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Within-woman change in regulatory T cells from pregnancy to the postpartum period

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Abstract

Regulatory T cells (Treg cells) are an important area of investigation in human health and disease. In this study, the trajectory of percentage of Treg cells (defined as CD4+CD25+Foxp3+CD127– lymphocytes) was measured in the blood of 208 women during pregnancy and up to 3 additional times in the postpartum period (1, 6 and 12 months postpartum). Whether the trajectory was affected by gravidity, parity, neonatal sex, pet exposure, maternal atopic and asthma status, smoking, maternal race or other pregnancy factors was examined. Multilevel models were fit using full maximum likelihood methods and included both random and fixed effects. Overall, percentages of Treg cells increased from the prenatal to the postpartum period. Among women who were not atopic, nulliparous women had lower percentages of Treg cells over time compared with parous women. Atopic women with pets in the home during pregnancy had lower percentages of Treg cells than atopic women who did not have pets. The trajectory was not affected by the other factors investigated. We conclude that within-woman change in percentages of Treg cells may vary by time in relation to delivery, as well as by maternal atopic status and exposure to pets and number of prior births. The data did not indicate an overall decline in Treg cells in the postpartum period. Future work to better identify the role of Treg cells in successful pregnancy would ideally include a set of well characterized women sampled serially starting prior to pregnancy and throughout the postpartum period.

INTRODUCTION

Regulatory T cells (Treg cells) are an important area of investigation in understanding human health and disease. Treg cells play a key role in immune self-reactivity and immune tolerance (Izcue, A. and Powrie, F. 2005; Sakaguchi, S. 2000); however, the precise mechanism by which they are regulated and their function are incompletely understood (Mellor, A.L. and Munn, D. 2004.; Somerset, D.A., et al. 2004; Powrie, F. and Maloy, K.J. 2003). The examination of the role of Treg cells in numerous health conditions, including allergic and autoimmune diseases and pregnancy, is ongoing. Details about patterns of Treg

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cells in successful pregnancy and the postpartum period continue to be revealed as markers to identify Treg cells become more specific (Somerset, D.A., et al. 2004; Sasaki, Y., et al. 2004; Heikkinen, J., et al. 2004; Sasaki, Y., et al. 2007; Mjösberg, J., et al. 2009; Wegienka, G., et al. 2009; Guerin, L.R., et al. 2009).

Analyses have suggested that decreasing levels of Treg cells in the postpartum period indicate a role for them in maternal alloantigen tolerance during pregnancy (Somerset, D.A., et al 2004). Other researchers have demonstrated that patterns of Treg cells in pregnancy may not be elevated in successful pregnancy (Mjösberg, J., et al 2009). Prior literature on this pattern from pregnancy through the postpartum period has been limited by the use of cross-sectional data (Somerset, D.A., et al. 2004; Sasaki, Y., et al. 2004; Heikkinen, J., et al. 2004; Sasaki, Y., et al. 2007; Mjösberg, J., et al. 2009) rather than the measurement of within-woman change using longitudinal data.

For this current investigation, we examined the trajectory of regulatory T cell percentages (defined as CD4+CD25+Foxp3+CD127- lymphocytes) measured in the blood of women from pregnancy through the postpartum period. We also examined how this trajectory was affected by gravidity, parity, sex of the neonate, pet exposure, maternal atopic and asthma status, smoking status, gestational age, delivery type, preeclampsia/pregnancy induced hypertension and maternal race.

MATERIALS AND METHODS

1. Study Population

The Wayne County Health, Environment, Allergy and Asthma Longitudinal Study (WHEALS) is an ongoing birth cohort study in southeastern Michigan designed to examine the relationships between early life exposures such as pets, infections or endotoxin and allergic diseases in early childhood. Women who were 21–49 years old, from a predefined geographic area, who were in their second trimester or later of pregnancy, were seeing a Henry Ford Health System (HFHS) medical group obstetrician at one of five clinics, and planned to stay in the Detroit area for at least two years after delivery were recruited for the study of their children's health. Recruitment for this sub-study occurred over a 12-month period when funding for Treg cell analyses in a subset of 226 mothers became available. Women completed an interview about their health histories at the time of recruitment and provided a venous blood sample. Home visits were conducted at 1, 6 and 12 months postpartum according to the WHEALS protocol. Additional blood samples were collected from the women at these postpartum visits. This research was approved by the HFHS IRB.

2. Interview

Covariates used in the analyses were based on maternal report in the WHEALS prenatal interview. Women were asked to report their race based on U.S. Census 2000 categories. Maternal report of the timing (month and year ended), duration (weeks or months) and outcome of each of their prior pregnancies was also recorded. Women were asked if they had ever been diagnosed with asthma. Current asthma was defined as ever having a doctor diagnosis of asthma and self-report of either an asthma attack or use of an asthma medication in the last 12 months.

Details on maternal smoking were also collected during the prenatal and subsequent interviews. Women were asked if they smoked at least one tobacco product of any type per day during their pregnancy or in the year after their pregnancy. We did not collect information on smokeless tobacco use.

Details on pet exposure were collected during the prenatal interview, as well as during the postpartum contacts. Women were asked whether they had owned or cared for any pets in their homes for more than one week since they learned they were pregnant. Pet keepers were then asked to identify the usual number of hours the pet was inside the home each day. For this report, we define those women who reported owning or caring for dogs or cats that spent 12 or more hours each day in the home for more than 1 week as being exposed.

We also reviewed the medical charts of the women for diagnoses of preeclampsia, HELPP syndrome, pregnancy induced hypertension (PIH), and gestational hypertension. We also recorded the delivery mode for each pregnancy, as well as gestational age at delivery.

Tobacco exposure and pet keeping were allowed to vary at each time point for our analyses.

3. Laboratory Measurements

Because the blood samples were being utilized for whole blood activation and analysis of intracellular cytokines for the parent WHEALS project, aliquots (0.5 ml) of whole blood collected in heparinized tubes were incubated for 6 hours at 37°C. Brefeldin A was added after two hours of incubation. The samples were then incubated with BD Fast Immune™ EDTA Solution (BD Biosciences, San Jose, CA) for 15 minutes followed by BD FACS Lysing Solution (BD Biosciences) for 10 minutes to lyse red blood cells and fix white blood cells. Treg cell analyses were performed on unstimulated blood samples and stored at -80°C until staining and flow cytometry analysis.

Fluorochrome-conjugated antibodies against the cell surface marker CD25 (anti-CD25 FITC, BD Biosciences) and the Treg activation marker CD127 (anti-CD127-Alexa Fluor 647, BD Biosciences), along with anti-CD4 PerCP-Cy5-5 (BD Biosciences) for detection of CD4+ T cells were added to each 100 µl staining aliquot. Following incubation (in the dark) with these cell surface marker antibodies, cells were then permeabilized by addition of Fixation/Permeabilization buffer (eBioscience, San Diego, CA) and incubated with fluorochrome-conjugated antibody against FOXP3 (anti-FOXP3 PE, eBioscience). Cells were incubated at room temp in the dark and fixed with 1% paraformaldehyde in PBS. Flow cytometry was performed within 24 hours of staining. We did not incorporate the CD127 marker until after prenatal sample collection began for this study. Because of this, some women had samples collected and analyzed for FOXP3, but not the additional measurement of CD127. The analyses include all samples that had a CD127 measurement.

Flow cytometry was performed with a BD LSR flow cytometer (BD Biosciences). Data were collected and analyzed using CellQuest Pro software (BD Biosciences). Matched isotype controls were used to set quadrants and regions of positive staining. Cells were gated on lymphocytes using forward and side light scatter properties and a minimum of 10,000 lymphocyte gated events were acquired. CD4+ T lymphocytes were analyzed with bivariate dot plots of CD25 vs FOXP3. CD4+CD25+FOXP3+ lymphocytes were then analyzed with bivariate dot plots of CD4 vs CD127. Events were finally separated as either CD127 positive or negative. Lymphocytes that were CD4+CD25+FOXP3+CD127- were considered to be Treg cells (Liu, W., et al. 2006; Seddiki, N., et al. 2006; Santner-Nanan, B., et al. 2008). Figure 1 demonstrates the gating strategy for identification of Treg cells. All Treg cell analyses were performed by a single immunologist (KRB). The coefficients of variation for these tests were within 5% for duplicate samples analyzed within the same flow cytometry run and within 15% for duplicate samples run on different days. Treg cells are expressed as a percentage of CD4+CD25+FOXP3+ lymphocytes that are CD127-.

Venous blood from the prenatal sample was analyzed to assess allergen-specific IgE. Plasma was isolated and stored at -80°C until assayed. Measurements of allergen-specific IgE were

performed following the standard manufacturer's protocols using the Pharmacia UniCAP system (Phadia, Portage, MI). Allergen-specific IgE was analyzed for: dust mite (*Dermatophagoides farinae*), dog, cat, Timothy grass (*Phleum pratense*), ragweed (*Ambrosia elatior*), mold (*Alternaria alternata*), hen's egg white and cockroach (*Blattella germanica*). One percent of all assays were repeated in a different assay run on a different day to provide estimates of interassay reliability. The geometric mean coefficient of interassay variation was 5.9% for all eight allergens. Sensitization was defined as a positive allergen-specific IgE result of ≥ 0.35 kU/L. Atopy was defined as having at least one allergen specific sensitization.

4. Statistical Methods

Firstborn status, first pregnancy, sex of the neonate, current asthma, atopy, pet exposure, tobacco smoking status, gestational age, delivery type, preeclampsia/pregnancy induced hypertension and self-reported African-American race were evaluated for their associations with the trajectory of Treg cells from pregnancy through the postpartum period.

All data from all possible time points were included in the analyses. To assess within-woman change in Treg cells over the study period, a time variable was defined as the number of months between the blood collection date and the birth of child. For blood collection during pregnancy, this was a negative number (of months); for blood collected after delivery, the number of months was positive. The time variable is thus anchored to delivery date.

Multilevel models (MLM) were fit using full maximum likelihood methods and included both random and fixed effects (called *mixed* effect models, PROC MIXED (SAS V9.2, Release 2, Cary, NC)). Random effects were necessary to account for the correlation within individual (repeated measures). The multilevel model approach is flexible enough to allow and account for variability in the spacing of collection points. This approach was important because while the study's intention was to meet with every woman at 1, 6 and 12 months postpartum, the dates were not exact. We employed a two-level MLM in order to address both within-individual change over time and between-individual change as affected by additional covariates (e.g.; maternal atopic status, race, etc). This allowed us to directly address our research aims.

To determine the best fitting models for our data, a series of nested and increasingly complex models were fit. Model fits were compared using a deviance statistic, and were further assessed by Akaike information criteria (AIC). Treg cell data were log-transformed prior to inclusion in the models due to non-normality.

RESULTS

Of the 208 women enrolled with Treg cells measured for at least 1 time point, 33 (15.9%) had only 1 sample included, 62 (29.8%) had exactly 2 samples included, 63 (30.3%) had 3 samples included and 50 (24.0%) had all four samples included. Details about the study population appear in Table 1. The study population was mostly African American (65.9%), and had a prior pregnancy (74%) and prior live birth (57.7%). Few women smoked during pregnancy and/or the postpartum period (10.6%) and more than half were atopic (59.2%). Approximately one-third kept a dog or cat in their home during pregnancy (35.1%), about half the children were female (48.6%) and 13.9% of the women had current asthma. Fourteen (6.7%) women had diagnoses of preeclampsia, HELLP syndrome, pregnancy induced hypertension (PIH), or gestational hypertension and 84 (40.3%) women delivered by cesarean section. Most women (194, 93.3%) delivered after reaching their 37th week of

gestation. Descriptive statistics about the timing of sampling and the number of samples included in the analyses from each time point are presented in Table 2.

Overall, percentages of Treg cells increased from the prenatal to the postpartum period (Figure 2). Factors influencing the trajectory varied depending on maternal atopic status; atopic women had a higher and curvilinear trajectory pattern compared with nonatopic women. Among women who were not atopic, nulliparous women had lower percentages of Treg cells over time compared with parous women (Table 3, Figures 3a and 3b). Among women who were atopic, the level of the trajectory varied by whether a pet was present in the home (Table 4, Figures 4a and 4b). Those atopic women with pets in the home during pregnancy had lower percentages of Treg cells than atopic women who did not have pets in the home during pregnancy.

The models indicate the magnitude of the differences between subgroups. For example, among non-atopic mothers, the parameter estimate for time since delivery is 0.04 (Table 3). The result of exponentiating 0.04 ($e^{0.04}$), or 1.04, can be interpreted as a 4% increase in percentage of Treg cells with each postpartum month and an overall decrease of 21% ($e^{-0.26}$) associated with delivering their firstborn. While calculations are more complex for the model for atopic women since there is a quadratic term in the model (Table 4), there is an overall decrease of 20% ($e^{-0.21}$) associated with pet exposure and 12% increase in the percentage of Treg cells from birth to 1 month postpartum, for example.

The overall trajectory was not affected by sex of the neonate, maternal race, gravidity, maternal asthma status, smoking status, delivery type, gestational age at delivery or preeclampsia, HELLP syndrome, PIH or gestational hypertension.

DISCUSSION

Our data indicate that within woman change in percentages of Treg cells may vary by time in relation to delivery, as well as by maternal atopic status and exposure to pets and prior births. The data do not support the conclusion that Treg cells always decrease in the postpartum period.

We think that these data may indicate that pregnancy “educates” the immune system in a way that persists into the postpartum period and that these pregnancy-associated immune changes may affect the immune status of women in their subsequent pregnancies. However, the effect of this pregnancy-induced change may wane over time in the postpartum period. Additionally, with respect to the results based on atopic status, recent investigations suggest that atopic individuals may have dysfunctional Treg cells (Bacchetta R, et al. 2007). Thus atopic women may have lower levels of Treg cells in the presence of allergen (dog/cat) compared with unexposed atopic women.

Placing our data in the context of the existing body of research is somewhat complicated. Laboratory methods have evolved to identify more specific markers of Treg cells, thus making direct comparisons to past publications difficult due to variability in the method of identifying Treg cells. Prior publications have tended to report an increase in levels of Treg cells in successful pregnancy with a peak in the first trimester and a decline prior to delivery into the postpartum period (Somerset, D.D., et al. 2004; Heikkinen, J., et al. 2004; Sasaki, Y., et al. 2004; Santner-Nanan, B., et al. 2009).

These results were found through the cross-sectional study of separate and unique populations of pregnant, postpartum and nonpregnant women. There were no analyses of within-woman change over time. Additionally, prior analyses used CD4+CD25+ or

CD4⁺CD25^{high} as markers of regulatory T-cells, which are not uniquely specific markers of Treg cells (Saito, S., et al., 2005; Mjösberg, J., et al. 2009).

Recently, Mjösberg et al. reported that circulating Treg cells (CD4^{dim}CD25^{high}Foxp3⁺) were reduced in the second trimester of human pregnancy. Those Treg cells, however, were still suppressive (Mjösberg, J., et al. 2009). The authors concluded that systemic tolerance during pregnancy is not facilitated by Treg activity. They suggested that earlier research in the role of Treg cells in pregnancy should be reevaluated.

Our results are more consistent with the conclusions of Mjösberg et al. (Mjösberg et al. 2009) and reach different conclusions than the other publications on Treg cells in pregnancy (Somerset, D.A., et al. 2004; Heikkinen, J., et al. 2004; Sasaki, Y., et al. 2004; Santner-Nanan, B., et al. 2009). Our data do not demonstrate an overall decrease in Treg cells in the circulating blood of postpartum women. Our data are novel in that we have examined within-woman and between women changes and the factors affecting these changes. The observed patterns and relationships can only be investigated in longitudinal data.

Unlike other studies, we examined various factors that affected the trajectories of Treg cells. These data indicate that the timing of sample collection in relation to delivery is also associated with variation in the percentage of Treg cells. Timing of sample collection should be considered in the design of future studies of Treg cells in pregnancy and the postpartum period. Future studies must also ensure that additional factors such as atopy and reproductive history are included in their analyses or at least considered in their study subject selection.

As with all statistical models (linear regression, logistic regression, etc.) used in analyses, the models presented are those that provide the best overall fit of the data. This does not mean that every woman had the exact same patterns or that all women had strictly increasing levels of Treg cells over time.

Ideally, we would have had more women with samples from every time point, as well as collected samples at more time points, including delivery. However, the additional time points were beyond the scope of this project. Also, the modeling approach used all available data from all women. Thus, no woman was excluded from the analyses for incomplete data. Additionally, prepregnancy samples would have been useful in our analyses. Collection of these samples would be difficult since large numbers of pregnancies are not planned and planned pregnancies may not be immunologically representative of all pregnancies. Although not all women contributed samples at all time points, our analytical methods did not require complete data for inclusion in the trajectory analyses. Additionally, the single greatest factor for women not contributing all four samples to the analyses is because of delayed implementation of CD127 staining. Original plans for this sub-study were to stain only for CD4, CD25 and Foxp3. However, after this sub-study was underway, staining for CD127 was implemented in our laboratory using then newly available commercial reagents.

An additional limitation of our study is that we did not conduct functional studies of these Treg cells. However, we did measure CD127 which is reported to be downregulated in active regulatory T cells and can therefore be used as a surrogate marker for functionality (Liu, W., et al. 2006; Seddiki, N., et al. 2006; Santner-Nanan, B., et al. 2008).

Future work to better identify the role of Treg cells in successful pregnancy would ideally include a set of well characterized women sampled serially starting prior to pregnancy throughout the postpartum period. This approach will provide the best evidence for drawing the strongest conclusions about these important immune system regulators and their role in successful pregnancy.

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Figure 1.

Gating Strategy for Treg Analysis: Lymphocytes were gated using forward scatter vs. side scatter characteristics (A). Gated lymphocytes were then examined for CD4 expression (B). Gated CD4+ lymphocytes were next analyzed for Fxp3 and CD25 expression (C), number represents the percentage of CD4+ lymphocytes that are CD25+ Fxp3+. CD4+ CD25+ Fxp3+ lymphocytes were then analyzed for CD127 expression (D), numbers represent the percentage of CD4+ CD25+ Fxp3+ cells that are CD127+(92.7%) or CD127– (7.6%). Lymphocytes negative for CD127 expression that were also CD4+ CD25+ Fxp3+ were counted as regulatory T cells.

Figure 2.

The overall trajectory of Treg cells from pregnancy through 12 months postpartum. The x axis is the months before and after delivery labeled “Birth”. The y axis is the percentage of CD4+CD25+Foxp3+ lymphocytes that are CD127-. The dotted lines are the 95% confidence intervals. The vertical lines at birth indicate a gap for which we are unable to estimate Treg cells at parturition.

Figure 3.

The overall trajectory of Treg cells among nonatopic women from pregnancy through 12 months postpartum is presented separately for nulliparous (Figure 3a) and parous (Figure 3b) women. The x axis is the months before and after delivery labeled “Birth”. The y axis is the percentage of CD4+CD25+Foxp3+ lymphocytes that are CD127⁻. The dotted lines are the 95% confidence intervals. The vertical lines at birth indicate a gap for which we are unable to estimate Treg cells at parturition.

Figure 4.

The overall trajectory of Treg cells among atopic women from pregnancy through 12 months postpartum is presented separately for those who did (Figure 4a) and did not have (Figure 4b) pets during pregnancy. The x axis is the months before and after delivery labeled “Birth”. The y axis is the percentage of CD4+CD25+Foxp3+ lymphocytes that are CD127-. The dotted lines are the 95% confidence intervals. The vertical lines at birth indicate a gap for which we are unable to estimate Treg cells at parturition.

Table 1

Details of the study population and their pregnancies (N=208)

	N	% of Total
African American	137	65.9
Not African American	71	34.1
First pregnancy	54	26.0
First livebirth	88	42.3
Smoked during pregnancy and/or the postpartum period	22	10.6
Atopic *	122	59.2
Kept dogs or cats in home during pregnancy	73	35.1
Baby was female	101	48.6
Mother had current asthma	29	13.9
Mother had preeclampsia, HELLP syndrome, pregnancy induced hypertension or gestational	14	6.7
Delivery was a c-section	84	40.3
Gestational age at delivery		
<32 Weeks	3	1.4
32–36 Weeks	11	5.3
37+ Weeks	194	93.3

* Atopic status is defined as having a specific IgE ≥ 0.35 ku/IL for at least one of the following allergens: dust mite, dog, cat, Timothy grass, ragweed, *Alternaria alternata*, egg white and cockroach. The atopic status of 10 women was determined using their 1 month postpartum samples due to inadequate sample volumes collected during pregnancy. Two women did not have sufficient blood collected to allow measurement of specific IgEs at any time point.

Time (in months) of sample collection in relation to birth (negative indicates time preceding birth and positive indicates time since birth). Samples with CD4+CD25+Foxp3+CD127− results included in the analyses are presented.

Table 2

Time	N	Median Months	Mean Months	Std dev.	Range
Pregnancy	121	−1.4	−1.4	0.9	−3.4 to −0.03
Approximately 1 month postpartum	140	1.1	1.3	0.8	0.6–4.6
Approximately 6 months postpartum	169	6.3	6.6	1.0	5.5–10.0
Approximately 12 months postpartum	116	12.0	12.4	1.1	11.6–18.1

Table 3

Level 2 model of Treg cells over time in non-atopic mothers.*

	Parameter Estimate	Standard Error	Pvalue
Intercept	1.77	0.09	<0.0001
Time since delivery	0.04	0.01	0.0002
Firstborn	-0.26	0.09	0.01

* Atopic status is defined as having a specific IgE ≥ 0.35 ku/IL for at least one of the following allergens: dust mite, dog, cat, Timothy grass, ragweed, *Alternaria alternata*, egg white and cockroach. The model indicates the magnitude of the differences between subgroups. For example, among non-atopic mothers, the parameter estimate for time since delivery is 0.04 (Table 3). The result of exponentiating 0.04 ($e^{0.04}$), or 1.04, can be interpreted as a 4% increase in percentage of Treg cells with each postpartum month and an overall decrease of 21% ($e^{-0.26}$) associated with delivering their firstborn.

Table 4

Level 2 model of Treg cells over time in atopic mothers.*

	Parameter Estimate	Standard Error	Pvalue
Intercept	1.84	0.08	<0.0001
Time since delivery	0.11	0.025	<0.0001
Time since delivery ²	−0.006	0.002	0.002
Pet present	−0.21	0.09	0.02

* Atopic status is defined as having a specific IgE ≥ 0.35 ku/IL for at least one of the following allergens: dust mite, dog, cat, Timothy grass, ragweed, *Alternaria alternata*, egg white and cockroach. The models indicate the magnitude of the differences between subgroups. Calculations are more complex for the model for atopic women since there is a quadratic term in the model (Table 4), there is an overall decrease of 20% ($e^{-0.21}$) associated with pet exposure and 12% increase in the percentage of Treg cells from birth to 1 month postpartum, for example.