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An Optimized Multi-parameter Flow Cytometry Protocol for Human T Regulatory Cell Analysis on Fresh and Viably Frozen Cells, Correlation with Epigenetic Analysis, and Comparison of Cord and Adult Blood

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Abstract

Multi-parameter flow cytometry analysis of T regulatory (Treg) cells is a widely used approach in basic and translational research studies. This approach has been complicated by a lack of specific markers for Treg cells and lack of uniformity in quantification of Treg cells. Given the central role of Treg cells in the inception and perpetuation of diverse immune responses as well as its target as a therapeutic, it is imperative to have established methodologies for Treg cell analysis that are robust and usable for studies with multiple subjects as well as multicenter studies. In this study, we describe an optimized multi-parameter flow cytometry protocol for quantification of human Treg cells from freshly obtained and viably frozen samples and correlations with epigenetic Treg cell analysis (TSDR demethylation). We apply these two methodologies to characterize Treg cell differences between cord blood and adult peripheral blood. In summary, the optimized protocol appears to be robust for Treg cell quantification from freshly isolated or viably frozen cells and the multi-parameter flow cytometry findings are strongly positively correlated with TSDR demethylation thus providing several options for characterization of Treg cell frequency and function in large translational or clinical studies.

Keywords

T regulatory cell; demethylation; flow cytometry; Foxp3; cord blood; epigenetics

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Introduction

Treg cells are a subset of CD4 T cells that generally serve the unique role of suppressing immune responses. Treg cells were initially believed to suppress autoimmune responses, however many lines of evidence now demonstrate that Treg cells are important in a broad spectrum of immune responses including autoimmunity, cancer, transplantation, and infectious diseases. As such, robust assays are necessary in order to definitively characterize Treg cell frequency and function in various clinical scenarios.

Initially, Treg cells were defined as CD4⁺ T cells with high levels of CD25 surface expression (Takahashi et al., 1998). Subsequent studies have focused on identifying unique markers for Treg cells. Proposed unique Treg cell markers to date have failed to fulfill the criteria of being exclusively expressed on Treg cells and have revealed that within the Treg cell population heterogeneity exists (Miyara et al., 2009; d’Hennezel et al., 2011). This includes the transcription factor Foxp3. Despite being necessary for the development and function of Treg cells, Foxp3 is transiently expressed in activated non-suppressor T cells (Tran et al., 2007; d’Hennezel and Piccirillo, 2011). More recent studies have utilized multiple surface and intracellular markers to identify Treg cells. To date, a rigorous analysis of multi-parameter flow cytometry for Treg cell quantification using various sample preparations and optimization steps has not been reported.

A separate line of investigation to distinguish Treg cells has demonstrated demethylation of CpG motifs in a defined region of the Foxp3 promoter (Treg specific demethylated region, TSDR) was present in Treg cells that have stable suppressor activity and a low potential for differentiation into other effector phenotypes (Baron et al., 2007; Floess et al., 2007; Polansky et al., 2008; Miyara et al., 2009; McClymont et al., 2011). Therefore, TSDR demethylation status is proposed to distinguish phenotypically stable and suppressive Treg cells from activated conventional T cells that transiently upregulate Foxp3. To date, epigenetic analysis has not been directly compared to multi-parameter flow cytometry Treg cell analysis.

The impact of immune immaturity on Treg cell development remains poorly defined. Nonetheless, it is generally believed that neonates are skewed toward a tolerant state that is mediated in part by Treg cells. There are limited studies comparing Treg cells in neonates versus adults. One confounding aspect of published studies is utilization of varied criteria to define Treg cells. It has been reported that fetal T cells express higher levels of Foxp3 (Steinborn et al., 2010) and are more readily converted to Foxp3⁺ suppressive T cells after activation (Mold et al., 2008). Other studies have suggested cord blood Treg cells are less functionally active than their adult counterpart (Schaub et al., 2008; Ly et al., 2009), have diminished Foxp3 expression (Chen et al., 2006; Miyara et al., 2009) and express Foxp3 in T cells that lack other Treg cell markers (Ly et al., 2009). Another group reported that cord blood is superior to adult sources for ex vivo expansion of highly suppressive Treg cells, implying cord blood has a higher frequency of stable Treg cells (Godfrey et al., 2005). A rigorous comparison in Treg cell frequency between cord and adult blood utilizing several methodologies has not been reported.

In summary, the ability to accurately determine the frequency of Treg cells and potential for differentiating into an inflammatory effector cell is an essential tool for characterizing the role of Treg cells in various disease states and therapeutic modalities. In this study, we report an optimized protocol for multi-parameter flow cytometry of Treg cells from freshly processed and viably frozen cells and its correlation with TSDR demethylation. A multi-parameter flow cytometry approach to quantifying Treg cells in samples with varied sample preparations and direct comparison to Treg cell epigenetic analysis has not been previously

reported. We apply these methods to human cord and adult blood samples to determine similarities and differences in Treg cells.

Materials and Methods

Blood samples

Deidentified cord blood was obtained from full term infants delivered via scheduled c-section. Deidentified adult blood was obtained on healthy adults (age range: 19-60 years; average age = 34.9, SD 14.1) in sodium heparin tubes. All studies were approved by the Institutional Review Board at the University of Wisconsin and Meriter Hospital, Madison WI. Peripheral blood mononuclear cells were isolated using Lymphocyte Separation Medium (Mediatech Inc, Manassas, VA) according to manufacturer's instructions. Cells were frozen in CryoStor CS10 freezing media (Biolife Solutions, Inc, Bothell, WA) according to manufacturer's instructions. Cells were thawed according to manufacturer's instructions, washed, counted, and rested overnight in RPMI media supplemented as previously published (Seroogy et al., 2004) at 37 °C and 5% CO₂. Thawed cells were only processed if ≥85% viable using trypan blue exclusion. The following day, cells were treated with 200U DNaseI (Promega, Madison, WI) in 10ml media in a 37 °C water bath for 30 min.

Flow cytometry

Isolated peripheral blood mononuclear cells (PBMCs) were incubated with 10 µl of a 1.2% human IgG solution (Baxter, Deerfield, IL) to block non-specific antibody binding and then stained in media with the following antibody/conjugate: CD3-PerCP (clone UCHT1), CD4-Pacific Blue (clone RPA-T4), FoxP3-Alexa Fluor 488 (clone 206D) (all from BioLegend, San Diego, CA), CD25-APC (clone 2A3), CD127-PE (clone hIL-7R-M21), (all from BD Biosciences, San Jose, CA). Cells were fixed using 2% formaldehyde (Polysciences, Inc, Warrington, PA) followed by permeabilization using 1x permeabilization buffer (eBioscience, San Diego, CA) then stained for Foxp3. This is discussed in more detail in the results section. Cells were acquired on a LSR II (BD Biosciences). The BD LSR II is calibrated daily by the University of Wisconsin Carbone Cancer Center Flow Cytometry Laboratory staff using the manufacturer's Cytometer Settings and Tracking calibration software and identical voltages are used for all acquisitions for all fluorescent channels on all samples. Data were analyzed using Flowjo software (Treestar, San Carlos, CA). Positive staining and gating strategy was determined by comparison to an isotype control.

DNA isolation, bisulfite-conversion, bisulfite sequencing and QPCR

$1-5 \times 10^5$ PBMCs were pelleted and stored at -80°C. Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Bisulfite conversion of genomic DNA, bisulfite sequencing of Foxp3, CD3γ and CD3δ regions, and GAPDH, and QPCR have been previously described (Sehouli et al., 2011).

Statistics

For correlation, Pearson's correlation coefficient was calculated. Student's t-test unpaired, two tailed with a p value < 0.05 considered significant.

Results

Foxp3 antibody titration is critical for optimal protein detection

It is widely accepted that antibodies must be titrated to establish the saturating concentration. We observed this to be particularly important for the Foxp3 antibody and

routinely found the optimal antibody concentration was higher than the manufacturer's recommendation. For this antibody, the optimal concentration in our hands using multiple lots was $9 \mu\text{l}=0.54 \mu\text{g}$ per 1×10^6 cells. Using this optimized concentration, we observed Foxp3 staining predominantly in the CD3+CD4+ T cells (Figure 1A), and a higher frequency of CD4+CD25+Foxp3+ T cells and higher mean fluorescence intensity (MFI) for Foxp3 with $9 \mu\text{l}$ of antibody (Figure 1B). When the concentration of antibody was increased above $9 \mu\text{l}$, we did not see what we considered a significant increase in Foxp3 frequency or MFI. Using the Alexa Fluor 488 fluorophore, we observe better distinction of Foxp3+ CD4+ T cells when CD25 gating is included. Under all staining conditions, CD4+CD25+Foxp3+ T cells were >93% CD127lo/- (data not shown).

Optimization of fixation and permeabilization reagents

While establishing the optimal anti-Foxp3 antibody concentration, we initially observed inconsistency with Foxp3 protein staining. Therefore, we compared various fixation and permeabilization protocols. In our hands, the optimal protocol is a combination of 2% ultrapure formaldehyde and commercially available permeabilization buffer containing 0.1% saponin (Figure 2).

Viably frozen cells can be used for multi-parameter flow cytometry Treg analysis

Analysis of large numbers of human blood samples and multicenter studies often necessitate the use of viably frozen cells. Our readouts for comparison included percentage of Foxp3 positivity and intensity of Foxp3 staining. We observed that quick thaw of viably frozen cells with immediate staining for Treg cell analysis led to suboptimal staining, particularly for Foxp3 MFI. By resting cells overnight after quick thaw before staining for flow cytometry, the Foxp3 MFI was significantly improved (Figure 3). The percentage of CD4+CD25+CD127lo/-Foxp3+ T cells was not significantly different between the experimental groups (Figure 3B). This suggests that the percentage of Foxp3 positive cells is preserved under both conditions, but Foxp3 staining is qualitatively suboptimal in the post-thaw conditions. Therefore, we incorporated a rest period prior to initiation of the staining protocol. After thawing, cells were washed in media and then incubated in media overnight at 37 C and 5% CO₂. For convenience, we rested the cells overnight and have not compared shorter lengths of time. Therefore, we conclude that multi-parameter flow cytometry to determine Treg cell frequency can be performed on viably frozen cells. Next we directly compared fresh samples to our optimized viably frozen cell protocol to determine consistency in quantification of Treg cells between the two sample preparations.

We compared fresh blood samples (<24 hours old) to viably frozen cord blood or adult peripheral blood mononuclear cells following our optimized viably frozen cell protocol. The frequency of Treg cells, defined as CD4+CD25+CD127lo/-, is on average lower in viably frozen samples compared to fresh samples (Figure 4A). To gate for this population, a CD25 gate is determined within the gated CD4+ T cells using an isotype control and the CD127lo/- gate is determined by plotting CD25 versus CD127 (see supplemental figure). The correlations between fresh and viably frozen samples for Treg cells defined as CD4+CD25+CD127lo/- or CD4+CD25+CD127lo/-Foxp3+ were both significantly positive (Figure 4B and C). Therefore, from these data, we conclude Treg cell frequency can accurately be determined in fresh and viably frozen samples. Our data suggests consistency in sample processing should be followed, for example all fresh samples or all viably frozen, for accurate comparisons between study subjects.

Often Treg cells are analyzed or FACS sorted based on CD25 staining intensity for quantification of Treg cells and subsequent downstream analyses such as functional assays, epigenetic analysis or transcriptional profiling. Therefore, we compared CD25 staining in

fresh versus viably frozen samples. CD25 staining was segregated into CD25^{bright} and CD25^{hi} based on gating strategy from previously published studies (Figure 5A) (Baecher-Allan and Hafler, 2005; Lundgren et al., 2005). We found both populations were clearly definable in fresh or frozen adult and cord blood. Again, there was strong correlation between these populations in fresh and frozen samples (Figure 5B). We did validate Foxp3 expression in these populations. There was a statistically significant difference in the percentage of Foxp3 positive cells between fresh and frozen in the CD25^{bright} population (fresh: mean 73.9 [SD 10.5] vs. frozen: mean 84.5 [SD 5.88]; $p = 0.03$, data not shown). The percentage of Foxp3 positive cells in the CD25^{hi} population did not differ significantly between fresh and frozen samples (fresh mean 90.8 [SD 6.6] vs. frozen mean 89.3 [SD 12.1], $p = 0.76$). These data suggest that FACS sorting, at least for CD25^{bright} cells from viably frozen cells should yield a relatively pure population of Foxp3 expressing T cells that is comparable in phenotype to freshly isolated cells.

Treg specific demethylated region, TSDR, is strongly associated with flow cytometry Treg cell analysis

Using an epigenetic approach, Treg cells and total T cells can be quantified from the genomic DNA of heterogeneous cell populations and TSDR demethylation is reported to correlate with suppressor activity (Baron et al., 2007; Floess et al., 2007; Polansky et al., 2008; Liu et al., 2010; Sehouli et al., 2011). The correlation between Treg cell frequency determined by multi-parameter flow cytometry and this epigenetic approach remains unknown. Therefore, we sought to look at the relationship between our optimized multi-parameter flow cytometry method for Treg cell quantification and QPCR epigenetic determination of Treg cells (Figure 6). The frequency of Treg cells by flow cytometry is expressed as the percentage within a CD3⁺ gate. The QPCR data is presented as the percentage of TSDR demethylation (Treg cell epigenetic signature) divided by CD3 locus demethylation within the genomic DNA sample. Using cord and adult blood samples and two different criteria for Treg cell phenotype, (CD4⁺CD25⁺CD127^{lo/-}, Figure 6A, and CD4⁺CD25⁺CD127^{lo/-}Foxp3⁺, Figure 6B) there was a statistically significant positive correlation between flow cytometry and QPCR approaches.

Application of epigenetic and multi-parameter flow cytometry Treg cell analysis in cord and adult blood: cord blood has a higher frequency of Treg cells

Lastly, we used the optimized multi-parameter flow cytometry assay alongside the epigenetic assay to look for differences between cord and adult blood Treg cells. As a first step to investigate for differences in Treg cells between cord and adult blood, Treg cell frequency was compared using multi-parameter flow cytometry. The gating strategy used for determining Treg cells with the optimized multi-parameter flow cytometry assay is shown in Figure 7A. Using a generous forward and side scatter gate from density gradient buffy coats, we noted increased monocytic cells in the cord blood compared to adult PBMCs (Figure 7A, first panel). We believe this is secondary to the previously reported finding that cord blood contains an increased frequency of monocytes compared to adult peripheral blood mononuclear cells (PBMC) (Manroe et al., 1979; Weinberg et al., 1985). Several phenotypes were used to define Treg cells: CD4⁺CD25⁺CD127^{lo/-}, CD4⁺CD25⁺CD127^{lo/-}Foxp3⁺, CD4⁺CD25⁺Foxp3⁺, and CD4⁺CD25^{hi}Foxp3⁺ (Figure 7A and data not shown). Treg cells defined as CD4⁺CD25⁺CD127^{lo/-} provided the strongest statistically significant difference between the samples with a higher frequency in cord blood compared to adult PBMCs (Figure 7B). When the three other defined criteria to compare Treg cells amongst cord or adult CD3⁺ T cells, we found the same trend, but these comparisons did not reach statistical significance (data not shown). The percentage of Foxp3⁺ cells within the CD4⁺CD25⁺CD127^{lo/-} T cells was comparable between cord and adult Treg cells (cord blood 73.3% (SD 13.6) vs. adult PBMC 80.9% (SD 6.9); $p = 0.88$).

Interestingly, the Foxp3 MFI was statistically lower in cord blood CD4+CD25+CD127lo/- FoxP3+ T cells than in adults (cord blood 887.1 (SD 190) vs adult PBMC 1076.5 (SD 170.9); $p = 0.03$, data not shown), a finding that is similar to previously published work (Miyara et al., 2009). The level of TSDR demethylation, determined as a ratio with the percentage of CD3+ T cell locus demethylation as denominator, was significantly higher in cord blood compared to adult blood (Figure 7C).

Discussion

In this study, we demonstrated an optimized multi-parameter flow cytometry protocol for quantification of Treg cells. Our method worked effectively using freshly processed or viably frozen blood cells and provides an approach with increased flexibility thus allowing for multicenter studies. Our data also demonstrated a strong correlation between the frequency of Treg cells determined by flow cytometry and the frequency of T cells with demethylation at TSDR. Lastly, using these two approaches we report that cord blood contains a statistically higher frequency of Treg cells compared to adult PBMCs.

To date, there have been limited studies published on protocols for Treg cell quantification. A recent study focused on anti-Foxp3 antibody choice (Grant et al., 2009). In this study, fix/permeabilizing reagents were reported to be interchangeable and fresh and frozen sample preparations were comparable. It is unknown why our results differ from this study. One difference is cells in the Grant et al. study were frozen for only 24 hours whereas in our study the samples had been held at -80 °C for weeks.

We believe this is the first study to directly compare multi-parameter flow cytometry and epigenetic analysis for Treg cells quantification. One study looked at melanoma patients pre and post therapy and measured Treg cells by flow cytometry and epigenetic analysis (Wieczorek et al., 2009). Trends were comparable using these two methodologies, but unlike our study direct relationships were not interrogated. For future studies, we propose using a multi-parameter flow cytometry approach along with epigenetic assays for rigorous quantification of human Treg cells. Alternatively, if sample is limiting, our findings support a role for solely using epigenetic assays for Treg analysis since our data demonstrated a strong association between Treg cell frequency using multi-parameter flow cytometry and TSDR demethylation. A recent publication compared TSDR demethylation with in vitro suppressor activity in cord blood and found a direct relationship (Liu et al., 2010). However, this result needs to be validated by other groups and is an area of ongoing investigation in our laboratory. We predict future additional testing will demonstrate TSDR demethylation, multi-parameter flow cytometry Treg cell analysis, and in vitro suppressor activity are all significantly correlated.

The strongest correlation between QPCR Treg/T cell and flow cytometry is found using % CD4+CD25+CD127lo/- (Figure 6A, $p = 0.0009$). If Foxp3 is included, the association is statistically significant, but less so (Figure 6B, $p = 0.05$). The correlation between %CD4+CD25+Foxp3+ T cells/CD3+ and QPCR Treg/T cell was statistically significant ($p = 0.011$, data not shown), whereas using Foxp3 expression alone in CD4+ T cells was not significantly correlated with QPCR Treg/T cell ($p = 0.317$, data not shown). Thus, these data suggest that flow cytometry may not be sensitive enough to detect all Foxp3 protein or there is turnover of Foxp3 protein resulting in transient states of open chromatin (TSDR) at Foxp3 promoter region and protein level below the level of detection by flow cytometry. Our findings suggest CD4+CD25+CD127lo/- may more accurately represent Treg cell frequency since it has the strongest correlation with TSDR demethylation.

Lastly in this study, we analyzed cord and adult blood Treg cells with a primary aim to determine if differences exist between these immunologically distinct groups. Published studies have not consistently demonstrated significant differences between cord and adult blood Treg cell frequency (Schaub et al., 2008; Ly et al., 2009). This inconsistency is likely secondary to differences in defining Treg cells. Our study is the first study to use multi-parameter flow cytometry and epigenetic analysis to directly compare Treg cells in cord and adult blood. Our results demonstrated that Treg cell frequency is higher in cord blood. The strongest statistically significant difference is observed using %CD4+CD25+CD127lo/- (Figure 7 A & B). When Foxp3 is added to this panel, the p value is trending toward significant (p = 0.07, data not shown). These findings are consistent with the fetal T cell environment being skewed toward a tolerant state that includes increased Treg cell frequency. It is possible that the addition of Foxp3 in defining Treg cells did not reach statistical significance secondary to our sample size, or a more interesting possibility is that cord blood T cells may have TSDR demethylation in the absence of any Foxp3 protein expression, have higher protein turnover, or have low level Foxp3 protein expression that is below the limit of detection by flow cytometry. Studies are ongoing in our laboratory aimed at identifying additional differences between cord and adult blood Treg cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Importance of titrating anti-Foxp3 antibody

PBMCs (1×10^6 cells) were stained as described in the materials and methods section with the manufacturer's recommended quantity of antibody (far left) and increasing amounts. A. Within the CD3+ gate, Foxp3 staining is shown in CD4+ and non-CD4+ T cells. B. For gated CD3+CD4+ T cells, FACS plots are shown for Foxp3 and CD25 staining. A representative experiment of 3 independently performed is shown.

Figure 2. Optimization of fixation and permeabilization conditions

PBMCs were stained as described in the materials and methods section using manufacturer provided fixation and permeabilization reagents, standard fix/perm (eBioscience, upper) or optimized combination (lower). FACS plots are shown for gated CD3+CD4+ T cells. Frequency of stained cells is shown in upper right quadrant for CD25 staining and Foxp3 or isotype control. A representative experiment of 3 independently performed is shown.

Figure 3. Impact of viably frozen cells on Treg staining

A. Viably frozen cells from cord or adult PBMCs were stained for Treg cells as described in the material and methods section either immediately after thaw (post-thaw) or after thawed, washed and rested O/N (rested O/N). Representative FACS plots are shown for cord and PBMCs on gated CD4+ T cells. B. The percentage of CD4+CD25+CD127lo/-Foxp3+/CD3+ T cells (top panel) and Foxp3 MFI within the CD4+CD25+Foxp3+ gate (bottom panel) compiled data between the two experimental groups are shown (N=5 per group).

Figure 4. Direct comparison of fresh vs. viably frozen cell preparation on Treg staining

A. A combination of fresh cord and adult PBMCs and paired viably frozen samples were stained for Treg cells using our optimized methodology and directly compared for frequency of Treg cells within gated CD4+ T cells (N=8). B. Pearson's correlation of CD4+CD25+CD127lo/- cell frequency between fresh or viably frozen samples. C. Pearson's correlation of CD4+CD25+CD127lo/-Foxp3+ cell frequency between fresh and viably frozen samples. The line fitting these data was generated using linear regression analysis.

Figure 5. Impact of viably frozen cell preparation on CD25 staining

A. Cord blood and adult PBMC representative FACS plots freshly obtained or frozen and rested overnight samples and gating strategy for CD25^{bright} (smaller box) and CD25^{hi} (larger box) Treg cells. B. Pearson's correlation of CD25^{bright} cell frequency (top) or CD25^{hi} cell frequency (bottom) between paired fresh and viably frozen samples (N=8). The line fitting these data was generated using linear regression analysis.

Figure 6. Relationship between multi-parameter flow cytometry for Treg cell quantification and epigenetic analysis

A. Pearson's correlation between CD4+CD25+CD127lo/- Treg cells within CD3+ gate determined by flow cytometry and percentage of TSDR demethylation divided by percentage of demethylated CD3 locus in PBMC by QPCR (N=18). B. Pearson's correlation between CD4+CD25+CD127lo/-Foxp3+ Treg cells within CD3+ gate determined by flow cytometry and percentage of TSDR demethylation divided by percentage of demethylated CD3 locus in PBMC by QPCR (N=18). The line fitting these data was generated using linear regression analysis.

Figure 7. Multi-parameter flow cytometry and epigenetic analysis for Treg cell quantification in cord blood vs. adult PBMCs

A. Representative FACS plots from cord and adult blood demonstrating gating strategies. To determine the frequency of Treg cells in blood cells, a mononuclear forward and side scatter gate is drawn followed by a CD3 gate (first and second panels). After the CD3 gate, a forward and side scatter gate is applied to eliminate non-lymphocytic cells and a forward width gate is applied to eliminate doublets followed by a CD4 gate. Then Treg cell frequency is determined by the described parameters. Shown in the third panel is CD25 and CD127 staining with Treg cell frequency determined by the lower right quadrant. B. Composite percentage of CD4+CD25+CD127lo/- T cells in cord blood compared to adult blood within the established CD3+ gate. C. Composite of percentage of genomic DNA with demethylation at TSDR divided by percentage of genomic DNA with demethylation of CD3 locus determined by QPCR (see materials and methods section). Cord blood (N=10); adult PBMC (N=8).