



Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jreprimm



Regulatory T cells vary over bleeding segments in asthmatic and non-asthmatic women

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ARTICLE INFO

Article history:

Received 6 July 2010

Received in revised form 1 December 2010

Accepted 16 March 2011

Available online xxx

Keywords:

Regulatory T cells

Menstrual cycle

ABSTRACT

Sex hormones may play an important role in observed gender differences in asthma incidence and severity. Regulatory T cells (Treg cells) are presumed to be involved in asthma and may vary with hormone levels. To investigate the effects of sex hormones on levels of Treg cells (percentage of CD4+CD25+Foxp3+ lymphocytes that are CD127[−]), a cohort of 13 women (6 with and 7 without an asthma diagnosis) had blood drawn multiple times over the course of a bleeding segment (bleeding interval plus the following bleeding-free interval) and collected urine samples daily for measurement of estrogen (estrone E1C) and progesterone (pregnanediol-glucuronide PDG) metabolites. The samples from non-asthmatic women indicated no association between bleeding segment day and Treg cells. Asthmatic women showed a 3% increase in Treg cell percentage with each successive day over the bleeding segment. Among non-asthmatic women, Treg cell percentages were not associated with PDG levels on the same day, or 1, 2 or 3 days before Treg cell measurement. E1C was positively correlated with the Treg cell percentage measured only on the same day – a 5% increase in E1C was associated with a 1.4% increase in Treg cell percentage. Among asthmatic women, only E1C was associated with Treg cell percentages after adjusting for PDG on the same day and 1 and 2 days before Treg cell measurement. A 5% increase in E1C was associated with a 2.3% increase in Treg cell percentage. A larger study of contiguous cycles to better determine within-woman cyclicity of the observed patterns is needed.

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1. Introduction

Women are more likely than men to ever have asthma or visit an emergency department or be admitted to a hospital for asthma or die due to asthma (National Center for Health Statistics; Centers for Disease Prevention and Control). Sex hormones may play an integral role in these disparities. Investigations of the role of sex hormones in asthma frequency, severity and outcomes in women have been plagued by poor study design, limited data collection, and small and select study populations. Limited reports in

the literature suggest that asthma symptoms worsen during times of hormonal change such as puberty, pregnancy, menopause and the premenstrual period (Balzano et al., 2001). Publications detailing the relationship between hormone patterns and underlying mechanisms related to asthma have not been well studied in women.

Currently, maintenance of airway homeostasis is believed to result from the suppressive action of a population of T cells, identified as CD4+CD25+Foxp3+CD127^{low/−}, and referred to as T regulatory cells (Treg cells) (Klein et al., 2010; Lloyd and Hawrylowicz, 2009). Treg cells can be divided into thymically derived natural Treg cells (nTreg cells) or inducible Treg cells (iTreg cells). These cells are identified via expression of a combination of extracellular (CD4+CD25^{bright}CD127^{lo}) and intracellular (FoxP3+)

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markers (Seddiki et al., 2006; Liu et al., 2006; Klein et al., 2010). The combination is necessary since human activated T cells normally upregulate CD25 and undergo a transient induction of FoxP3 (Gavin et al., 2006). Only in combination do the markers clearly identify a population of T cells with suppressive activity. The importance of Treg cells in immune regulation is their ability to induce “tolerance to self-antigens and many harmless environmental antigens” (Robinson, 2010).

If sex hormones are related to gender differences in asthma, and Treg cells are also related to asthma, we could expect that Treg cells would vary with sex hormone levels. We would also expect there to be differences in the associations between hormones and Treg cells depending on asthma status, and this serves as the overall objective of this work. While an earlier report indicated that Treg cells may vary over the human menstrual cycle (Arruvito et al., 2007), detailed analyses of within-woman change in Treg cells over the menstrual cycle by asthma status are not available.

To determine the effects of sex hormones on levels of Treg cells, we had four main objectives in our study population of asthmatic and non-asthmatic women:

- 1) To determine whether Treg cells vary over the menstrual cycle;
- 2) to determine whether any observed patterns of Treg variability are the same before and after the estimated day of ovulation;
- 3) to explore whether the changes in absolute levels of hormones (estrogen and progesterone metabolites) and/or the ratios of those hormones are associated with changes in levels of Treg cells; and
- 4) to determine whether any observed associations differ for asthmatic and non-asthmatic women.

We investigated these objectives using a cohort of 13 women who had their blood drawn multiple times over the course of a bleeding segment (bleeding episode plus bleeding-free interval) and collected urine samples daily for measurement of estrogen (estrone E1C) and progesterone (pregnanediol-glucuronide PDG) metabolites.

2. Materials and methods

2.1. Study population

Women were recruited from employees of Henry Ford Health System, Detroit, Michigan. Advertisements were placed in the weekly system announcements seeking women who were having menstrual periods and were not taking hormones for any reason. Women were required to speak English well enough to provide written informed consent. This research was approved by the HFHS IRB. The goal was to recruit a study population enriched with asthmatic women to allow comparisons between asthmatic and non-asthmatic women.

2.2. Study protocol

The study lasted six weeks, and women were asked to begin the study a few days prior to when they expected to begin their next menstrual period. Women also were asked to complete a baseline interview about their basic and reproductive health, including the number of prior pregnancies and live births. Women also were asked whether they had ever been diagnosed with asthma by a doctor and to report all medication they were taking for any reason. At this time, the first of a series of blood samples was collected from the women.

Blood samples were collected from the women up to three times per week. Samples were collected Monday through Friday. Women contributed no more than 18 blood samples over the course of the study; no woman was sampled on more than two consecutive days. Participants were also asked to collect a daily sample of their first morning urine. Samples were then frozen immediately until analyzed for urinary metabolites of estrogen (E1C) and progesterone (PDG).

For the purposes of this study, we subscribed to the terminology recommended by the WHO and defined a bleeding segment as a bleeding episode followed by a bleeding-free interval (Belsey and Farley, 1988). The term bleeding segment is used because ovulation is estimated and not observed.

2.3. Measurement of Treg cells

All samples were analyzed for CD4, CD25, Foxp3 and CD127. Aliquots (0.5 ml) of whole blood collected in heparinized tubes were incubated with BD FastImmune™ EDTA Solution (BD Biosciences, San Jose, CA) for 15 min followed by BD FACS Lysing Solution (BD Biosciences) for 10 min to lyse red blood cells and fix white blood cells. Treg analyses were performed on unstimulated blood samples only. Samples were frozen at -80°C until staining and flow cytometry analysis was performed.

For each blood sample, a 100 μl aliquot was incubated with the following antibodies to identify regulatory T cells: anti-CD4 PerCP-Cy5.5, anti-CD25 FITC, and anti-CD127 Alexa Fluor 647 (all BD Biosciences). Following incubation with these cell surface marker antibodies, cells were then permeabilized by addition of Fixation/Permeabilization buffer (eBioscience, San Diego, CA) and incubated with anti-Foxp3 PE (eBioscience). Cells were incubated at room temp in the dark and fixed with 1% paraformaldehyde diluted in PBS. Flow cytometry was performed within 24 h of staining.

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 minimize non-specific 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 versus Foxp3. CD4⁺CD25⁺Foxp3⁺ lymphocytes were then analyzed with bivariate dot plots of CD4 versus CD127. Events were then divided into CD127 pos-

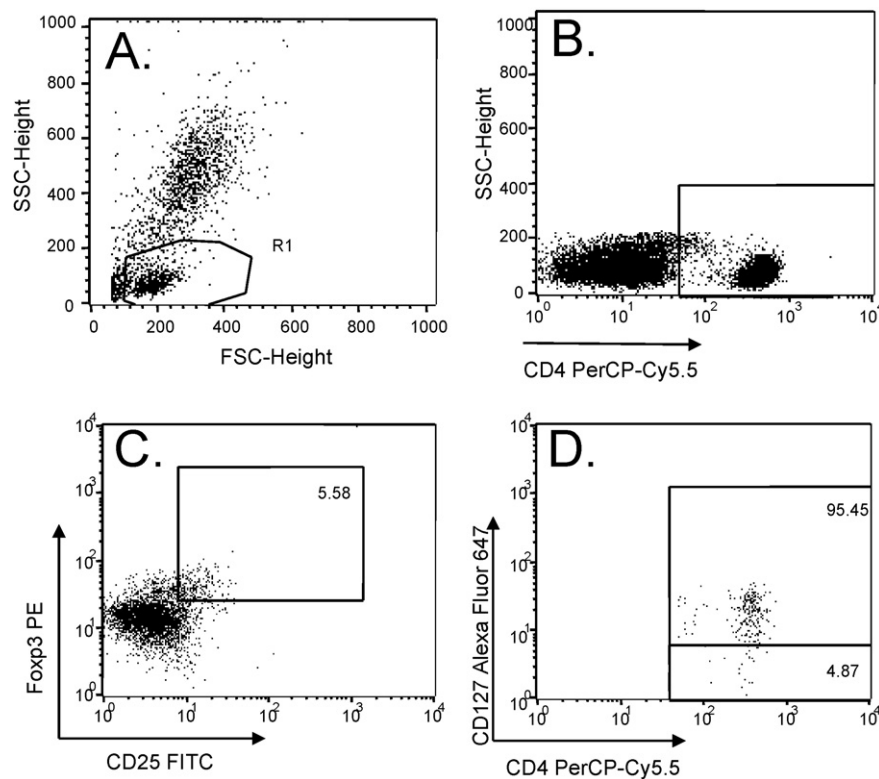


Fig. 1. Lymphocytes were gated using forward scatter versus side scatter characteristics (A). Gated lymphocytes were then examined for CD4 expression (B). Gated CD4⁺ lymphocytes were next analyzed for Foxp3 and CD25 expression (C), number represents the percentage of CD4⁺ lymphocytes that are CD25⁺Foxp3⁺. CD4⁺CD25⁺Foxp3⁺ lymphocytes were then analyzed for CD127 expression (D), numbers represent the percentage of CD4⁺CD25⁺Foxp3⁺ cells that are CD127⁺(95.45) or CD127[−](4.87). Lymphocytes negative for CD127 expression and were also CD4⁺CD25⁺Foxp3⁺ were counted as regulatory T cells.

itive and negative (Fig. 1). CD127 negative cells contain both the CD127 dim and CD127 negative cell populations. Lymphocytes that were CD4⁺CD25⁺Foxp3⁺CD127[−] were considered Treg cells (Liu et al., 2006; Seddiki et al., 2006; Hartigan-O'Connor et al., 2007; Klein et al., 2010). All Treg analyses were reviewed 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 (percentage of CD4⁺CD25⁺Foxp3⁺ lymphocytes that are CD127[−]).

2.4. Measurement of hormones

Numerous epidemiologic studies have measured daily hormones to study fertility or ovarian function (Wilcox et al., 2000; Santoro et al., 2003). The methods, which utilize first daily urine samples collected by the women and placed in their home freezer, are well-established and have been proven successful in women of all ages and of various ethnic and socioeconomic backgrounds. Urine samples were processed at the CLASS laboratory at the University of Michigan using methods described below. We used the algorithm of Baird et al. (1991) to establish an estimated ovulation day using the daily hormone measurements. These methods have been published in detail previously. Although

ovulation is estimated, we refer to the period prior to the estimated date of ovulation as the follicular phase and the period after as the luteal phase. The hormone metabolites were adjusted for creatinine levels (ratio of metabolite to creatinine).

2.4.1. Urinary estrone conjugate and urinary pregnanediol-glucuronide assays

The estrone-conjugate (E1C) assay and the pregnanediol-glucuronide (PDG) assay are competitive immunoassays (Santoro et al., 2003; Randolph et al., 2003) with manual steps and an off-line incubation. Urine samples and quality control preparations were pre-diluted in potassium phosphate buffer. Standards, samples, and quality control preparations were pipetted by hand into 12 × 75 polypropylene tubes. First antibody, rabbit anti-E1C antibody, and estrone-glucuronide labeled with dimethylacridinium ester (DMAE) (E1C) or rabbit anti-PDG (Munroe et al., 1991) antibody and PDG labeled with DMAE (PDG) were added and then the tubes were incubated for several hours at room temperature. All tubes were then placed on a Bayer Diagnostic ACS-180 automated analyzer with a goat anti-rabbit antibody covalently coupled to paramagnetic particles (PMP) for analysis. The assays were standardized against a dual standard prepared with estrone-glucuronide and estrone-sulfate or PDG obtained from SIGMA Co. The laboratory reported the

following for E1C and PDG, respectively: inter-assay CV: 11.00%, intra-assay CV: 7.77%; and inter-assay CV: 16.31%, intra-assay CV: 7.65%.

2.4.2. Urinary creatinine assay

Creatinine was measured with a spectrophotometric assay that used the Hamilton Microlab AT Plus to pipette standards, controls, unknowns, diluent, and reagents (picric acid and sodium hydroxide) into a microtiter plate. The plate was then incubated for 15 min at room temperature, the light absorbance read at 490 nm, and the concentrations of the unknowns calculated using the SpectraMax 340 PC Plate Reader (Spencer, 1986; Weber and van Zanten, 1991). The assay was standardized against creatinine obtained from the Sigma Co. The laboratory reported the following: inter-assay CV: 11.4%, intra-assay CV: 4.3%.

2.5. Statistical methods

To assess within-woman change in Treg cells over a bleeding segment, a time variable was defined in terms of days in relation to estimated ovulation date. For blood collection during the follicular phase, days were expressed as negative numbers; for blood collected in the luteal phase, the number of days was positive. The time variable was thus anchored to ovulation date and 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)). MLMs have been used in other disciplines including growth trajectories for intimate partner violence recurrence (MacMillan et al., 2009), change in functional status associated with dialysis (Tamura et al., 2009), and study of the effects of events around the time of stenting or endarterectomy on health status at 1 year (Brott et al., 2010).

Random effects accounted 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 blood collection varied in spacing within-woman and between women. 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.; asthma status, age, parity, etc.). Five of 6 asthmatic women took asthma medication during the study. Thus, no additional analyses of the effects of asthma medication usage were included as there were not enough women with asthma who were not taking medication ($n = 1$) to assess separate effects of asthma status and medication use. Treg cells were log-transformed prior to inclusion in the models due to non-normality. This modeling approach allowed us to directly address our research aims.

Models were then created to examine the relationships between Treg cells and E1C and PDG (alone and adjusted for the other) and the ratio of E1C/PDG regardless of phase (follicular or luteal). The specific levels of the sex hormones in the models were selected from different time windows: sex hormone levels from the same day as the Treg cells, as well as the levels from 1, 2 and 3 days before the levels of

Table 1

Health information of 13 women included in analyses.

	N	%
Had doctor diagnosis of asthma	6	46.2
Was taking asthma medication during study	5	38.5
Had prior pregnancy	9	69.2
Had prior live birth	9	69.2

Treg cells. Hormone levels were log transformed to meet model requirements.

3. Results

Seventeen women were enrolled in the study that occurred September through December 2009. One woman was excluded after enrolling in the study because of difficulty in obtaining venous blood specimens. Three women were excluded because the date of ovulation could not be estimated. Three women did not have complete bleeding segments, and analyses were run with and without these women. Of the 13 women in the analyses, 6 (46.2%) reported a physician diagnosis of asthma, 5 of whom took asthma medication during the study. The asthmatic women reported having asthma symptoms or wheezing in the last year and/or use of asthma medication (daily controller or rescue inhaler) in the last year. The age range of the women was 24–48 years with a mean (SD) age of 36.1 (8.0) years and 9 (69.2%) women had a prior pregnancy (all with at least one live birth) (Table 1).

Of the 13 women, 4 completed all 18 blood draws, 5 completed exactly 17 blood draws, and all 13 women completed at least 14 blood draws. Samples collected after the first complete bleeding segment were excluded; the 13 women contributed 134 measurements of Treg cells that were collected on days that ranged from their first bleeding day to 13 days after the estimated ovulation date. The estimated day of ovulation ranged from day 5 to day 18 of the bleeding segment. The distribution of Treg cells over the bleeding segments by asthma status appears in Fig. 2.

The best fit model for the overall trajectory of Treg cells was the mean model (a flat line without increase or decrease) (model intercept = 2.06, standard error (SE) = 0.08, $p < 0.01$). There was no effect of estimated ovulation on this trajectory (interaction $p = 0.80$). However, there was an effect of asthma in the model (interaction between asthma status and bleeding segment day $p = 0.003$). Thus, we stratified the trajectory models by asthma status (Table 2). The model for the samples from

Table 2

Models of log transformed percentages of Treg cells (CD4+CD25+Foxp3+CD127– as a percentage of CD4+CD25+Foxp3+ lymphocytes) over the bleeding segment ($n = 13$) by prior doctor diagnosis of asthma.

	Beta (SE)	p-Value
No prior asthma diagnosis ($n = 7$)		
Intercept	1.98 (0.12)	<0.001
Number of days relative to ovulation	–0.01 (0.01)	0.35
Prior asthma diagnosis ($n = 6$)		
Intercept	2.18 (0.09)	<0.001
Number of days relative to ovulation	0.03 (0.01)	0.005

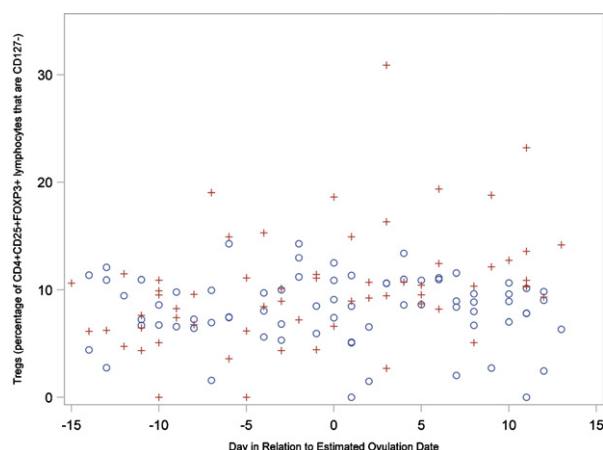


Fig. 2. Treg cells by menstrual segment day. The figure is a scatterplot of levels of Treg cells expressed as a percentage of CD4+ CD25+ Foxp3+ lymphocytes that are CD127– (Y axis). The day of measurement in relation to the estimated day of ovulation defines the X axis. A negative day indicates the sample was collected prior to the estimated ovulation date. Samples from asthmatic women are indicated with a + and samples from women without reported asthma are indicated with an open circle.

women who were not asthmatic does not indicate a statistically significant effect of timing of sample. However, the model for the samples from asthmatic women indicates an association between bleeding segment day and Treg level; the model estimates a slight, but statistically significant increase over time. Among asthmatic women, there was an overall increase in Treg cells of about 3% with each successive day over the bleeding segment. Adjustments for age and whether the woman ever had a prior live birth did not alter the associations (data not shown).

Among non-asthmatic women, PDG levels measured on the same day, 1 day before, 2 days before or 3 days before the Treg percentages were not associated with the Treg level (Table 3). E1C was positively correlated with the Treg percentage measured only on the same day (Table 3). Parameter estimates for the models should be interpreted as the percent change in Treg percentage associated with a 1% increase in the hormone measurement. Estimated changes in percentages associated with a 5% increase in the hormone are included in Table 3. For example, a 1% increase in E1C is associated with as 0.28% increase in the Treg percentage, a 5% increase in E1C is associated with a 1.4% increase in Treg percentage and a 100% increase in E1C is associated with a 21.4% increase in Treg cells. Details for a 50% change are provided in Table 3.

Among asthmatic women, E1C and PDG were individually associated with Treg percentages on the same day and 1 and 2 days before Treg cells were measured. However, only E1C was associated with Treg percentages after adjusting for PDG (Table 3). Parameter interpretation is the same as in the models for the non-asthmatic women. For example, even after adjusting for the PDG level, a 1% increase in E1C was associated with a 0.47% increase in Treg cell percentage, a 5% increase in E1C was associated with a 2.3% increase in Treg cell percentage and a 100% increase in E1C is associated with a 38.5% increase in Treg cells. Details for a 50% change are provided in Table 3.

Results were unchanged with the exclusion of samples from the three women who did not have complete bleeding segments.

4. Discussion

Researchers have well quantified the disparities between women and men with respect to asthma. Based on statistics from 2005, compared with men, women are more likely to have asthma (prevalence: 8.8% versus 6.4%), visit an emergency department for asthma (age-adjusted: 65 visits/10,000 persons versus 62 visits/10,000 persons), and be admitted to a hospital for asthma (age-adjusted: 19/10,000 persons versus 14/10,000 persons) (Centers for Disease Control and Prevention). However, actual explanations for these differences are still lacking. Due to observable gender differences, most researchers think hormones play a role in asthma development, symptoms and exacerbation, but mechanisms have not been proven (Carey et al., 2007; Haggerty et al., 2003).

Our data suggest that Treg percentages increase over the menstrual cycle among asthmatic women. The overall trajectory does not mean that the levels are strictly increasing in perpetuity, but only a multi-cycle study would provide insight into cyclicity. Additionally, an increase in the estrogen metabolite E1C is associated with increased Treg percentages, while the progesterone metabolite PDG was not associated with Treg cells after controlling for E1C levels. This was true during both phases of the bleeding segment and to a lesser extent, among non-asthmatic women. Larger increases in hormone levels, such as those whose magnitudes could be observed around ovulation or prior to menses, were associated with significant increases in Treg cell percentages – increases that exceeded our laboratory's CV for Treg cells measurement.

The observed differences between asthmatic women and non-asthmatic women are interesting. E1C was more strongly associated with percentages of Treg cells in asthmatic women. There could be an additional factor associated with being asthmatic that modifies the association between E1C and Treg cells. For example, Prieto and Rosenstein (2006) reported that unstimulated Treg cells proliferated only slightly in response to estradiol (E2) while cell proliferation increased in an E2 dose-dependent manner if cells were activated by CD3/CD28 engagement.

Others have investigated the relationship between sex hormones and Treg cells. Arruvito, L., et al., 2006 reported that in fertile, nonpregnant women, Treg cells (CD4+CD25+Foxp3+ cells) were correlated with E2 ($R^2 = 0.48$, $p < 0.001$) but not progesterone; Treg cells were highest in the late follicular phase and lowest in the luteal phase based on a series of 5 blood samples. This increase was strongly correlated with an increase in serum estrogen. The authors suggest that this preovulatory increase could be necessary for induction of immune tolerance that facilitates successful implantation. Our data did not indicate this luteal phase decrease. However, their analyses were conducted using a cross-sectional approach that did not take full advantage of the longitudinal data and may explain this discrepancy. They also discarded samples from women they concluded did not ovulate (had low levels of

Table 3Models of log transformed percentages of Treg cells (CD4+CD25+Foxp3+CD127– as a percentage of CD4CD25+Foxp3+ lymphocytes).^a

Time of hormone measurement in relationship to Treg measurement	No prior asthma diagnosis (n = 7)			Prior asthma diagnosis (n = 6)		
	Parameter estimate (SE)	Percent change in percentage of Treg cells associated with 5% change in hormone level	Percent change in percentage of Treg cells associated with 50% change in hormone level	Parameter estimate (SE)	Percent change in percentage of Treg cells associated with 5% change in hormone level	Percent change in percentage of Treg cells associated with 50% change in hormone level
Same day						
A. E1C/PDG ratio	0.01 (0.05)	0.05	0.41	–0.11 (0.09)	–0.54	–4.36
B. PDG	0.04 (0.06)	0.20	1.64	0.20 (0.07) [†]	0.98	8.45
C. E1C	0.28 (0.12) [†]	1.38	12.02	0.57 (0.29) [†]	2.82	26.00
D. PDG	0.02 (0.06)	0.10	0.81	0.14 (0.07)	0.69	5.84
E1C	0.27 (0.12) [†]	1.33	11.57	0.47 (0.32) [‡]	2.32	20.99
1 day before Treg measurement						
A. E1C/PDG ratio	0.04 (0.07)	0.20	1.64	–0.09 (0.08)	–0.44	–3.58
B. PDG	–0.03 (0.08)	–0.15	–1.21	0.20 (0.09) [†]	0.98	8.45
C. E1C	0.09 (0.11)	0.44	3.72	0.63 (0.19) [†]	3.12	29.10
D. PDG	–0.03 (0.08)	–0.15	–1.21	0.11 (0.07)	0.54	4.56
E1C	0.11 (0.08)	0.54	4.56	0.54 (0.20) [†]	2.67	24.48
2 days before Treg measurement						
A. E1C/PDG ratio	–0.02 (0.07)	–0.10	–0.81	–0.10 (0.10)	–0.49	–3.97
B. PDG	–0.0004 (0.07)	–0.002	–0.02	0.22 (0.11) [†]	1.08	9.33
C. E1C	–0.13 (0.09)	–0.63	–5.13	0.64 (0.27) [†]	3.17	29.63
D. PDG	0.01 (0.10)	0.05	0.41	0.15 (0.11)	0.73	6.27
E1C	–0.14 (0.10)	–0.69	–5.84	0.54 (0.27) [†]	2.67	24.48
3 days before Treg measurement						
A. E1C/PDG ratio	0.004 (0.08)	0.02	0.16	–0.09 (0.13)	–0.44	–3.58
B. PDG	0.01 (0.11)	0.05	0.41	0.19 (0.10)	0.93	8.01
C. E1C	0.09 (0.24)	0.44	3.72	0.46 (0.26)	2.27	20.50
D. PDG	–0.001 (0.09)	–0.005	–0.04	0.14 (0.16)	0.69	5.84
E1C	0.10 (0.19)	0.49	4.14	0.38 (0.29)	1.87	16.66

^a Four models are presented for each time period of hormones: (A) only the ratio is in the model; (B) only PDG is in the model; (C) only E1C is in the model; and (D) both PDG and E1C are in the model. Model parameters can also be directly interpreted as the percent change associated with a 1% change in the covariate (i.e.; hormone level).

[†] $p < 0.05$.

[‡] $p = 0.055$.

progesterone indicative of anovulation) and did not include CD127 in their definition of Treg cells.

Prieto and Rosenstein (2006) investigated the percentage of Treg cells defined as CD4+CD25^{high} T cells in blood samples collected once during cycle days 6–9 (called follicular phase) and once during cycle days 20–24 (called luteal phase) in women who reported usual menstrual cycle lengths of 28 ± 2 days. They reported that levels of 17 β -estradiol were not associated with the percentages of Treg cells based on a *t*-test comparing means of Treg percentages for follicular and luteal phase samples. The low number of study participants ($n = 8$) may not have facilitated identification of small statistical differences.

Mjösberg et al. (2009) reported that the levels of Foxp3+ cells were reduced in CD4dimCD25^{high} cells isolated from PBMCs and cultured with 17 β -estradiol and progesterone for three days in 27 healthy nonpregnant women. They reported that progesterone was mainly the cause of these changes. They also stated that the effects of the hormone culturing were the same in the secretory and proliferative phases of the menstrual cycle. It is unclear how the phases were established from questionnaire data.

While we included only 13 women in our analyses, the use of daily hormone measurements and up to 14 blood

samples over a single bleeding segment for each woman are novel strengths. Additionally, our use of longitudinal analyses provides evidence of patterns. We were also able to examine whether absolute levels or ratios of hormone levels over specific time windows (one, two and three days before Treg cells were measured) were associated with Treg levels. A larger study population and multiple consecutive bleeding segments from individual women would greatly improve our understanding of the cyclic patterns of the observed associations.

In summary, E1C but not PDG was found to be associated with Treg levels, and the strongest associations were found in asthmatic women. Future studies should examine contiguous cycles from a larger group of women to better determine within-woman cyclicity of the observed patterns.

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