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Research paper

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

L. Nettenstrom [a], K. Alderson [a], E.E. Raschke [b], M.D. Evans [c], P.M. Sondel [a], S. Olek [b], C.M. Seroogy [a,*]

[a] Department of Pediatrics, University of Wisconsin, Madison, WI, United States
[b] Epiontis GmbH, Berlin, Germany
[c] Department of Biostatistics and Medical Informatics, University of Wisconsin, Madison, WI, United States

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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 the 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 the 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 the characterization of Treg cell frequency and function in large translational or clinical studies.

1. 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.

* Corresponding author at: 1111 Highland Avenue, 4139 WIMR, Madison, WI 53705-2275, United States. Tel.: +1 608 263 2652; fax: +1 608 265 0164.
E-mail address: cmseroogy@wisc.edu (C.M. Seroogy).

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A separate line of investigation to distinguish Treg cells has demonstrated that 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 that 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.

2. Materials and methods

2.1. 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 the manufacturer's instructions. Cells were frozen in CryoStor CS10 freezing media (Biolife Solutions, Inc., Bothell, WA) according to the manufacturer's instructions. Cells were thawed according to the manufacturer's instructions, washed, counted, and rested overnight in RPMI media supplemented as previously published (Seroogy et al., 2004) at 37 °C and 5% CO_2. Thawed cells were only processed if ≥85% viable using trypan blue exclusion. The following day, cells were treated with 200 U DNaseI (Promega, Madison, WI) in 10 ml media in a 37 °C water bath for 30 min.

2.2. 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), and 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 1× permeabilization buffer (eBioscience, San Diego, CA) and 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 were determined by comparison to an isotype control.

2.3. DNA isolation, bisulfite-conversion, bisulfite sequencing and QPCR

1–5×10^5 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).

2.4. Statistics

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

3. Results

3.1. 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 that 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 μl = 0.54 μg per 1×10^6 cells. Using this