Increased Prevalence of IL-17-Producing Peripheral Blood Lymphocytes in Pre-eclampsia

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Problem
Systemic inflammation is a dominant component in the pathogenesis of pre-eclampsia. Besides the imbalance of Th1 and Th2 cells, alterations of the prevalence of Th17 and regulatory T cells have also been suggested to contribute to inflammation. We aimed to describe the prevalence of these four CD4 lymphocyte subtypes in pre-eclampsia and normal pregnancy, along with that of IL-17-producing CD8 and NK cells.

Method of study
Twenty pre-eclamptic and 22 normal pregnant women were enrolled in this study. Using flow cytometry, we determined the prevalence of IL-17-producing cells among the CD4, CD8 and NK cell subsets. Furthermore, we measured the prevalence of CD4+ Tregs, and Th1/Th2 cells were characterized using cell surface chemokine receptor markers.

Results
We demonstrated that there is a shift not only in the Th1/Th2 but also in the Th17/Treg balance favouring skewness towards a pro-inflammatory status in pre-eclampsia. The proportion of CD8 and NK cells that express IL-17 was also higher in pre-eclampsia.

Conclusion
The prevalence of IL-17-producing CD4, CD8 and NK cells is elevated in pre-eclampsia, indicating that both the innate and adaptive arms of the immune system are involved in the development of the exaggerated maternal systemic inflammation observed in this pregnancy-specific disorder.

Introduction

Pre-eclampsia (PE) affects at least 5% of pregnancies globally and is a leading cause of both maternal and perinatal morbidity and mortality, even in developed countries. This systemic syndrome is characterized by hypertension and proteinuria that develop in the second half of pregnancy in a previously normotensive woman. In most of the cases, these symptoms disappear after delivery.

Systemic inflammation is considered to be a dominant component in the pathogenesis of this pregnancy-specific disorder. Redman et al.[1] suggest that the clinical features of pre-eclampsia are best described as a cytokine-mediated excessive maternal inflammatory response. An important feature of systemic inflammation in PE is the absence of Th2 skewness characteristic for healthy pregnancy and thus the predominance of a Th1-type immunity and pro-inflammatory cytokines.

Saito et al.[2] reported on their observations regarding higher prevalence of IFN-γ and lower prevalence of IL-4-producing CD4 lymphocytes among peripheral blood mononuclear cells (PBMCs) of PE women compared to healthy pregnant women. Furthermore, the percentage of Th1 and Th2 cells and the Th1/Th2 ratio correlated with IFN-γ and IL-4 secretion levels. In another study, this group observed increased production of IL-2, IFN-γ and TNF-alpha by PBMCs in PE and, interestingly, a positive correlation between mean blood pressure and Th1 cytokines.[3] The shift to a predominant Th1-type immunity in PE is reinforced by other experiments on intracellular cytokine measurements in T cells and NK cells, as well as by assessment of cytokine secretion levels of PBMCs isolated from PE patients.[4–6]

The recent discovery of a distinct T-helper subset, referred to as Th17 cells, led to the transformation of the Th1/Th2 paradigm of immunity into a four-component paradigm. This novel viewpoint incorporates Th1, Th2, Th17 and regulatory T cells (Tregs) as elements of a complex and mutually interacting network.[7] Indeed, besides the imbalance of Th1 and Th2 cells, alterations of the prevalence of Th17 and Treg cells have also been suggested to be of importance in the development of systemic inflammation in PE.[8]

Th17 cells produce IL-17 and other pro-inflammatory cytokines. IL-17 has been proposed to have an important role in the development of autoimmune disorders (including rheumatoid arthritis and multiple sclerosis) and in the induction and maintenance of chronic inflammation.[9] Th17 cells, this recently identified subpopulation of CD4 lymphocytes, originate from a developmental lineage that is distinct from both Th1 and Th2 cells. A recent report demonstrated that the prevalence of Th17 cells is elevated in the peripheral blood of PE patients compared to healthy pregnant women in the third trimester of pregnancy.[10]

The effect of Th17 cells on the inflammatory balance is opposed by CD4+ regulatory T cells. While higher than normal number of Tregs is a risk factor for cancer[11] and chronic infection,[12] lower than normal Treg prevalence may contribute to autoimmune diseases[13] and exaggerated inflammation including that characteristic for PE. Indeed, a number of groups including ours demonstrated that the prevalence of peripheral Tregs is lower in PE compared to healthy pregnancy.[14–16] However, contradictory data suggesting no alteration with regard to the prevalence of Tregs in PE also exist.[17]

In addition to their distinct role in the regulation of the inflammatory status, Th1, Th2, Th17 and regulatory T cells mutually influence one another through the production of different cytokines. For instance, an increase in Th17/Treg cells ratio may contribute to the shift of T cells towards the Th1 direction because IL-17 induces the production of other pro-inflammatory cytokines,[18] while the inhibitory effect of Tregs on Th1 cells is decreased at the same time. Hence, the simultaneous study of the prevalence of these four CD4 lymphocyte subtypes may be of major interest in PE.

Therefore, in this study, we aimed to describe the prevalence of Th1, Th2, Th17 and Treg lymphocytes in PE and healthy third trimester pregnancy. 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.[19,20] Emerging evidence suggests that these IL-17-producing lymphocyte subsets, especially NK cells, largely contribute to the inflammatory status.[21] Thus, as an extension of our study, we also examined the prevalence of IL-17-producing CD8 and NK cells in PE for the first time in the literature.

Materials and methods

We took peripheral blood samples from 20 PE and 22 healthy pregnant women with a median of 36 weeks of gestation. Clinical characteristics of the study participants are shown in Table I. PE was defined by increased blood pressure (≥140 mmHg systolic or ≥90 mmHg diastolic on ≥2 occasions at least 6 hr apart) that occurred after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥0.3 g/24 hr or ≥1 + on dipstick in the absence of urinary tract infection). PE was regarded as severe if any of the following criteria was present: blood pressure≥160 mmHg systolic or ≥110 mmHg diastolic, or proteinuria≥5 g/24 hr (or ≥3 + on dipstick). Early onset of PE was defined as onset of the disease before 34 weeks of gestation. Foetal growth restriction (IUGR) was diagnosed if the foetal birth-weight was below the 10th percentile for gestational age and gender, based on Hungarian birthweight percentiles. Exclusion criteria were multifoetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal

Table I Clinical characteristics of normal pregnant and pre-eclamptic women

Characteristics	Normal pregnant women $n = 22$	Pre-eclamptic women $n = 20$
Age (years)	33.5 (30–36)	32.5 (27.5–34)
No. of smokers during pregnancy	0 (0%)	2 (10%)
No. of primiparous women	11 (50%)	13 (65%)
Systolic blood pressure (mmHg)	110 (106–114)	160* (149–180)
Diastolic blood pressure (mmHg)	68 (60–70)	100* (99–110)
Gestational age at blood collection (weeks)	36 (34–37)	36 (30–38)
Gestational age at delivery (weeks)	39 (38–40)	37* (31–39)
Foetal birthweight (g)	3200 (3020–3640)	2825* (1365–3450)
No. of IUGR	0 (0%)	3 (15%)
No. of early onset PE	–	8 (40%)
No. of severe PE	–	12 (60%)

*P values <0.05 were regarded significant.
Data are expressed as median (interquartile range) for continuous variables and as number (percentage) for categorical variables.
IUGR, intrauterine growth restriction; PE, pre-eclampsia.

or foetal infection and foetal congenital anomaly. Informed consent was obtained from all subjects, and our study was reviewed and approved by an independent ethical committee of the institution (IRB No. 188/2008). The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

PBMCs were separated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 27 min, $400 \times g$, 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 phosphate-buffered saline. Cells then were suspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA).

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 hr at 37°C in an atmosphere containing 5% CO_2. Then, cells were 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. They were then stained with PE-conjugated IL-17A mAb (eBioscience, San Diego, CA, USA) for 30 min at room temperature. After washing, cells were analysed on a BD FACSAria flow cytometer (BD Biosciences). Fifty thousand cells were recorded. The population of lymphocytes was gated from PBMCs according to forward scatter characteristics and side scatter characteristics. Isotype-matched PE-conjugated mouse IgG1 antibody was used as a control (eBioscience).

For the detection of regulatory T cells and cell surface chemokine receptors, PBMCs were 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). CXCR3 was used for the detection of the Th1 subset, while CCR4 was applied for the detection of Th2 cells. After washing, cells were fixed with fixation/permeabilization solution and treated with permeabilization buffer according to the manufacturer's instructions (eBioscience). They were then stained with PE-conjugated FoxP3 mAb (eBioscience) for 30 min at 4°C. After washing, cells were analysed on a BD FACSAria flow cytometer as described earlier. Isotype-matched PE-conjugated mouse IgG1 antibody was used as a control (eBioscience).

Data are expressed as median and quartiles. Comparisons between sample populations were made with Mann–Whitney *U*-test, as a test of normality (performed according to Kolmogorov–Smirnoff) indicated non-normal distribution of data. For

correlation analysis, the Spearman test was applied. Two-tailed *P*-values <0.05 were considered significant. Statistics were calculated using STATISTICA software (version 8.0; StatSoft, Inc., Tulsa, OK, USA).

Results

Our results are summarized in Table II and Fig. 1.

The prevalence of CXCR3+ CD4 cells was higher in PE than in normal pregnancy, whereas that of CCR4+ CD4 cells was not significantly altered. The ratio of CXCR3+/CCR4+ CD4 cells was also significantly higher in PE compared to controls. The proportion of Th17 cells was elevated, whereas that of CD4+ CD25+ FoxP3+ regulatory T cells was lower in PE compared to healthy pregnant women. As a result, the ratio of Th17/Treg cells was increased in PE compared to controls. Furthermore, the prevalence of IL-17+ cells was elevated in the CD8 and NK cell subsets in PE. Additionally, we examined the relationship of the prevalence of IL-17-producing cells to those expressing CXCR3 or CCR4 in the CD4 subset in PE and controls, but could not reveal a correlation.

In the group of pre-eclamptic patients, no statistically significant differences were observed in the proportion of IL-17-producing lymphocytes between patients with mild and severe pre-eclampsia [3.47 (2.41–4.40) % versus 4.15 (3.16–4.73) % for IL-17+ CD4 cells, 5.79 (5.07–6.77) % versus 7.45 (5.62–12.99) % for IL-17+ CD8 cells, 1.97 (1.21–2.35) % versus 1.59 (1.22–1.90) % for IL-17+ NK cells, respectively]. Similarly, no difference was detected in the prevalence of IL-17-producing lymphocytes between patients with late and early onset of the disease or between pre-eclamptic patients with and without foetal growth restriction (data not shown).

Discussion

In our study, we applied a comprehensive approach investigating the four major CD4 lymphocyte subsets (i.e. Th1, Th2, Th17 and Treg cells) to characterize immune polarization in PE compared to healthy pregnancy. We demonstrated that there is a shift not only in the Th1/Th2 but also in the Th17/Treg balance towards the pro-inflammatory direction in PE.

The lack of Th2 shift characteristic for physiological pregnancy and thus a predominance of Th1-type immunity have been documented in PE by several studies.[2–6] More recently, Santner-Nanan et al.[10] reported that the prevalence of Th17 cells is higher in PE compared to healthy pregnancy, which is in line with our present findings. This group also demonstrated that the prevalence of Th17 cells is elevated in the peripheral blood of non-pregnant subjects in comparison with healthy pregnant women, and similar to that observed in pre-eclamptic patients. Simultaneously with higher than normal Th17 numbers, we found that the prevalence of Tregs is lower in PE, resulting in an elevated Th17/Treg ratio. Lower than normal Treg prevalence may contribute to exaggerated inflammation and the loss of control over activated T cells.

Owing to the production of IL-17 and the induction of other pro-inflammatory cytokines, the effect

Table II Prevalence of the investigated lymphocyte subsets (in proportion to the parent populations) in normal pregnancy and pre-eclampsia

Subset	Normal pregnancy *n* = 22	Pre-eclampsia *n* = 20	*P*
CD4 cells			
CXCR3+ (%)	6.87 (5.60–8.81)	10.75 (7.49–15.90)	0.002
CCR4+ (%)	11.45 (10.33–16.25)	11.80 (9.66–14.85)	0.420
CXCR3+/CCR4+	0.51 (0.40–0.64)	1.00 (0.56–1.44)	<0.001
IL-17A+ (%)	2.81 (2.34–3.17)	3.84 (2.99–4.73)	0.004
CD25+ FoxP3+ (%)	4.66 (4.17–5.72)	3.07 (2.62–3.45)	0.002
IL-17A+/CD25+ FoxP3+	0.55 (0.50–0.70)	1.40 (0.87–2.40)	<0.001
CD8 cells			
IL-17A+ (%)	5.04 (3.78–6.11)	6.10 (5.18–8.95)	0.019
NK cells			
IL-17A+ (%)	0.96 (0.63–1.17)	1.74 (1.21–2.01)	<0.001

Data are expressed as median (interquartile range). *P* values <0.05 were regarded significant.

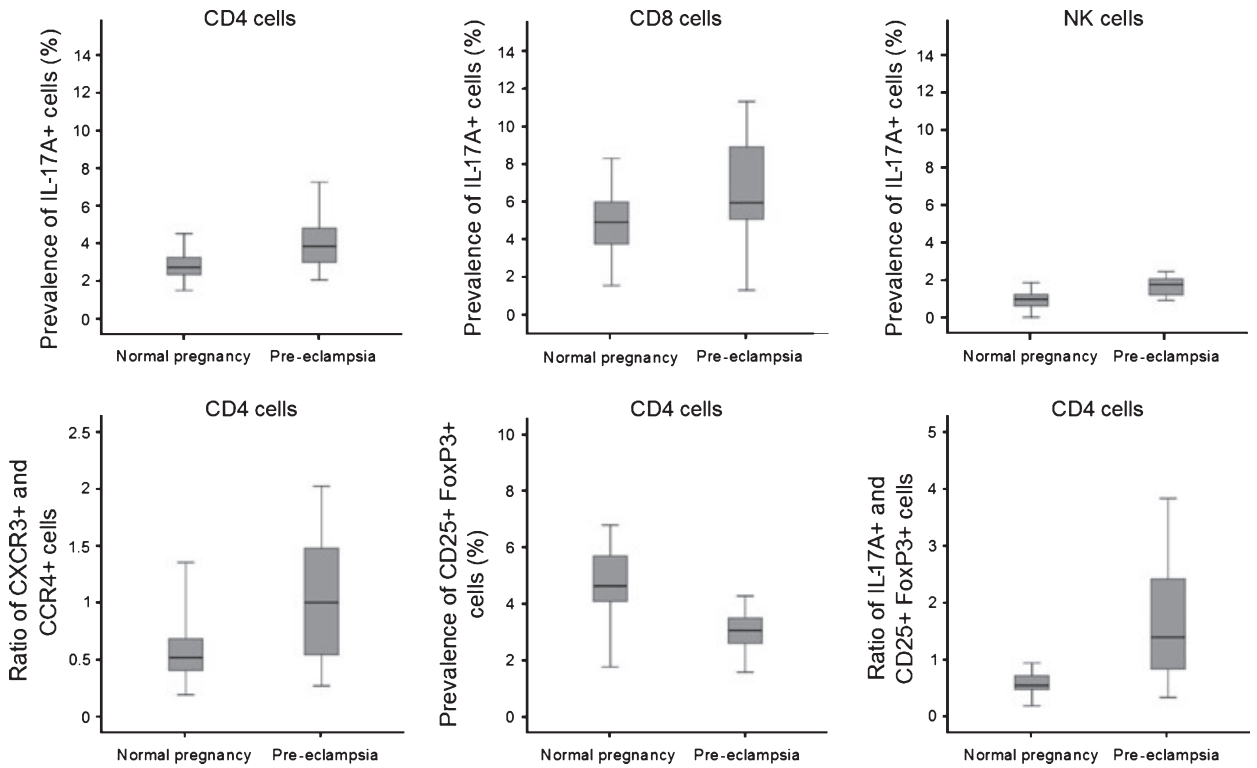

Fig. 1 Boxplots representing the prevalence of IL-17A-producing lymphocyte subsets, the ratio of CXCR3+ and CCR4+ CD4 cells, the prevalence of CD4+ regulatory T cells (CD25+ FoxP3+) and the ratio of CD4+ IL-17A+ to CD4+ Treg cells in normal pregnancy and pre-eclampsia. Horizontal line: median, box: interquartile range, whisker: range. $P < 0.05$ for all comparisons.

of Th17 cells on the inflammatory balance is opposing to that of the immunosuppressive Tregs. Interestingly, Th17 cells and Tregs originate from the same developmental lineage that is distinct from Th1 and Th2 cells. An exclusive dichotomy was observed in their generation: either Treg or Th17 cells develop from the ancestor cells depending on whether they are activated in the presence of TGF-beta or TGF-beta plus inflammatory cytokines.[9] Thus, the changes in TGF-beta signalling might partly be responsible for the increased Th17/Treg ratio in PE. Furthermore, it is also possible that the lower prevalence of Tregs in PE is because of the phenomenon that a portion of this subset has differentiated into Th17 cells. This theory might also explain the described increase in the prevalence of Th17 cells in PE.[22]

Besides the findings in PE, the alterations of Th17 cells have also been observed in other pregnancy-related disorders, suggesting that the balance of Th17 cells and Tregs and not only that of Th1 and Th2 cells has crucial effects on the inflammatory status in human pregnancy. Ito et al.[23] recently demonstrated the importance of IL-17-producing cells in the pathomechanism of pre-term labour. Nakashima et al.[24] found that the prevalence of decidual IL-17-producing cells is significantly higher in inevitable abortion cases (but not in missed abortion) than in normal pregnancy, indicating that these cells might be involved in the induction of inflammation in the late stage of abortion, but not in the early stage.

In another study, Wang et al.[25] showed that the prevalence of Th17 cells in the peripheral blood and decidua is increased in unexplained recurrent spontaneous abortion patients. They observed that the expression of a Th17-associated factor, RAR-related orphan receptor gamma (ROR-γ or RORc), is also higher in peripheral blood lymphocytes and decidua of these patients. This factor is required for the differentiation of Th17 cells and probably acts through the down-regulation of the expression of Fas ligand and IL-2.[26] In a recent study, Jianjun et al.[27] found that the mRNA level of this factor in peripheral blood mononuclear cells (and decidua) of PE

patients is elevated when compared with healthy pregnant women. Therefore, the increased expression of this transcription factor may partly be responsible for the increased prevalence of Th17 cells in peripheral blood of PE patients.

Apart from CD4 lymphocytes, CD8 and NK cells were also demonstrated to produce IL-17.[19,20] Our results show – for the first time in the literature – that in addition to CD4 cells, the prevalence of CD8 and NK cells that express IL-17 is also higher in PE. IL-17 production by these lymphocyte subsets might contribute to the development of a systemic pro-inflammatory environment.

Sargent et al.[21] proposed that the innate rather than the adaptive immune system controls immune regulation during human pregnancy and that NK cells are of central importance to this process. The aberrant activation of NK cells both systematically and locally in the placenta is of major significance in the pathogenesis of PE. We found that the prevalence of IL-17-expressing NK cells is elevated in PE compared to healthy pregnancy, which might play a role in the aberrant activation of NK cells in this disorder.

In our measurements, we characterized the prevalence of Th1 and Th2 cells in PE and healthy pregnancy with cell surface chemokine receptor markers. CXCR3 was used for the detection of the Th1 subset, while CCR4 was applied for the detection of Th2 cells. We demonstrated a shift towards the Th1 direction in PE based on these markers.

The altered ratio of Th17/Treg cells may contribute to the shift of T lymphocytes towards the Th1 direction in PE, as IL-17 induces the production of other pro-inflammatory cytokines, such as IFN-γ.[18] However, we could not detect a correlation between the prevalence of IL-17-producing and CXCR3+ cells in the CD4 subset. Thus, the effect of Th17 cells and IL-17 on the inflammatory status is more likely to be exerted in a direct manner rather than through the modulation of the Th1/Th2 balance in PE.

Our results suggest the importance of the pro-inflammatory cytokine, IL-17, and the significance of the altered ratio of Th17/Treg cells in the pathogenesis of PE. This effect is probably exerted in a direct manner rather than through the modulation of the Th1/Th2 balance. Based on our results, it is difficult to establish whether this finding is rather a cause or a consequence in the development of PE. However, given the complexity of the aetiology, this characteristic alteration might be an important hallmark and a contributing factor of immune dysfunction in PE. Nevertheless, a limitation of our study is its small sample size. To determine the biological significance of the observed differences in the pathogenesis of PE, further studies are needed involving measurement of circulating IL-17 levels.

Conclusion

In conclusion, the prevalence of IL-17-producing circulating T (helper and cytotoxic) and NK cells is increased in pre-eclampsia, indicating that both the innate and adaptive arms of the immune system are involved in the development of the exaggerated maternal systemic inflammation observed in this pregnancy-specific disorder.

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