

# Peripheral T<sub>h</sub>1/T<sub>h</sub>2/T<sub>h</sub>17/regulatory T-cell balance in asthmatic pregnancy

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## Abstract

Asthma is a common chronic disease that may complicate pregnancy and a risk factor for complications; however, immunological mechanisms of the bilateral interactions between asthma and pregnancy are not fully understood. Healthy gestation is characterized by a sensitive balance of T<sub>h</sub>1/T<sub>h</sub>2/T<sub>h</sub>17/regulatory T (Treg) cells that may be altered in asthmatic pregnancy. The aim of this study was to describe the prevalence of these cell subsets in asthmatic compared with healthy pregnancy. The prevalence of T<sub>h</sub>1, T<sub>h</sub>2, T<sub>h</sub>17 and Treg lymphocytes was identified by cell surface and intracellular marker staining in blood samples of 24 healthy non-pregnant (HNP), 23 healthy pregnant (HP), 15 asthmatic non-pregnant (ANP) and 15 asthmatic pregnant (AP) women using flow cytometry. The T<sub>h</sub>1/T<sub>h</sub>2 cell ratio was decreased in both HP and ANP compared with HNP women; however, no further decrease was observed in the AP group. The T<sub>h</sub>17/Treg ratio was decreased in HP, but not in AP women, compared with HNP data. Healthy pregnancy increased Treg cell prevalence compared with HNP data (4.64% versus 2.98%;  $P < 0.05$ ), and this pregnancy-induced elevation was absent in AP women (2.52% versus 4.64%;  $P < 0.05$ ). T<sub>h</sub>17 cell prevalence was similar in the HP and HNP groups (2.78% versus 3.17%;  $P > 0.05$ ). Asthma increased T<sub>h</sub>17 prevalence in non-pregnant patients (3.81% versus 3.17%;  $P < 0.05$ ), and this asthma-specific increase of T<sub>h</sub>17 cell prevalence was also observed in AP patients (AP versus HP: 3.44% versus 2.78%;  $P < 0.05$ ). The abnormal asthma-dependent T<sub>h</sub>17 elevation together with blunted Treg increase may play a role in the compromised immune tolerance characterizing asthmatic pregnancy.

**Keywords:** asthma, pregnancy, Tc17, T<sub>h</sub>17, Treg

## Introduction

Allergic asthma is an inflammatory disease characterized by T<sub>h</sub>2-type inflammation that leads to bronchial hyperresponsiveness, airway obstruction and—in some cases—tissue remodeling. Regulatory T cells (Tregs) have also been shown to influence inflammatory responses in asthma: Tregs inhibit CD4<sup>+</sup> CD25<sup>−</sup> effector T cells, suppress T<sub>h</sub>2 responses to allergens and prevent airway eosinophilia, mucous hypersecretion and airway hyperresponsiveness (1). In non-pregnant atopic asthmatics, a reduction of peripheral Treg cell prevalence was observed (2, 3).

The recent discovery of a distinct T helper subset, referred to as T<sub>h</sub>17 cells based on their IL-17 production, led to the transformation of the T<sub>h</sub>1/T<sub>h</sub>2 paradigm of immunity into a novel viewpoint that incorporates T<sub>h</sub>1, T<sub>h</sub>2, T<sub>h</sub>17 and

Treg cells as elements of a complex and mutually interacting network (4). IL-17 has been proposed to have an important role in the development of autoimmune disorders (such as rheumatoid arthritis and multiple sclerosis) and in the induction and maintenance of chronic inflammation (5). The effect of T<sub>h</sub>17 cells on the inflammatory balance is opposed by CD4<sup>+</sup> Tregs. A recent study by Zhao *et al.* (6) showed that abnormal T<sub>h</sub>17 immunity may be involved in the pathogenesis of allergic asthma. Besides the elevation of T<sub>h</sub>2 cell prevalence, they demonstrated that the percentage of T<sub>h</sub>17 cells as well as the concentration of T<sub>h</sub>17-related cytokines was higher in peripheral blood of uncontrolled, mostly moderate to severe persistent allergic asthmatics than in healthy controls.

Pregnancy is characterized by an immunological tolerance that attenuates maternal immune responses against paternal antigens expressed by the fetus (7). A component of this tolerance is skewness toward T<sub>h</sub>2-type immunity (8, 9). Importantly, Tregs are key players in the maintenance of peripheral tolerance to paternal antigens during pregnancy (10, 11). Diminished numbers of Treg cells in pregnancy were associated with immunological rejection of the fetus as well as preeclampsia and low fetal birth weight (11–13). Alteration of T<sub>h</sub>17 cell prevalence was also observed in adverse pregnancy outcomes such as preterm labor (14) and recurrent spontaneous abortion (15). In our recent study, we demonstrated that the imbalance of T<sub>h</sub>17/Treg cells is of importance in the development of systemic inflammation in preeclampsia (16). These findings suggest that in addition to T<sub>h</sub>1 and T<sub>h</sub>2 cells, T<sub>h</sub>17 and Treg cells also have crucial effects on the outcome of pregnancy. Moreover, although IL-17 was first identified in CD4 cells, later on other immune cells including CD8 and NK cells were also shown to produce this cytokine (17, 18), and emerging evidence suggests that these IL-17-producing lymphocyte subsets, especially NK cells, largely contribute to the inflammatory status in pregnancy-related disorders (19).

Asthma is one of the most common chronic diseases complicating pregnancy, affecting 3.7–8.4% of all pregnancies (20). It represents an increased risk for maternal and fetal morbidities, including preterm delivery, cesarean delivery, gestational hypertension, preeclampsia, low birth weight and neonatal mortality (21, 22). Pregnancy also has an effect on the course and control level of asthma, causing deterioration of symptoms in one-third of pregnant women (23). More severe asthma before pregnancy (24), maternal obesity (25) and female gender of the fetus (26) are risk factors for worsening asthma symptoms during pregnancy. In our earlier studies, maternal asthma control was found to be related to peripheral T cell-mediated inflammation in asthmatic pregnancy (27), and pregnancy-specific increase in Treg prevalence was diminished in pregnancies complicated with asthma (28). However, mechanisms of the bidirectional interactions between asthma and pregnancy—including the alteration of T cell-mediated immune responses—are not fully elucidated. Hence, a study evaluating the balance of T<sub>h</sub>1/T<sub>h</sub>2/T<sub>h</sub>17/Treg cells in asthmatic pregnant (AP) patients may be of importance for better understanding the mechanisms characterizing asthmatic pregnancy.

In this study, we aimed to describe the alterations in the balance of T<sub>h</sub>1, T<sub>h</sub>2, T<sub>h</sub>17 and Treg lymphocytes, along with the prevalence of IL-17-producing CD8+ and NK cells, in asthmatic compared with healthy pregnancy.

## Methods

### Study participants

A total of 77 women of reproductive age [24 healthy non-pregnant (HNP), 23 healthy pregnant (HP) women and 15 asthmatic non-pregnant (ANP), 15 AP patients] were enrolled in our study. Participants' characteristics are summarized in Table 1. All patients had been diagnosed with allergic asthma according to current international guidelines (29) at least 6 months before pregnancy and were

regularly controlled with standard measures for asthma for at least 6 months. All of them reported a history of allergic disease (Table 2). Exclusion criteria were current smoking or >5 pack-years of smoking history, any other chronic disease, acute exacerbation of asthma, multiple gestation and acute infection within 3 weeks or systemic corticosteroid treatment within 6 weeks before participation in our study. Asthma was treated according to the current guideline (29). All patients had mild or moderate persistent disease and inhaled corticosteroids (ICS) were prescribed to all of them. Asthma control, lung function and the level of exhaled nitric oxide (FeNO) were assessed in asthmatic patients only.

### Study design

The study had a cross-sectional design. HP subjects were recruited when attending their scheduled visit at the First Department of Obstetrics and Gynecology (Semmelweis University, Budapest). Patients with asthma were enrolled at their visit at the outpatient clinic of the Department of Pulmonology (Semmelweis University, Budapest). All patients were asked not to use their medication for 12 h before sampling. Lung function values, use of asthma medication and clinical symptoms were all recorded by the attending physicians. Healthy age-matched female volunteers were recruited from students and staff of Semmelweis University. Non-pregnant women were in the early follicular phase of the menstrual cycle (between cycle days 3 and 5), and none of them received hormonal contraception. Written informed consent was obtained from all subjects, and our study was reviewed and approved by an independent ethical committee of the institution. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

### Lung function and asthma control evaluation

Forced expiratory volume in 1 s (FEV<sub>1</sub>), peak expiratory flow (PEF) and airway resistance (R<sub>aw</sub>) were measured by means of electronic spirometer and plethysmography (PDD-301/s; Piston, Budapest, Hungary) according to the American Thoracic Society guidelines (30). Three technically acceptable maneuvers were performed, and the highest one was recorded. Asthma control was assessed by using the Asthma Control Test (ACT) recommended by the current guideline (29).

### Measurement of FeNO

Exhaled nitric oxide was measured using NIOX MINO® Airway Inflammation Monitor (NIOX MINO®; Aerocrine AB, Solana, Sweden) according to the latest guidelines (31).

### Flow cytometry measurements

Peripheral blood mononuclear cells (PBMCs) were separated by standard density gradient centrifugation (Ficoll Paque; Amersham Biosciences AB, Uppsala, Sweden; 27 min, 400 × *g*, 22°C) from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer; BD Biosciences, San Jose, CA, USA). This cell suspension was washed twice in PBS. Cells were then suspended in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) and the following procedures were performed.

**Table 1.** Age and obstetrical data of subjects

|                                              | HNP, <i>n</i> = 24 | HP, <i>n</i> = 23 | ANP, <i>n</i> = 15 | AP, <i>n</i> = 15 |
|----------------------------------------------|--------------------|-------------------|--------------------|-------------------|
| Age (years)                                  | 32 (27–34)         | 33 (29–36)        | 34 (32–37)         | 33 (27–35)        |
| Gestational age at sample collection (weeks) | N/A                | 31 (21–35)        | N/A                | 29 (20–33)        |
| Gestational age at delivery (weeks)          | N/A                | 39 (38–40)        | N/A                | 40 (38–40)        |
| Birth weight (g)                             | N/A                | 3220 (2985–3670)  | N/A                | 3285 (2650–3610)  |

Data are expressed as median (interquartile range). *P* > 0.05 for all comparisons. N/A, not applicable.

**Table 2.** Clinical data of patients with asthma (ANP and AP women)

|                                                                | ANP, <i>n</i> = 15 | AP, <i>n</i> = 15  |
|----------------------------------------------------------------|--------------------|--------------------|
| BMI (kg/m <sup>2</sup> )                                       | 25.9 (22.7–29.2)   | 28.2 (26.7–31.1)   |
| FEV <sub>1</sub> (% of predicted)                              | 90.0 (84.0–97.5)   | 88.0 (83.0–102.0)  |
| PEF (% of predicted)                                           | 79.0 (74.5–91.5)   | 86.0 (74.0–95.0)   |
| R <sub>aw</sub> (% of predicted)                               | 121.0 (88.5–135.5) | 102.0 (82.0–130.0) |
| FeNO (ppb)                                                     | 29.0 (12.5–47.0)   | 18.0 (12.0–39.0)   |
| Number of patients using prescribed ICS regularly and properly | 10 (66%)           | 12 (80%)           |
| Daily dose of ICS (beclomethasone equivalent; µg)              | 450 (400–800)      | 500 (400–800)      |
| ACT total score                                                | 22.0 (19.5–24.5)   | 22.0 (13.0–24.0)   |

Data are expressed as median (interquartile range) for continuous variables and as number (percentage) for categorical variables. *P* > 0.05 for all comparisons. BMI, body mass index; FeNO, fraction of exhaled nitric oxide; R<sub>aw</sub>, airway resistance.

- (1) One portion of the cells was stained for 30 min at room temperature with PE Cy7-conjugated CD4 and APC Cy7-conjugated CD8 mAbs (PharMingen, San Diego, CA, USA) along with PerCP-conjugated CD56 mAb (BioLegend, San Diego, CA, USA). After washing, FACS Lysing Solution (BD Biosciences) was added, and after removal, cells were treated with FACS Permeabilizing Solution (BD Biosciences) for 10 min at room temperature. For the detection of regulatory T cells and cell surface chemokine receptors, another portion of PBMCs was stained for 30 min at 4°C with PE Cy7-conjugated CD4, FITC-conjugated CD25 and APC-conjugated CXCR3 mAbs (PharMingen) along with PerCP-conjugated CCR4 mAb (BioLegend). After washing, cells were fixed with Fixation/Permeabilization solution and treated with Permeabilization Buffer according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).
- (2) For intracellular cytokine detection, PBMCs were stimulated with 10 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich) and 1 µg/ml of ionomycin (Sigma-Aldrich) in the presence of 10 µg/ml of brefeldin A (BFA; Sigma-Aldrich) for 4 h at 37°C in an atmosphere containing 5% CO<sub>2</sub> before the staining procedure.
- (3) The first portion of cells was stained with PE-conjugated IL-17A and APC-conjugated IL-4 mAbs (eBioscience) along with FITC-conjugated IFN-γ mAb (BioLegend) for 30 min at room temperature. The other portion of PBMCs was stained with PE-conjugated FoxP3 mAb (eBioscience) for 30 min at 4°C.

After washing, cells were analyzed on a BD FACSaria flow cytometer (BD Biosciences); 200 000 cells were recorded. The gating strategy for the detection of lymphocyte subsets is shown on Fig. 1. The population of lymphocytes was gated from PBMCs according to Forward Scatter Characteristics

and Side Scatter Characteristics. This was followed by the gating of the CD4+, CD8+ or CD56+ subsets. Then, within these subsets, CXCR3+, CCR4+, CD25+ FoxP3+, IFN-γ+, IL-4+ and IL-17+ cells were gated, respectively. Isotype-matched PE-, APC- and FITC-conjugated mouse IgG1 antibodies were used as controls (eBioscience and BioLegend, respectively). Assessment of the investigated cell subtypes was performed among the CD4+, CD8+ or CD56+ lymphocyte subsets, respectively, as well as among the overall lymphocyte population.

### Statistics

Data are expressed as median and quartiles. Comparisons between sample populations were made with Kruskal–Wallis tests and Mann–Whitney tests, as a test of normality (performed according to Kolmogorov–Smirnov) indicated non-normal distribution of data. *P* values < 0.05 were considered significant. The sample size was estimated to achieve 80% power with 0.45 effect size to detect differences in T-cell subsets between the four groups. Statistics were calculated using the R software (R Development Core Team; R Foundation for Statistical Computing, Vienna, Austria).

### Results

#### Clinical data

Ages in the four groups, as well as gestational age at sampling and delivery in the pregnant groups were comparable (Table 1). Asthma severity and the level of asthma control were the same in the two asthmatic groups: no differences in FEV<sub>1</sub> and PEF values, FeNO levels as well as in ACT total scores were observed (Table 2). Asthma was well controlled in most cases with a median ACT total score of 22 in both asthmatic groups. Eighty percent of AP and 66% of ANP

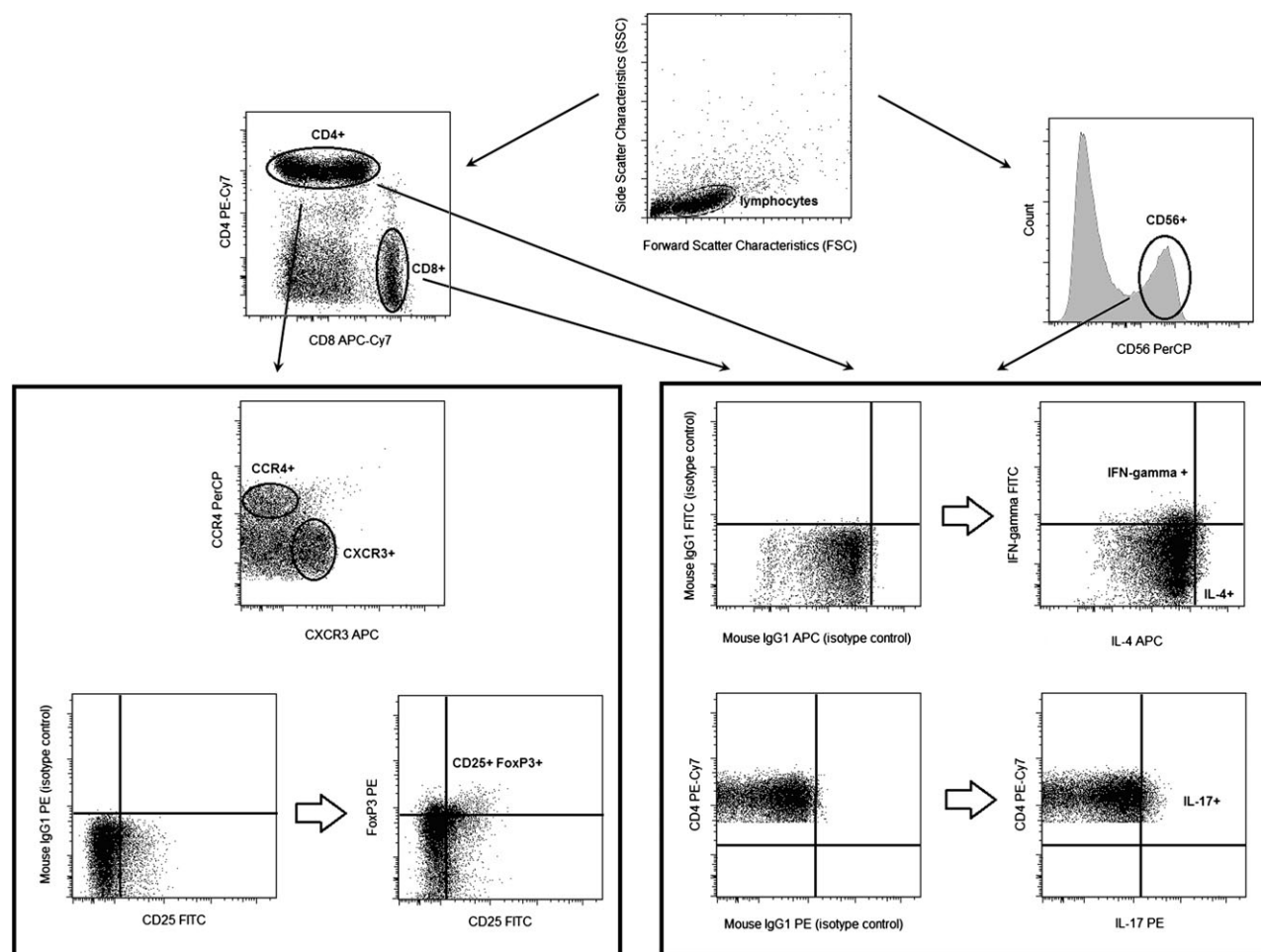


**Fig. 1.** Gating strategy for the detection of the investigated lymphocyte subsets. APC, allophycocyanin; PerCP, peridinin chlorophyll protein.

patients used ICS therapy regularly and strictly according to the prescription. Similar median birth weights observed in newborns of healthy and asthmatic mothers (Table 1) may be the consequence of well-controlled asthma in most patients.

#### Lymphocyte subsets

Our results are summarized in Table 3. The prevalence of the CD4<sup>+</sup> CXCR3<sup>+</sup> subset ( $T_h1$  cells, cell surface marker detection) was lower in the HP, ANP and AP groups than in the HNP group, whereas that of CD4<sup>+</sup> CCR4<sup>+</sup> cells ( $T_h2$  subset, cell surface marker detection) did not differ between the four groups. The ratio of CXCR3<sup>+</sup>/CCR4<sup>+</sup> CD4 cells showed a similar—but not significant—trend in the study groups to the alteration of CD4<sup>+</sup> CXCR3<sup>+</sup> subset. The prevalence of CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> lymphocytes ( $T_h1$  cells, intracellular cytokine detection) was lower in HP, ANP and AP samples compared with HNP data (Fig. 2a), while the CD4<sup>+</sup> IL-4<sup>+</sup> prevalence ( $T_h2$  cells, intracellular cytokine detection) was elevated among HP, ANP and AP women compared with HNP controls (Fig. 2b). Healthy pregnancy and asthma alone both decreased the IFN- $\gamma$ <sup>+</sup>/IL-4<sup>+</sup> CD4 ( $T_h1/T_h2$ ) cell ratio, but no further decrease was observed in asthmatic pregnancy (Fig. 2c).

Asthma itself increased CD4<sup>+</sup> IL-17<sup>+</sup> ( $T_h17$ ) cell prevalence, as it was significantly higher in ANP samples compared with HNP (Fig. 2d). HP did not change  $T_h17$  prevalence compared with HNP control data. Supporting compromised pregnancy-specific immune tolerance as a result of asthma,  $T_h17$  prevalence was significantly higher in the AP group than in the HP group. Moreover, healthy pregnancy was accompanied by a significant elevation of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory T-cell prevalence compared with HNP subjects, and this elevation of Tregs was absent in AP patients (Fig. 2e). As a result, the ratio of  $T_h17$ /Treg cells decreased in HP but not in AP subjects in comparison with HNP controls. In the AP group, the  $T_h17$ /Treg ratio was significantly higher than in HP women (Fig. 2f), which also suggests an attenuated immune tolerance caused by asthma that complicates pregnancy.

The prevalence of CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup> IL-4<sup>+</sup> (Tc1 and Tc2) lymphocytes was altered neither in the HP nor in the AP group. However, the prevalence of CD8<sup>+</sup> IL-17<sup>+</sup> (Tc17) cells among CD8<sup>+</sup> lymphocytes was higher in HP than in HNP samples and this pregnancy-induced elevation of Tc17 cell prevalence was not detectable in the AP group. Here, the prevalence of Tc17 was comparable to that

**Table 3.** Prevalence of the investigated lymphocyte subsets [in proportion to the parent populations as well as the overall lymphocyte population (ly)] in HNP, HP, ANP and AP women

| Subset                        | HNP, <i>n</i> = 24  | HP, <i>n</i> = 23      | ANP, <i>n</i> = 15   | AP, <i>n</i> = 15     |
|-------------------------------|---------------------|------------------------|----------------------|-----------------------|
| CD4+ CXCR3+/CD4+ (%)          | 27.65 (25.00–29.80) | 21.50 (19.00–25.30)*** | 24.10 (18.70–27.30)* | 22.70 (20.20–25.20)** |
| CD4+ CXCR3+/ly (%)            | 9.97 (7.36–12.77)   | 8.59 (7.13–11.27)*     | 8.78 (6.98–10.70)*   | 8.60 (6.70–11.00)*    |
| CD4+ CCR4+/CD4+ (%)           | 11.45 (10.25–15.05) | 10.70 (8.45–14.20)     | 12.80 (10.70–15.80)  | 13.60 (9.90–15.80)    |
| CD4+ CCR4+/ly (%)             | 4.22 (3.13–5.41)    | 3.94 (2.91–5.75)       | 4.94 (4.40–6.32)     | 4.51 (4.02–5.34)      |
| CD4+ CXCR3+/CD4+ CCR4+        | 2.18 (1.98–2.82)    | 2.03 (1.73–2.69)       | 1.71 (1.46–2.16)     | 1.67 (1.38–2.21)      |
| CD4+ IFN $\gamma$ /CD4+ (%)   | 28.05 (17.50–33.50) | 19.00 (16.50–22.30)*   | 18.20 (15.30–23.10)* | 18.50 (14.20–23.90)*  |
| CD4+ IFN $\gamma$ /ly (%)     | 9.43 (6.12–11.07)   | 5.61 (3.23–6.97)***    | 5.26 (4.30–6.97)**   | 4.84 (4.41–6.87)**    |
| CD4+ IL-4+/CD4+ (%)           | 2.42 (1.13–2.92)    | 3.21 (2.26–3.77)*      | 3.08 (2.50–3.72)*    | 3.64 (3.03–4.81)***   |
| CD4+ IL-4+/ly (%)             | 0.75 (0.38–1.15)    | 0.83 (0.56–1.29)*      | 0.94 (0.72–1.23)*    | 1.16 (1.03–1.24)**    |
| CD4+ IFN $\gamma$ /CD4+ IL-4+ | 11.95 (6.21–18.00)  | 5.92 (4.09–8.88)**     | 5.83 (4.39–7.45)**   | 4.72 (3.85–5.30)***   |
| CD4+ IL-17+/CD4+ (%)          | 3.17 (2.86–3.49)    | 2.78 (2.35–3.06)       | 3.81 (3.37–5.68)**   | 3.44 (3.13–3.75)##    |
| CD4+ IL-17+/ly (%)            | 1.18 (0.98–1.44)    | 0.75 (0.44–0.98)**     | 0.82 (0.56–0.96)*    | 0.52 (0.30–0.71)***   |
| CD4+ CD25+ FoxP3+/CD4+ (%)    | 2.98 (2.28–3.49)    | 4.64 (4.04–5.73)**     | 2.64 (1.82–3.72)#    | 2.52 (1.57–3.69)##    |
| CD4+ CD25+ FoxP3+/ly (%)      | 1.11 (0.69–1.39)    | 1.86 (0.85–2.45)*      | 1.09 (0.70–1.78)#    | 0.76 (0.69–1.35)##    |
| CD4+ IL-17+/CD4+ CD25+ FoxP3+ | 0.98 (0.84–1.65)    | 0.55 (0.52–0.74)**     | 1.74 (1.00–2.34)###  | 1.17 (0.87–2.31)###   |
| CD8+ IFN $\gamma$ /CD8+ (%)   | 2.87 (1.83–4.57)    | 2.72 (1.94–4.71)       | 2.63 (2.01–3.54)     | 2.10 (1.73–2.63)      |
| CD8+ IFN $\gamma$ /ly (%)     | 0.33 (0.22–0.53)    | 0.25 (0.10–0.38)       | 0.32 (0.24–0.47)     | 0.27 (0.22–0.47)      |
| CD8+ IL-4+/CD8+ (%)           | 3.03 (2.16–4.29)    | 4.53 (2.89–6.57)       | 2.43 (1.43–3.45)###  | 2.66 (2.10–4.43)      |
| CD8+ IL-4+/ly (%)             | 0.31 (0.25–0.48)    | 0.36 (0.21–0.41)       | 0.30 (0.14–0.52)     | 0.31 (0.23–0.62)      |
| CD8+ IL-17+/CD8+ (%)          | 2.43 (1.68–3.68)    | 4.82 (3.60–6.17)**     | 1.66 (0.90–2.14)###  | 2.13 (1.51–3.53)#     |
| CD8+ IL-17+/ly (%)            | 0.30 (0.21–0.43)    | 0.28 (0.21–0.42)       | 0.20 (0.10–0.41)     | 0.26 (0.16–0.42)      |
| CD56+ IFN $\gamma$ /CD56+ (%) | 2.76 (2.07–3.27)    | 3.16 (1.95–7.05)       | 2.55 (1.58–2.87)     | 1.88 (1.22–2.32)##    |
| CD56+ IFN $\gamma$ /ly (%)    | 0.51 (0.15–1.10)    | 0.38 (0.19–0.67)       | 0.16 (0.13–0.48)     | 0.15 (0.10–0.21)*, #  |
| CD56+ IL-4+/CD56+ (%)         | 2.21 (1.68–2.85)    | 2.70 (1.59–4.10)       | 1.77 (1.28–2.71)     | 2.15 (1.49–2.82)      |
| CD56+ IL-4+/ly (%)            | 0.45 (0.19–0.60)    | 0.30 (0.16–0.41)       | 0.22 (0.09–0.44)     | 0.46 (0.43–0.48)      |
| CD56+ IL-17+/CD56+ (%)        | 1.60 (1.07–2.52)    | 0.98 (0.62–1.23)*      | 1.01 (0.22–2.50)     | 1.46 (0.73–3.32)      |
| CD56+ IL-17+/ly (%)           | 0.39 (0.06–0.58)    | 0.09 (0.06–0.13)       | 0.06 (0.01–0.30)     | 0.09 (0.04–0.25)      |

Data are presented as median (interquartile range). *P* values <0.05 were regarded significant. \**P* < 0.05 versus HPN; \*\**P* < 0.01 versus HPN; \*\*\**P* < 0.001 versus HPN; #*P* < 0.05 versus HP; ##*P* < 0.01 versus HP; ###*P* < 0.001 versus HP.

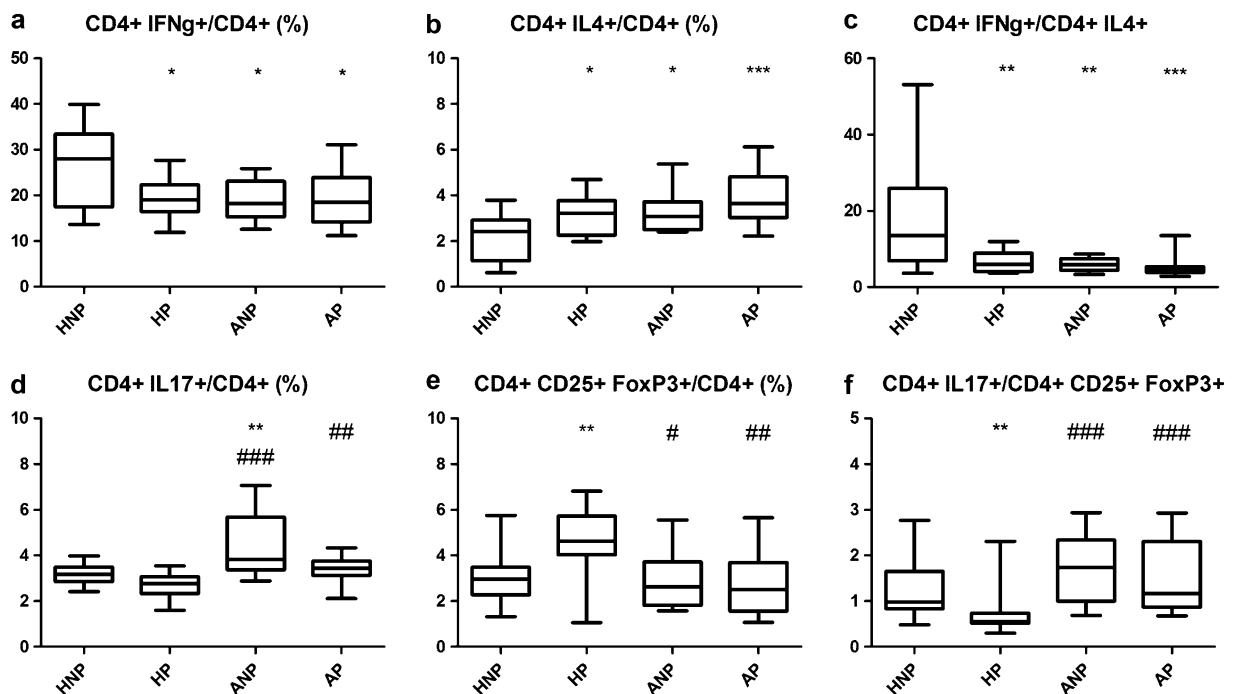


**Fig. 2.** Box plots representing the prevalence of IFN- $\gamma$ + (a), IL-4+ (b), IL-17+ (d) and CD25+ FoxP3+ (e) cells among CD4+ lymphocytes as well as the  $T_H1/T_H2$  (c) and  $T_H17/Treg$  (f) ratios in HNP, HP, ANP and AP women. Horizontal line, median; box, interquartile range; whisker, range. *P* values <0.05 were regarded significant. \**P* < 0.05 versus HPN; \*\**P* < 0.01 versus HPN; \*\*\**P* < 0.001 versus HPN; #*P* < 0.05 versus HP; ##*P* < 0.01 versus HP; ###*P* < 0.001 versus HP.

observed in ANP and significantly lower than that in HP (Fig. 3b). Since the prevalence of  $T_h17$  and  $Tc17$  cells changed in opposite directions both in AP and HP women, we further analyzed the prevalence of  $CD4^+$  IL-17 $^+$  and  $CD8^+$  IL-17 $^+$  lymphocytes. After the initial assessment of the  $T_h17$  and  $Tc17$  cell prevalence among  $CD4^+$  and  $CD8^+$  lymphocytes, respectively (Fig. 3a and b), we also determined the prevalence of these subsets among the overall lymphocyte population (Fig. 3d and e) along with the frequency of  $CD4^+$  IL-17 $^-$  and  $CD8^+$  IL-17 $^-$  cells in the overall lymphocyte population (Fig. 3g and h). Our findings revealed two different mechanisms that explain the opposite alterations of  $T_h17$  and  $Tc17$  prevalence in the study groups. While  $CD4^+$  IL-17 $^+$  cell prevalence showed differences among the four groups when examined in the overall lymphocyte population (Fig. 3d), and also among  $CD4^+$  cells (Fig. 3a), the frequency of  $CD4^+$  IL-17 $^-$  cells remained the same in all study

groups (Fig. 3g). Therefore, the detected differences regarding the prevalence of  $T_h17$  ( $CD4^+$  IL-17 $^+$ ) cells show absolute alterations (since  $T_h17$  cell numbers were altered not only among  $CD4^+$  cells but also in the overall lymphocyte population and  $CD4^+$  IL-17 $^-$  prevalence was the same throughout the study groups). In contrast,  $Tc17$  cell frequencies did not show significant difference if assessed among the overall lymphocyte population (Fig. 3e); only the prevalence of  $CD8^+$  IL-17 $^-$  cells differed among the study groups (Fig. 3h) and the direction of IL-17 $^-$  cell alteration was the opposite of that seen for  $Tc17$  cell prevalence in the  $CD8^+$  subset (Fig. 3b). Therefore, the prevalence of  $Tc17$  cells does not show absolute alterations, only virtual ones, as a consequence of different  $CD8^+$  IL-17 $^-$  cell frequencies in the four groups.

Finally,  $CD56^+$  IL-4 $^+$  cell numbers were comparable throughout the four groups, while the prevalence of  $CD56^+$

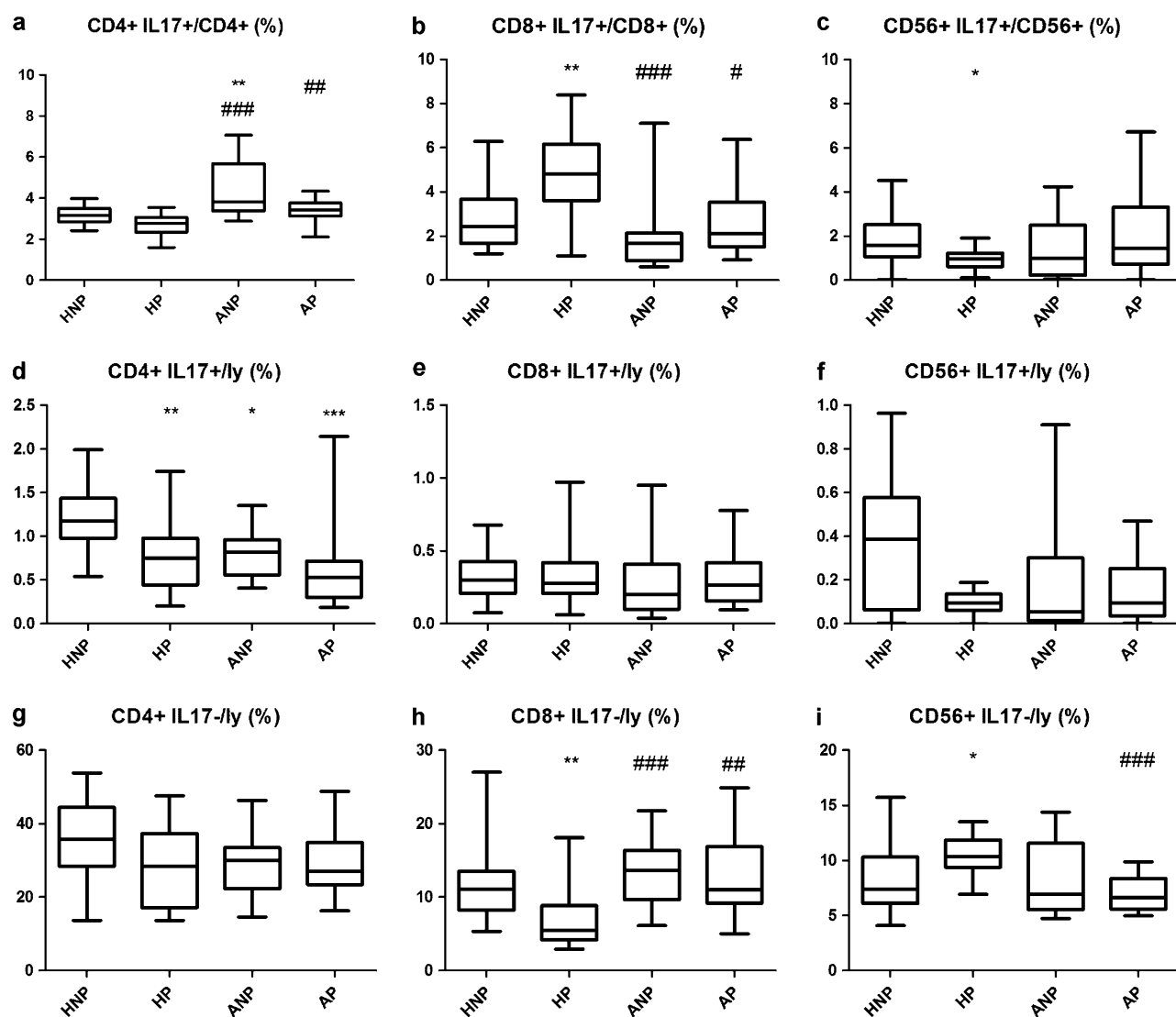


**Fig. 3.** Box plots representing the prevalence of IL-17 $^+$  cells among  $CD4^+$ ,  $CD8^+$  and  $CD56^+$  lymphocytes (a–c) and in the overall lymphocyte population (d–f), as well as the prevalence of IL-17 $^-$  cells in the overall lymphocyte population (g–i) in HNP, HP, ANP and AP women. Horizontal line, median; box, interquartile range; whisker, range.  $P$  values  $< 0.05$  were regarded significant. \* $P < 0.05$  versus HNP; \*\* $P < 0.01$  versus HNP; \*\*\* $P < 0.001$  versus HNP; # $P < 0.05$  versus HP; ## $P < 0.01$  versus HP; ### $P < 0.001$  versus HP.

IFN- $\gamma$ + (NK) cells was lower in asthmatic than in HP subjects. Regarding CD56+ IL-17+ cells, their prevalence (within CD56+ cells) was lower in HP than in HNP samples, and this decrease was missing in asthmatic pregnancy (Fig. 3c). Similarly, CD56+ IL-17+ cell prevalence within the overall lymphocyte population showed a trend ( $P = 0.09$ ; Fig. 3f) toward a decrease in the HP compared with the HNP group. However, since CD56+ IL-17+ cell prevalence was higher in healthy pregnancy than in HNP controls (Fig. 3i), alterations observed in CD56+ IL-17+ cell prevalence might partially be the result of different CD56+ IL-17+ cell frequencies.

Apart from IL-17+ cells, the analysis of other investigated cell subtypes within the overall lymphocyte population did not alter the results compared with the values obtained among the respective lymphocyte subsets (i.e. CD4+, CD8+ and CD56+ lymphocytes) (Table 3).

#### Relationship of lymphocyte subsets and clinical outcomes

No correlation was observed between any of the lymphocyte subsets and ACT values, FeNO levels or neonatal birth weight in ANP, AP or HP patients.

#### Discussion

This study examined the prevalence of  $T_h1$ ,  $T_h2$ ,  $T_h17$  and Treg lymphocytes and that of IL-17-producing CD8 and NK cells in asthmatic and HP women. While the prevalence of  $T_h1$ ,  $T_h2$  and Treg cells has previously been investigated in our earlier studies (27, 28) in asthmatic and healthy pregnancy, the current findings are the first to provide data in a comprehensive approach on the  $T_h17$  subset, together with IL-17-producing CD8 and NK cell subsets.

Both allergic asthma and physiological pregnancy are traditionally considered as  $T_h2$ -polarized conditions. Indeed, in our results, both chemokine receptor cell surface marker expression and intracellular cytokine production indicate that the prevalence of  $T_h1$  lymphocytes is lower in healthy gestation and ANP patients compared with HNP controls, while pregnancy in well-controlled asthma does not further influence  $T_h1$  cell prevalence. In case of the  $T_h2$  subset, while cell surface marker measurements suggested similar prevalence, intracellular cytokine staining for IL-4 indicated that the prevalence of this T-cell subset is elevated in HP and asthma compared with HNP individuals. Again, no further increase was detected in pregnancy complicated with mostly well-controlled asthma, resulting in similar  $T_h1/T_h2$  ratio in AP and HP groups (Fig. 4a). This is not in line with our earlier published data on uncontrolled asthma exaggerating both  $T_h1$  and  $T_h2$  immune responses in pregnancy (27), when a significant amount of peripheral IFN- $\gamma$  and IL-4-producing T cells was detected in mostly not well-controlled AP women and a negative correlation was observed between IFN- $\gamma$  and IL-4-positive T cells and birth weight of newborns, which pointed at fetal growth retardation related to active asthma-associated maternal immune reactions. In that study, the  $T_h1/T_h2$  ratio was increased in asthmatic compared with healthy pregnancy (27) (Fig. 4b). The apparent contradiction between our current and previous data may be the result of different asthma control levels in

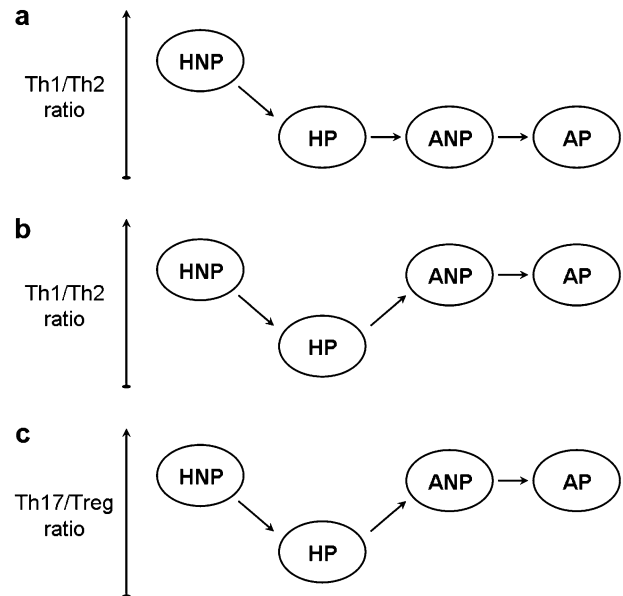


**Fig. 4.** Schematic presentation of the alterations of  $T_h1/T_h2$  ratio caused by mostly controlled (a) and mostly uncontrolled [b, (27)] asthma, and alteration of  $T_h17/Treg$  ratio (c) caused by mostly controlled asthma in HNP, HP, ANP and AP women.

the two examinations (our current study involved partially or well-controlled patients, while in the previous one, the majority of asthmatics were uncontrolled). The difference in the treatment guidelines in that time and nowadays together with different control levels of both pregnant and not pregnant asthmatic patients might all contribute to the differences between our previous and current data. However, the current findings and our other investigations (32) suggest that the immunosuppressive effect of uncomplicated pregnancy may attenuate lymphocyte activation and the inflammatory response characteristic for asthma in mostly well-controlled patients. Furthermore, the evaluation method used in this earlier study was different from the method of the current study, which might further contribute to the differences of data. In our previous study, total lymphocyte counts were given, while here, the cell prevalence was calculated within the CD4+ subset.

In this study, increased prevalence of  $T_h17$  cells was observed in asthma both when the non-pregnant and the pregnant groups were compared. Asthma-dependent  $T_h17$  elevation may play a role in the altered immunological tolerance in pregnancies of asthmatics, which might interfere with physiological intrauterine growth (this hypothesis, however, is not supported by the observed similar birth weights in AP and HP in our study). The other component responsible for the asthma-related impairment of immune tolerance characteristic for healthy pregnancy is the decrease in Treg prevalence in asthmatic compared with HP women. This is in line with our recent study finding lower prevalence of peripheral Treg cells in asthmatic compared with HP women in their second and third trimester (28). Therefore, the decreased  $T_h17/Treg$  ratio observed in healthy pregnancy is suspended in asthmatic gestation and is rather comparable to that in non-pregnant asthmatics (Fig. 4c).

Previous studies demonstrated the importance of peripheral  $T_h17/Treg$  ratio besides that of  $T_h1/T_h2$  ratio in HP and reported an elevation of  $T_h17/Treg$  ratio in pregnancies with adverse outcomes, such as preterm labor, spontaneous abortion or preeclampsia (14–16). Interestingly, we found no correlation between  $T_h17$  prevalence and clinical data of AP patients (such as birth weight of newborn, ACT scores, FEV<sub>1</sub> or PEF values). Similarly, our previous observations demonstrated a lack of correlation between Treg prevalence and neonatal birth weight in pregnancies of mostly well-controlled asthmatics (28). Therefore, in spite of the demonstrated  $T_h1/T_h2/T_h17/Treg$  imbalances in our present and previous studies (28), in order to determine the potential value of these immunological changes in the prediction of asthmatic pregnancy outcomes, further prospective longitudinal studies would be needed, enrolling both controlled and uncontrolled AP women.

As we found no correlation between  $T_h17$  or Treg prevalence and any clinical data of AP patients, the alteration of these subsets does not adequately explain the clinical fact that pregnancy causes deterioration, improvement or no change in asthmatic symptoms in 1 one-third of the cases, respectively (23).

Since CD8 and NK cells may also contribute to the regulation of the inflammatory response in asthmatic pregnancy and have also been shown to produce IL-17, we assessed the intracellular expression of this cytokine in these subsets as well. Our results indicated an apparent elevation in the prevalence of Tc17 and decrease in the prevalence of IL-17-expressing NK cells subsets in HP compared with HNP, and these changes were not detectable in the AP group. However, this apparent increase in Tc17 cell prevalence in HP compared with HNP controls proved to be the consequence of decreased CD8+ IL-17– cell frequency (that resulted in a higher proportion of Tc17 cells among the CD8+ subset). Although in AP the frequency of CD8+ IL-17– cells was increased when compared with HP, the absolute number of Tc17 cells remained the same resulting in a virtual decrease of Tc17 cells in the CD8+ subset of AP. Since Tc17 cells have been described to have lower cytotoxic activity in comparison with CD8+ IL-17– cells in non-pregnant status (33), the decrease in the latter subset and a relative increase in Tc17 prevalence might be regarded as part of the immunosuppressive mechanisms characterizing healthy but not asthmatic pregnancy. However, since no studies are known to determine the cytotoxicity of Tc17 cells in pregnancy, this explanation remains hypothetical.

Lower prevalence of IL-17-producing NK cells (among CD56+ cells) in HP women (compared with HNP) was confirmed by a similar trend when evaluated among the overall lymphocyte population and may be considered as a cellular sign of immune tolerance characterizing healthy pregnancy. Lack of this change in AP may be regarded as asthma-induced attenuation of the pregnancy-specific immune tolerance; however, data on the role of this cell subset in the pathogenesis of asthma or maintenance of pregnancy is scarce. Notably, observed differences in the prevalence of IL-17-producing NK cells might be influenced by increased CD56+ IL-17– cell frequency in healthy but not in asthmatic pregnancy (compared with HNP data).

Lower prevalence of IFN-gamma+ NK cells in asthmatic compared with HP subjects might also be a part of the immunological imbalance contributing to impaired pregnancy outcomes in asthmatic pregnancies since recent data establish the role of this cell subset in vascular development characterizing normal pregnancy (34).

In summary, the sensitive balance of  $T_h1$ ,  $T_h2$ ,  $T_h17$  and Treg cells that characterizes healthy pregnancy is altered by concomitant well or partially controlled asthma. Healthy pregnancy and asthma are both characterized by lower  $T_h1/T_h2$  cell ratios, but we observed no further decrease in asthmatic pregnancy. The prevalence of circulating  $T_h17$  cells is higher and that of Treg cells is lower in asthmatic than in HP women, resulting in a decreased  $T_h17/Treg$  ratio in healthy, but not in asthmatic pregnancy, compared with the HNP state. Theoretically, this may play a role in the altered immunological tolerance in pregnancies of asthmatics. Nevertheless, based on our current results, it is hard to establish the clinical importance and possible future diagnostic and therapeutic relevance of these alterations. Therefore, further studies are needed to determine the role of circulating  $T_h17$  cells in the pathogenesis of maternal and perinatal complications of asthma in pregnancy.

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