Cytokine production by monocytes, NK cells and lymphocytes is different in preeclamptic patients as compared with normal pregnant women

Abstract

Objective: To measure cytokine production in ex vivo stimulated leukocyte populations of women with normal pregnancy and those with preeclampsia. Methods: Whole blood from preeclamptic and normal pregnant women was stimulated with LPS or PMA/Ca-ionophore. The percentages of IFNγ- and IL2-, 4- and 10-producing lymphocytes and NK cells and the percentages of TNFα-, IL1β- and IL12-producing monocytes were measured by flowcytometry. Results: In women with preeclampsia, there was a significantly increased percentage IL4-producing cytotoxic T cells. Also, a significant decreased percentage IL2-producing T helper cells and IL12-producing monocytes was seen as compared with normal pregnancy. Conclusion: Th1 cytokine production of lymphocytes and monocytes appears to be decreased in our group of preeclamptic patients compared with normal pregnant women.
Introduction

In normal pregnancy, the immune system plays a crucial role in maintaining a healthy equilibrium between the mother and the foetus. Macrophages, monocytes, and especially natural killer (NK) cells in the decidua play an important role in implantation and trophoblast invasion (Loke and King 2000). Also peripheral immune responses seem to play a role in the maintenance of pregnancy; it has been shown that during pregnancy the Th1/Th2 ratio of cytokine production of peripheral T lymphocytes is decreased (Reinhard et al. 1998; Saito et al. 1999a; Veenstra van Nieuwenhoven et al. 2002). This shift in cytokine production of lymphocytes is associated with a shift from a cellular immune response to a humoral immune response. Also, the decreased production of interferon-gamma (IFNγ) by NK cells in pregnant women is in line with the decreased Th1/Th2 ratio during pregnancy (Veenstra van Nieuwenhoven et al. 2002).

Not only the specific immune response, but also the non-specific immune system, with important cells like monocytes and granulocytes, changes during pregnancy. Monocytes and granulocytes show an activated phenotype in normal pregnancy as compared with these cells from non-pregnant women (Sacks et al. 1998; Naccasha et al. 2001; Luppi et al. 2002). We, and others, have previously shown that also monocyte function is altered during pregnancy: monocyte cytokine production changes during pregnancy (Sakai et al. 2002; Veenstra van Nieuwenhoven et al. 2003; Sacks et al. 2003). The changes in monocyte cytokine production are in line with the notion that monocytes are activated during pregnancy (Veenstra van Nieuwenhoven et al. 2003).

Preeclampsia is a common obstetric syndrome affecting about 3% to 4% of the pregnant women. The signs of this syndrome usually occur after the 20th week of pregnancy and most frequently near term. The syndrome is characterized by hypertension, proteinuria, oedema, and at times, coagulation abnormalities and liver dysfunction (Cunningham et al. 1997). Endothelial dysfunction is thought to play a major role in the pathogenesis of preeclampsia and hypertension and proteinuria may be regarded as secondary signs. The primary pathology, although unknown, is most likely localized within the placenta because resolution almost always occurs after delivery; however, the condition may sometimes even occur shortly after delivery. The events leading to preeclampsia are not completely known, but it has been suggested that abnormal activation of the immune system may play a role in the etiology of this syndrome (Faas et al. 2001; Sacks et al. 2001). Indeed, it has been shown that lymphocyte cytokine production is different in preeclamptic pregnancies as compared with normal pregnancies. The ratio of Th1/Th2 cytokine production is found to be increased among women with preeclampsia as compared with normal pregnant women (Saito et al. 1999b). Like in normal pregnancy, cells of the non-specific immune system are also phenotypically activated in preeclampsia (Sacks et al. 1998). It appeared, however, that the cells of the non-specific immune response are more activated in preeclampsia as compared with normal pregnancy, as can be seen by expression of surface markers (Holthe et al. 2004). Consistent with the increased phenotypical activation of monocytes during preeclamptic pregnancy, it has been shown that monocyte IL12 production is increased in preeclampsia as compared with normal pregnancy (Sakai et al. 2002). However, there are no other data to suggest that monocyte function is altered during preeclampsia.
Although a few studies suggest that the Th1/Th2 ratio is increased in preeclampsia versus normal pregnancy, very little is known about cytokine production of monocytes and NK cells in preeclamptic patients. Therefore, in the present study we evaluated the stimulated cytokine production of monocytes and NK cells as well as the stimulated cytokine production of lymphocytes. We hypothesize that, during preeclampsia, not only lymphocyte cytokine production, but also monocyte and NK cell cytokine production is shifted towards type 1 cytokine production. Whole blood from women with preeclampsia and from women with normal healthy pregnancy with foetus of the same gestational age was incubated with LPS (to stimulate monocytes) or PMA and Ca-ionophore (to stimulate lymphocytes and NK cells). We measured intracellular monocyte TNF$\alpha$ and IL1$\beta$, since these are the most important cytokines, which are produced upon monocyte activation (Van der Poll et al. 1992). Both cytokines are mainly produced by monocyte-macrophages, and are the principal mediators of the inflammatory response and tissue destruction in many immuno-inflammatory diseases (Dayer and Burger 1994).

We also measured monocyte IL12, which is a well-known inducer of Th1 cytokines by lymphocytes and forms a link between innate and adaptive immunity (Trinchieri 2003). In lymphocytes and NK cells we measured intracellular IL2, IFN$\gamma$, (well-known type 1 cytokines) and IL4, IL10 (both well-known type 2 cytokines).

**Material and methods**

**List of reagents for cell activation and cell staining:**
The following reagents were used: Monensin (Sigma); FACS$^\text{tm}$ lysing solution (Becton Dickinson); FACS$^\text{tm}$ permeabilizing solution (Becton Dickinson); Complete Roswell Park Memorial Institute (RPMI) 1640 (GIBCO) medium supplemented with 60$\mu$g/ml Gentamycin; Washing buffer (phosphate buffered saline with 0.5% Bovine Serum Albumin and 0.1% NaN$_3$); Fixation buffer (0.5% paraformaldehyde in phosphate buffered saline); phorbol myristate acetate (PMA; Sigma); Calcium ionophore A-23187 (Sigma); Endotoxin (LPS) (2$\mu$g/mL; 055.B5 E.coli, BioWhittaker, Walkersville, MD, USA).

**Subjects:**
Preeclampsia was defined by hypertension associated with proteinuria according to the definition of the ISSHP (Brown et al. 2001). Hypertension was defined as a blood pressure of more than 140 mmHg (systolic) or at least 90 mmHg (diastolic) on two or more occasions and at least 4 to 6 hours apart in women known to be normotensive beforehand. Proteinuria is defined as excretion of 300 mg or more protein, using urinary dipsticks (Brown et al. 2001). Proteinuria was found in two or more samples taken at least 4 to 6 hours apart.

Nulliparous women who were diagnosed as preeclamptic were recruited from our outpatient clinic. They were all admitted to the hospital for at least 4 days. Clinical signs of preeclampsia did not worsen during hospitalization and the patients did not progress to severe preeclampsia and did not need any medication, such as anti-hypertensive treatment.

These patients (n = 15; age range: 18-35 years) were matched for gestational age with normal pregnant women (n = 19; age range: 18-35 years). The normal pregnant women were recruited from the outpatient clinic. These women were regularly checked by their midwives and their pregnancy remained normal. Patient
characteristics are shown in Table 1. All women (normotensive and preeclamptic) were nulliparous and delivered at term.

After obtaining institutional approval of the local ethics committee, and after signing informed consent, blood samples were taken from all women. For both groups, exclusion criteria were evidence of treatment with antibiotics or fever as well as the presence of any known diseases.

Blood samples (20 mL) were obtained in 2 vacutainer tubes, one of which contained sodium heparin, and which was used to evaluate intracellular cytokine production; the other contained EDTA and was used for white blood cell (WBC) counts (using a microcell counter: Model Sysmex F800, TOA Medical Electronics CO, Ltd, Kobe, Japan).

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Systolic tension (median in mmHg (range))</th>
<th>Diastolic tension (median in mmHg (range))</th>
<th>Proteinuria (mean in g/L)</th>
<th>Maternal age (median in years (range))</th>
<th>Ammenorhoe (median in weeks (range))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pregnancy (n = 19)</td>
<td>115 (90-135 mmHg)</td>
<td>75 (55-85 mmHg)</td>
<td>&lt; 0.3 g/L</td>
<td>27.1 (18-35)</td>
<td>37.2 (33-40)</td>
</tr>
<tr>
<td>Preeclampsia (n = 15)</td>
<td>140 (130-160 mmHg)</td>
<td>95 (90-100 mmHg)</td>
<td>(0.3-0.6 g/L)</td>
<td>26.8 (18-35)</td>
<td>36.1 (33-41)</td>
</tr>
</tbody>
</table>

Table 1. Patient characteristics

Sample Processing:
Antibodies:
The following monoclonal antibodies were used, and unless stated otherwise, all purchased from IQ Products Groningen, The Netherlands: Cy-Q labeled mouse anti-human CD3 (clone B-B11); Fluorescein isothiocyanate (FITC) labeled mouse anti-human CD8 (clone MCD8); FITC-labeled mouse anti-human CD14 (clone UCHM1); Phycoerythrin (PE) labeled mouse anti-human IFNγ (clone 45-15), PE-labeled mouse anti-human IL2 (clone N7-48A), PE-labeled mouse anti-human IL4 (clone 8F-12), PE-labeled mouse anti-human IL10 (clone B-N10), PE-labeled mouse isotype control IgG1 (clone MCG1); FITC-labeled mouse anti-human CD94 (clone HP-3B1; Coulter Immunotech, Hamburg, Germany); Phycoerythrin (PE) labeled mouse anti-human TNFα (clone Mab11; Pharmingen), PE-labeled mouse anti-human IL12 (clone C11.5; Pharmingen), PE-labeled mouse anti-human IL1β (clone AS10; Becton Dickinson).

Incubation:
Immediately following sampling, 3 mL heparinized whole blood was mixed with 3 mL of RPMI and stimulated with PMA (40 nM) and calcium ionophore (2 nM) in a sterile polystyrene tube. Another 3 mL heparinized whole blood was mixed with 3 mL of RPMI and stimulated with 2 µg/mL LPS in a sterile polystyrene tube. Three millilitre of heparinized whole blood was used as unstimulated control and only mixed with 3 mL of RPMI in a sterile polystyrene tube. Samples were incubated for 4 hours.
at 37°C and 5% CO₂. In both the stimulated and unstimulated samples, monensin (3μM) was added to enable accumulation of the cytokines in the golgicompex by interrupting intracellular processes (Jung et al. 1993).

Sample Labeling:

Monocytes:
After stimulation, the samples were aliquoted into polystyrene tubes (0.2 mL per tube). Samples were labeled according to methods previously described (Veenstra van Nieuwenhoven et al. 2003). In brief: after incubation with anti-CD14, the red blood cells were lysed and white blood cells were permeabilized. Then samples were incubated with anti-TNFα or anti-IL1β or anti-IL12 or isotype control IgG1 at saturating dilutions. Cells were fixed with ice-cold fixation buffer and kept at 4 °C in the dark until measured (within 24 hours).

Lymphocytes and NK cells:
After stimulation, simulated and unstimulated samples were aliquoted into polystyrene tubes (0.2 mL per tube). Samples were labeled according to methods previously described (Veenstra van Nieuwenhoven et al. 2002). In brief: the red blood cells were lysed and the white blood cells were permeabilized. Stimulated and unstimulated aliquots were incubated with anti-CD3, anti-CD8 (cytotoxic T lymphocytes) and either anti-IFNγ, anti-IL2, anti-IL4, anti-IL10 or isotype control IgG1. Or aliquots were incubated with anti-CD3, anti-CD94 and either anti-IFNγ, anti-IL2, anti-IL4, anti-IL10 or isotype control IgG1 at saturating dilutions. The expression of the CD94 molecule is restricted to NK-lymphocytes and to a small T-lymphocyte subpopulation (Sivori et al. 1996). Since it has been shown that almost all peripheral NK cells express CD94 (Jacobs et al. 2001; Jacobs et al. 2004), we were able to study NK-lymphocytes by selecting CD94+/CD3- cells. Cells were fixed with ice-cold fixation buffer and kept at 4 °C in the dark until measured (within 24 hours).

Flow Cytometry:
Cells were analyzed using the Coulter Epics Elite flow cytometer (Argon-ion 488 nm laser). To minimize day-to-day variation of the measurements, the PE fluorescence (i.e. the label for the cytokine antibodies) was calibrated before each experiment using Standard Brite Beads (Coulter, Luton, UK). For each individual, two thousand lymphocytes were acquired by live gating on lymphocytes using forward and sidescatter characteristics and CD3+ cell signal. Data were saved for later analysis. Also for each individual, two thousand monocytes were acquired by live gating on monocytes using CD14+ cell signal and saved for later analysis. In the samples for NK cells, thousand NK cells were acquired by gating lymphocytes using forward and sideward scatter and CD3+/CD94+ cell signal. Analysis was performed using Winlist 3.0, 32-bit (Verity Software House, Topsham, ME).

Intracellular Cytokines:
During analysis of lymphocytes and NK cells, a gate was set on lymphocytes using forward and sidescatter characteristics. Secondary gates were set on CD3+/CD8- (Th cells) and CD3+/CD8+ (Tc cells) or CD3+/CD94+ (NK cells) cells. For monocytes a gate was set on the CD14+ signal (see figure 1). For monocytes, Th, Tc and NK cells, single parameter fluorescence histograms were defined for evaluation of intracellular cytokine production. During analysis of all samples, it was found that the isotype control completely overlapped the unstimulated
sample, indicating that cells in the unstimulated sample did not produce cytokines. Using the unstimulated control sample, linear gates were set in the histograms so that at least 99% of the unstimulated cells were negative for cytokine production. This gate was copied to the histogram for stimulated cells. Results are expressed as percentage positive cells in the stimulated blood sample and mean channel brightness of the positive cells, as a measure of the total amount of cytokines produced.

Figure 1
A: After whole blood-stimulation with PMA and CA-ionophore, lymphocytes (selected in a gate) can be demonstrated separately in a forward scatter (FSC) and sideward scatter (SSC) dot plot. This gate was copied to a CD8-FITC/CD3-Cy-Q dotplot (fig. 1B).
B: To analyze cytokine production (for instance IFNγ) by for example Th lymphocytes, Th lymphocytes (CD8/CD3+ cells) were selected in a CD8-FITC/CD3Cy-Q dot plot. 
C: These selected cells were then copied to a single-parameter fluorescence histogram, which was used to evaluate the number of IFNγ- (PE) positive cells in this lymphocyte region. Using the unstimulated control sample (black area), linear gates were set in the histogram (M1) so that at least 99% of the unstimulated cells were negative for IFNγ production. The percentage positive cells were evaluated from the histogram of the stimulated cells (grey line); results are expressed as percentage of positive cells or mean intensity of staining in the stimulated blood sample.

Differential Blood Cell Counts:
Using forward and sidescatter characteristics, a gate was set on the total leukocyte population. Using sidescatter characteristics as well as the CD14 antibody (see figure 2), we defined lymphocytes (low side scatter, CD14 negative), monocytes (strong CD14+ cells) and granulocytes (low CD14+ cells with a high sidescatter) in an unstimulated aliquot of each sample. The percentages of lymphocytes, monocytes and granulocytes of the total leukocyte population were evaluated. Within the lymphocyte population also the percentage of CD3+, CD3+/CD8+ and CD3+/CD8- and CD3+/CD94+ (NK cells) cells were evaluated. The absolute number of cells within of the various cell populations was calculated by multiplying the percentages with total number of white blood cells.
Figure 2
A: In an unstimulated sample, in a FCS and SSC plot, the total leukocyte population was gated.
B: To evaluate the percentage of monocytes, granulocytes and lymphocytes, the gated cells (leukocytes) were copied to a CD14-FITC/SSC dot plot, and monocytes (CD14<sup>bright</sup>), granulocytes (CD14<sup>dim</sup>/high SSC) and lymphocytes (CD14/<sub>low</sub> SSC) were gated. The total number of cells within each population was assessed by multiplying the percentage by the total number of white blood cells.

Statistics:
Results in the tables are expressed as medians. Results in the figures are expressed as individual points. In these figures medians are indicated by the horizontal lines in each figure.
To evaluate differences between preeclampsia and normal pregnancy, Mann-Whitney U test was used for the total white blood cell counts, for differential cell counts, and for the percentage of cytokine-producing cells. A difference of P < 0.05 was considered statistically significant. To evaluate the relation between cytokine production and mean blood pressure we used a linear regression analysis (least sum of squares method). R<sup>2</sup> and the slope were calculated and it was tested whether the slope was significantly different from zero.
**Results**

*Cell Counts:*
Table 2 shows the total WBC counts and differential cell counts in normal pregnant women and in women with preeclampsia. Results are expressed as median total number of cells and median percentage from white blood cell population (top panel) or median percentage from lymphocyte population (lower panel). The total WBC counts did not differ between preeclamptic and normal pregnant women. The differential counts for granulocytes, monocytes and lymphocytes also did not differ between the groups. Within the lymphocyte population, only the percentage of NK cells was significantly decreased in preeclamptic women as compared with normal pregnant women (medians 7.19% compared with 4.19%, $P = 0.033$).

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy</th>
<th>Preeclamptic pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total number of cells (*10⁹ cells/L)</td>
<td>percentage of white blood cell population (Median (range))</td>
</tr>
<tr>
<td>White blood cells</td>
<td>8.2 (6.6-12.8)</td>
<td>9.3 (5.2-21.1)</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>6.57 (5.8-10.5)</td>
<td>3.04 (1.73-5.65)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.25 (0.04-0.51)</td>
<td>0.30 (0.06-0.47)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.31 (0.44-2.1)</td>
<td>15.52 (6.22-18.65)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy</th>
<th>Preeclamptic pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total number of cells (*10⁹ cells/L)</td>
<td>percentage of lymphocyte population (Median (range))</td>
</tr>
<tr>
<td>T helper cells</td>
<td>0.56 (0.21-1.0)</td>
<td>43.13 (35.97-49.04)</td>
</tr>
<tr>
<td>T cytotoxic cells</td>
<td>0.34 (0.12-0.48)</td>
<td>26.78 (18.44-35.15)</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.09 (0.02-0.2)</td>
<td>7.19 (3.15-9.47)</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>1.01 (0.38-1.61)</td>
<td>69.00 (61.00-79.00)</td>
</tr>
<tr>
<td>T helper/T cytotoxic ratio</td>
<td>1.75 (1.10-2.85)</td>
<td>1.64 (1.14-4.34)</td>
</tr>
</tbody>
</table>

Median total white blood cell counts, median total and median percentage differential cell counts from white blood cells (upper panel) and median total number and percentages of lymphocytes subpopulations (lower panel) in normal pregnancy (left columns) compared with preeclamptic pregnancy (right columns). * significantly different from normal pregnant women (Mann-Whitney U test $P < 0.05$).

**Monocyte cytokine production in preeclamptic women compared with normal pregnant women**
Figure 3 shows the percentage of TNFα-, IL1β- and IL12-producing monocytes for each individual. The percentage IL12-producing monocytes in preeclamptic women was significantly decreased as compared with the percentage IL12-producing monocytes in normal pregnant women (median 2.40% compared with 5.50%, $P = 0.027$). The percentage of IL1β-producing monocytes and the percentage TNFα-
producing monocytes did not differ between normal pregnant women and preeclamptic women. The mean channel brightness (figure 4), i.e. the total cytokine production, of TNFα positive cells was significantly decreased in preeclamptic women versus normal pregnant women (median 551.75 (165.59-1807.87) versus 345.87 (40.74-1263.99), P = 0.036). No difference in the mean channel brightness for IL12 and IL1β between preeclamptic and normal pregnant women was found.

Figure 3: Percentage of cytokine-producing monocytes in normal pregnant women compared with preeclamptic women. Each symbol represents an individual. * = significantly different from normal pregnant women (Mann-Whitney U test P < 0.05)
PE = preeclamptic pregnancy, NP = normal pregnancy, dash = median

Figure 4: The intensity of staining of the cytokine-producing monocytes. Each symbol represents an individual. * = significantly different from normal pregnant women (Mann-Whitney U test P < 0.05), PE = preeclamptic pregnancy, NP = normal pregnancy, dash = median

Percentage cytokine-producing lymphocytes and NK cells in preeclamptic women compared with normal pregnant women:
Figure 5A-D shows the percentage IFNγ- (fig. 5A), IL2- (fig. 5B), IL4- (fig. 5C) and IL10- (fig. 5D) producing NK cells and lymphocytes for each individual. No significant differences were seen in percentage IFNγ-producing Th cells in normal pregnant women as compared with preeclamptic women (fig. 5A). Also the percentage of IFNγ-producing Tc cells was similar between normal pregnant women and women with preeclampsia (fig. 5A). Comparing normal pregnant women with preeclamptic women no significant differences were seen in the percentage IFNγ-producing NK cells (fig. 5A).
In Th cells of preeclamptic women a significant decrease was seen in the percentage IL2-producing cells as compared with normal pregnant women (18.26 % compared with 40.86%, P = 0.01) (fig. 5B). The percentage of IL2-producing Tc cells and NK cells were similar between normal pregnant women and preeclamptic women (fig. 5B).

The percentage IL4-producing Th lymphocytes showed no significant differences between normal pregnant women and preeclamptic women (fig. 5C). However, in preeclamptic women there is a significant increase in the percentage IL4-producing Tc cells as compared with normal pregnant women (1.88 % compared with 1.00%, P = 0.01), while the percentage NK cells producing IL4 was not different between preeclamptic and normal pregnant patients (fig. 5C).

The percentage of IL10-producing Tc, Th and NK cells did not differ between normal pregnant women and preeclamptic women (fig. 5D).

The mean channel brightness of the positive cells, i.e. the total cytokine production, for IFN\(_\gamma\), IL2, IL4 or IL10 did not differ between normal healthy pregnant women and preeclamptic women (results not shown).

**Figure 5:** Percentage of IFN\(_\gamma\)- (A), IL2- (B), IL4- (C) and IL10- (D) producing Th cells, Tc cells and NK cells in preeclamptic pregnancy (PE) compared with normal pregnancy (NP). Each symbol represents an individual. * = significantly different from normal pregnant women (Mann-Whitney U test P < 0.05)

NP = normal pregnancy, PE = preeclamptic pregnancy, Th = T helper lymphocytes, Tc = T cytotoxic lymphocytes, NK = NK cells, dash = median
Relation between percentage cytokine-producing cells (lymphocytes, NK cells and monocytes) in preeclamptic women and mean blood pressure in preeclamptic women:

In the present group of preeclamptic women, we found no relation between mean blood pressure and stimulated cytokine production of either monocytes or lymphocytes (results not shown).

Discussion

Results of the present study demonstrate that, in our group of preeclamptic patients, the cytokine production by monocytes and lymphocytes differs from the cytokine production in normal healthy pregnant women. The WBC counts did not differ between normal pregnant and preeclamptic women. Most percentages of cells and differential cell counts did not differ between the groups. Only the percentage of NK cells of the lymphocyte population was significantly decreased in preeclamptic patients as compared with normal pregnant controls. This is in contrast to other studies in which increased numbers of NK cells were found in preeclamptic patients (Matthiesen et al. 1999; Borzychowski et al. 2005). The differences between our study and the other studies may be the severity of preeclampsia. Our group of preeclamptic patients had mild preeclampsia, while in the other papers, preeclampsia was much more severe (see also below).

Monocytes play a key role in immune responses by processing antigens and determining the nature of lymphocyte response by producing cytokines. In normal pregnancy and in preeclampsia, monocytes are phenotypically activated (Sacks et al. 1998). We studied the function of monocytes in preeclampsia versus normal pregnancy, by in vitro stimulation of whole blood with LPS. We observed a significant decrease in percentage IL12-producing monocytes in preeclampsia. Although no difference in percentage TNFα-producing cells was found between preeclamptic and normal pregnant women, the mean channel brightness for TNFα was significantly decreased in preeclamptic women versus normal pregnant women, indicating that the amount of TNFα produced per cell was decreased in preeclamptic women. No difference in IL1β production by monocytes or mean channel brightness was found between preeclamptic patients and normal healthy pregnant women. We thus show a different cytokine expression pattern between monocytes from normal healthy pregnant women and preeclamptic women. These data (decreased monocyte IL12 and TNFα production), however, are not consistent with our hypothesis of a shift towards Th1 cytokine production during preeclampsia. One of the most important functions of IL12 is to induce the production of IFNγ from lymphocytes and NK cells and IL12 can therefore also be considered as a Th1 cytokine. At first sight the decreased production of IL12 in preeclamptic monocytes appears to be a sign of less activated monocytes rather than an increased activation of monocytes. However, decreased monocyte IL12 production can also be a sign of LPS tolerance. LPS tolerance is a phenomenon observed in activated monocytes and characterized by decreased cytokine production in response to LPS-stimulation (Ertel et al. 1995; Van der Poll et al. 1996; Veenstra van Nieuwenhoven et al. 2003).

Interestingly, also in the rat model for preeclampsia (Faas et al. 1994), which we developed in our lab, we have shown that monocytes of these preeclamptic rats become LPS tolerant (Faas et al. 2004). Also, in more severe forms of preeclampsia, LPS
tolerance was observed (Beckmann et al. 2004; Luppi et al. 2006). Although these two studies did not evaluate endotoxin-induced monocyte IL12, Luppi and DeLoia (2006) found endotoxin-induced IL1β to be decreased in their preeclamptic patients compared with normal pregnant women. Endotoxin-induced TNFα was not different between normal and preeclamptic women. In this study, they had a mixed population of patients, some had early onset preeclampsia (before 34 weeks and diastolic blood pressure above 110 mmHg), some had milder disease, as reflected by lower blood pressure and later onset of disease. In total, their group had more severe disease than our group of patients. Beckman and colleagues studied women with very severe early onset preeclamptic women (mean diastolic pressure 180 mmHg) and proteinuria (mean 4g/24 hours), as well as decreased birthweight (including patients with HELLP syndrome), and found endotoxin-induced TNFa to be decreased in preeclamptic patients compared with normal pregnant women (Beckmann et al. 2004). Unfortunately, they did not measure either IL1β or IL12. The difference between our study and the two other studies may be the severity of preeclampsia. It turned out that our group of patients showed a relatively mild preeclampsia as compared to the other studies. Our group of patients all showed a mild disease, characterized by diastolic pressure between 90 and 110 mmHg and proteinuria from 0.3 to 0.6g/L, and none of the patients had elevated liver enzymes or decreased platelets. Mild disease was also shown by the fact that birth weights were all in the normal range and that all women delivered at term. It may be speculated that LPS tolerance is a feature of preeclampsia, but that the tolerance presents itself in various ways, depending on the severity of the disease.

In lymphocytes from preeclamptic women, which were stimulated with PMA and Ca-ionophore, we observed a slight, but significant increase in percentage IL4-producing Tc cells. We also found a significant decrease in percentage IL2-producing Th cells as compared with lymphocytes from normal pregnant women. This may suggest a shift away from Th1 type cytokine production rather than a shift towards Th1 type cytokine production. Therefore, these results are also in contrast to our hypothesis. In contrast to our study, others found a shift towards Th1 type cytokine production in preeclampsia as compared with normal pregnancy (Saito et al. 1999a; Saito et al. 1999b; Darmochwal-Kolarz et al. 2002; Orange et al. 2003). Also in contrast to our study, in which we found no effect of preeclampsia on NK cell cytokine production, Darmochwal-Kolarz et al. found an increased IFNγ production in NK cells of preeclamptic women as compared with normal healthy pregnant women (Darmochwal-Kolarz et al. 2002). The difference between the above mentioned studies and our study may again be the severity of preeclampsia at almost comparable gestational age of pregnancy. As indicated above it turned out that our group of preeclamptic patients had relatively mild preeclampsia as compared with the other studies. In the other papers, patients showed severe preeclampsia as defined by much higher diastolic and systolic blood pressure (mean diastolic blood pressure around 110 mmHg or more), severe proteinuria (> 0.6g/L) and some included patients with elevated liver enzymes, sometimes combined with decreased platelets. Most of the time, pregnancies delivered preterm by caesarean section. Although the group of patients in our study may be of less clinical importance as compared to patients with severe preeclampsia, this group is still of clinical interest. This is a relatively large group of preeclamptic patients that absorb clinical care in all western countries, since they stay in hospital for at least a few days to observe the development of severity of their disease.
It may be hypothesized that mild preeclampsia is associated with a shift towards Th2 type cytokine production, while severe preeclampsia is associated with a shift towards type 1 cytokine production. This hypothesis is corroborated by a study of Hayakawa and colleagues, who induced experimental preeclampsia in pregnant mice by inoculating them with splenocytes stimulated with either IL12 or IL4 (Hayakawa et al. 2000). Both types of splenocytes induced preeclampsia-like symptoms, but the symptoms induced by IL4 stimulated splenocytes were milder than those induced by IL12 stimulated splenocytes. If indeed a shift towards type 1 cytokine production is associated with severe preeclampsia and a shift towards type 2 cytokine production with mild preeclampsia, a relation between the clinical signs of preeclampsia and the production of cytokines is to be expected. This has been shown in severe preeclampsia, where production of Th1 type cytokines was positively related with mean blood pressure (Saito et al. 1999b; Sakai et al. 2004). In the present study, however, we found no relation between mean blood pressure and cytokine production of either stimulated lymphocytes or monocytes.

Several theories for altered immune response in preeclampsia may be suggested. One theory is that altered tryptophan metabolism may play a role in the dysregulation of immune responses during pregnancy and preeclampsia (Kudo et al. 2003). However, in general, it is thought that factors produced or shed from the placenta may activate immune cells. Suggested factors included cytokines (Henessey et al. 2003) or oxygen free radicals (Zusterzeel et al. 2001; Holthe et al. 2004; Burton and Jauniaux 2004) and syncytiotrophoblast microvillus membranes (STBM) (Knight et al. 1998). It has been shown that increased amounts of STBM are shed from the placenta during preeclampsia as compared with normal pregnancy (Knight et al. 1998). It may be suggested that these STBM generate circulating immune complexes (Feinberg 2006), which in their turn may activate the inflammatory response. Although it remains unclear from the present study why in mild preeclampsia the immune response is associated with a Th2 profile, it may be suggested that this Th2 profile is involved in the inflammatory reaction of mild preeclampsia. In a Th2 environment, the formation of immune complexes is increased, which may increase the activation of the inflammatory response in mild preeclampsia.

Although the mechanism remains obscure, we showed that in our group of preeclamptic patients, which turned out to be relatively mild preeclamptic, both specific and non-specific immune responses appeared to shift their cytokine production away from Th1 cytokine production.
References


