

HORMONE INDUCED IMMUNOLOGIC RESPONSES IN ASTHMA

Enhanced Generation of Suppressor T Cells in Patients with Asthma Taking Oral Contraceptives

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Introduction. A dysregulation of regulatory T cells (Tregs) could play a major role in the pathogenesis of bronchial asthma. Sex-dependent differences as well as the impact of hormonal changes in the incidence and severity of asthma are widely recognized. Emerging evidence suggests that asthma symptoms are alleviated in female patients taking hormone oral contraceptives (OCs). The impact of OCs on the generation of induced Tregs (iTregs) was assessed in a cohort of female patients with asthma. **Methods.** Thirteen patients were included in this pilot study. During three distinct phases of their menstrual cycles, we measured exhaled nitric oxide (eNO) levels, forced expiratory volume at 1 second (FEV1s), asthma control test (ACT) score, sex steroid hormone levels in serum, natural Tregs in peripheral blood, and the ability of CD4⁺ T cells to generate iTregs ex vivo. **Results.** The luteal serum levels of estradiol and progesterone negatively correlated with the proportion of iTregs generated ex vivo in patients not taking OCs. In addition, physiological doses of estradiol and progesterone prevented the acquisition of a suppressor T cell phenotype in vitro. Interestingly, patients taking OCs had reduced serum sex hormone levels associated with higher iTreg induction, a better ACT score, and a tendency toward lower eNO levels. **Conclusions.** Our results identify an impact of sex hormones on the capacity of T cells to polarize towards a regulatory phenotype and suggest the regulation of peripheral T cell lineage plasticity as a potential mechanism underlying the beneficial effects of OCs in women with asthma.

Keywords CD4 T cells, estradiol, induced regulatory T cells, menstrual cycle, progesterone

INTRODUCTION

The T helper type 1 (Th1) versus T helper type 2 (Th2) dichotomy of T cell responses has been considered for many years as a cornerstone of the adaptive immune system. An exaggerated Th2-driven response is believed to lead to the inflammatory allergic reaction of asthma, which is a highly complex, multifactorial disease characterized by airway hyperresponsiveness, inflammation, and infiltration of different immune cell types, including eosinophils, mast cells, and T lymphocytes. Particularly, Th2 cytokines induce the production of immunoglobulin E and mast cells, activation and recruitment of eosinophils, airway hyperreactivity, and mucus secretion (1). Clinical observations and epidemiological studies suggest a role for sex hormones in the incidence and/or regulation of symptom severity in numerous Th2-related pathologies (2). Indeed, while the incidence of asthma is higher in boys before puberty, the manifestations of asthma are

predominant in females after puberty (3–7) and about 30–40% of female patients experience perimenstrual worsening of symptoms (8, 9). Moreover, the use of oral contraceptives (OCs) has been shown to improve symptoms in some women with asthma (10–12). The capacity of the sex steroid hormones estradiol and progesterone as contributing factors in asthma is being actively addressed, (13) but the underlying immunoregulatory mechanisms have not been fully elucidated yet.

Additional T helper expression profiles that challenge the Th1/Th2 paradigm have been identified. The Th17 population has been implicated in neutrophilic asthma and resistance to steroids in mouse models of asthma (14, 15). Of major importance are Tregs, which make up 1–5% of the T cells in the peripheral blood of adult mice and humans (16). Tregs can inhibit or suppress the effector function of T helper cells and their tolerance to environmental allergens (17), thus preventing the induction or reducing the severity of asthma. In fact, a perturbed Treg compartment has been associated with autoimmunity, inflammatory disorders, and cancer development (18). In addition to the “natural” Tregs (nTregs) that stem as a separate lineage in the thymus, induced Tregs (iTregs) can be generated in peripheral tissues from conventional CD4⁺ T cells. Interestingly, recent evidence

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supports the prevailing relevance of iTregs in mucosal tissues and inflammatory processes (19–21). As such, any potential mechanism altering the generation, maintenance, or function of iTregs might be relevant to the etiology, pathogenesis, and/or evolution of asthma. In fact, the pathological Th2 response of asthma patients has been associated with the reduced number or suppressive activity of circulating CD4⁺CD25⁺FoxP3⁺ Tregs (22–24). Consequently, there is a growing interest in the mechanisms that regulate iTreg function and differentiation to therapeutically manipulate their enormous potential in immune and inflammatory conditions, including asthma (25, 26).

In this study, we investigate the impact of OC intake on the ability of T cells to generate suppressor iTregs in asthma patients.

METHODS

Patients with Asthma and Controls

Thirteen female patients with asthma were recruited at the University of Kentucky Clinics. Their asthma severity was mild to moderate persistent according to the National Asthma Education and Prevention Program (NAEPP) treatment guidelines (27). All patients were followed for two consecutive months during three specific phases of the menstrual cycle for a total of six study visits. The three time points studied were menses (days 1–5 of the menstrual cycle), mid-cycle (days 12–16), and luteal phase (days 23–27). One cycle (three visits) from one of the patients was removed because pregnancy was suspected. In each visit, we drew approximately 20 mL of a heparinized blood sample. In addition, prick skin testing was performed at the initial visit to assess the presence of atopy. All the study subjects had well-controlled asthma at the initial study visit according to the NAEPP guidelines. Six patients were taking combination (estradiol and progesterone or equivalent) OCs, and the remaining seven patients were not under any hormone-based contraceptive treatment (non-OC). Patients were non-smokers, did not exhibit other medical conditions, and did not receive systemic steroid treatment 4 weeks prior to the study enrolment or during the study. The study protocol was approved by the Medical Institutional Review Board of the University of Kentucky (approval number 06–0578), and written informed consent was obtained from all the study participants.

Fresh buffy coats from 26 healthy adults (mean age 34, range 19–45), anonymous blood donors with no gender identification, were obtained from the Kentucky Blood Center (Lexington, KY, USA) and included for control purposes.

Asthma Clinical Parameters

All the study subjects underwent spirometry in each visit according to the American Thoracic Society criteria (28). Forced expiratory volume in 1 second (FEV₁s) was measured with a MicroLab portable spirometer (Micro Direct, Inc., Lewiston, ME, USA) at each visit. Asthma control

test (ACT) questionnaires were filled out by every subject on each visit (29). Modified ACT questionnaires were constructed allowing reported symptoms to only reflect those experienced since the previous study visit; therefore, an assessment could be made on the level of asthma symptoms during each phase of the menstrual cycle. Fractional exhaled nitric oxide (eNO) levels were measured with the Aerocrine NIOX MINO system (Morrisville, NC, USA) (30) in accordance with recommendations of the American Thoracic Society (31) at a flow rate of 250 mL per second. Subjects were instructed to refrain from green leafy vegetables, processed meat products, and smoked meat 24 hours prior to visits to minimize the interference with eNO measurements.

Serum Hormone Measurement

Serum levels of estradiol and progesterone were measured using the Immulite 1000 (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) following manufacturer's instructions.

Generation of iTregs Ex Vivo

The method for the polarization of CD4⁺ T cells toward a regulatory phenotype has been described in detail elsewhere (32, 33). Briefly, negatively isolated (Stem Cell Technologies) CD4⁺ T cells were placed under polarizing conditions for 5 days: supplemented Rowsell Park Memorial Institute medium with 5 ng/mL recombinant human Interleukin-2 (IL-2) 2 ng/mL recombinant human Transforming Growth Factor (TGF)-β1 (eBioscience, San Diego, CA, USA), and plate-bound anti-CD3 antibodies (OKT3 clone; Bio X Cell, West Lebanon, NH, USA). For some experiments, different concentrations of estradiol (17β-Estradiol) or the synthetic progesterone analog Norgestrel (Cayman Chemical) were added to the polarizing media at the beginning of the assay.

Flow Cytometry

Cells were stained with antibodies against CD25, CD45RA (Milty, Miltenyi Biotec USA; Auburn, CA, USA), CD4, FoxP3, and CTLA-4 (BD Biosciences; Franklin Lakes, NJ, USA). All antibodies were monoclonal and directly conjugated with fluorescent markers. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA) and analyzed using FlowJo software (Tree Star Inc.; OR, USA) by an examiner blind to the group assignments of the patients and the menstrual time points. Live cells were gated based on their forward and side scatter profiles.

Statistics

Data were analyzed using a two-way ANOVA with repeated measurements, the Student's *t*-test or the Mann–Whitney *U*-test using Prism 5 software (GraphPad Software Inc; La Jolla, CA, USA). *P*-values for the correlation analyses were calculated using an *F*-test. Values of *p* < .05 were considered to be statistically significant. Data are presented as mean ± SEM.

TABLE 1.—Demographic data and clinical parameters of the patients.^a

Characteristic	Patients taking OCs	Non-OC patients
N	6	7
Sex, Female (Male)	6 (0)	7 (0)
Age ^b , years	28 (21–34)	30 (23–35)
ACT score ^b	22.4 (20.5–25)	21.4 (17–24.5)
FEV1 ^b , % of predicted	99.3 (89.5–112.5)	96.8 (75–116)
eNO ^b , ppb ^c	16.5 (7–32)	25.9 (11.5–74.5)

Note: ^aAveraged clinical parameters obtained in the first visits of each menstrual cycle (i.e., averaged data from visits 1 and 4).

^bData presented as mean (range).

^cParts per billion.

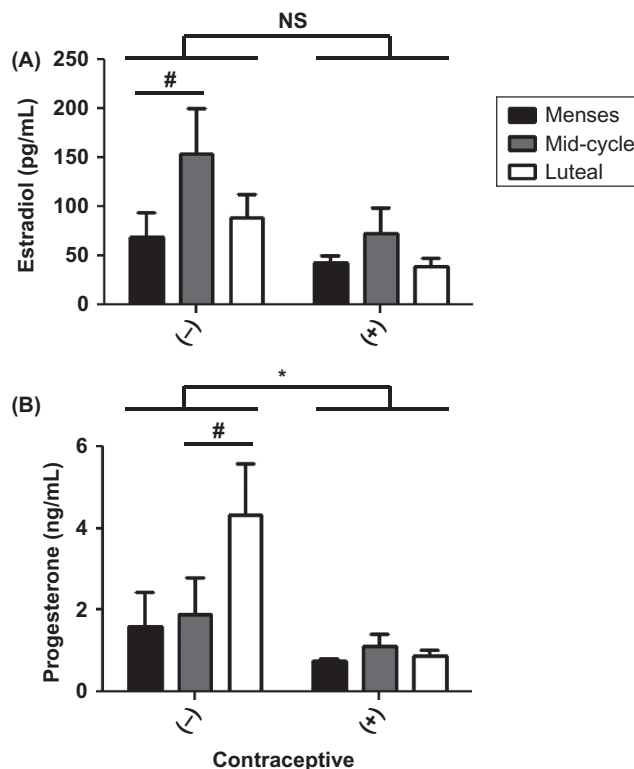


FIGURE 1.—Women with asthma taking OCs have reduced levels of sex hormones in serum. Serum levels of the sex hormones estradiol (A) and progesterone (B) in asthmatic patients taking (+) ($n = 6$) and not taking (—) OCs ($n = 7$) measured by ELISA at three different phases of the menstrual cycle: Menses, mid-cycle, and luteal. The asterisk indicates a statistically significant difference between the two groups of patients measured by a two-way ANOVA with repeated measurements. Statistically significant differences between cycle phases were calculated with a paired Student's t -test and are indicated by the symbol #. NS: Not significant.

RESULTS

CD4⁺ T Cells from Female Patients with Asthma Taking OCs Exhibit Improved Ability to Generate iTregs Ex Vivo

Demographic data of the patients with asthma included in this study are shown in Table 1. Data were collected at three different points of the menstrual cycle: follicular, mid-cycle, and luteal phases, for two consecutive cycles. Averaged clinical parameters obtained in their first visit of each menstrual cycle are also shown in Table 1. As

expected in non-OC patients, serum levels of the steroid sex hormones estradiol and progesterone peaked at mid-cycle and luteal phases, respectively (Figure 1). Patients using OCs showed an overall decrease in the serum levels of estradiol and progesterone and the absence of pronounced peaks at any of the menstrual cycle phases (Figure 1).

We first looked for alterations in the frequency of nTregs in the patients' peripheral blood by flow cytometry. We used a stringent CD4⁺CD25^{hi} gating strategy and found similar levels of Tregs between OC and non-OC patients (Figure 2A and B), which resembled the values observed in our cohort of normal donors (data not shown). Moreover, no significant differences were observed in the frequencies of nTregs at the different phases of the menstrual cycle (Figure 2B).

Next, we purified peripheral blood CD4⁺ T cells to assess their ability to adopt a regulatory phenotype ex vivo using a previously established strategy. Upon Treg polarization, we were able to gate on CD45RA⁺CD25^{hi} T cells to obtain a population enriched with FoxP3⁺CTLA4⁺CD127⁺ cells that exhibit suppressor activity (32–34) (Supplementary Figure 1 and data not shown). After 5 days in culture and under these Treg polarizing conditions, T cells from non-OC patients with asthma generated a lower percentage of CD45RA⁺CD25^{hi}FoxP3⁺ iTregs than T cells from patients taking OCs (Figure 2C and D). Of note, the majority of the cells using this gating strategy expressed CTLA-4 ($87.1 \pm 1.92\%$), further supporting the bona fide regulatory phenotype of these cells (35). The levels of iTregs generated ex vivo did not correlate with the frequency of CD4⁺CD25^{hi} nTregs present in the initial T cell pool (Supplementary Figure 2), which argues against an expansion of nTregs in vitro. Interestingly, T cells from healthy donors cultured under the same conditions yielded a frequency of CD45RA⁺CD25^{hi}FoxP3⁺ iTregs ($11.2 \pm 2.39\%$, $n = 22$) that resembled those observed for women with asthma taking OCs (Figure 2D).

High Estradiol and Progesterone Levels in Serum during the Luteal Phase Associate with Reduced Ability to Generate iTregs

Since a higher proportion of iTregs was observed in the patients who exhibited lower levels of sex hormones in serum (i.e., patients taking OCs), we wondered whether estradiol or progesterone affected the ability of CD4⁺ T cells to polarize toward a regulatory phenotype. We compared serum hormone levels at each menstrual cycle phase with the percentages of CD45RA⁺CD25^{hi} iTregs generated ex vivo in both the groups of patients. We found that, for non-OC patients, the percentage of iTregs generated in culture negatively correlated with the serum levels of estradiol ($r = -0.96$, $p < .001$) and progesterone ($r = -0.86$, $p < .05$) during the luteal phase (Figure 3). No correlations between hormone serum levels and the frequency of iTregs were observed in the follicular phase, during menses, or for patients taking OCs (data not shown).

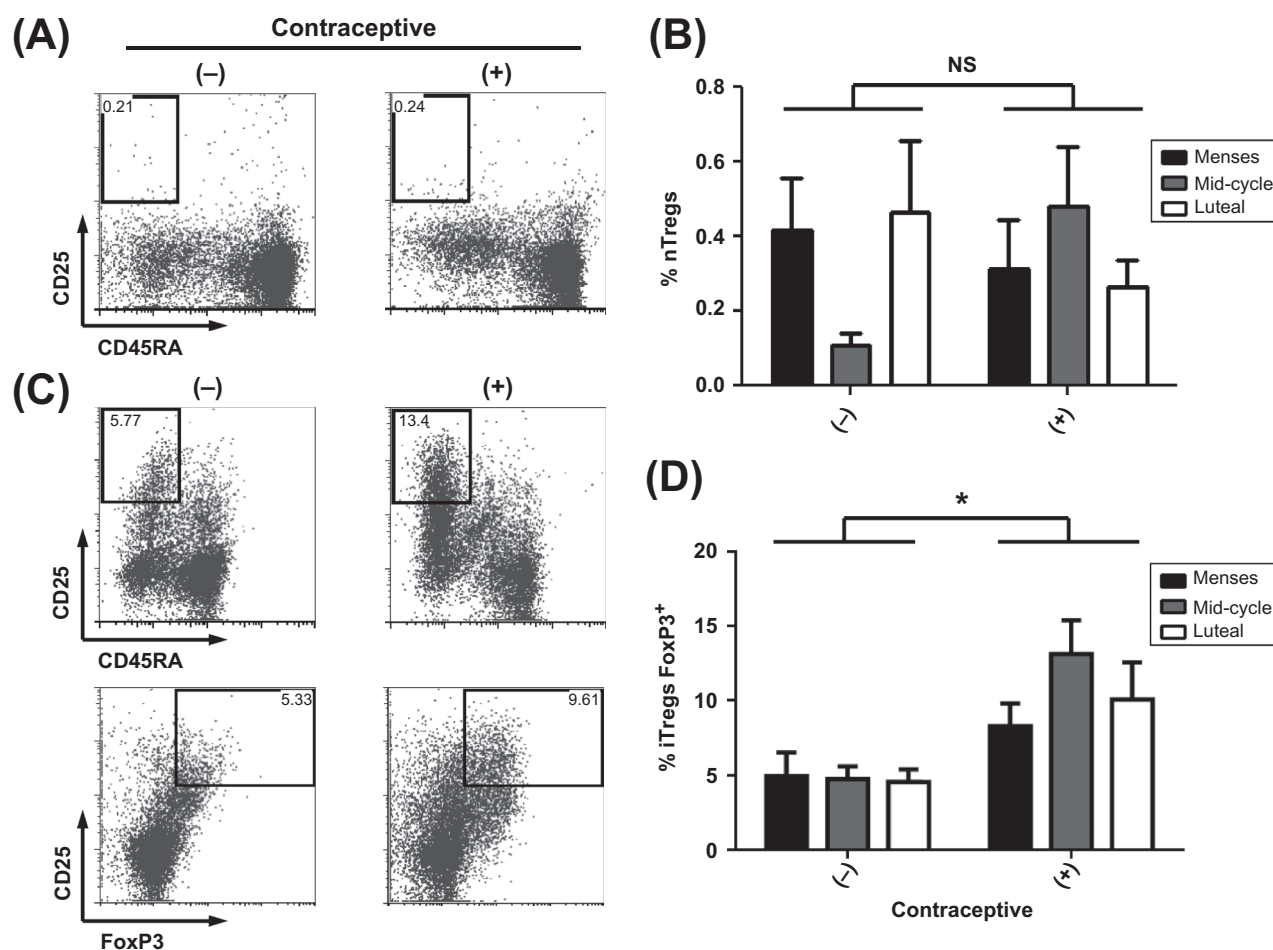


FIGURE 2.—CD4⁺ T cells from women with asthma taking OCs can generate greater levels of iTregs. (A) Representative flow cytometry plots showing the gates on CD45⁺CD25^{hi} cells that were used to measure the frequency of nTregs in peripheral blood of patients taking (+) and not taking (-) OCs. CD4⁺ gate was applied. (B) Mean frequencies of nTregs for OC (n = 6) and non-OC (n = 7) patients during the three menstrual cycle phases: Menses, mid-cycle and luteal. (C) Representative flow cytometry plots showing the gating strategy used to measure the percentages of CD45⁺CD25^{hi}FoxP3⁺ iTregs generated ex vivo for OC (+) and non-OC (-) patients. Only cells that belonged to both gates (using Boolean logic) were considered as iTregs. (D) Mean frequencies for both groups of patients during the three menstrual cycle phases: Menses, mid-cycle, and luteal. Data from the two groups of patients were analyzed with a two-way ANOVA with repeated measurements, excluding patients with missing data (1 patient per group). One asterisks (*) indicates a value of $p < .05$. NS: not significant.

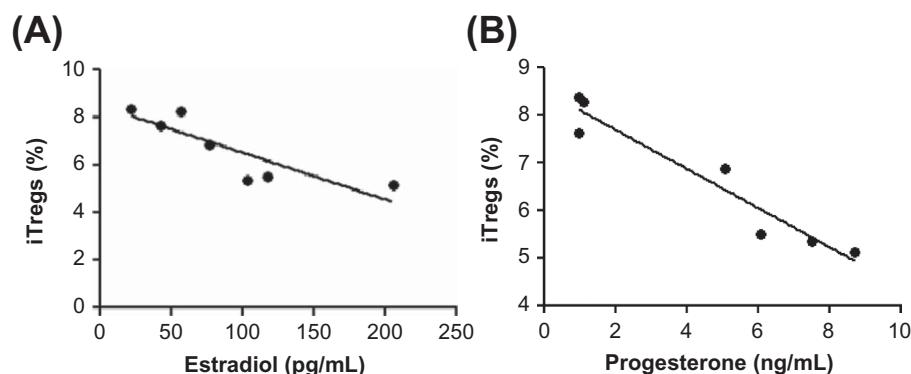


FIGURE 3.—Luteal sex hormone levels negatively correlate with the levels of iTregs generated ex vivo in patients not taking OCs. The serum levels of estradiol (A) and progesterone (B) at the luteal phase were plotted against the frequency of CD45⁺CD25^{hi} iTregs generated ex vivo for each patient with asthma not taking OCs (n = 7).

Estradiol and Progesterone Prevent the Generation of iTregs In Vitro

We further investigated the direct impact of estradiol and progesterone in the differentiation of iTregs by adding these hormones to our in vitro system in doses that

resembled the levels observed in the patients' sera: 10, 50, and 250 pg/mL estradiol (Figure 4B) or 0.5 and 2 ng/mL progesterone (Figure 4C). Similar to our in vivo findings, when CD4⁺ T cells from healthy donors were cultured for 5 days under Treg polarizing conditions in the

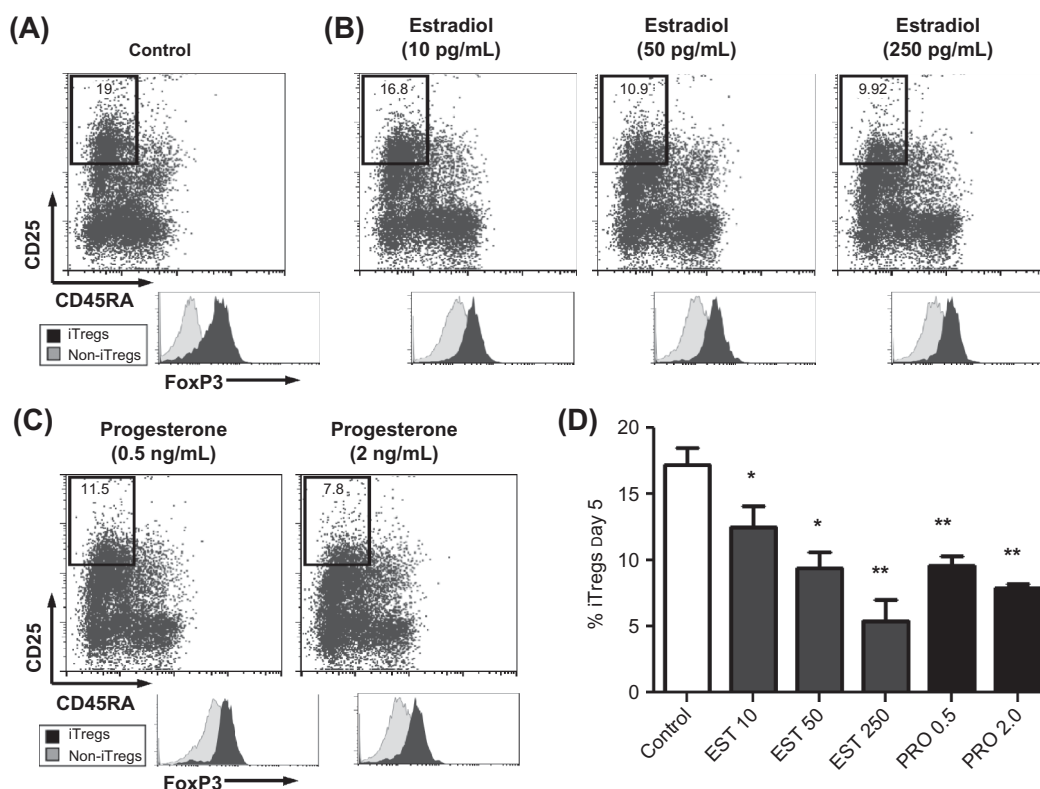


FIGURE 4.—Exogenous estradiol and progesterone reduce the frequency of iTregs generated *in vitro*. Representative flow cytometry charts showing the percentage of iTregs (CD45RA⁺CD25^{hi} gate) generated *in vitro* in the absence of hormonal treatment (A) or in the presence of different concentrations of estrogen (B) or progesterone (C). The histograms underneath the dot plots show the expression of FoxP3 in the iTreg (black) and non-iTreg cells (gray). (D) Mean frequencies of iTregs generated in each condition ($n = 3-4$). Each culture condition was compared against the control without hormonal treatment and statistically significant differences were calculated with a paired Student's *t*-test. One asterisk (*) indicates a value of $p < .05$ and two asterisks (**) a value of $p < .01$.

presence of either hormone, the ability to generate CD45RA⁺CD25^{hi} iTregs was reduced in a dose-dependent manner (Figure 4D).

Oral Contraceptives Influence on Asthma Parameters

We next wondered whether the relationship between serum hormone levels and iTreg development correlated with changes in clinical parameters in the patients. The asthma severity was assessed by evaluating the levels of eNO, FEV₁s, and ACT score. When comparing the measurements obtained during the luteal phase, where a clear correlation between serum hormone levels and iTreg generation had been established, we found that the female patients with asthma using OCs exhibited significantly higher ACT scores (Figure 5G) and significantly lower eNO levels (Figure 5H) when compared with the non-OC patients. In contrast, we found similar FEV₁s levels for both the groups of patients (Figure 5I). Similar results were observed at the mid-cycle phase of the menstrual cycle (Figure 5D–F).

DISCUSSION

We evaluated the ability of the peripheral blood CD4⁺ T cells from a small cohort of women with well-controlled mild to moderate persistent asthma to generate iTregs *ex vivo*. We found a negative correlation between luteal

serum levels of estrogen and progesterone and the frequency of iTregs generated in the culture. The ability of T cells to differentiate into iTregs also declined when we cultured CD4⁺ T cells isolated from healthy donors with physiological concentrations of estrogen or progesterone. These results suggest that the presence of female sex hormones may directly contribute to the perturbed development of iTregs and could perpetuate the asthmatic inflammation in an at-risk female population. Consistent with this possibility, the lower serum levels of circulating estrogen and progesterone in women with asthma using OCs were associated with higher percentages of *ex vivo*-generated iTregs when compared with non-OC patients. Interestingly, a reduction in the frequency of peripheral blood Tregs was reported during the second trimester of pregnancy, which we replicated *in vitro* by treating peripheral blood mononuclear cells of non-pregnant women with estrogen or progesterone (36).

In this study, we only included women with well-controlled mild to moderate asthma, and did not select for women with fluctuations in asthma symptoms during their menstrual cycle, which translated into a small range of ACT scores in our cohort. We found small, yet significant, differences in ACT scores and eNO levels between OC and non-OC patients. These results are consistent with previous reports (37), suggesting that OC use may improve asthma symptoms (higher ACT scores and lower eNO levels). Of note, ACT scores have been shown to

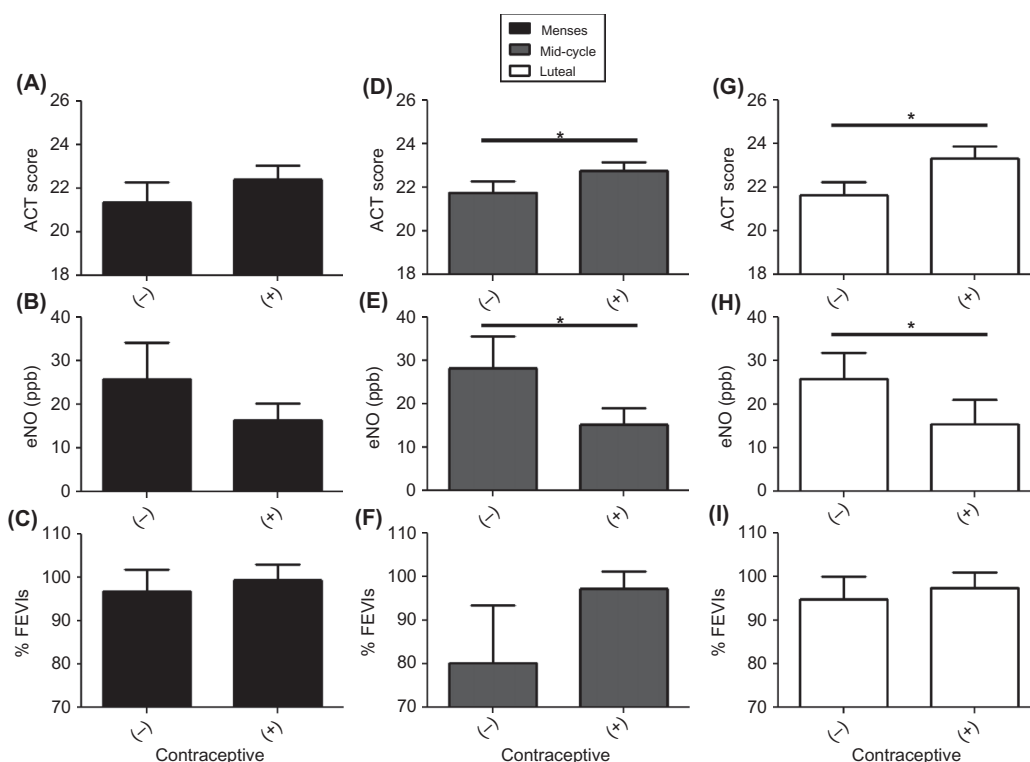


FIGURE 5.—Women with asthma taking OCs report better ACT scores. Mean ACT scores (A, D, G), eNO levels (B, E, H), and FEV1s (C, F, I) during the menses (A–C), mid-cycle (D–F), and luteal (G–I) phases of the menstrual cycle for patients with asthma taking (+) ($n = 6$) and not taking (–) ($n = 7$) OCs. The asterisk indicates a statistically significant difference between both the groups of patients using the Mann–Whitney U -test.

negatively correlate with eNO levels in patients with asthma (38), and changes in ACT scores of as little as 1.88 have been considered a better asthma control rating (39). All patients in our cohort exhibited FEV1s > 80% of predicted, and the fluctuations were not significant in either OC or non-OC patients, similar to previous observations (37). Our results, though, further support the idea of sex steroid hormones as allergic disease modifiers through a mechanism that involves the blockage of iTreg differentiation. Asthma exacerbations have been observed in the ovulatory phase when the levels of estrogen are high (40). The effects of estrogen on Treg suppressor activity and proliferation have been conducted with contradictory results, probably due to the complexity of estrogen-mediated T cell effects; while estrogen treatment increased the proliferation of TCR/CD28-stimulated human Tregs (41) and induced the expression of FoxP3 in TCR/CD28-treated murine T cells (42), estrogen-treated dendritic cells reduced the proliferation of murine Tregs but potentiated their suppressor capacity (43).

The *in vitro* iTreg polarization of CD4⁺ T cells from women with asthma was performed in the absence of sex hormones. Since a negative correlation was found between serum hormone levels present during CD4⁺ T cell isolation and the frequency of iTregs generated 5 days later in culture, we believe that the T cells were already primed toward a phenotype that facilitated (or prevented) their differentiation into iTregs. In agreement with others (41), we did not observe significant differences in the levels of

circulating nTregs at different phases of the menstrual cycle, and we also found similar levels of nTregs between patients taking OCs and not taking OCs. However, a limitation of our study is that the effector function of iTregs was not determined, and thus, changes in their suppressor activity cannot be ruled out as potential contributors to the observed clinical differences.

The manipulation of peripheral T cell plasticity in pathological systems might represent a novel strategy for future therapeutic approaches. The conversion of memory pathogenic Th2 cells into Tregs was recently achieved through TGF- β stimulation in the presence of retinoic acid and rapamycin, and these “converted” Tregs were able to migrate to the airways and suppress Th2-mediated responses (27). Further studies are necessary to evaluate whether treatment with sex steroid hormones can induce the conversion of pathogenic Th2 or Th17 cells into Tregs. For instance, the possible conversion of Th17 cells into Tregs could be of great importance in patients with steroid-resistant asthma. We did not look for the potential mechanisms of action of estrogen and progesterone in the development of iTregs. It is known that T cells express estrogen receptor alpha (ER α) (44), that estrogen may affect T cell function through non-genomic, ER α -independent mechanisms (45), and that progesterone promotes the development of Th2 cells and cytokines (46). Tamoxifen is an ER ligand that can act as a full agonist, partial agonist, or even as an antagonist of the ER depending on the target tissue (47). The fact that Tamoxifen behaved in an

agonistic manner in our in vitro system (Supplementary Figure 3) further supports the involvement of functional ERs in iTreg differentiation.

Others have reported alterations in the percentages of Tregs present in the bronchoalveolar lavage fluid of patients with asthma (48, 49) and in the peripheral blood of allergic individuals (50). However, to our knowledge, this is the first report evaluating the generation of iTregs in asthma patients. In spite of the small sample size tested in this study, and the fact that our donor pool of healthy subjects did not exclude males or OC users, our findings suggest a link between patients' clinical symptoms and serum hormone levels. A better understanding of the impact that sex steroid hormones have on iTreg generation could help us clarify patient-to-patient variability in the efficacy of current treatments and benefit future therapies for a plethora of pathologies, including asthma.

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DECLARATION OF INTEREST STATEMENT

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