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Temporal Biological variability in dendritic cells and regulatory T cells in peripheral blood of healthy adults

Maleewan Kitcharoensakkul, M.D.[1], **Leonard B. Bacharier, M.D.**[1], **Huiqing Yin-Declue, Ph.D.**[2], **Jonathan S Boomer, Ph.D.**[2], **Dana Burgdorf, R.N.**[2], **Brad Wilson, M.A.**[3], **Kenneth Schechtman, Ph.D.**[3], and **Mario Castro, M.D., M.P.H.**[2]

[1]Division of Allergy, Immunology and Pulmonary Medicine, Department of Pediatrics, St. Louis Children's Hospital, Washington University School of Medicine. Address: Campus box 8116, One Children's Place St. Louis, Missouri, 63110, USA

[2]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Washington University School of Medicine. Address: 660 S. Euclid Ave, Campus box 8052, St. Louis, MO, 63110, USA

[3]Division of Biostatistics, Washington University School of Medicine. Address: 660 S. Euclid Ave, Campus box 8067, St. Louis, MO, 63110, USA

Abstract

Background—Studies evaluating circulating dendritic cells (DCs) and natural and induced regulatory T cells (nTregs, iTregs) are often obtained at a single time point and difficult to interpret without understanding their intrinsic day-to-day biologic variability.

Methods—We investigated the day-to-day variability in quantifying DCs, nTregs (FoxP3+CD25+CD4+) and cytokine production by iTregs (Granzyme B –GZB, Th1/2 cytokines following CD3 plus CD46 *in vitro* activation) from peripheral blood mononuclear cells (PBMC) collected on three consecutive days in healthy adults. Intraclass correlation coefficients (ICC) were used to evaluate intra-individual variability.

Results—In 10 healthy adults, the %PBMC of plasmacytoid (pDC) and myeloid (mDC1 and mDC2) were 0.27±0.12, 0.22±0.10, and 0.02±0.02, with ICC 0.91, 0.90, and 0.17 respectively. Natural Tregs (3.27±1.27% CD4+ cells) had an ICC of 0.86. Inducible Tregs (GZB-positive, 35.3±17.7% CD4+ cells) had an ICC of 0.77. The ICC for IL-10, TNF-α, IFN-γ, IL-4, and IL-5 production by iTregs were 0.49, 0.63, 0.68, 0.74, and 0.82, respectively. There were no significant changes in ICC (<0.1) after adjusting for age, gender and atopy except for IL-4. Substantial variability for iTregs was determined for the control condition (PBS with IL-2).

Corresponding author: Mario Castro, MD, MPH, Division of Pulmonary and Critical Care Medicine, Department of Pediatrics, Washington University School of Medicine, 660 S. Euclid Ave, Campus Box, St. Louis, MO 63110 castrom@dom.wustl.edu Telephone number: +1-314-362-6904, Fax number: +1-314-362-2307.

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Conclusions—No meaningful day-to-day biologic variability was observed for the quantification of nTregs, pDC and mDC1 in normal adults; however, there was substantial variability in measuring mDC2 proportions and iTreg production of IL-10. These results suggest obtaining an average of several measurements over time to determine the most representative value of these biologic measures.

Keywords

dendritic cells; regulatory T cells; variability; human

1. Introduction

Dendritic cells (DCs) and regulatory T cells (Tregs) are subsets of cells that play important roles in immune regulation (Kornete and Piccirillo, 2012; Smigiel et al., 2014). Although the measurement of DCs and Tregs is common in studies of various disease states (Silver et al., 2009; Wu and Xu, 2014), the interpretation of these results is complicated by possible intra-individual biological variability (IBV) within subjects over time. Prior studies of biological variability of lymphocyte subsets have been performed (Backteman and Ernerudh, 2007; Tosato et al., 2013); however, to the best of our knowledge, this has not been performed for the IBV of Tregs or DCs in healthy individuals.

2. Material and methods

2.1 Blood samples and cell processing

Ten healthy adults were enrolled into the study. Exclusion criteria included the presence of allergic diseases, history of smoking, recent uses of immunosuppressive medications, or upper or lower respiratory tract symptoms. Allergy testing by skin prick tests using respiratory allergen extracts (GREER®, Lenoir, NC) or by blood tests for ImmunoCAP (Missouri-Illinois Regional Allergen Panel, Core Lab, St Louis Children's hospital, MO) to common respiratory allergens were performed in all participants. Peripheral blood was collected into EDTA (Ethylenediaminetetraacetic acid) tubes for 3 consecutive days and processed by a single operator within one hour of blood collection. Except one subject, the time of day at which three measures on a particular subject were collected differed by at most 39 minutes (median difference of time of blood draw 15 minutes, range 5 – 213 minutes). The majority of our patients had blood draws in the morning between 0900AM-1145AM, except two subjects (blood collection at 1050AM-0230 PM in one subject and 0215PM-0230 PM on the other). Human subjects' research approval was obtained by the Washington University Medical Center Institutional Review Board with participants providing written informed consent.

2.2 Identification of natural Tregs using CD4, CD25 and FoxP3 staining

Natural Tregs (nTregs, $CD4^+CD25^+Foxp3^+$ cells) were identified by the Human Regulatory T cell Whole Blood Staining Kit (eBioscience, San Diego, CA). Briefly, 100 μl of fresh whole blood was incubated with FITC-conjugated anti-human CD4 (RPA-T4) and APC-conjugated anti-human CD25 (BC96). Red blood cells were lysed in RBC lysis buffer followed by pelleting of cells by centrifugation at 450×g for 5 minutes. After addition of

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wash buffer, cells were pelleted by centrifugation at 450×g for 5 min and resuspended in Foxp3 LWB Fixation/Permeabilization solution. Cell pellets were washed twice, as indicated above, and labeled with anti-human Foxp3 or IgG isotype control. Following incubation, cells were washed and resuspended in wash buffer. Cell analysis was performed using the FACSCaliber 4-color flow cytometer (BD Biosciences). 10,000 gated events were collected based upon forward vs side scatter with data analysis performed using FlowJo software, version 9.2 (Ashland, OR). Prior to the sample collection on FACSCalibur, quality control was performed using BD FACS calibrite beads (cat #340486) and FACSComp software (BD Biosciences) to calibrate the instrument.

2.3 Measurement of Granzyme B and cytokine production of CD46-induced Tregs

Induced Tregs (iTregs) were generated after peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. The PBMC were washed, aliquoted in freezing media (20% DMSO in Fetal Bovine Serum (FBS) plus 5% glucose in FBS), and stored in liquid nitrogen. T lymphocytes (CD4+) were isolated by magnetic bead separation (CD4-positive isolation kit, Miltenyl Biotec, Auburn, CA). *In vitro* stimulation was performed by plate-bound anti-CD3 (10ug/mL, OKT3) and soluble anti-CD46 (10 μg/mL, TRA-2-10) or PBS. Human CD3 antibody (OKT3) was purchased from ATCC (Manassas, VA) and human CD46 antibody (TRA-2-10) was obtained from Rheumatic Diseases Core Center, Washington University School of Medicine (Saint Louis, MO). Briefly, $CD4^+$ T lymphocytes (5.5×10^4 cells/well) were added to antibody coated 96 well plates (Roswell Park Memorial Institute) in RPMI1640 medium (Sigma-Aldrich, St Louis, MO) with 10% fecal calf serum (GE Healthcare Life Sciences, Logan, Utah) plus 200 mM L-glutamine and 25 U/mL recombinant human IL-2 (Chiron, Emeryville, CA). After incubation at 37 °C in 5% CO_2 for 3 days, cell viability was assessed under the light microscope using Trypan blue exclusion. Viability was >98% on average and across conditions. An increase in cell size, an indicator of cellular proliferation, was also observed by light microscopy and an increase in FS/SS on the flow cytometer. Supernatants and cells were then collected for cytokine and granzyme B (GZB) analysis. Experiments were performed in duplicate with data presented as the mean +/− standard deviation.

Cytokine levels (IL-10, IL-2, IL-4, IL-5, TNF-α, and IFN-γ) were determined by cytometric bead array (Human Th1/Th2 kit, BD Biosciences, San Jose, CA). Data were obtained on a FACSCalibur flow cytometry (BD Biosciences) with analysis performed by FCAP Array Software v3 (BD Biosciences). Intracellular GZB was assayed via flow cytometry according to methods outlined for intracellular staining (eBioscience). Briefly, cells were harvested, fixed and permeabilized in fixation and permeabilization buffer (eBioscience). Cells were labeled with FITC-conjugated anti-granzyme B antibody (GB11, eBioscience) or isotype control antibody. After antibody labeling, cells were analyzed by FACSCalibur (BD Biosciences) and CellQuest™Pro version 5.2.1 software (Becton-Dickinson Corporation, Mountainview, CA).

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2.4 Identification of DC subsets

Dendritic cells were isolated from PBMC using the Blood Dendritic Cell Enumeration kit (Miltenyl Biotec). Dendritic cell subsets were identified by anti-BDCA-2^+ ($CD11c^-$/$CD123^+$high) for pDCs, anti-BDCA-1^+ ($CD11c^+$ high/$CD123^+$ low) for mDC1 and anti-BDCA-3^+ ($CD11c^+$low/$CD123^-$) for mDC2(Dzionek et al., 2000). Samples were also labeled with CD19-PE-Cy2 and CD14-PE-Cy5 (eBiosciences), for exclusion of B cells and monocytes. Cells were analyzed by FACSCalibur (BD Biosciences).

2.5 Statistical analysis

The intra-class correlation coefficients (ICC) were generated to evaluate IBV of samples obtained across 3 days and were adjusted for age, gender, and atopic status using SAS software, version 9.3 (SAS Institute, Cary, NC)

3. Results and discussion

Of 10 healthy subjects (age 42.6 ± 10.2 years), 7 were female, 6 were atopic and race/ethnicity was as follows: 7 Caucasians, 2 African Americans and 1 Hispanic. Table 1 demonstrates the IBV of DCs, nTreg and GZB expression in iTreg and $CD4^+$ cells in these healthy subjects. There was substantial IBV in the measurement of mDC2 (ICC=0.17), likely due to the rare events of these cells in peripheral blood.

Table 2 demonstrates the IBV of cytokine production from iTreg in healthy subjects. There was substantial IBV in the measurement of iTreg production of IL-10 (ICC=0.49, Table 2). Little IBV exist in the quantification of mDC1 (ICC=0.90), pDC (ICC=0.91) and nTregs (ICC=0.86). Age and gender had minimal effects on biological variability (ICC adjusted Table 1 & 2). The ICC of induced Treg production of IL-4 was affected by atopic status, with decreases in the ICC of >0.1. Surprisingly, there is considerable variability of IL-10, TNF-α, and IFN-γ production in the PBS/IL2 control condition (ICC <0.5). Due to the 4–5 ng/mL limit of detection for both IL-4 and IL-5 in the CBA, the variability of IL-4 and IL-5 in the PBS/IL2 control condition is not meaningful (Morgan et al., 2004). To evaluate the role of circadian effects, we calculated the ICC after adjusting for the mean time of day at which data were collected and there were no significant changes in ICC after adjustment (mean change of ICC 0.02).

Limitations of this study include small sample size, presence of IL-2 - a survival factor in control conditions, lack of cell viability and apoptosis assessment within each study subject across all experimental conditions, and data collection at three time points. Although the flow cytometer gates were set using isotype controls, background noise within the gate for each population of cells was not determined and therefore could account for the variation of rare populations of cells as with mDC2 (Rovati et al., 2008). The variability in mDC2 is unlikely to be a sampling issue, as the observed range of % mDC2 in this study is consistent with a previously published report (Rovati et al., 2008).

We conclude human immunologic experiments involving rare event cell populations should take into consideration the temporal biological variability for each measurement reported.

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Therefore, repeat measurements should be obtained from study participants at multiple time points to ensure accuracy of the reported findings.

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Abbreviations

DCs	Dendritic cells
pDC	plasmacytoid Dendritic cells
mDC1	myeloid Dendritic cell subpopulation 1
mDC2	myeloid Dendritic cell subpopulation 2
Tregs	Regulatory T cells
nTreg	natural Tregs
iTreg	CD3/CD46 induced Tregs
IBV	Intra-individual Biological Variability
PBMCs	Peripheral Blood Mononuclear Cells
FBS	Fetal Bovine Serum
GZB	Granzyme B
ICCs	Intraclass Correlation Coefficient

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Highlights

- There is substantial day-to-day variability in measuring mDC2 *in vivo* and iTreg production of IL-10 *in vitro* in healthy adults.
- There is a low intra-individual variability in measuring nTregs, pDCs and mDC1 in healthy adults.
- Repeat measurements over time of human biologic samples should be performed to determine the most representative values.

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Table 1

The intra-individual variability of DC, nTreg cells, intracellular granzyme B expression of iTregs, and $CD4^+$ cells in healthy adults

DC subsets	**%leukocytes**	**Unadjusted ICCs**	**ICCs adjusted by age, gender and atopy***
mDC1	0.22 ± 0.10	0.90	0.82
mDC2	0.02 ± 0.02	0.17	0
pDC	0.27 ± 0.12	0.91	0.92
Tregs	**%CD4+ cells**	**Unadjusted ICCs**	**ICCs adjusted by age, gender and atopy***
Natural Tregs	3.27 ± 1.27	0.86	0.88
GZB expression of CD46-induced Tregs	35.3 ± 17.7	0.77	0.78
GZB expression of $CD4^+$cells in PBS/IL2	0.83 ± 0.62	0.42	0.53

Data is shown as mean ± standard deviation

Abbreviations: DC- dendritic cells (BDCA-1+ for mDC1, BDCA-3+ for mDC2 and BDCA-2+ for pDC), nTreg- natural regulator T cells, iTreg- CD46-induced regulatory T cells, PBMC- peripheral blood mononuclear cells, ICCs- Intra-class correlation coefficients, GZB- granzyme B

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Table 2

The intra-individual variability of cytokine production of iTregs and CD4+ cells in healthy adults

Cytokine levels	pg/ml	ICC	ICCs adjusted by age	ICC adjusted by gender	ICC adjusted by atopy	ICC adjusted by age, gender and atopy
CD46-induced Tregs						
IL-10	2103 ± 1128	0.49	0.49	0.52	0.53	0.59
IFN-γ	13200 ± 4942	0.68	0.71	0.64	0.67	0.70
TNF-α	1503 ± 794	0.63	0.66	0.66	0.57	0.65
IL-4	18.3 ± 21.3	0.74	0.75	0.71	0.57	0.55
IL-5	38.1 ± 26.3	0.82	0.83	0.78	0.83	0.82
PBS/IL2 (Control condition)						
IL-10	110 ± 88	0.40	0.42	0.25	0.33	0.23
IFN-γ	1154 ± 2021	0.40	0.43	0.36	0.29	0.34
TNF-α	29.4 ± 29.4	0.37	0.39	0.40	0.41	0.48
IL-4	0.01 ± 0.03	0.41	0.34	0.43	0.42	0.41
IL-5	1.47 ± 2.87	−0.0037	0.034	−0.032	0.039	0.048

Data is shown as mean ± standard deviation. Limit of detection of cytokines= 4–5 ng/mL.