+}$ events were deemed positive using that buffer set. $CD25^{hi}$ events are known to be $CD152^{+}$ (10).

Finally and most importantly, Foxp3 staining was heterogeneous using the cells from the same donor, depending upon the kit used. The $CD25^{+}Foxp3^{+}$ gate was set based on FMO controls for the CD25 staining and on $CD127^{+}CD25^{-}$ "non-T_{reg}" cells for Foxp3 (Fig. 1, rows 5-7). $CD4^{+}$ T cells were analyzed for their CD127 and CD25 expression and a gate based on CD127 and CD25 FMO controls was set on the $CD127^{+}CD25^{-}$ $CD4^{+}$ T cells (Fig. 1, row 5). The Foxp3 gate was set on the $CD127^{+}CD25^{-}$ cells (Fig. 1, row 6) and further applied to the general $CD4^{+}$ T cell population (Fig. 1, row 7). Results ranged from 0.44 to 2.8% $CD25^{+}Foxp3^{+}$ events within the $CD4^{+}$ gate (Fig. 1, row 7). The PCH101 clone used with the eBioscience Foxp3 buffer yielded the highest number of $Foxp3^{+}$ events (2.8%, Fig. 1, row 7, first column). Staining with the 259D/C7 clone used in combination with the BD Foxp3 buffer yielded the second highest proportion of positive events (1.8%, Fig. 1 row 7 column 6). The same 259D/C7 clone used with the Caltag buffer yielded lower staining (0.44%, Fig. 1 row 7 last column), highlighting the need for efficient fixation and permeabilization conditions, as the Caltag buffer is not specifically designed for Foxp3 staining. The lowest Foxp3 staining was obtained with the Imgenex kit (0.24%, Fig. 1 row 7 column 3). Increasing the amount of 3G3 antibody only increased background staining, with a shift of the whole $CD4^{+}$ population toward the

Foxp3$^+$ gate (data not shown). The BioLegend sets gave lower numbers of Foxp3$^+$ events than the 259D/C7 antibody used with the BD Foxp3 buffer set, with 1.2 and 0.51% compared to 1.8% of the CD4$^+$ events falling in the CD25$^+$Foxp3$^+$ gate (Fig. 1, row 7 columns 4-6).

Comparison of the anti-Foxp3 antibodies used in different fixation and permeabilization conditions

In a second set of experiments, each of the six anti-Foxp3 antibodies were used in combination with each of the fixation/permeabilization buffer sets previously described (Fig. 2A). Using a CD25$^+$Foxp3$^+$ gate based on FMO controls for the CD25 staining and on CD127$^+$CD25$^-$ "non-T_{reg}" CD4$^+$ T cells for the Foxp3 staining, the level of Foxp3 staining was compared across the different staining conditions and overall, the best antibody-buffer combination was the 259D/C7 clone from BD Pharmingen used in combination with the eBioscience buffer (4.4%) (Fig. 2A, row 6, column 1). The PCH101 clone used with the Imgenex (3.2%), eBioscience (2.8%), and BD-Foxp3 (2.0%) buffers followed (Fig. 2A, row 1, columns 1, 2, and 4). Then the 259D/C7 clone used with the BD-Foxp3 (1.8%) and BioLegend (1.6%) buffers yielded high staining as well (Fig. 2A, row 6). The 236A/E7 clone from eBioscience gave the best staining when used with either the BioLegend buffer (1.4%) or its own buffer (1.2%) (Fig. 2A, row 2 columns 1 and 3). The staining obtained with the 3G3 clone from Imgenex was low overall regardless of which buffer was used (from 0.09 to 0.45%) (Fig. 2A, row 3). The 206D clone from BioLegend gave the highest staining when used with its own buffer (1.2%), then with the BD-Fox3 buffer (0.97%), and Imgenex buffer (0.73%) (Fig. 2A, row 4). The best staining using the 150D clone from BioLegend was obtained with the Imgenex buffer (0.74%), the eBioscience buffer (0.54%), then the BioLegend buffer (0.51%), and the BD-Foxp3 buffer (0.49%) (Fig. 2A, row 5). All other buffers used with these two BioLegend clones gave lower staining. The use of the Caltag buffer resulted in the lowest levels of Foxp3 staining (from 0.02 to 0.44%, Fig. 2A, column 6).

Comparison of anti-Foxp3 antibodies used to stain frozen PBMCs from 10 individuals

In a third set of experiments, we used all clones of anti-Foxp3 antibodies in combination with the eBioscience buffer to stain PBMCs from 10 different individuals (Fig. 2B). Across the 10 individuals, the highest levels of Foxp3 staining were found using 259D/C7 clone (mean = 6.9%), then PCH101 clone (mean = 5.1%), and 236A/E7 clone (mean = 4.7%). The 206D clone yielded intermediate levels of Foxp3 staining (mean = 3.7%), and the 150D clone yielded lower levels of Foxp3 staining (mean = 1.7%, $p<0.001$ for 150D vs. 259D/C7). Overall, Clone 3G3 yielded the lowest levels of Foxp3 staining (mean = 0.3%, $p<0.0001$ for 3G3 vs. PCH101, 259D/C7, or 236A/E7 and $p<0.001$ for 3G3 vs 206D) (Fig. 2B). These results are consistent with published reports of T_{reg} cell frequency in healthy subjects, ranging from 1.5 to 4.5% of CD4$^+$ T cells (26-31). Notably, the same two individuals had the highest (• symbol for donor 1) and lowest (▲ symbol for donor 10) frequencies of Foxp3$^+$ cells across the different antibody conditions, with the relative order of intermediate frequencies mostly consistent as well.

Technical challenges of Treg identification

Variability of CD25 staining depending on the buffer system used—As mentioned earlier, staining for CD25 was highly variable and dependent on the combination of anti-CD25 antibody and buffer set used, with 4.2 to 35.3% of CD4$^+$ cells staining positive for CD25 (Fig. 1, row 3). The lowest level of CD25 staining was observed when the M-A251 clone of CD25 antibody was used in combination with the BioLegend buffer set. To address the possible interference of the BioLegend buffer set with clone M-A251 of CD25 antibody, a comparative study of CD25 staining was done using clone BC96 (from BioLegend) (Fig. 3A, lower panel) in parallel with clone M-A251 (Fig. 3A, upper panel). CD25 staining improved using the BC96 clone provided by BioLegend (from 1.4 to 13.2% of CD4$^+$ cells being CD25$^+$) (Fig. 3A, left

dot-plots). The rest of the analysis was changed accordingly, with lower levels of $CD152^+CD127^-$ events (from 41% of $CD25^+$ events with M-A251 to 21% with BC96) and higher levels of $CD127^+CD152^-$ events (from 32 to 51% of $CD25^+$ cells) obtained with the BC96 clone compared to the M-A251 clone (Fig. 3A, middle dot-plots). The percentage of $Foxp3^+CD25^+$ events within the $CD4^+$ population increased considerably as well (Fig. 3A, right dot-plots, from 0.68 to 2.7%). These observations highlight the need to validate staining panels prior to study commencement and the need to maintain consistent buffer conditions within a study.

"Non-specificity" of some clones of anti-Foxp3 antibodies with a Foxp3 gate based on isotype controls—The results obtained and displayed in this paper were analyzed using a Foxp3 gate based on $CD127^+CD25^-$ $CD4^+$ T cells known to be "non- T_{reg}" cells. However, and even though the use of isotype controls is controversial, the Foxp3 gate could have been set based on isotype controls. Analyzing the same samples using a $Foxp3^+$ gate set on isotype controls provided slightly different results (Fig. 3B). Depending on the Foxp3 antibody-buffer set combination used, when the Foxp3 gate was set based on isotype controls, a shift of the $CD4^+$ population toward the $CD25^+Foxp3^+$ gate was observed and could have been interpreted as "non-specific" staining. This observation was made when using the PCH101 clone from eBioscience with every buffer set, with $CD25^+Foxp3^+$ events ranging from 6.1 to 11.3% of $CD4^+$ T cells (Fig. 3B, columns 1-5) compared to 0.12 to 3.2% of $CD4^+$ T cells when results were analyzed with a gating strategy based on $CD127^+CD25^-$ "non- T_{reg}" $CD4^+$ T cells (Fig. 2, row 1, columns 1-5). The same observation was made when using clone 259D/C7 from BD Pharmingen with the Imgenex buffer (22% versus 1.4% comparing Fig. 3B, column 6 to Fig. 2, row 6, column 2). No shift of the negative population toward the positive gate was observed using any of the other antibody-buffer set combinations for which the Foxp3 gates could be reproducibly set based on isotype controls (data not shown). A gating strategy based on $CD127^+CD25^-$ "non- T_{reg}" $CD4^+$ T cells, instead of on isotype controls, appeared to eliminate the "non-specificity" of certain clones of Foxp3 antibody.

Comparison of anti-Foxp3 antibodies used to stain fresh versus frozen PBMCs from four individuals

PBMCs were isolated from four normal control donors: half of the cells were harvested for overnight freezing when fresh cells were immediately stained with all Foxp3 antibodies used in combination with the eBioscience Foxp3 buffer. Frozen cells were thawed the following day and processed in the same way as fresh cells. The acquisition of the fresh and frozen stained cells was performed on the same day on the LSRII. A side-by-side analysis of the frequencies of $Foxp3^+$ events collected in the $Foxp3^+CD25^+$ and $Foxp3^+CD25^-$ gates is presented in Figure 4. The PCH101 clone yielded higher frequencies of $Foxp3^+$ events collected in the $Foxp3^+CD25^+$ gate in frozen cells than in fresh cells (p=0.03). The 3G3 clone on the contrary yielded higher frequencies of $Foxp3^+$ events collected in the $Foxp3^+CD25^+$ gate in fresh cells than in frozen cells (p=0.005). The 236A/E7 clone yielded slightly higher frequencies of $Foxp3^+$ events collected in the $Foxp3^+CD25^-$ gate in frozen cells than in fresh cells (p=0.05). No other significant differences were observed when comparing the other clones using fresh versus frozen cells.

Comparison of the Foxp3 staining using PCH101 and 259D/C7 clones coupled to different fluorochromes

Different fluorochromes coupled to PCH101 antibodies (PCH101-FITC, -Alexa488, -PE, and -Alexa647) from eBioscience and 259D/C7 antibodies (259D/C7-Alexa488, -PE, and -Alexa647) from BD Pharmingen were compared for the staining of fresh and frozen PBMCs from four normal donors (Fig. 5). The separation between $Foxp3^+$ and $Foxp3^-$ populations was better when PCH101 was used to stain fresh cells versus frozen cells (p value fresh vs. frozen

for the ratio $MFI_{Foxp3+}/MFI_{Foxp3-}$ < 0.05 for PCH101-PE, and Alexa647). However, separation of $Foxp3^+$ and $Foxp3^-$ populations was observed when PCH101 antibodies were used on fresh as well as on frozen PBMCs, with a significantly clearer separation observed when PCH101 clone was coupled to Alexa647 compared to PE or FITC (Fig. 5A, $MFI_{Foxp3+}/MFI_{Foxp3-}$ ratio for fresh or frozen PCH101-Alexa647 vs. –FITC, $p < 0.0001$; fresh PCH101-Alexa647 vs. PE, $p < 0.05$; fresh PCH101-PE vs. - FITC, $p < 0.05$; frozen PCH101-Alexa647 vs. PE, $p < 0.001$). The separation between $Foxp3^+$ and $Foxp3^-$ populations was better when 259D/C7 was used to stain frozen cells versus fresh cells (p value fresh vs. frozen for the ratio $MFI_{Foxp3+}/MFI_{Foxp3-}$ < 0.05 for 259D/C7-Alexa647).Staining using the 259D/C7 clone coupled to PE resulted in significantly better separation of Foxp3 events than staining using the 259D/C7 clone coupled to Alexa488 or Alexa647 (Fig. 5B, $MFI_{Foxp3+}/MFI_{Foxp3-}$ ratio fresh or frozen 259D/C7-PE vs. -Alexa488 or -Alexa647, $p < 0.05$).

DISCUSSION

The purpose of this study was to determine the best combination of antibody and fixation/permeabilization buffer for the staining of the Foxp3 transcription factor in cryo-preserved PBMCs from blood donors. T_{reg} cells have been described as $CD4^+CD25^{hi}$ T cells expressing most specifically the forkhead/winged helix transcription factor gene *Foxp3* (7). However, no marker can be used to uniquely define the T_{reg} subset in humans, and additional studies have demonstrated the importance of markers such as CD152 and CD127 in identifying functional T_{reg} cells (6,11,12). The T_{reg} panel used in this study consisted of all of the above markers. While trying to assemble this complex panel, optimization steps were undertaken and highlighted the need for a larger comparative study of the commercially available Foxp3 staining kits.

A few general conclusions resulted from this study. First, depending on the kit or the antibody used, the variability in the Foxp3 staining was striking. This highlighted the need to use the same staining conditions over the course of a particular study, and especially necessary when measuring inter- or intra-donor variation over time. Second, not only Foxp3 staining, but also CD25, and consequently CD127 and CD152 staining, differed depending on assay conditions; using the BioLegend buffer, CD25 staining using the M-A251 clone was lower compared to the staining obtained with other buffers, with only the brightest CD25 events collected in the $CD25^+$ gate. As a consequence, the CD152 staining among $CD25^+$ events was higher, as only $CD25^{high}$ events were collected and the expression of CD152 is increased on $CD25^{high}$ events (29,32). Third, in order to ensure quality of results and objectivity of interpretation, multiparameter flow cytometric immunophenotyping studies require reproducible staining conditions, standardized instrument calibration processes (24,25), and consistent gating strategies. The different gating approaches used to set the $Foxp3^+$ gate in this study illustrate the importance of the gating strategy on subsequent data analysis and interpretation. While it was possible to set the $Foxp3^+$ gate based on an isotype control for most of the antibodies used with most of the buffer sets, it was impossible to achieve reproducibility and specificity of this gate set using an isotype control with the PCH101 clone no matter the buffer combination used. This observation was reported by others (22,23). The same phenomenon was observed when the 259D/C7 antibody was used with the Imgenex or BioLegend buffer sets and was also reported by others (10). In cases where the entire $CD4^+$ T cell population increases in intensity in the Foxp3 staining, gates cannot be set based on isotype controls. If the specificity and reproducibility of the gating cannot be achieved when the gate is set based on isotype controls, the subjectivity of the operator can be overcome by setting the gate based on the $CD127^+CD25^-$ "non-T_{reg}" $CD4^+$ T cells for each individual.

Overall, this study compared different clones of antibodies used in varied staining conditions and found that most of them indentified a population of $CD4^+CD25^+Foxp3^+$ T cells. The

staining conditions that yielded the most distinct population of putative T_{reg} cells were achieved using the 259D/C7, PCH101, 236A/E7, and 206D antibodies. These antibodies yielded statistically higher levels of Foxp3 cells than 150D and 3G3 antibodies. Notably, the PCH101 clone from eBioscience revealed distinct populations of T_{reg} cells but required analysis using gates set on "non-T_{reg}" populations rather than isotype controls.

Experiments using fresh versus frozen cells yielded slightly different Foxp3 staining, with clone PCH101 staining more $Foxp3^+$ events within the $CD25^+Foxp3^+$ gate when used to stain frozen cells compared to fresh cells, while clone 3G3 showing the opposite trend. Better separation of the $Foxp3^+$ versus $Foxp3^-$ populations was observed when fresh cells were used with the PCH101 clone and when frozen cells were used with the 259D/C7 clone. These observations raise the potential that the freeze/thawing process affects the epitope recognized by these clones. The choice of fluorochromes also influenced the quality of Foxp3 staining. Antibodies coupled to the Alexa647 or PE fluorochromes allowed clearer separation of $Foxp3^+$ and $Foxp3^-$ populations compared to antibodies coupled to FITC or Alexa488. This study provides a useful starting point for the generation of Foxp3 staining panels for identification of T_{reg} cells in human samples, highlighting the need to consider the antibody source, fluorochrome, and staining buffer to achieve optimal results.

Acknowledgments

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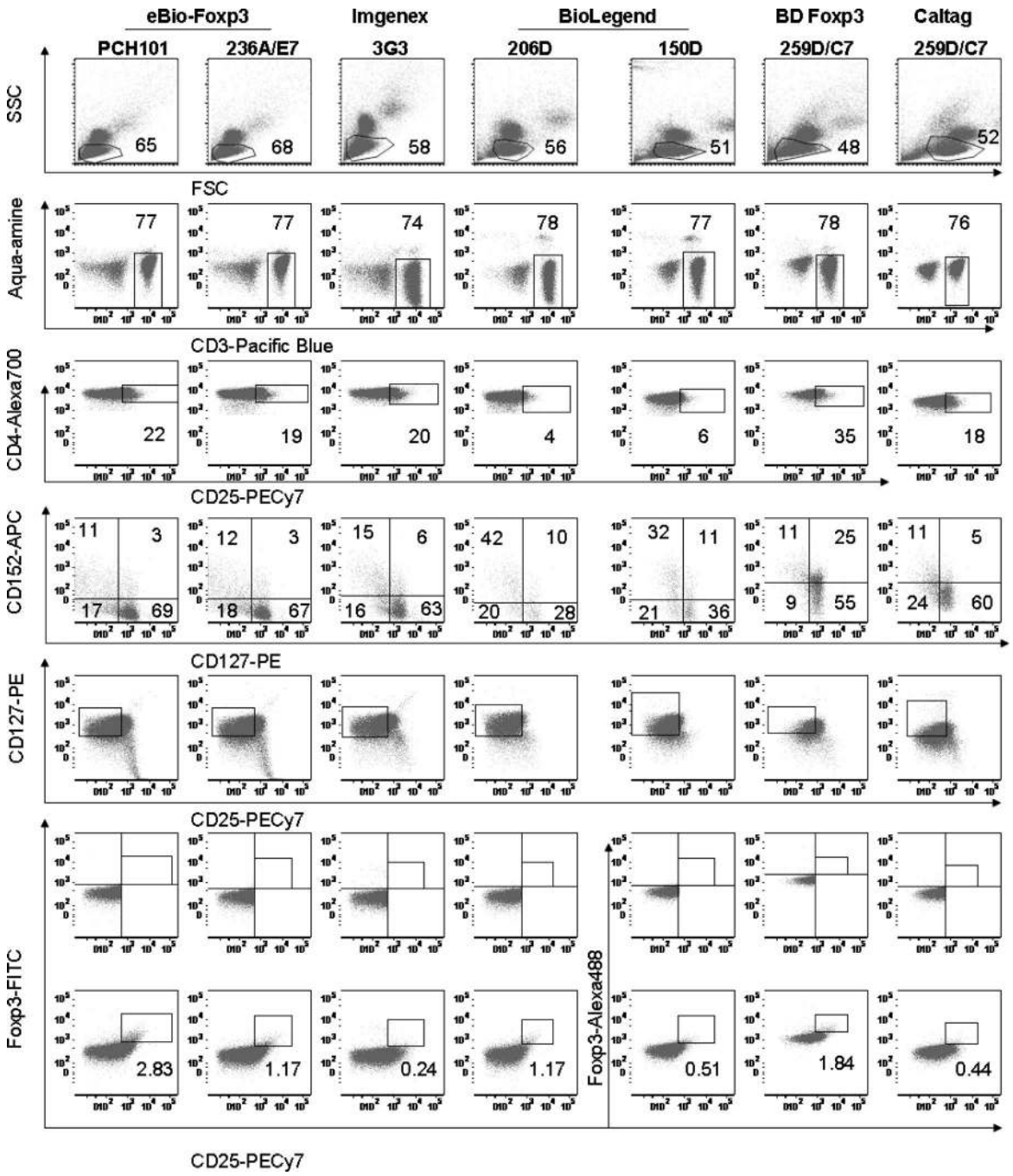


Figure 1. Comparison of Foxp3 staining kits with a Foxp3 gate based on CD127$^+$CD25$^-$ "non-T$_{reg}$" CD4$^+$T cells

(**A**) Gating strategy and results obtained for staining with the T$_{reg}$ panels using the PCH101 (column 1) and 236A/E7 (column 2) antibodies with the eBioscience fixation/permeabilization buffer, the 3G3 antibody with the Imgenex buffer (column 3), the 206D (column 4) and 150D (column 5) antibodies with the BioLegend buffer, the 259D/C7 antibody with the BD Foxp3 (column 6), or the Caltag buffers (column 7). Lymphocyte populations were gated based on SSC/FSC characteristics and the numbers displayed are percentages of total events (row 1). Viable CD3$^+$ events were gated based on exclusion of aqua amine-reactive dye and displayed numbers are CD3$^+$ events expressed as percentages of lymphocytes (row 2). Among CD3$^+$

events, $CD4^+$ (gates not shown) and $CD4^+CD25^+$ events were gated (row 3), with $CD4^+CD25^+$ events expressed as percentages of $CD4^+$ cells. $CD4^+CD25^+$ events were further analyzed for their CD152/CD127 expression (row 4). Finally, $CD3^+CD4^+$ cells (row 3) were analyzed for Foxp3 and CD25 expression (row 7). The CD25, CD152, and CD127 gates were based on FMO controls. The Foxp3 gates were set (row 6) based on $CD127^+CD25^-$ "non-T_{reg}" $CD4^+$ T cells (see gate in row 5) and applied to the $CD4^+$ T cell population (row 7). Displayed numbers are $CD25^+Foxp3^+$ events expressed as percentages of $CD4^+$ events.

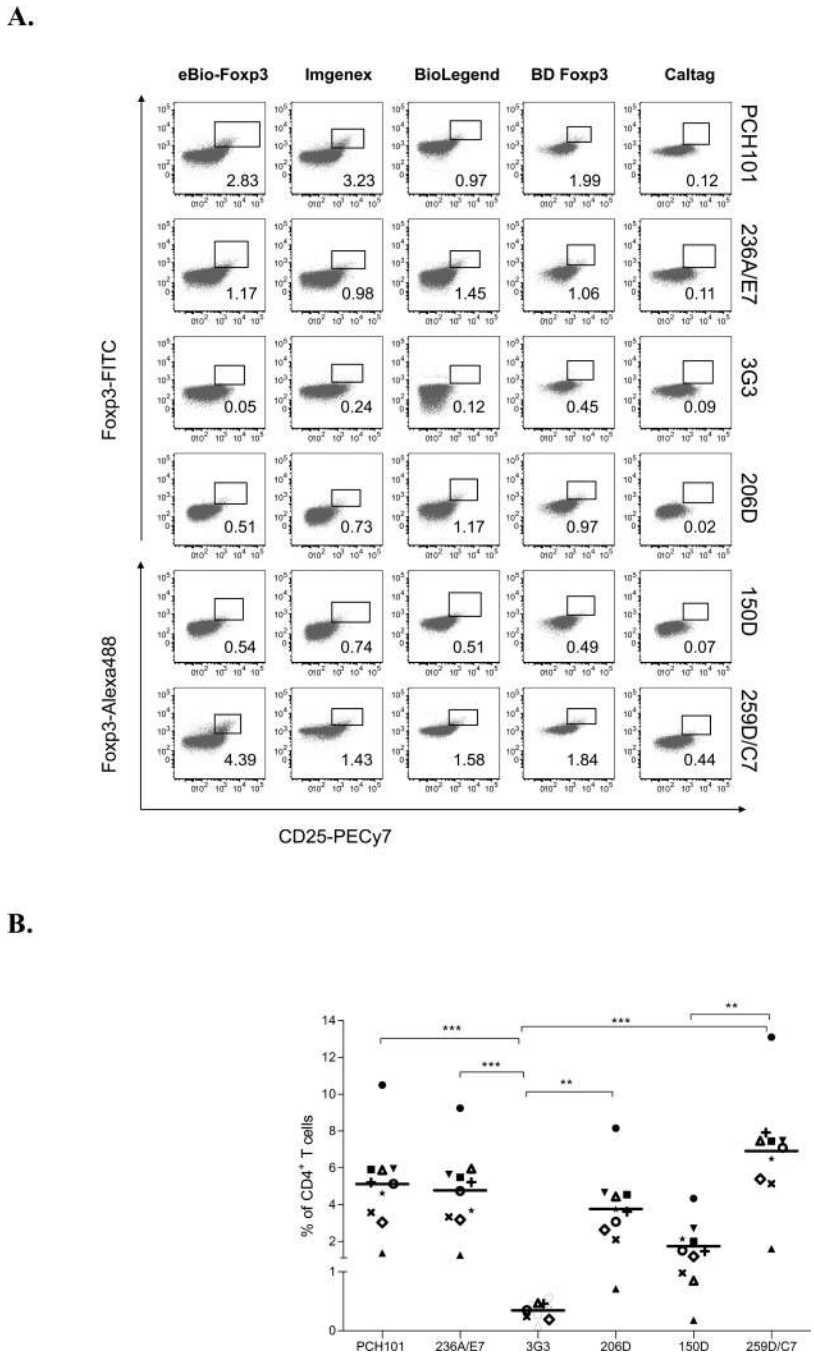


Figure 2. Comparison of the anti-Foxp3 antibodies with a Foxp3 gate based on CD127$^+$CD25$^-$ "non-T$_{reg}$" CD4$^+$ T cells

(A) In different fixation and permeabilization conditions. The FITC-conjugated clones PCH101 (row 1), 236A/E7 (row 2), 3G3 (row 3), and 206D (row 4) and the Alexa488-conjugated clones 150D (row 5) and 259D/C7 (row 6) were used as part of independent T_{reg} panels in combination with the eBioscience Foxp3 (column 1), Imgenex (column 2), BioLegend (column 3), BD Foxp3 (column 4), or Caltag (column 5) buffers. Within CD3$^+$ events from the viable lymphocyte population, CD4$^+$ events were gated and analyzed for their Foxp3 and CD25 expression. Displayed numbers are CD25$^+$Foxp3$^+$ events expressed as percentages of CD4$^+$ T cells. (B) In the same fixation and permeabilization conditions using

frozen PBMCs from 10 normal individuals. The FITC-conjugated clones PCH101, 236A/E7, 3G3, 206D, and the Alexa488-conjugated clones 150D and 259D/C7 were used in combination with the eBioscience Foxp3 buffer to stain frozen PBMCs from 10 different individuals. Within $CD3^+$ events from the viable lymphocyte population, $CD4^+$ events were gated and analyzed for their Foxp3 and CD25 expression. The CD25 gates were based on FMO controls. The Foxp3 gates were set based on $CD127^+CD25^-$ "non-T_{reg}" $CD4^+$ T cells. Results are displayed with a unique symbol representing each individual, and denote the percentage of $CD4^+$ T cells that were $CD25^+Foxp3^+$. The Kruskal-Wallis test followed by the Dunn's test for multiple comparisons were used to compare the mean percentages of $CD25^+Foxp3^+$ events within $CD4^+$ T cells using the different clones. *** p <0.0001 and ** p <0.001.

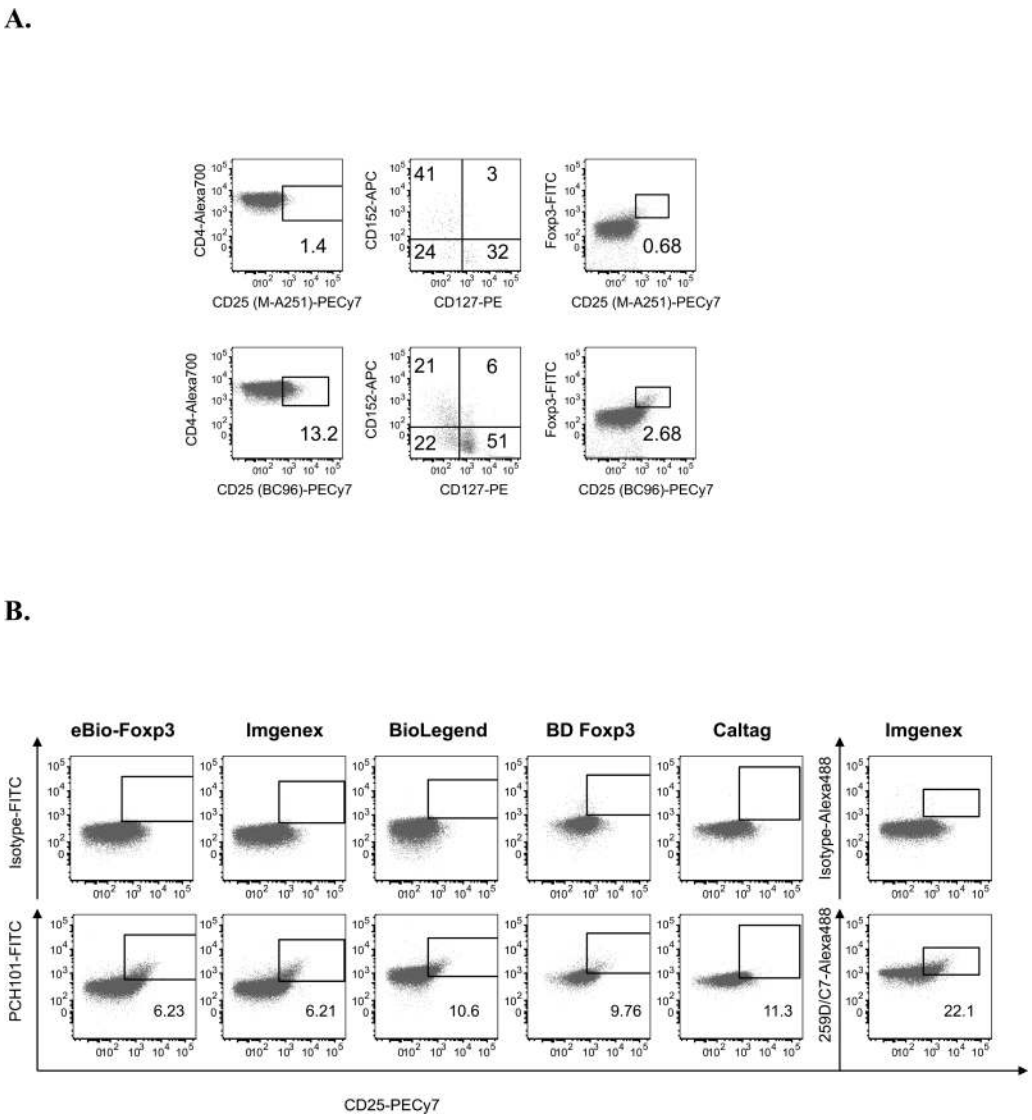


Figure 3. CD25 and Foxp3 staining technical challenges

(**A**) Comparison of CD25 staining using clone M-A251 (row 1, as used in Fig. 1 and 2) versus clone BC96 (row 2) with the BioLegend buffer set along with Foxp3 clone 206D to stain frozen PBMCs from another individual. CD25 gates were set based on FMO tubes. Foxp3 gates were set based on $CD127^{+}CD25^{-}$ "non-T_{reg}" $CD4^{+}$ T cells. (**B**) "Non-specificity" of the Foxp3 staining when the Foxp3 gates are set based on isotype controls. Staining frozen PBMCs from the same individual as in Fig. 1 and 2, the FITC-conjugated clone PCH101 (row 2, columns 1-5) was used as part of independent T_{reg} panels in combination with the eBioscience Foxp3 (column 1), Imgenex (column 2), BioLegend (column 3), BD Foxp3 (column 4), or Caltag (column 6) buffers. The Alexa488-conjugated clone 259D/C7 was used in combination with the Imgenex buffers (row 2, column 6). Within viable $CD3^{+}$ events, $CD4^{+}$ events were gated and analyzed for CD25 and Foxp3 expression. The CD25 gates were set based on FMO controls, and the Foxp3 gates were set based on isotype controls (row 1). Displayed numbers are $CD25^{+}Foxp3^{+}$ events expressed as percentages of $CD4^{+}$ T cells.

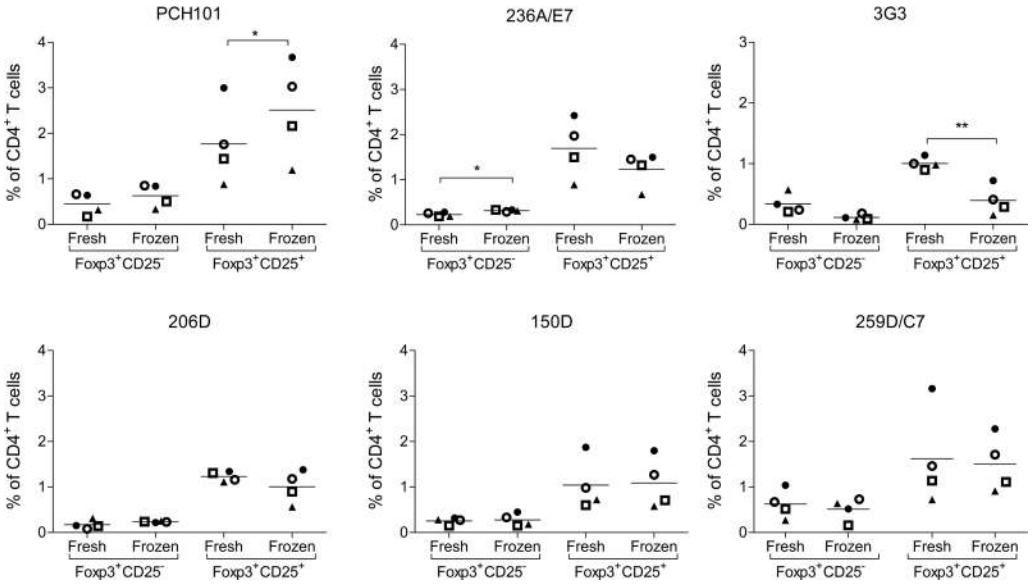
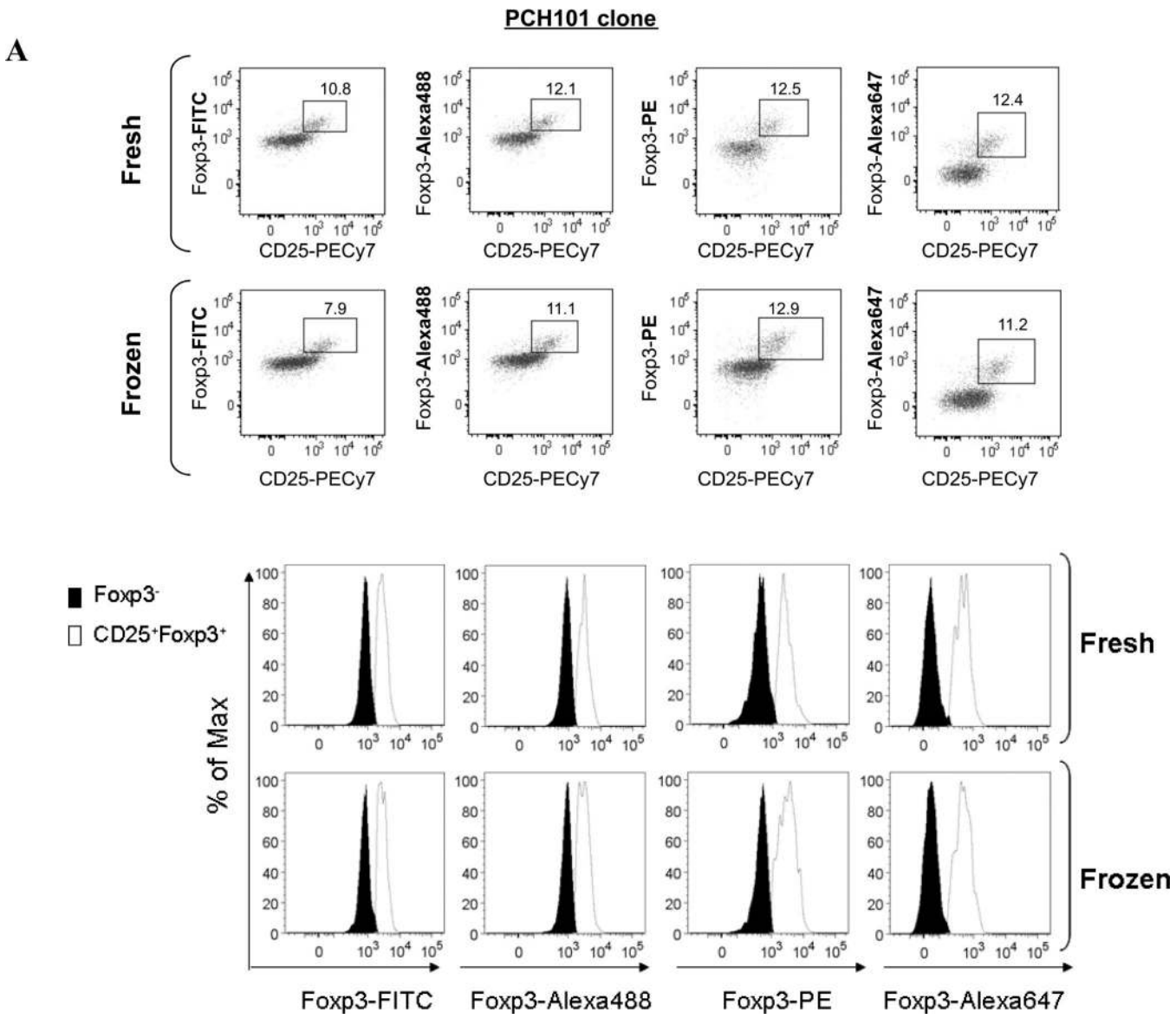


Figure 4. Comparison of Foxp3 staining using fresh versus frozen PBMCs from four donors

The FITC-conjugated clones PCH101, 236A/E7, 3G3, 206D, and the Alexa488-conjugated clones 150D and 259D/C7 were used in combination with the eBioscience Foxp3 buffer to stain fresh versus frozen PBMCs from four other individuals. CD25 gates were based on FMO controls and Foxp3 gates were set based on $CD127^{+}CD25^{-}$ "non-T_{reg}" $CD4^{+}$ T cells. Results are displayed with a unique symbol representing each individual and denote the percentage of $CD4^{+}$ T cells that were $CD25^{-}Foxp3^{+}$ or $CD25^{+}Foxp3^{+}$. The Student t-test for paired comparisons was used to compare the mean percentages of $CD25^{-}Foxp3^{+}$ or $CD25^{+}Foxp3^{+}$events in the fresh versus frozen PBMCs. ** p <0.001 and * p <0.05.



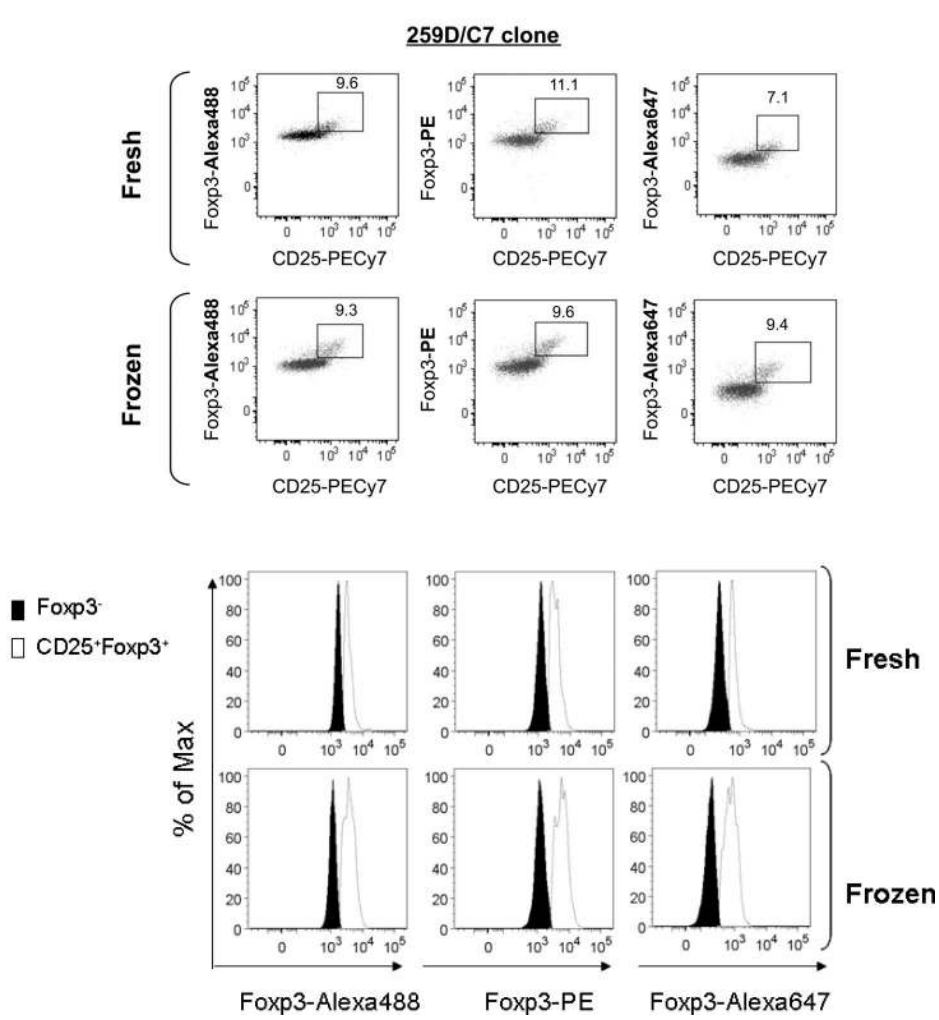


Figure 5. Comparison of Foxp3 staining using PCH101 and 259D/C7 clones coupled to different fluorochromes

(**A**) Fresh and frozen PBMCs from four normal individuals were stained with the PCH101 clone coupled to FITC, Alexa488, PE, or Alexa647 fluorochromes, and (**B**) with the 259D/C7 clone coupled to Alexa488, PE, or Alexa647 fluorochromes. CD25 gates were set based on FMO controls, and Foxp3 gates were set based on $CD127^{+}CD25^{-}$ "non-T_{reg}" cells. Dot-plots are from one representative donor, displayed numbers are $CD25^{+}Foxp3^{+}$ events expressed as percentages of $CD4^{+}$ T cells. Histograms are from one representative donor and display populations of $Foxp3^{-}$ events (filled histograms) and $CD25^{+}Foxp3^{+}$ events (unfilled histograms).

Table 1

LSR II configuration and fluorochromes

Laser	Detector	LP Mirror	BP Filter	Reagent	Fluorochrome
Blue	A	735	780/60	CD25	PE-Cy7
Blue	B	685	695/20	-	-
Blue	C	635	670/14	-	-
Blue	D	552	575/26	CD127 Foxp3	PE
Blue	E	505	530/30	Foxp3	FITC/Alexa 488
Violet	A	505	525/50	AARD[a]	AARD
Violet	B	-	440/40	CD3	Pacific Blue
Red	A	685	720/45	CD4	Alexa 700
Red	B	-	660/20	CD152 Foxp3	APC Alexa 647

[a] **AARD :** Aqua amine-reactive dye

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