Regulatory T and B cells in asthmatic women: variations from pregnancy to postpartum

Treg and Breg: pregnancy to postpartum

Martins C, PhD

Corresponding address: Immunology, NOVA Medical School, Campo dos Mártires da Pátria, 1169-056 Lisbon, Portugal

Mailing address: catarina.martins@nms.unl.pt

Affiliation: 1 CEDOC, Chronic Diseases Research Center, NOVA Medical School|FCM; Universidade Nova de Lisboa, Lisbon, Portugal

Lima J, MD

Affiliation: 2 Ginecologia e Obstetrícia, Hospital CUF Descobertas, Lisbon, Portugal; 1 CEDOC, Chronic Diseases Research Center, NOVA Medical School|FCM, Universidade Nova de Lisboa, Lisbon, Portugal

Nunes G, BSc

Affiliation: 1 CEDOC, Chronic Diseases Research Center, NOVA Medical School|FCM; Universidade Nova de Lisboa, Lisbon, Portugal

Borrego LM, MD, PhD

Affiliation: 1 CEDOC, Chronic Diseases Research Center, NOVA Medical School|FCM; Universidade Nova de Lisboa, Lisbon, Portugal; 4 Imunoalergologia, Hospital CUF Descobertas, Lisbon, Portugal

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.18176/jiaci.0086
Abstract

Background: Allergic asthma and rhinitis are common in pregnancy. The immune mechanisms underlying the effects of pregnancy in asthma and vice-versa are not completely understood.

Objectives: This work aimed to study the evolution of regulatory T and B cells in asthmatic pregnant women, from late pregnancy till postpartum.

Methods: Four groups of women were enrolled for this study: third trimester pregnant women, asthmatic (n=24) and healthy (n=43), and non-pregnant women, asthmatic (n=33) and healthy (n=35). Pregnant women were also evaluated postpartum (>6 weeks after delivery). Blood samples were taken from each woman and flow cytometry was used to characterize circulating regulatory T and B cells. Foxp3 expression was assessed within CD4\textsuperscript{Dim}CD25\textsuperscript{hi} regulatory T cells.

Results: In asthmatic and healthy pregnant women, regulatory T cells did not oscillate significantly from pregnancy to postpartum, but CD24\textsuperscript{hi}CD38\textsuperscript{hi} regulatory B cells, decreased in pregnancy, rose significantly postpartum. Foxp3 expression in regulatory T cells was also impaired during pregnancy in asthmatic and healthy pregnant women, recovering postpartum. Nevertheless, asthmatic pregnant women presented higher Foxp3 expression than healthy pregnant women (p=0.007), probably due to the use of control medication.

Conclusions: Women with controlled asthma present variations in regulatory cell subsets during pregnancy and postpartum. The similar pattern observed for Foxp3 expression and CD24\textsuperscript{hi}CD38\textsuperscript{hi} regulatory B cells during this period corroborates the interaction established between regulatory T and B cells in immune responses. Considering the immunomodulatory potential of these immune mediators, more studies are needed to evaluate their relation with asthma and rhinitis complications in pregnancy.

Resumen

Antecedentes: El asma y la rinitis alérgica son enfermedades comunes durante el embarazo. A pesar de ello, no están completamente esclarecidos los mecanismos inmunológicos del embarazo implicados en el asma y viceversa.

Objetivos: Este trabajo tuvo como objetivo el estudiar la evolución de los linfocitos T y B reguladores en mujeres asmáticas embarazadas, desde fases tardías del embarazo hasta después del parto.

Métodos: Se incluyeron cuatro grupos de mujeres para este estudio: mujeres embarazadas en su tercer trimestre, asmáticas (n = 24) y sanas (n = 43), y mujeres no embarazadas, asmáticas (n = 33) y sanas (n = 35). Las mujeres embarazadas también fueron evaluadas después del parto (> 6 semanas después del parto). Se tomaron muestras de sangre de cada mujer y se realizó citometría de flujo para caracterizar los linfocitos T y B reguladores circulantes. La expresión de Foxp3 se evaluó en los linfocitos T reguladores CD4<sup>Dim</sup>CD25<sup>Hi</sup>.

Resultados: En las mujeres embarazadas, tanto sanas como asmáticas, los linfocitos T reguladores no oscilaron de manera significativa desde el embarazo hasta después del parto. Sin embargo, en los linfocitos B reguladores CD24<sup>Hi</sup>CD38<sup>Hi</sup>, se observó una disminución durante el embarazo que aumentó significativamente después del parto. La expresión de Foxp3 en los linfocitos T reguladores también se vio alterada durante el embarazo tanto en las mujeres embarazadas asmáticas como en las sanas, normalizándose en el posparto. No obstante, las mujeres asmáticas embarazadas presentaron niveles de expresión de Foxp3 superiores a los de las mujeres embarazadas sanas (p = 0,007), probablemente debido a la utilización de medicación de control.

Conclusiones: Las mujeres con asma controlada, durante el embarazo y después del parto, presentan variaciones en los diferentes subtipos linfocitos reguladores. El similar comportamiento que se observa para la expresión de Foxp3 y los linfocitos B reguladores CD24<sup>Hi</sup>CD38<sup>Hi</sup> apoya la interacción que se establece en la respuesta inmunitaria, entre los linfocitos T y B reguladores, durante este período. Teniendo en cuenta el potencial inmunomodulador de estos mecanismos, se necesitan más estudios para evaluar su relación con las complicaciones del asma y la rinitis durante el embarazo.

Palabras clave: Atopia, embarazo, postparto, linfocitos humanos T reguladores, linfocitos humanos B reguladores, citometría de flujo
**Introduction**

For women in fertile age, allergic asthma and rhinitis can present risks for pregnancy [1]. Disease course may improve, remain unchanged, or worsen during pregnancy, and asthma exacerbations may have a significant impact in fetal development and birth weight, besides being related with preterm delivery [1]. Also, maternal asthma is a risk factor for the development of asthma in asthmatic women’s progeny [2].

Pregnancy challenges the immune system, as it must assume tolerance towards the semi-allogenic fetus, without compromising mother defenses. Th₂ cells, hallmark of atopic diseases, are typically associated to gestation and appear to contribute to the maintenance of pregnancy [3]. Nonetheless, more than biased Th₂ immune response, pregnancy presents rather strict regulatory mechanisms that balance cytokine production [4].

Regulatory T cells (Tregs) are known to be expanded during early pregnancy [5], and seem to be decreased and/or functionally impaired in asthmatics [6, 7]. Yet, Tregs and IL10, an anti-inflammatory cytokine secreted by these cells, have been implicated in asthma’s amelioration [8]. Recently, B cells have been considered to have an important role in Th₂ responses and airway inflammation [9]. As for regulatory B cells (Bregs), its impaired regulatory activity was also reported in allergic asthma [10], and Bregs have a close relation to Tregs [11].

Modifications in T and B cell subsets in healthy [12-14] and asthmatic [15, 16] pregnant women have been reported. However, not much is known about B cells neither about the immune profile in postpartum, when an immune reactivation is believed to happen [17].

Our aim was to monitor pregnant women with asthma, from late pregnancy till postpartum in order to characterize Tregs, Bregs and IL10 expression, and the impact of pregnancy on these immune parameters.
Methods

Study Subjects

Sequential pregnant women with atopic asthma (AP), healthy pregnant women (HP), as well as non-pregnant women with atopic asthma (ANP) and healthy non-pregnant women (HNP) of reproductive age followed at CUF Descobertas Hospital were enrolled for this study. All women were informed about the nature of the study and provided written informed consent at recruitment. Blood samples were collected once for non-pregnant women and twice for pregnant: the pregnancy time point occurred in the third trimester of gestation (between weeks 31 and 36 of gestation) and the postpartum time point at least 6 weeks after delivery. All women completed questionnaires for demographic and clinical data in each time point. Diabetes; hypertension; autoimmune diseases; any active infectious disease including hepatitis and HIV; any other allergic (except for atopic dermatitis and allergic rhinitis) or respiratory disease; and smoking for the last 6 months before sample collection, represented exclusion criteria for all groups. Multiple gestation, pregnancy complications and prenatal use of any medication (other than vitamins, folic acid, and iron supplements) were exclusion criteria for HP. The same criteria were applied to AP, except for therapeutics.

AP and ANP groups included women with both atopic asthma and rhinitis, physician diagnosed according to current international guidelines [18-20], with documented sensitization to aeroallergens (by skin prick tests and/or specific IgE quantification). Immunotherapy, performed in the past or present, was considered an exclusion criteria for AP and ANP. Asthma and rhinitis were treated according to the current guidelines [19, 20]. All AP and ANP were under inhaled corticosteroid (ICS) therapy (low-median dose, 200-400 ug beclomethasone /daily), and/or long-acting Beta-agonists (LABA) and antileukotrienes. At recruitment, AP and ANP presented no asthma exacerbation for at least 6 weeks before sample collection. Asthma symptoms were evaluated according to GINA guidelines [19, 20], using daytime symptoms, night waking due to asthma, reliever needed for symptoms and activity limitation due to asthma in the assessment of disease control. Table 1 resumes demographic and anthropometric data.
The study was approved by CUF Descobertas Hospital and NOVA Medical School Ethics Committees. All investigations were conducted according to the Declaration of Helsinki.

**Regulatory T and B cells**

Peripheral blood samples were collected into EDTA-containing tubes using standard aseptic venipuncture techniques. Cells were processed less than 24 hours after collection.

For Tregs phenotyping, the Human FoxP3 Buffer Set (BD Pharmingen, San Jose, CA) was used, according to the manufacturer’s instructions. First, cells were lysed with BD FACS Lysing (BD Biosciences), and incubated with anti-CD25 PE (clone BC96, Biolegend, San Diego, CA) and anti-CD4 PerCP Cy5.5 (clone SK3, Biolegend). After being fixed and permeabilized with the reagents supplied in the kit set, cells were stained with Anti-Foxp3 Alexa Fluor 488 (clone 259D/C7, BD Pharmingen). At least 10,000 CD4+ T cells were acquired.

For the evaluation of Bregs, the panel of mAbs included anti-CD19 PerCP Cy5.5 (clone HIB19, Biolegend), anti-CD24 PE (clone ML5, Biolegend), anti-CD27 FITC (clone O323, Biolegend) and anti-CD38 APC (clone HIT2, Biolegend). A lyse-wash protocol was used. Briefly, cells were incubated for 15 minutes with monoclonal antibodies (mAbs), and then lysed with BD FACS Lysing (BD Biosciences). Acquisition was performed after a wash step with PBS. At least 2,000 CD19+ B cells were acquired.

**Cell culture for intracellular cytokine stimulation**

Heparin whole blood was used to evaluate IL10 expression in T and B cells. Cells were stimulated with PMA (50ng/mL; Sigma Aldrich, St. Louis, MO), calcium ionophore (1µg/mL, Sigma Aldrich) and LPS (10µg/mL, Sigma Aldrich) [21], and incubated for 5h, at 37°C in a 5% CO₂ atmosphere, in the presence of Brefeldin A (1.0µg/ml, BD Pharmingen). For each sample, unstimulated tubes were incubated in parallel, and were further used as stimulation and staining controls. The protocol was performed according to the instructions of Cytofix-Cytoperm kit (BD Pharmingen). Briefly, the initial surface staining step included an incubation with anti-CD3-FITC (clone SK7, BDBiosciences), anti-CD8 APC (clone SK1, Biolegend) and anti-CD19 PerCP Cy5.5 (clone HIB19, Biolegend). After fixing and permeabilizing, cells were marked with anti-IL10 PE (clone JES3-19F1, Biolegend). A minimum of 2,000 B cells (CD19+) or 10,000 T cells (CD3+) were acquired.
Multicolor Flow Cytometry and Data Analysis

Flow cytometry was performed in a 4-color BD FACSCalibur (BD Biosciences, San Jose, CA), equipped with a 488nm blue laser and a 647nm red laser. Equipment setup, calibration and quality control protocols were performed to assure trough time stability in measurements. Figure 1 presents the gating strategy for Treg assessment and Foxp3 expression (assessed in geometric mean values of Mean Fluorescence Intensity (MFI) units). To reduce the impact of day-to-day variation on this evaluation, a ratio between the MFI of Foxp3 in CD4^{dim}CD25^{Hi} Tregs and the MFI of Foxp3 in CD4^{-} Lymphocytes was performed. The gating strategy for Bregs subsets and IL10 secretion are presented in Figures 2 and 3. All evaluations were performed directly in whole blood.

Statistical Analysis

Absolute frequencies were used in categorical variables, expressed also as percentages, and analyzed by Fisher’s exact test or Chi-square. D’Agostino & Pearson test was carried out to assess normality of distributions. Data normally distributed were presented as mean± standard deviation, otherwise as median and interquartile range. Multiple group evaluation was performed with One-way ANOVA (ANOVA I) or Kruskal-Wallis test, followed by Tukey’s or Dunn’s multiple comparison tests, respectively. Regarding paired groups, data were analyzed with paired Student’s t-test or Wilcoxon test. Unpaired Student’s t-test or Mann–Whitney test were used to compare each 2 independent groups.

Statistical significance was defined by a p-value <0.05. All data were analyzed using GraphPad Prism software, version 6.01 for Windows (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com). Tukey box-and-whiskers graphs, also obtained with GraphPad Prism, were used to present results.

Results

Demographic and anthropometric data

Demographic and anthropometric evaluations are summarized in Table 1. Analyzing all groups of women (AP, n=24; ANP, n=32; HP, n=43; HNP, n=35) for demographic data, statistical significance was observed in the comparison of Body Mass Index (BMI). As for BMI, Kruskal-Wallis test presented a
significant p-value, with the subsequent Dunn’s multiple comparisons reporting significantly higher BMI values in pregnant women (p<0.001 for HP vs HNP and p=0.001 for AP vs ANP). Regarding parity, AP and HP groups were comparable.

Asthma and/or rhinitis complications were reported as illustrated in Table 1: 4 (16.7%) AP presented asthma exacerbations and 2 (8.3%) presented rhinitis (nasal symptoms) in the 3rd trimester of pregnancy. Postpartum, 1 AP (4.2%) reported asthma exacerbation and 2 AP (8.3%) reported rhinitis (nasal symptoms). None of these complications required hospitalization or treatment at emergency department. Though medicated during pregnancy, postpartum only 13 AP (54.2%) remained under therapeutics (ICS therapy and/or LABA and antileukotrienes).

Final postpartum measurements were carried out on median 44 (8) days after delivery.

Increase of CD4+CD25HiFoxp3+ Tregs in ANP and in AP postpartum

In the comparison of non-pregnant asthmatic and non-asthmatic patients, significant differences were observed in CD4+CD25HiFoxp3+ Tregs (p=0.002), increased in ANP (Figure 4.a).

Following the evolution of pregnant women from 3rd trimester of pregnancy to postpartum, no differences were reported either within AP or HP in the paired evaluation of time points. Comparing Tregs of 3rd trimester pregnant women vs non-pregnant women, no significant differences were found for the comparisons of AP vs ANP and HP vs HNP. Nevertheless, in postpartum, Tregs were increased in both AP and HP compared to HNP (AP vs HNP, p=0.041; HP vs HNP, p=0.005), which seems to point towards an increase of Tregs after pregnancy.

FoXP3 expression is increased in asthmatic women, but oscillates from pregnancy to postpartum

We also studied the levels of FoXP3 expression in CD4DimCD25Hi Tregs (Figure 4.a). In ANP, FoXP3 expression was augmented (p<0.001) compared to HNP.

During pregnancy, and at least until delivery (data not shown), decreased FoXP3 expression within CD4DimCD25Hi Tregs was observed in both AP and HP. This decrease was more pronounced in HP, with poorer expression levels then the ones observed in AP (p=0.007). Both groups of women significantly down regulated FoXP3 expression in the third trimester of pregnancy, compared to non-pregnant women (vs ANP and vs HNP; p<0.001). Postpartum, FoXP3 expression levels increased significantly in AP and HP, compared to the values observed in the 3rd trimester of pregnancy (p<0.001). AP reached
expression levels similar to the ones observed in ANP; HP recovered their Foxp3 expression levels as well, reaching values similar to HNP.

**CD24^HiCD38^Hi regulatory B cells are augmented postpartum in AP and HP**

As displayed in table 2, CD24^HiCD27^ Bregs showed similar levels in all groups of women studied, and in all time points. Thus, no differences were observed either in their monitoring from pregnancy to postpartum in AP and HP (Figure 4.b). CD24^HiCD38^Hi Bregs, a transitional subset of B cells, displayed mostly pregnancy-associated changes. First, considering ANP vs HNP, comparable values were obtained. No differences were reported regarding comparisons of AP vs HP, in the time points analyzed. Along pregnancy, CD24^HiCD38^Hi Bregs fluctuate with the same pattern observed for Foxp3 expression: reduced proportions of circulating CD24^HiCD38^Hi Bregs during pregnancy in AP and HP (compared to non-pregnant controls; p≤0.004). Postpartum, both AP and HP raised significantly the proportions of CD24^HiCD38^Hi Bregs, compared to the levels observed in the 3rd trimester of pregnancy (p<0.001), but also compared to ANP and HNP (p≤0.003).

In 3rd trimester, a positive correlation was found between CD24^HiCD38^Hi Bregs and the levels of Foxp3 expression in HP (p=0.002, r=0.457; Spearman correlation test), but significance is lost postpartum.

In ANP, the percentages of IL10-secreting T cells (CD3, CD4 and CD8) were increased compared to HNP (p=0.024 for CD3; p=0.034 for CD4; p=0.009 for CD8). However, 3rd trimester AP presented only significantly increased percentages of IL10-secreting B cells, but not T cells, compared to both HP (p=0.022) and HNP (p=0.008). Postpartum, IL10-secreting T cells (CD3 and CD8) were again augmented in AP compared to HNP (p=0.046 for CD3; p=0.016 for CD8), but also IL10-secreting B cells (p=0.031). Curiously, in postpartum, HP presented higher percentages of IL10-secreting B cells compared to HNP (p=0.019), possibly sustaining an impaired IL10 production by B cells in healthy pregnancy. Within the pregnant women groups (AP and HP), paired analyses revealed no significant changes from pregnancy to postpartum in AP, but a significant increase of IL10-secreting B cells in HP in the later evaluation (p=0.037), as displayed in figure 4.c.
Discussion

Regulatory cells have an important role in immune tolerance and inflammation. To our knowledge this is the first study to report variations of regulatory T and B cell subsets in women with asthma, from the third trimester of pregnancy till postpartum, addressing also IL10 secretion profiles. It seems that though Tregs do not oscillate expressively along this period, CD24\textsuperscript{hi}CD38\textsuperscript{hi} Bregs decrease in the third trimester of pregnancy, and present an important increase postpartum, in both asthmatic and healthy women. Moreover, the reduction of Foxp3 expression in Tregs in late pregnancy (and at least until delivery), was also overcome 6 weeks after delivery. Considering IL10 production, women with asthma presented distinguishing features from healthy, with more IL10 producing T cells than HNP. In the 3\textsuperscript{rd} trimester of pregnancy, IL10-secreting B cells were augmented in AP, compared to HP, but no differences were reported in postpartum.

Tregs have been involved in several human pathologies. Their number increases during early pregnancy and declines from mid-gestation onwards [5]. As observed in our study, other authors reported similar Tregs percentages in third trimester pregnant women and non-pregnant controls [5, 22]. Wegienka et al. [23], reported a progressive augment of Tregs percentages from the prenatal period until the first 12 months postpartum, in both atopic and non-atopic patients, similarly to our findings. Despite our monitoring timings are not the same, we also found that at 6 weeks after delivery, both AP and HP presented higher percentages of Tregs compared to healthy non-pregnant controls. Different strategies for cell identification, distinct gestational periods or confrontation with animal models are also possible additional biases.

Our results shown that pregnant and non-pregnant women, with controlled asthma, present higher percentages of Tregs than HNP, which are maintained in postpartum. Some studies have already reported an increased Tregs population in children with asthma under ICS treatment, compared to non-asthmatic age-matched controls [24]. Nevertheless, Bohacs et al. [15] reported comparable Tregs in healthy non-pregnant and asthmatic (pregnant and non-pregnant) women, although asthmatics presented higher values. Similar values for Tregs were reported in asthmatic patients taking ICS, independently of the regularity on its intake. We also observed comparable values in treated and untreated AP in postpartum (data not shown). Taking into consideration that all AP had been under ICS during pregnancy, the similarity in postpartum values in both groups of patients (treated and untreated)
may suggest that the impact of ICS in circulating Tregs frequency may be sustained even after the
treatment is stopped. Another effect of ICS is the upregulation of Foxp3 expression. Karagiannidis et al.
[25] reported that ICS-treated asthmatic patients presented significantly higher values of Foxp3
expression than healthy controls. By contrast, Provoost et al. [26] only reported augmented expression
in ICS treated asthmatics compared to untreated patients, but not compared to healthy controls.
Methodological differences can justify these opposite results; nevertheless, our data seem to be in
accordance with Karagianiddis [25]. Not only ANP presented higher Foxp3 expression than HNP, but
also, during pregnancy, AP showed higher levels compared to HP. Yet, an important decrease in Foxp3
expression in pregnancy was observed in both AP and HP. Recently, progesterone receptors have been
identified in Tregs [27], supporting the immunomodulatory potential of pregnancy hormones. Without
compromising the regulatory potential of Tregs during pregnancy, and even promoting their
proliferation [28] and proportions [29], progesterone and 17β-estradiol are thought to reduce Foxp3
expression on Tregs in second trimester pregnant women [30]. Our data support these findings,
extending this decrease to the third trimester of gestation. Furthermore, we have shown that ICS-
treated AP also have this pregnancy-derived decrease of Foxp3 expression, though maintaining higher
values than HP. Recognizing that Foxp3 expression can be induced in activated T cells, and that our
study did not assess the functional status of Tregs, we can only speculate a functional normality for
these cells in AP. Considering the protective role of Tregs towards asthma manifestations [31], AP
presenting controlled asthma at recruitment is in favor of a normal function of regulatory cells in these
patients. Nevertheless, further studies are needed to complete and confirm our observations.
In postpartum, Foxp3 expression levels normalized in AP and HP, reaching non-pregnant controls,
reinforcing that pregnancy induces modulation of Foxp3 expression.
Bregs evaluation in asthmatic pregnant women constitutes the innovation of our study. The regulatory
capacities of B cells have been recently reported [32], though there’s a lack of consensus on their
phenotype [33]. Both CD24HiCD27+ and CD24HiCD38Hi B cell subsets have been considered to have
regulatory functions, namely by the secretion of IL10 [10, 34]. Patients with asthma and/or AR have
shown decreased frequencies of CD24HiCD27+ Bregs [10, 35], but increased CD24HiCD38Hi Bregs has also
been spotted in asthmatic patients without medication [10]. In our study, Bregs subsets presented no
differences comparing asthmatic to non-asthmatic patients, but unlike the previous study, our patients
were under medication. As defended for Tregs [24], our data suggest that the beneficial effects of therapeutics (such as ICS) in the course of atopic diseases can also be related to the normalization of Bregs frequencies in treated patients.

Interestingly, CD24^hi^CD38^hi^ Bregs are modulated by pregnancy. Pregnancy is associated with B cell lymphopenia and B cell lymphopoiesis arrestment [13], which probably decreases circulating transitional B cell subsets such as CD24^hi^CD38^hi^ Bregs. These modifications were importantly overcome in postpartum, with CD24^hi^CD38^hi^ Bregs reaching levels significantly higher than those observed in non-pregnant controls, traducing maternal immune system’s recovery after gestation. Interestingly, in mice, transitional B cells express high levels of prolactin receptors and hyperprolactinemia has been shown to increase transitional but not mature B cells [36]. A prolactin-mediated response can thus explain the accumulation of transitional B cells postpartum, when the hormone levels raise up to 30 times, compared to pre-pregnancy levels [37].

It is believed that the promotion of Tregs differentiation is one of the mechanisms by which CD24^hi^CD38^hi^ Bregs operate in healthy individuals [34]. Thus, the elevation of CD4^+^CD25^hi^Foxp3^+^ Tregs postpartum in HP and AP, also reported by other authors [23], may be mediated by CD24^hi^CD38^hi^ Bregs. Recent experimental data also concluded that CD24^hi^CD38^hi^ Bregs promote Foxp3 expression in co-cultured CD4 T cells [34]. In our study, Foxp3 expression and CD24^hi^CD38^hi^ Bregs evolved similarly from pregnancy to postpartum, and there was a positive correlation between these parameters in HP, reinforcing the idea of Bregs modulating Tregs.

The profile of CD24^hi^CD38^hi^ Bregs and Foxp3 expression is comparable in asthmatic and non-asthmatic women, sustaining a pregnancy-derived pattern, independently of either therapeutics or asthma and rhinitis.

Studies approaching IL10 production by PBMCs during pregnancy described similar levels before pregnancy and in late pregnancy [38]. In line with these reports, our data showed that IL10-secreting T and B cells were similar in 3rd trimester HP women, compared to HNP. However, in postpartum, HP presented increased frequencies of IL10^+^ B cells after LPS stimulation. The increase of circulating CD24^hi^CD38^hi^ Bregs in postpartum (the subset in which IL10 secretion is mainly described in B cells) [32, 34], could be a possible explanation. Other studies addressed the production of IL10 in asthmatic and allergic women, usually analyzing total PBMC secretion and serum levels [39, 40]. Although no
differences in PBMC production were identified comparing asthmatic and non-asthmatic women [39], IL10 serum levels were increased in allergic mothers (at delivery) and their children [40]. As far as we know, we report for the first time the distinct profiles of IL10-secreting T and B cells in asthmatic pregnant and non-pregnant women. We stress out that ANP presented increased IL10-secreting T cells, but during pregnancy IL10-secreting B cells were increased in asthmatic women, compared to healthy ones. Nonetheless, Tregs can secrete IL10, as well as other subsets of T cells, such as Th2 cells [4]. Th2 cells, associated to both pregnancy and allergic diseases, may support the similar levels observed amongst AP, ANP and HP. However, during pregnancy, AP have a distinct capacity of B cells to secrete IL10, compared to HP and HNP, presenting similar (vs HP) or even lower (vs HNP) frequencies of CD24^{hi}CD38^{hi} Bregs. Corroborating what was observed in ANP, in postpartum, AP also presented increased IL10-secreting B and T cells, compared to HNP.

At follow-up, we realized that several AP women stopped therapeutics during postpartum. Considering the distinguishing features of immune profiles in postpartum, we compared AP with and without therapeutics, and concluded that studied parameters were similar in both subgroups, being independent from therapeutics.

Monitoring AP pre-pregnancy and throughout more pregnancy time points would have been ideal, thought difficult to accomplish from a financial and practical point of view. Nevertheless, important immune events are known to occur during the 3rd trimester of pregnancy, recovering in the postpartum, as we were also able to identify in our study.

Overall, we conclude that pregnant women with controlled asthma present a similar profile towards healthy pregnant women, though with few distinctive features regarding mostly Foxp3 expression. The changes observed in postpartum reinforce the idea of being pregnancy-dependent, probably hormone driven. More studies will clarify if these parameters can be used to assess complications during pregnancy in women with asthma and eventually influence the immune profile of the asthmatic women’s offspring.
Acknowledgements

The authors would like to thank Tiago Domingues for technical support on statistical analysis.

Conflicts of interests

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of the paper. The authors have no financial sources to declare for this work.

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References


Figure 1. Gating strategies for the identification of distinct regulatory T cells subsets.

A-B. The first step of the analysis of Tregs was the identification of CD4⁺ T cells, recognized as the CD4⁺ cells within the lymphocyte gate. C-D. Identification of CD4⁺CD25⁺Foxp3⁺ regulatory T cells with dot plots of FMO (C) and Foxp3 (D) tubes. E. CD4 vs CD25 dot plot, showing the identification of CD4⁺CD25⁺ regulatory T cells. F. Histogram with Foxp3 expression within CD4⁺CD25⁺ regulatory T cells (grey line), overlaid on Foxp3 expression within CD4⁻ Lymphocytes (black line). Geo MFI means were further in the ratio MFI of Foxp3 in CD4⁺CD25⁺/MFI of Foxp3 in CD4⁻ Lymphocytes.
Figure 2. Gating strategies for the identification of distinct regulatory B cells subsets.

B cells were identified as the CD19+ population within the lymphocyte gate, as displayed in dot plots A and B. C and D dotplots present the identification of regulatory B cells subsets according to their expression of CD24, CD27 and CD38 (CD24HiCD27+Bregs and CD24HiCD38Hi Bregs).
Figure 3. Gating strategies for the identification IL10 secretion in T and B cells.

A-B. T cells were identified according their positive expression of CD3 as shown in the CD3/SSC (Side Scatter) dot plot, and were further gated in CD8 negative T cells (CD4) and CD8 positive T cells (CD8), according to their expression of CD8. C-D. CD19+ B cells (gated as shown in Figure 2) and CD4+ and CD8+ T cells (gated as shown in Figure 3 – A and B) were analyzed for the expression of IL10, after stimulation with PMA+Ionomycin and LPS. Unstimulated samples (C) were used to assess the cutoff for the expression of IL10 after stimulation in B cells, CD3, CD4 and CD8 T cells (D).
Figure 4. Immune parameters in HNP, HP, ANP and AP

a) Representative Tukey box-and-whiskers of CD4^+CD25^{Hi}Foxp3^+ Regulatory T cells subsets frequencies and Foxp3 expression within CD4^{Dim}CD25^{Hi} Tregs in HNP, HP, ANP and AP, including the postpartum evaluation of pregnant women.

b) Representative Tukey box-and-whiskers of circulating Regulatory B cells subsets frequencies in HNP, HP, ANP and AP, including the postpartum evaluation of pregnant women.

c) Representative Tukey box-and-whiskers of IL-10 secreting T and B cells in HNP, HP, ANP and AP, including the postpartum evaluation of pregnant women.

All comparisons performed with Mann-Whitney U, except paired groups comparisons (AP vs AP PP; and HP vs HP PP), which were performed with Wilcoxon test.

Central line: median; box: interquartile range; whiskers: range; dots: outliers.

* p<0.05; ** p<0.001. AP – Asthmatic pregnant women; ANP – Asthmatic non-pregnant women; HP – Healthy pregnant women; HNP – Healthy non-pregnant women; PP – postpartum.
4.b

**CD19+CD24\textsuperscript{Hi}CD27\textsuperscript{+} B regs**

**CD19+CD24\textsuperscript{Hi}CD38\textsuperscript{Hi} B regs**

% of CD19\textsuperscript{+} B cells

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doi: 10.18176/jiaci.0086
4. C

**CD3⁺ IL₁₀⁺ T cells**

**CD4⁺ IL₁₀⁺ T cells**

**CD8⁺ IL₁₀⁺ T cells**

**CD19⁺ IL₁₀⁺ B cells**

* denotes statistical significance.
Table 1. Demographic and anthropometric comparisons between the groups of women recruited.

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<th>AP n=24</th>
<th>ANP n=32</th>
<th>HP n=43</th>
<th>HNP n=35</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age in years, median (IQR)</td>
<td>34 (4)</td>
<td>36 (6)</td>
<td>32 (5)</td>
<td>35 (4)</td>
<td>0.063(^a)</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>24 (100.0)</td>
<td>32 (100.0)</td>
<td>42 (97.7)</td>
<td>35 (100.0)</td>
<td>0.545(^b)</td>
</tr>
<tr>
<td>Black</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (2.3)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>BMI in kg/m(^2), median (IQR)</td>
<td>26.2 (4.5)</td>
<td>21.9 (5.0)</td>
<td>25.6 (4.1)</td>
<td>21.1 (2.4)</td>
<td>&lt;0.001(^a)</td>
</tr>
<tr>
<td>Education, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic / High school</td>
<td>2 (8.3)</td>
<td>5 (16.1)</td>
<td>4 (9.3)</td>
<td>7 (2.3)</td>
<td>0.296(^b)</td>
</tr>
<tr>
<td>Higher education</td>
<td>22 (91.7)</td>
<td>26 (83.9)</td>
<td>39 (90.7)</td>
<td>23 (76.7)</td>
<td></td>
</tr>
<tr>
<td>Parity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuliparous</td>
<td>12 (50.0)</td>
<td>-</td>
<td>24 (55.8)</td>
<td>-</td>
<td>0.799(^d)</td>
</tr>
<tr>
<td>Multiparous</td>
<td>12 (50.0)</td>
<td>-</td>
<td>19 (44.2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Smoke exposure during pregnancy, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (12.5)</td>
<td>-</td>
<td>7 (16.3)</td>
<td>-</td>
<td>1.000(^d)</td>
</tr>
<tr>
<td>No</td>
<td>22 (91.7)</td>
<td>-</td>
<td>36 (83.7)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gestational age (complete weeks) at evaluation, mean (sd)</td>
<td>33 (0.9)</td>
<td>-</td>
<td>33 (1.1)</td>
<td>-</td>
<td>0.265(^c)</td>
</tr>
<tr>
<td>Gestational age (complete weeks) at parturition, mean (sd)</td>
<td>39 (1.1)</td>
<td>-</td>
<td>39 (1.1)</td>
<td>-</td>
<td>0.296(^c)</td>
</tr>
<tr>
<td>Weight at birth in grams, mean (sd)</td>
<td>3262 (329)</td>
<td>-</td>
<td>3199 (402)</td>
<td>-</td>
<td>0.493(^c)</td>
</tr>
<tr>
<td>Baby Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (50.0)</td>
<td>-</td>
<td>21 (48.8)</td>
<td>-</td>
<td>1.000(^d)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (50.0)</td>
<td>-</td>
<td>22 (51.2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Days after labour in postpartum, median (IQR)</td>
<td>44 (8)</td>
<td>-</td>
<td>44 (5)</td>
<td>-</td>
<td>0.589(^b)</td>
</tr>
<tr>
<td>Breast feeding, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>23 (95.8)</td>
<td>-</td>
<td>33 (76.7)</td>
<td>-</td>
<td>0.082(^d)</td>
</tr>
<tr>
<td>No</td>
<td>1 (4.2)</td>
<td>-</td>
<td>10 (23.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sensibilization to aeroallergens, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. pteronyssinus</em></td>
<td>15 (62.5)</td>
<td>21 (65.6)</td>
<td>-</td>
<td>-</td>
<td>1.000(^d)</td>
</tr>
<tr>
<td><em>D. farinae</em></td>
<td>12 (50.0)</td>
<td>18 (56.3)</td>
<td>-</td>
<td>-</td>
<td>0.788(^d)</td>
</tr>
<tr>
<td><em>Lepidoglyphus destructor</em></td>
<td>2 (8.3)</td>
<td>9 (28.1)</td>
<td>-</td>
<td>-</td>
<td>0.093(^d)</td>
</tr>
<tr>
<td><em>Grass polens</em></td>
<td>25 (20.8)</td>
<td>14 (43.8)</td>
<td>-</td>
<td>-</td>
<td>0.092(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Kruskal-Wallis Test, \(^b\) Chi-square test, \(^c\) Unpaired Student’s t test, \(^d\) Fisher exact test, \(^e\) Mann-Whitney U.

Table 2. Percentages and absolute counts of T and B cell subsets analyzed in AP, ANP, HP and HNP women.

<table>
<thead>
<tr>
<th>Cellular Subset</th>
<th>AP (n=24)</th>
<th>AP PP (n=24)</th>
<th>ANP (n=32)</th>
<th>HP (n=43)</th>
<th>HP PP (n=43)</th>
<th>HNP (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulatory T cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;Hi&lt;/sup&gt; Fox p3&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>6.14 (1.54)</td>
<td>5.90 (2.43)</td>
<td>6.88 (2.17)</td>
<td>6.07 (3.44)</td>
<td>6.63 (2.85)</td>
<td>5.48 (1.69)</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;Hi&lt;/sup&gt; Fox p3&lt;sup&gt;+&lt;/sup&gt; (Cells/µL)</td>
<td>52 (22)</td>
<td>65 (29)</td>
<td>83 (33)</td>
<td>50 (25)</td>
<td>71 (39)</td>
<td>56 (31)</td>
</tr>
<tr>
<td>Foxp3 expression in CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;HI&lt;/sup&gt;</td>
<td>1.64 (1.26)</td>
<td>1.94 (0.46)</td>
<td>2.09 (0.43)</td>
<td>1.49 (0.19)</td>
<td>1.93 (0.30)</td>
<td>1.80 (0.25)</td>
</tr>
<tr>
<td><strong>Regulatory B cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD24&lt;sup&gt;HI&lt;/sup&gt;CD27&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>25.47 (16.75)</td>
<td>27.26 (20.46)</td>
<td>31.77 (13.37)</td>
<td>26.92 (15.02)</td>
<td>25.76 (14.30)</td>
<td>27.46 (49.80)</td>
</tr>
<tr>
<td>CD24&lt;sup&gt;HI&lt;/sup&gt;CD27&lt;sup&gt;+&lt;/sup&gt; (Cells/µL)</td>
<td>45 (47)</td>
<td>59 (45)</td>
<td>102 (97)</td>
<td>44 (41)</td>
<td>57 (47)</td>
<td>54 (40)</td>
</tr>
<tr>
<td>CD24&lt;sup&gt;HI&lt;/sup&gt;CD38&lt;sup&gt;HI&lt;/sup&gt; (%)</td>
<td>1.87 (2.97)</td>
<td>7.13 (8.84)</td>
<td>3.84 (2.74)</td>
<td>1.42 (1.38)</td>
<td>5.62 (5.58)</td>
<td>3.63 (2.48)</td>
</tr>
<tr>
<td>CD24&lt;sup&gt;HI&lt;/sup&gt;CD38&lt;sup&gt;HI&lt;/sup&gt; (Cells/µL)</td>
<td>3 (4)</td>
<td>14 (25)</td>
<td>12 (7)</td>
<td>2 (2)</td>
<td>11 (14)</td>
<td>8 (8)</td>
</tr>
<tr>
<td><strong>IL-10 secreting cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL10&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;-&lt;/sup&gt; T cells (%)</td>
<td>0.83 (0.69)</td>
<td>0.91 (0.70)</td>
<td>0.94 (0.46)</td>
<td>0.77 (0.47)</td>
<td>0.83 (0.50)</td>
<td>0.72 (0.57)</td>
</tr>
<tr>
<td>IL10&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;-&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt; (CD8&lt;sup&gt;-&lt;/sup&gt;) T cells (%)</td>
<td>1.00 (0.76)</td>
<td>1.03 (0.78)</td>
<td>1.04 (0.71)</td>
<td>0.92 (0.54)</td>
<td>0.95 (0.53)</td>
<td>0.84 (0.54)</td>
</tr>
<tr>
<td>IL10&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;-&lt;/sup&gt;CD8&lt;sup&gt;-&lt;/sup&gt; T cells (%)</td>
<td>0.66 (0.69)</td>
<td>0.65 (0.86)</td>
<td>0.73 (0.51)</td>
<td>0.49 (0.55)</td>
<td>0.59 (0.47)</td>
<td>0.40 (0.59)</td>
</tr>
<tr>
<td>IL10&lt;sup&gt;+&lt;/sup&gt;CD19&lt;sup&gt;-&lt;/sup&gt; B cells (%)</td>
<td>1.26 (0.67)</td>
<td>1.24 (0.83)</td>
<td>1.04 (0.73)</td>
<td>1.00 (0.90)</td>
<td>1.17 (0.91)</td>
<td>0.92 (0.70)</td>
</tr>
</tbody>
</table>

All results are presented as median (IQR).