# CYTOKINES AND IMMUNE BALANCE IN PREECLAMPSIA

A survey of some immunological variables and methods in the study of preeclampsia

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To progress

# ABSTRACT

Preeclampsia is one of the most feared pregnancy complications, with a risk of maternal and fetal death and with no ideal therapy readily available. The cause of this strictly pregnancy-related disease is still unknown and is therefore a great challenge to all researchers in the field of pregnancy-related pathophysiology.

Today, the dominating theory of the origin of preeclampsia is defective initial placentation with insufficient penetration of the trophoblasts, leading to impaired maternal blood flow through narrow spiral arteries. However, the cause of this defective trophoblast behavior is not known. The maternal immune system has been proposed to have an influence on both the placentation and the subsequent systemic reactions. Therefore, it is very interesting to study the maternal immune system during preeclampsia, in hope of achieving a better understanding of this puzzling disease.

Earlier studies have suggested that normal pregnancy requires a shift to a Th2/anti-inflammatory type of immunity, at least directed towards the fetus and placenta, while some pregnancy complications, such as preeclampsia, could be due to a skewed Th1/pro-inflammatory type of immunity. However, the results from earlier studies designed to test the Th1/Th2 hypothesis in preeclampsia have not been consistent. Therefore, the aim of this thesis was to examine if established preeclampsia is associated with increased innate inflammatory responses and a deviation of adaptive responses towards Th1 when compared with normal pregnancy.

Enumerations of cytokine-producing cells from peripheral blood did not show any difference in the production of IFN-$\gamma$, IL-4, IL-10 and IL-12 between women with preeclampsia and normal pregnancies. However, a decrease in the spontaneously produced levels of IL-5 was detected in cell cultures on peripheral blood mononuclear cells in women with preeclampsia. Furthermore, a decreased production of IL-10 in response to paternal antigens, believed to represent the fetus, was also detected for the preeclamptic women.

Serum analysis showed increased levels of the pro-inflammatory mediators IL-6 and IL-8 during preeclampsia. Also, preeclamptic women displayed increased serum levels of the soluble IL-4 receptor, but no difference in the levels of IL-4 compared to normal pregnant women. This was an elusive finding, since the receptor was originally thought to reflect the levels of IL-4, but has recently been shown to have both agonistic and antagonistic properties on the IL-4 levels. Further studies of the local immune responses in the placenta showed no difference in the immunohistochemical staining of IL-4 and TNF-$\alpha$ between women with preeclampsia and women with normal pregnancies. In general, there were no hallmarks of abnormal morphology in the placental sections examined, regardless of diagnosis.

In conclusion, the decreased levels of IL-10 in response to paternal antigens and the systemically increased levels of IL-6 and IL-8 suggest a specific decrease in anti-inflammatory responses towards fetal antigens, together with a systemic activation of pro-inflammatory mediators during preeclampsia. Furthermore, the decreased production of IL-5 also indicates, at least partly, decreased Th2 responses in the established preeclampsia.

# CONTENTS

## LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Jonsson Y., Ekerfelt C., Berg G., Nieminen K., Sharma S., Ernerudh J. and Matthiesen L. (2004).** Systemic Th1/Th2 cytokine responses to paternal and vaccination antigens in preeclampsia: no differences compared with normal pregnancy. *Am J Reprod Immun* 51 (4): 302–310.

II. **Jonsson Y., Matthiesen L., Berg G., Ernerudh J., Nieminen K., Ekerfelt C. (2005).** Indications of an altered immune balance in preeclampsia: A decrease in *in vitro* secretion of IL-5 and IL-10 from blood mononuclear cells and in blood basophil counts compared with normal pregnancy. *J Reprod Immunol* 66 (1): 69–84.

III. **Jonsson Y., Rubèr M., Matthiesen L., Berg G., Nieminen K., Sharma S., Ernerudh J. and Ekerfelt C.** Cytokine mapping of sera from women with preeclampsia and from women with normal pregnancies. 2005, accepted for publication in *J Reprod Immunol*.

IV. **Jonsson Y., Ekerfelt C., Olsson H., Boij R., Berg G, Ernerudh J. and Matthiesen L.** Immunohistochemical studies of interleukin-4 (IL-4) and tumor necrosis factor α (TNF-α) in placentas from preeclamptic and normal pregnancies. Manuscript.

# ABBREVIATIONS

| | | | |
|---|---|---|---|
| APC | Antigen presenting cell | TGF | Transforming growth factor |
| CD | Cluster of differentiation | TMB | Tetramethylbenzidine |
| CM | Cell culture medium | TNF | Tumor necrosis factor |
| DC | Dendritic cell | TT | Tetanus toxoid |
| ELISA | Enzyme-linked immunosorbent assay | Th | T helper lymphocyte |
| ELISPOT | Enzyme-linked immunospot assay | Treg | Regulatory T lymphocyte |
| GM-CSF | Granulocyte-macrophage colony stimulating factor | VCAM | Vascular adhesion molecule |
| HRP | Horseradish peroxidase | | |
| HLA | Human leukocyte antigen | | |
| ICAM | Intracellular adhesion molecule | | |
| IHC | Immunohistochemistry | | |
| IFN | Interferon | | |
| IUGR | Intrauterine growth restriction | | |
| MCP | Monocyte chemotactic protein | | |
| MHC | Major histocompatibility complex | | |
| MIP | Macrophage inflammatory protein | | |
| MLC | Mixed leukocyte culture | | |
| MLC-pool | Pooled leukocytes from different donors. Used as stimuli in paper I-II | | |
| NK-cell | Natural killer cell | | |
| PBMC | Peripheral blood mononuclear cells | | |
| PFA | Paraformaldehyde | | |
| PHA | Phytohemagglutinin | | |
| PIBF | Progesterone inhibitory factor | | |
| PMN | Polymorphonuclear leukocytes | | |
| PRR | Pattern-recognition receptor | | |
| PPD | Purified protein derivate of *Mycobacterium tuberculosis* | | |
| RA | Rheumatoid arthritis | | |
| RANTES | Regulated on activation, normal T-expressed and secreted | | |
| SLE | Systemic lupus erythematosus | | |
| Streptavidin-RPE | Streptavidin–R-phycoerythrin | | |

# INTRODUCTION

## PREECLAMPSIA

Preeclampsia is one of the most feared pregnancy complications, with a risk of maternal and fetal death and with no ideal therapy readily available. The cause of this strictly pregnancy-related disease is still unknown and is therefore a great challenge to all researchers in the field of pregnancy-related pathophysiology.

Today, the dominating theory of the origin of preeclampsia is defective initial placentation with insufficient penetration of the trophoblasts, leading to impaired maternal blood flow through narrow spiral arteries, see Figure I. However, the reason for this defective trophoblast behavior is not known. A possible mechanism is some kind of immunologic dysfunction, resulting in an unwanted hampering of normal trophoblast activity.

The diagnosis of preeclampsia requires elevated blood pressure and proteinuria, but a number of other symptoms are often associated with the disease; for further details see Table I.

The inadequately transformed uterine spiral arteries lead to a superimposed maternal systemic syndrome triggered by the placental ischemia, which results in widespread endothelial activation by placentally derived factors (Loke and King 1995). More specifically, the levels of damaging lipid peroxides are increased even during normal pregnancy, but in preeclampsia the production of antioxidants is also decreased, resulting in endothelial cell damage (Stark 1993, Symonds and Symonds 1998). Together with placental damage, in the form of hypoperfusion, this leads to maternal vasoconstriction and pathological lesions in the liver, kidney and placental bed. The renal lesion leads to retention of sodium and water, but most of the fluid accumulates in the tissues instead of in the vascular system. At the same time, the increased retention of sodium increases the vascular sensitivity to angiotensin II, which promotes further vasoconstriction and more tissue damage; thus the vicious circle continues with even more restricted blood flow to the placenta.

The invading extravillous trophoblasts are in close contact with maternal immune cells at the implantation site in the decidua. Therefore, human leukocyte antigen (HLA) expression in extravillous cells is of importance in the recognition of the semi-allogenic fetus by the mother. Maternal natural killer (NK) cells, macrophages and T-cells interact with the extravillous trophoblast, and the interplay between the trophoblast cells and the maternal immune cells

may be one of the regulatory mechanisms behind the degree of vascular transformation and trophoblast invasion (King, et al. 2000), see also Figure III. Furthermore, it has been shown that the maternal immune system is involved in maternal endothelial dysfunction (Roberts, et al. 1989) and the subsequent systemic reaction seen in preeclampsia (Saito and Sakai 2003).

Further arguments to support the idea of the involvement of the maternal immune system in the development of preeclampsia come from the primipaternity theory. This hypothesis holds that the risk of developing preeclampsia is highest in the first pregnancy, and a previous normal pregnancy is associated with a lowered incidence of preeclampsia in the subsequent pregnancy. The protective effect of multiparity is, however, possibly lost with a change of partner (Dekker and Robillard 2003, Need 1975, Robillard, et al. 1999), indicating that a protective memory response could be generated against paternal antigens during normal pregnancy, thus strengthening the significance of adaptive responses in preeclampsia. However, this has been questioned by others (Skjaerven, et al. 2002, Trogstad, et al. 2001, Verwoerd, et al. 2002), who report that change of paternity is not associated with an increased risk of preeclampsia; the key risk factors might rather be the inter-pregnancy interval and the length of sexual co-habitation.

Because of the proposed influence of the maternal immune system on placentation and the subsequent systemic reactions, it is of great interest to study the maternal immune system during preeclampsia in the hope of achieving a better understanding of this puzzling disease.

## Table I Definitions of preeclampsia and IUGR

**Definition of preeclampsia and IUGR according to the World Health Organization**

Preeclampsia is a syndrome defined by hypertension and proteinuria and may be associated with other signs and symptoms.
Preeclampsia occurs after the $20^{th}$ gestational week.

**Diagnosis criteria**

**Moderate preeclampsia:**
- Systolic blood pressure $\geq$ 140 mmHg and/or a diastolic pressure $\geq$ 90 mmHg, measured at separate occasions at least 4 hours apart.
- Proteinuria in a 24-hour protein excretion $\geq$ 300 mg or 1+ on two random urine samples collected 4 hours apart.
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**Severe preeclampsia:**
- Systolic blood pressure $\geq$ 160 mmHg and/or diastolic pressure $\geq$ 110 mmHg, measured at separate occasions at least 4 hours apart.
- Proteinuria in a 24-hour protein excretion $\geq$ 5 g or 3+ on two random urine samples collected 4 hours apart.

**Other common symptoms:**
- Cerebral dysfunction (blurred vision, scotoma, headache, cerebrovascular accidents).
- Epigastric or right upper quadrant pain.

- Renal failure or oliguria $\leq$ 500 ml in 24 hours.
- Pulmonary edema.
- Impaired liver function (serum transaminase levels two times normal or greater).
- Thrombocytopenia ($\leq$ 100,000 platelets/mm$^3$).
- Coagulopathy.
- Fetal growth restriction.
- Eclampsia (generalized convulsions).
- HELLP; hemolysis, elevated liver enzymes, low platelets.

**Intrauterine growth restriction (IUGR):**
Intrauterine growth restriction is defined as fetal weight below the $10^{th}$ percentile for gestational age.
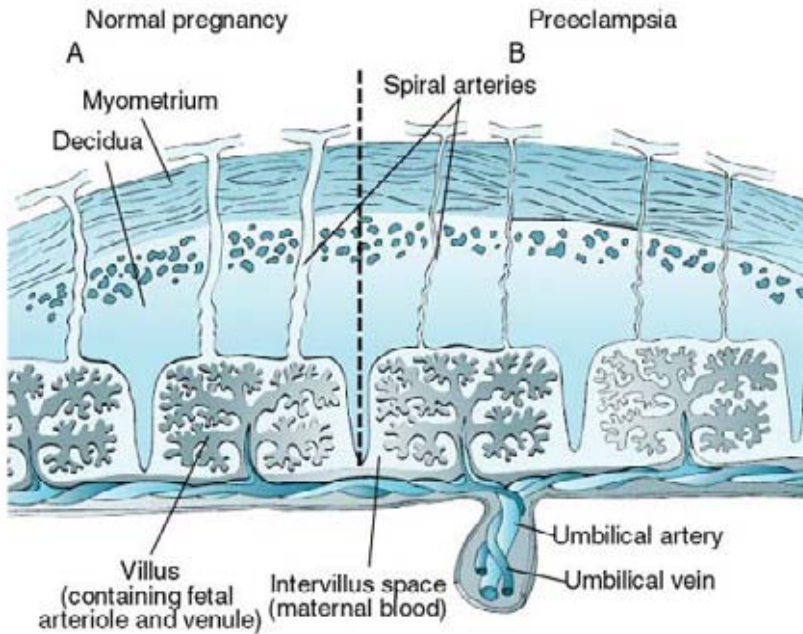
Figure I Normal and preeclamptic placenta.

During normal placentation (A), the spiral arteries become invaded by trophoblasts. As a result they become high-throughput vessels which adequately can support the feto-placental unit. In preeclampsia (B), the trophoblast invasion is shallow, which results in constricted spiral arteries and reduced blood flow to the feto-placental unit.

## IMMUNOLOGY

## Basic immunology

The human immune system can be divided into innate and adaptive immunity. Innate immunity provides a rapid and first line of defense against invading microorganisms. The major cell types included in innate immunity are macrophages, natural killer (NK) cells and the granulocytes: mast cells, neutrophils, eosinophils and basophils, as shown in Figure II (Abbas and Lichtman 2005, Janeway 2005).

These cells recognize microorganisms by their pattern-recognition receptors (PRRs) and they respond immediately by phagocytosis of microorganisms, eradication of infected cells, and cooperation with the adaptive immune response (Abbas and Lichtman 2005, Janeway 2005). The innate immune cells

initiate immune responses and start to direct the adaptive immune responses, as well as aiding the adaptive immune response in the removal of pathogens targeted by the adaptive immune response. It takes 4–7 days before the initial adaptive immune response takes effect, and during this time the innate immune response has a critical role in controlling infections (Janeway 2005).

Activated macrophages and NK cells secrete cytokines and chemokines, which initiate the inflammatory response and aid the destruction of phagocytosed particles (Abbas and Lichtman 2005, Janeway 2005). Furthermore, the macrophages have the capacity to present antigens from phagocytosed particles. The adaptive immunity reacts slower than the innate immunity, but has more long-lived and highly evolved antigen-specific protective responses, such as antibody production and cell-mediated immunity, reviewed by Tosi (2005).

The cells included in adaptive immunity are B- and T-lymphocytes, and these cells are also responsible for the immunological memory. B-lymphocytes are involved in the humoral defense against extracellular antigens by producing specialized antibodies upon stimulation; furthermore, these cells have the capacity to present antigens to the T-cells. A "helping" T-cell response is needed to induce B-cell proliferation and differentiation toward protein antigens. T-lymphocytes, with the αβ-receptor, have a cell-mediated response where they respond to a foreign antigen presented on human leukocyte antigen (HLA) (Abbas and Lichtman 2005).

T-cells destroy intracellular pathogens by killing infected cells and by activating macrophages, as mediated by the Th1 subset. However, T-lymphocytes also have a central role in the destruction of extracellular pathogens by activating B-cells, and this is the specialized role of the second subset of CD4^{+} T-cells, called Th2 cells. Only a few antigens with special properties can activate naïve B-lymphocytes on their own. Most antigens require an accompanying signal from helper T-cells before they can stimulate B-cells to proliferate and differentiate into cells secreting antibody (Janeway 2005).

The T-lymphocytes expressing αβ-receptors are further divided into CD4^{+} T-helper cells and CD8^{+} cytotoxic T-cells, depending on their type of antigen recognition and subsequent action. Cytotoxic T-cells mediate their function by "docking" onto a cell presenting foreign peptide antigen on HLA class I molecules, and induce apoptosis of that cell. T-helper cells direct the reaction of other cells of the innate and adaptive immune system more than they participate themselves in an immune reaction. They recognize antigens presented on HLA class II molecules on antigen-presenting cells (APC). The T-helper cells practice their effector functions on other immune cells by secreting cytokines and, as

discussed below, different types of cytokines have different effector functions (Abbas and Lichtman 2005).
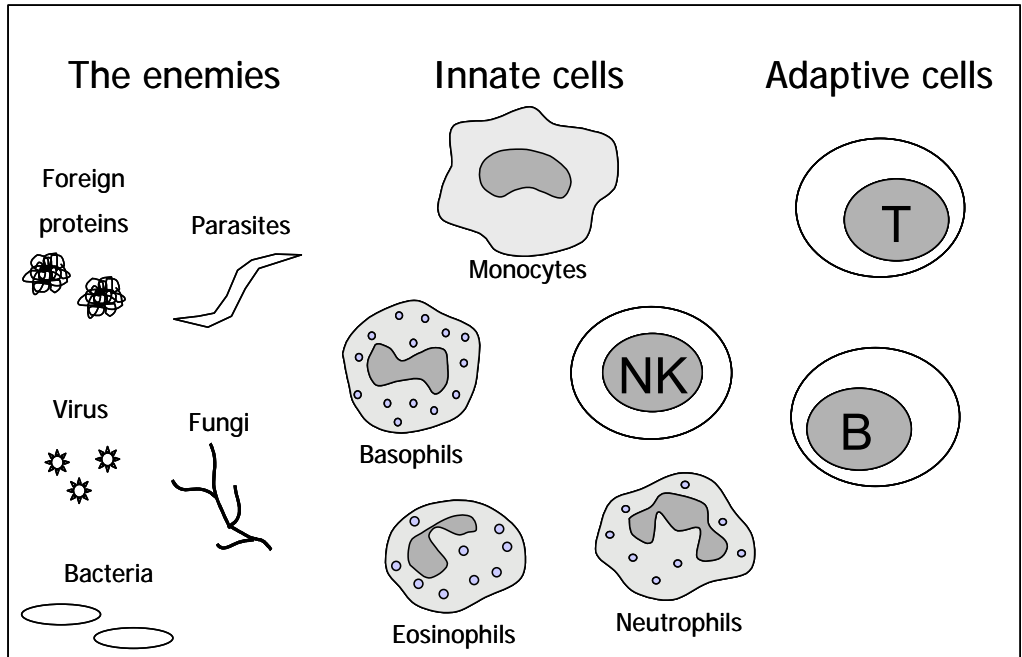


Figure II The effector cells of the immune system and their targets.

The immune defense consists of leukocytes of different types, which by complex interactions cooperate in eliminating foreign structures.


## Th1/Th2 reactions and cytokines

The response from the T-helper lymphocytes present in the human immune system can be divided into two separate and counterbalancing reactions, depending on the type of cytokines that are produced. Human CD4^{+} positive T-lymphocytes were originally shown to secrete cytokines in two distinct profiles, i.e., T-helper (Th) type 1 and type 2 (Th2) responses (Del Prete, et al. 1991, Romagnani 1994, Salgame, et al. 1991). However, this Th1/Th2 concept, originally described in mice (Mosmann and Coffman 1989), is not as distinct in humans.

The signature cytokine of human Th1 cells is interferon (IFN)-γ and lymphotoxin (LT), while Th2 cells are defined by their production of interleukin (IL)-4, IL-5 and IL-9. The cytokines produced by these T-cells determine the effector function as well as participate in the development and expansion of each subset. That is, the IFN-γ produced by the Th1 cells promotes further Th1 differentiation and inhibits Th2 cells. *Vice versa*, the IL-4 produced by the Th2

cells promotes Th2 differentiation and, accompanied by the anti-inflammatory IL-10, produced mainly by activated macrophages, inhibits the activation of Th1 cells. Thus, the Th1 and Th2 cells amplify their own subset and each cross-regulates the other. Once an immune response develops along one pathway, it becomes increasingly polarized in that direction (Abbas and Lichtman 2005, Janeway 2005). However, immune cells other than Th1 and Th2 cells as well as other cells in the body can produce cytokines; for details see Table II. Therefore, in general terms of the entire immune system the Th1/Th2 responses are called type 1 and type 2 responses, but for simplicity, in this thesis these responses will be called Th1 and Th2.

The main effector function of the Th1 cells is phagocyte-mediated and cytotoxic defense against infections, especially with intracellular microbes. The main effector function of the Th2 cells is IgE- and eosinophil/mast cell-mediated immune reactions, as well as inhibition of phagocytosis (Abbas and Lichtman 2005, Opal and DePalo 2000, Romagnani 2000).

## Pro- and anti-inflammatory cytokines

Just as for the Th1/Th2 concept, the balance between the effects of pro- and anti-inflammatory cytokines is thought to determine the outcome of the disease (Dinarello 2000, Opal and DePalo 2000). The timing and the location of pro- and anti-inflammatory cytokines plays a role in the outcome of a disease, as shown by Widhe et al. (2002), where patients with non-chronic neuroborreliosis showed higher levels of the pro-inflammatory cytokine tumor necrosis factor (TNF)-$\alpha$ in cerebrospinal fluid compared to patients with the chronic form of the disease. Furthermore, the non-chronic patients showed higher serum levels of the anti-inflammatory cytokine transforming growth factor (TGF-$\beta$)$_2$ than chronic patients. These findings can be interpreted as implying that an early pro-inflammatory reaction is needed in the central nervous system to get rid of the infection, but at the same time serum levels of TGF-$\beta_2$ rise to control the systemic immune response. In line with this reasoning, the chronic patients failed to mount the initial necessary pro-inflammatory response and the subsequent systemic anti-inflammatory response.

The damaging effect of excessive amounts of pro-inflammatory cytokines is also seen in sepsis. Normally, secreted TNF-$\alpha$ helps to contain an infection locally, but in the case of sepsis TNF-$\alpha$ is released systemically, which leads to vasodilatation and loss of plasma volume owing to increased vascular permeability, resulting in shock. In septic shock, disseminated intravascular coagulation is also triggered by TNF-$\alpha$, leading to hypercoagulation in capillaries and massive consumption of clotting protein (Janeway 2005).

As reviewed in Dinarello (2000), the concept of pro- and anti-inflammatory cytokines is based on whether genes coding for the synthesis of mediator molecules are up-regulated during an immune response. Pro-inflammatory genes are those coding for phospholipase $A_2$ ($PLA_2$), cyclooxygenase (COX)-2 and inducible NO synthase, since these are the enzymes that increase the synthesis of platelet-activating factor and leukotrienes, prostanoids and NO. The cytokines IL-1 and TNF (and in some cases IFN-γ) are effective in stimulating the expression of these genes. Chemokines, such as IL-8, are chemotactic and facilitate the passage of leukocytes into the tissue from the vascular compartment. In addition, they are also pro-inflammatory molecules. Anti-inflammatory cytokines block or suppress the intensity of the pro-inflammatory cascade. For example, IL-4, IL-10, IL-13 and TGF-β suppress the production of IL-1, TNF, IL-8 and vascular adhesion molecules. Therefore, the balance between the effects of the pro-inflammatory and anti-inflammatory cytokines is thought to determine the outcome of the disease.

The immunological markers (mainly cytokines and chemokines) analyzed in this thesis are described in Table II. The chemokines have systematic names (CXC and CC), but in this thesis the traditional names will be used.


## Regulatory T-cells

Another cell type involved in the outcome of immune reactions is the regulatory T-cell. There exist different types of regulatory T-cells identified up to now and more subsets are continuously being discovered; however, one type of regulatory T-cells (Treg) are the $CD4^+CD25^+$ self-reactive T-cells which suppress other, harmful, auto-reactive T-cells by cell–cell contact and/or by producing IL-10 and TGF-β (Akl, et al. 2005, Janeway 2005, Kronenberg and Rudensky 2005, Thompson and Powrie 2004). The suppression mediated by regulatory T-cells is essential for the induction and maintenance of tolerance to self-antigens (Akl, et al. 2005, Thompson and Powrie 2004).

Furthermore, T-helper lymphocytes producing high amounts of TGF-β, IL-10 and IL-4 have been termed as type 3 cells (Th3). (Bolton 2005, Janeway 2005, Romagnani 2000) These cells also display regulatory functions in that they actively suppress antigen-specific responses after re-challenge with antigens. The cytokine secretion in response to antigen-specific stimulation inhibits the development of Th1 responses, and is associated with low levels of antibody and a virtual absence of inflammatory T-cell responses (Bolton 2005, Janeway 2005). Besides preventing autoimmune T-cell responses, Tregs are also believed to be involved in the suppression of other inflammatory responses, e.g., infection and allergy.

IMMUNOLOGY OF THE NORMAL PREGNANCY

Among all the other changes that a woman's body undergoes during pregnancy, her immune system also has to undergo adaptations, since the maternal immune system likely plays a part in the placentation process as well as in the maintenance of pregnancy, as described below. In the late luteal phase of the menstrual cycle and early gestation, the decidua is infiltrated by large numbers of CD56-positive NK-cells (Dekker and Sibai 1998, Loke and King 1995), but their numbers decline as the pregnancy progresses (Dekker and Sibai 1998, Saito 2001). The number of macrophages and T-cells remains relatively constant throughout gestation. CD56 cells have been proposed as promoting trophoblast growth and invasion by secreting cytokines, especially leukemia inhibitory factor (LIF), in response to the HLA-G expressed on cytotrophoblastic cells (Dekker and Sibai 1998, Saito 2001). Furthermore, it has been shown that the expression of LIF is associated with Th2 responses (Piccinni, et al. 1998).

At the adaptive level it is believed that the immune system during normal pregnancy is associated with a Th2 shift, as suggested by Wegmann et al. (1993). The idea of weakened cell-mediated immunity and increased humoral immunity is supported by clinical findings in pregnant women, where women with rheumatoid arthritis (RA), a cell-mediated autoimmune disorder, experience temporary remission of symptoms during pregnancy (Nelson and Ostensen 1997, Ostensen and Villiger 2002, Wegmann, et al. 1993). On the other hand, women with systemic lupus erythematosus (SLE), an autoantibody-mediated disease, may experience worsened symptoms of the disease during pregnancy (Nossent and Swaak 1990, Wegmann, et al. 1993). Furthermore, Sacks et al. (1999) has proposed that the alterations of adaptive immune responses during pregnancy are accompanied by increased innate immune responses.

A majority of women experiencing unexplained recurrent spontaneous abortions (RSA) shows increased peripheral blood mononuclear cell (PBMC) production of IFN-γ and TGF-β in response to trophoblast antigens, while PBMCs from women not prone to RSA respond with IL-10, reviewed by Raghupathy (1997). In addition, Piccinni et al. (1998) showed that decidual cells from women with RSA had a lower production of IL-4, IL-10 and LIF in comparison with decidual cells from normal pregnant women. This suggests that Th2 type immune responses play a significant role in successful gestation and that Th1 type of immune responses are inconsistent with successful pregnancies (Piccinni, et al. 1998, Raghupathy 1997). Furthermore, Wegmann et al. (1993) also report a Th2 cytokine dominance in decidual cell culture supernatants from normal pregnancy.

Further laboratory findings of increased production of IL-4 and IL-10 and decreased production of IL-2 and IFN-γ from phytohemagglutinin (PHA, mitogen)-stimulated PBMCs supports the idea of a Th2 shift during normal pregnancy (Marzi, et al. 1996). Reinhard et al. (1998) also found increased levels of IL-4 and decreased levels of IFN-γ and IL-2 in T-lymphocytes from women with normal pregnancies compared to non-pregnant women. In addition, Ho et al. (2001) detected higher spontaneous production of IL-10 than IFN-γ in PBMC cultures from normal human pregnancies. More specifically, the maternal immune system has been shown to have an increase of fetus-specific Th2 type responses, i.e., increased numbers of IL-4-secreting PBMCs in response to paternal antigen in normal human pregnancies as compared to non-pregnant controls (Ekerfelt, et al. 1997).

Taken together, these findings suggest that normal pregnancy requires a Th2/anti-inflammatory type of immunity, at least directed toward the fetus and placenta, while pregnancy complications such as RSA could be due to a skewed Th1/pro-inflammatory type of immunity.


IMMUNOLOGY OF PREECLAMPSIA

In contrast to normal pregnancy, there are indications of increased inflammatory responses and also of an immune deviation toward Th1 in the established preeclamptic pregnancy (Dekker and Sibai 1999). Roberts et al. (1989) were one of the first to suggest that mediators released from the preeclamptic placenta are responsible for the endothelial damage seen in preeclampsia. Subsequent to the damage, the injured endothelium initiates a dysfunctional cascade of coagulation, vasoconstriction and intravascular fluid redistribution that results in the clinical syndrome of preeclampsia. As reviewed by Redman et al. (1999), TNF-α can activate the endothelial cells and present damage similar to that seen in preeclampsia.

Furthermore, Redman et al. (1999) suggest that preeclampsia is an excessive maternal inflammatory response to pregnancy. Indeed, preeclampsia is associated with systemic maternal inflammation (reviewed by (Redman and Sargent 2003)), which at the adaptive level has been suggested to be dominated by T-helper (Th) type 1 responses, as reviewed in Saito and Sakai (2003).


## Th1/Th2 in preeclampsia

Results from studies designed to test the Th1/Th2 hypothesis in preeclampsia have not been consistent. Saito et al. (1999a) measured the number of Th1 (CD4$^+$IFN-γ$^+$IL-4$^-$) and Th2 (CD4$^+$IFN-γ$^-$IL-4$^+$) cells during normal pregnancy and preeclampsia, and found that the Th2 cells dominated during normal

pregnancy while Th1 cells dominated during preeclampsia. In the same study, the PBMC production of IFN-γ and IL-4 was measured and the secreted levels were found to correlate with the number of Th1/Th2 cells. That is, normal pregnant women showed a significantly higher production of IL-4 and lower production of IFN-γ than women with preeclampsia. Darmochwal-Kolarz et al. (1999) has also found increased production of IFN-γ in stimulated PBMCs.

In contrast, Gratacos et al. (1998) found that the serum levels of IL-4 and IL-10 did not differ between women with normal pregnancies and women with preeclampsia. In line with this, Henriques et al. (1998) were able to detect IL-4 in placentas, but were unable to detect any differences between placentas from preeclamptic pregnancies and those from normal pregnancies, while Omu et al. (1995) detected increased levels of IL-4 in serum from women with preeclampsia compared to normal pregnant women. Furthermore, Daniel et al. (1998) detected similar plasma levels of IFN-γ between women with preeclampsia and women with normal pregnancies.

## Pro- and anti-inflammatory balance during preeclampsia

Likewise as for the studies of Th1 and Th2 balance, the reports on pro- and anti-inflammatory cytokines in preeclampsia differ from each other. The plasma levels of the pro-inflammatory cytokines IL-6 and TNF-α have been shown to be increased during preeclampsia when compared to normal pregnancy (Conrad, et al. 1998, Vince, et al. 1995), although no differences were seen for IL-1β and IL-10 (Conrad, et al. 1998). In addition, Kupferminc et al. (1996) found increased levels of IL-6 in plasma from women with preeclampsia compared to those with normal pregnancies.

In contrast, Al-Othman et al. (2001) found no differences in the levels of IL-6 in maternal serum from preeclamptic pregnancies compared to normal pregnancies. Furthermore, in studies on plasma levels of IL-10 and TGF-$\beta_1$ Benian et al. (2002) found increased levels of these markers during preeclampsia as compared to normal pregnancy.

On studies of the serum levels of IL-12, Dudley et al. (1996) found increased levels of the p40 subunit of IL-12 in women with preeclampsia, but no differences in the intact p70 dimer between women with preeclampsia and those with normal pregnancies. Although the levels of IL-12 p70 did not always reach detection levels, Daniel et al. (1998) found increased levels of the IL-12 dimer in plasma from preeclamptic women compared to normal pregnant women.

When measuring the stimulated cytokine production in PBMC cell culture supernatants, Darmochwal-Kolarz et al. (1999) found increased levels of IL-2

and decreased levels of IL-10 in PBMCs from women with preeclampsia compared to women with normal pregnancies.

Gratacos et al. (1998) measured the serum levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and found decreased levels during preeclampsia compared to normal pregnancy. Based on studies of murine pregnancies where this cytokine is found in high concentrations at the maternal–fetal interface and injection of high levels decreases fetal death in resorption-prone animals, they suggested that GM-CSF may also be of importance in human pregnancy. Furthermore, Sacks et al. (1998) found a pattern of increased cell surface markers and reactive oxygen species in leukocytes akin to that of sepsis in women with preeclampsia.

## Possible causes for the shifts in immune balance seen in preeclampsia

An earlier report has shown production of TNF-α in pure choriocarcinoma cell lines (Jar and JEG-3, equivalent to cytotrophoblastic cells) (Yang, et al. 1993). Furthermore, Wang and Walsh (1996) found increased levels of TNF-α from preeclamptic placentas compared to placentas from normal pregnancies. These findings indicate that the placenta is involved in the production of TNF-α.

The ischemia in the placenta during preeclampsia can also lead to the production and release of cytokines into the maternal circulation. As reviewed by Conrad and Benyo (1997), it is highly possible that the ischemia leads to the production of TNF-α and IL-1. These cytokines are capable of producing endothelial cell activation and dysfunction.

Apoptotic trophoblast and syncytiotrophoblastic microparticles are constantly shed from the placenta during pregnancy, with increased circulating levels during preeclampsia, as reviewed by Sargent et al. (2003). Furthermore, it is possible that these trophoblastic cells might evoke an immune response in the mother (Dekker and Sibai 1998). Curiously, Neale et al. (2003), detected increased apoptosis of trophoblastic cell lines that were exposed to serum from preeclamptic women; this may in turn indicate a vicious circle involving the trophoblasts and general maternal systemic effects during preeclampsia, as detected by the influence of their serum on the cell cultures. Furthermore, the excessive amounts of syncytiotrophoblastic microparticles might be the cause of the Th1 activation in preeclampsia (Sargent, et al. 2003). These particles have been shown to inhibit endothelial cell function and to cause them to release pro-inflammatory factors. In addition, the monocytes can take up and process these particles with the possible result of increased production of the pro-inflammatory cytokines TNF-α and IL-12, the latter inducing Th1.
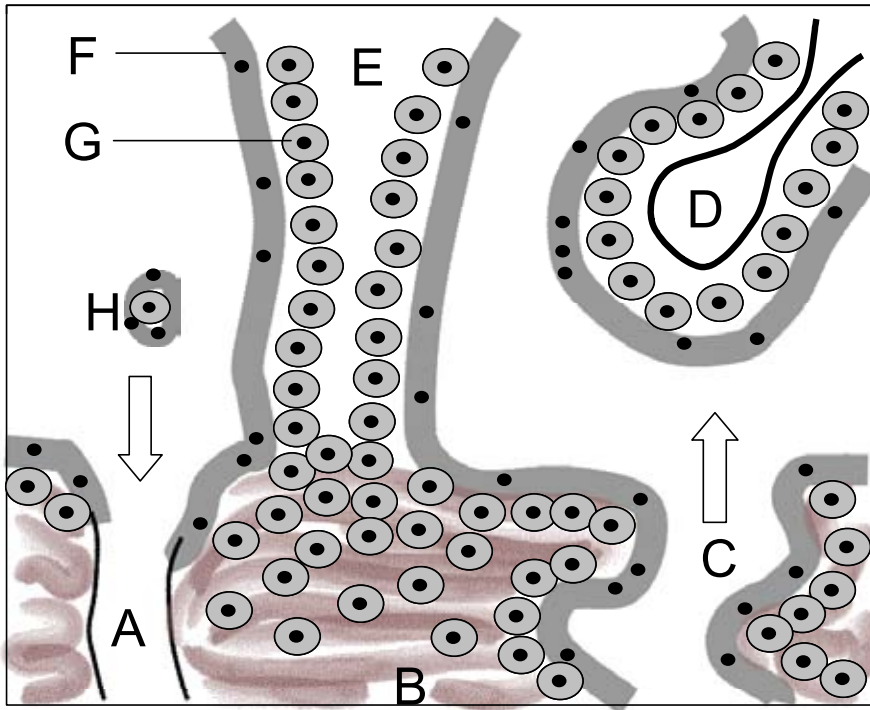
Figure III Schematic illustration of the trophoblast populations at the implantation site of the placenta.

Maternal side: (A) vein, (B) decidua and (C) spiral artery. The endothelium and media of the decidual part of the spiral artery are normally replaced by trophoblasts and fibrinoid material as a result of trophoblast invasion. Fetal side: (D) Villi with fetal circulation, (E) anchoring villi, (F) syncytiotrophoblastic layer, (G) cytotrophoblasts and (H) syncytiotrophoblastic particle from the placenta, which is deported via the uterine veins to the maternal circulation.

## AIM

As discussed above, the origin of the preeclamptic symptoms is elusive and many factors can trigger events leading to preeclampsia, e.g., immunological reactions, chronic hypertension and renal disease. Of all the possible triggering events, the focus of this thesis was to monitor immunological aberrations in established preeclampsia.

Hypothesis: Preeclampsia is associated with increased innate inflammatory responses and a deviation of adaptive responses toward Th1 compared with normal pregnancy.

Table II Description of immune molecules studied in papers I–IV

| Cytokine | Mainly produced by | Major functions | Functional class | Reference |
|---|---|---|---|---|
| IL-1β | Monocytes/macrophages, endothelial cells, neutrophils and others | Activate T-lymphocytes by enhancing the production of IL-12 and expression of IL-2 R. Augments B-cell proliferation and increases immunoglobulin synthesis. Interacts with CNS to produce fever, lethargy, sleep and anorexia. Induces production of TNF-α and IL-6. Induces the gene expression for PLA$_2$ and COX-2, which leads to PGE$_2$ and leukotriene synthesis. | Pro-inflammatory cytokine | (Borish and Steinke 2003, Dinarello 2000) |
| IL-2 | Activated T-lymphocytes | Induces clonal T-lymphocyte expansion. Growth factor for T-cells. Activates NK-cells, B-lymphocytes, cytotoxic T-cells and macrophages. | Pro-inflammatory | (Borish and Steinke 2003, Romagnani 2000) |
| IL-4 | Th2 lymphocytes, B-lymphocytes, eosinophils, basophils, stromal cells, mast cells | Promotes Th2 lymphocyte development. Downregulates Th1 responses. Maintains allergic responses. Activates and induces isotype switch to IgE and IgG4 in B-lymphocytes. Influences T-lymphocyte growth, differentiation and survival. Enhances expression of MHC class II molecules and CD23 on macrophages. Downregulates the monocyte/macrophage production of IL-1, IL-6, IL-8, IL-12, TNF-α and MIP-1α. Induces expression of VCAM-1 on endothelial cells. Stimulates the synthesis of the cytokine inhibitor IL-1ra. | Th2 | (Borish and Steinke 2003, Opal and DePalo 2000) |
| IL-5 | Th lymphocytes, mast cells | Stimulates eosinophil production. Chemotactic and activating for eosinophils. Upregulates eosinophil responses to chemokines, αδβ2 integrins, i.e., promotes adherence to VCAM-1. Maturates cytotoxic T-lymphocytes and differentiates basophiles. | Th2 | (Borish and Steinke 2003) |

16

| Cytokine | Mainly produced by | Major functions | Functional class | Reference |
|---|---|---|---|---|
| IL-6 | Mononuclear phagocytes, T- and B-lymphocytes, fibroblasts, endothelial cells, hepatocytes and others | Differentiation of B-cells to mature and immunoglobulin secreting plasma cells. Mediates T-cell activation, growth and differentiation. Similar actions as IL-1, although it inhibits IL-1, TNF, IFN-γ, GM-CSF and MIP-2 synthesis and induces acute-phase protein response. | Pleiotropic cytokine with both anti- and pro-inflammatory actions | (Borish and Steinke 2003, Opal and DePalo 2000) |
| IL-8 (CXCL8) | Mononuclear phagocytes, endothelial and epithelial cells, T-cells, eosinophils, neutrophils, fibroblasts and hepatocytes | PMN chemoattractant. Stimulates neutrophil degranulation, respiratory burst and adherence to endothelial cells by CD11b/CD18. | Pro-inflammatory | (Borish and Steinke 2003, Opal and DePalo 2000) |
| IL-10 | Monocytes, T helper lymphocytes, B-lymphocytes, cytotoxic T-cells and mast cells | Inhibits production of IFN-γ and IL-2 from Th1 lymphocytes and IL-4 and IL-5 from Th2 lymphocytes. Inhibits the production of IL-1β, IL-6, IL-8, IL-12 and TNF-α from NK-cells. Inhibits monocyte/macrophage production of IL-1, IL-6, IL-8, IL-12, TNF-α, GM-CSF, MIP-1α and MIP-2α. Inhibits monocyte CD23, ICAM-1 and B7 expression. The B7 inhibition is primarily responsible for the inhibition of Th1 and Th2 cytokine production. IL-10 inhibits cytokines associated with cellular immunity and allergic inflammation, while stimulating humoral and cytotoxic immune responses. | Mainly anti-inflammatory | (Borish and Steinke 2003, Opal and DePalo 2000) |
| IL-12 | Monocytes, macrophages, B-lymphocytes, DC, Langerhans cells, neutrophils and mast cells | The biological active form is a heterodimer of p40 and p35 called p75. Activates and induces NK-cells. Proliferates T-helper and cytotoxic lymphocytes. Induces IFN-γ production. | Th1 inducing | (Borish and Steinke 2003, Curfs, et al. 1997) |
| IL-12 p40 | Identical sources as IL-12 | The p40 subunit is homologous to the soluble IL-6 receptor. Acts as competitive antagonist by binding to IL-12 receptors without eliciting a transducing signal. | | (Borish and Steinke 2003) |

| Cytokine | Mainly produced by | Major functions | Functional class | Reference |
|---|---|---|---|---|
| **IL-13** | Th1 and Th2 lymphocytes | Shares homology with the function of IL-4 and shares the IL-4 type 1 receptor. Has similar effects as IL-4 on mononuclear phagocytic cells, endothelial cells and B-cells, i.e., VCAM-1 and IgE isotype switch, but has minimal effects on T-cell function. Downregulates the production of IL-1, IL-8, MIP-1α and TNF-α from monocytes. | Th2 > Th1 | (Borish and Steinke 2003, Opal and DePalo 2000) |
| **IL-15** | Mononuclear phagocytic cells, epithelium, fibroblasts, placenta and others | Similar action as IL-2. Chemotactic and growth factor for T-lymphocytes. Differentiates NK-cells, stimulates B-lymphocyte growth and differentiation. | Pro-inflammatory | (Borish and Steinke 2003) |
| **IL-17** | T-helper lymphocytes, activated T-cells (predominantly memory CD4$^+$CD45RO$^+$), eosinophils | Activates macrophages, fibroblasts and stromal cells (including the expression of ICAM-1 and IL-6, IL-8, IL-11, C-CSF, PGE-2 and NO). T-cell mediated inflammation | T-cell mediated inflammation | (Borish and Steinke 2003, Curfs, et al. 1997) |
| **IFN-α** | Monocytes, macrophages, B-lymphocytes and NK-cells | Antiviral activity; inhibits viral replication, protects uninfected cells. Stimulates antiviral immunity by cytotoxic lymphocytes and NK-cells. Upregulates MHC class I, mediates antitumor activity. Induces Th1 in viral infection. | Th1 inducing | (Biron 2001, Borish and Steinke 2003) |
| **IFN-γ** | T-helper lymphocytes, cytotoxic T-cells, NK-cells macrophages and dendritic cells | Increases MHC I and II expression. Stimulates antigen presentation, cytokine production and effector functions by monocytes. Stimulates killing by NK-cells and neutrophils. Induces ICAM-1. Inhibits IL-4 production and actions. Modest antiviral activity. | Th1 | (Borish and Steinke 2003) |

| Cytokine | Mainly produced by | Major functions | Functional class | Reference |
|---|---|---|---|---|
| **TNF-α** | Macrophages, dendritic cells, neutrophils, activated lymphocytes, NK-cells, endothelial cells and mast cells | Increases adhesion molecules on endothelial cells (ICAM-1, VCAM-1 and E-selectin). Activates neutrophils. Induces vascular leakage, has negative inotropic effects and is the primary endogenous mediator of toxic shock and sepsis. Induces production of IL-1, IL-6 and IL-10. | Pro-inflammatory cytokine | (Borish and Steinke 2003) |
| **GM-CSF** | T-cells (Th2), fibroblasts, endothelial cells, monocytes/macrophages, mast cells, neutrophils, eosinophils | Maturates DC, neutrophils and macrophages. Synergizes with other CSFs to support the production of platelets and erythrocytes. Activates mature neutrophils and mononuclear phagocytic cells. Prolongs the survival and contributes to the activity of eosinophils. | | (Borish and Steinke 2003, Curfs, et al. 1997) |
| **MCP-1 (CCL2)** | Monocytes/macrophages, fibroblasts, B-cells, endothelial cells and smooth muscle cells | Chemotactic for monocytes. Regulates cytokine production in monocytes and the expression of adhesion molecules on macrophages. Augments cytostatic activity. Enhanced during inflammation and infection. Induces basophil histamine release and promotes Th2 immunity. | Pro-inflammatory | (Borish and Steinke 2003, Curfs, et al. 1997) |
| **MIP-1α (CCL3)** | T- and B-lymphocytes. Monocytes, mast cells, fibroblasts and neutrophils | Chemotactic for monocytes, T-cells, neutrophils and eosinophils. Activates production of IL-1, IL-6 and TNF from monocytes and expression of β1-integrins on endothelial cells. Augments the cytolytic responses of cytotoxic T- and NK-cells. | Pro-inflammatory | (Borish and Steinke 2003, Curfs, et al. 1997) |
| **MIP-1β (CCL4)** | T- and B-lymphocytes. Monocytes, mast cells, fibroblasts and neutrophils | Chemotactic for monocytes, T-lymphocytes. Activates production of IL-1, IL-6 and TNF from monocytes and expression of β1-integrins on endothelial cells. Stimulates the adhesion of T-lymphocytes to endothelial cells. Augments the cytolytic responses of cytotoxic T- and NK-cells. | Pro-inflammatory | (Borish and Steinke 2003, Curfs, et al. 1997) |

| Cytokine | Mainly produced by | Major functions | Functional class | Reference |
|---|---|---|---|---|
| **RANTES** (CCL5) | T-lymphocytes, platelets, renal epithelium, mesangial cells | Chemotactic for monocytes, $CD4^+CD45\ RO^+$ T-cells, eosinophils and basophils. Mediates histamine release from basophils Augments the cytolytic responses of cytotoxic T- and NK-cells. | Pro-inflammatory | (Borish and Steinke 2003, Curfs, et al. 1997) |
| **Eotaxin** (CCL11) | Fibroblasts, endothelial cells, eosinophils and T-lymphocytes | Chemotactic for eosinophils and potent initiator of the respiratory burst and actin polymerization in eosinophils. Chemotactic and stimulates histamine and leukotriene release from basophils. Chemotactic for Th2 lymphocytes. | Pro-inflammatory | (Gutierrez-Ramos, et al. 1999, Simon, et al. 2004) |
| **sCD14** | Monocyte/macrophage cell surface glycoprotein | Membrane bound CD14 is a receptor for bacterial derived molecules, such as LPS and also involved in recognition and phagocytosis of apoptotic cells. CD14 is shed from the cell surface at inflammation, thus increased serum levels reflect activation of monocytes/macrophages. | | (Scherberich and Nockher 2000) |
| **sIL-4R** | Cell surface receptor expressed on a variety of cells | Normally the IL-4 receptor is expressed on cells of both hematopoietic and non-hematopoietic origin. But it can be shed from the cell surface or perhaps be produced due to alternative splicing of mRNA. <br><br> The soluble receptor can antagonize the activity of IL-4, protect IL-4 from degradation or act as a carrier protein. Thus, the exact function of the soluble receptor is difficult to predict. | | (Garrone, et al. 1991, Jung, et al. 1999, Kruse, et al. 1999) |

## MATERIAL AND METHODS

### PATIENT MATERIAL

Samples were collected, after informed consent, from women with normal pregnancies and from women with preeclamptic pregnancies. The latter group was recruited with the exclusion criteria of diabetes, chronic hypertension, multiple gestation, renal impairment or autoimmune diseases.

## Papers I–III

The same patient material was used for paper I-III, in order to analyze the number of cytokine-secreting cells (paper I), the levels of cytokines in cell culture supernatants (paper II) and cytokine levels in serum (paper III). For these analyses, blood samples were drawn in the morning from 15 women with preeclampsia and from 15 women with normal pregnancies and their respective male partners (father to be); for details see Table III. Diagnosis of preeclampsia was made according to the World Health Organization (WHO) criteria, see Table I.

Blood samples from the preeclamptic women were taken after diagnosis and admission to hospital, whereas blood samples from the healthy pregnant women were taken at the Antenatal Care Clinic at the pregnancy week matching a preeclamptic patient $\pm$ 2 gestational weeks. Four preeclamptic patients received Dexacortal (glucocorticoid) treatment ($2 \times 7.5$ mg within 24 hours) prior to the time of blood sample collection. The time between treatment and sampling varied between 1 and 11 days, median 6.5 days. Samples were also taken with an even distribution over the time of the year.

Table III Data on the participating women for papers I–III

| | Severe preeclampsia | Moderate preeclampsia | All preeclamptic | Normal pregnancy | p value |
|---|---|---|---|---|---|
| No. of patients | 7 | 8 | 15 | 15 | – |
| Maternal age, years (range) | 28 (26–30) | 29 (27–31) | 28 (26–31) | 29 (23–43) | 0.86 |
| Gestational weeks at sampling (range) | 32 (29–34) | 36.5 (33–41) | 34 (29–41) | 33 (29–37) | 0.34 |
| No. of parous women | 1 | 1 | 2 | 2 | >0.99 |
| No. with positive phadiatope | 1 | 1 | 2 | 7 | 0.11 |

Values are given as median values with range within parentheses. Mann–Whitney *U*-test was used for comparisons between groups and Fisher's exact test was used for calculation of distribution of the number of parous women and of positive phadiatope test.

## Paper IV

For paper IV, placental biopsies were collected from 11 women with severe preeclampsia and 12 normal pregnant women after informed consent. All but two women, who also participated in papers I–III, were recruited for the immunohistochemical study of paper IV. Of the preeclamptic women, five had their pregnancies affected by intrauterine growth restriction (IUGR). The diagnosis of preeclampsia and IUGR were made according to WHO criteria, see Table I. All of the preeclamptic women were previously healthy and all women, both preeclamptic and normal pregnant, did not have any interfering disease during the pregnancy. However, in order to stimulate fetal lung maturation, seven of the women with preeclampsia received glucocorticoid treatment prior to the time of sample collection. The time between treatment and sampling varied between 1 and 7 days (median 5 days). Data on the participating women are shown in Table IV.

Since it has been shown that TNF-α and IL-6 levels increase during labor (Opsjon, et al. 1993) all biopsies were collected from women with caesarian sections before the onset of labor. The reasons for caesarian sections were: pelvic disproportion, breech presentation or humanitarian reasons for the women with normal pregnancies, and fetal and/or maternal indications in the preeclamptic women.

The placental biopsy was taken immediately after delivery of the placenta. Biopsies of 1 cm$^3$ were taken from the central fetal part of the placenta, facing the decidual side, then fixed in neutral buffered 4% formaldehyde and embedded in paraffin.

Table IV Data on the participating women for paper IV

|  | Severe preeclampsia | Normal pregnancies | *p* value |
|---|---|---|---|
| No. of patients | 11 | 12 | – |
| Maternal age, years (range) | 31 (20–37) | 30.5 (24–39) | 0.69 |
| Gestational weeks (range) | 30 (27–37) | 39 (38–40) | <0.00 |
| Parity (range) | 0 (0–2) | 1 (0–2) | 0.25 |
| IUGR | 5 | 0 | 0.01 |

Values are given as median values with range within parentheses. Mann–Whitney *U*-test was used for comparisons between groups and Fisher's exact test was used for calculation of distribution of IUGR.

HANDLING OF THE BLOOD SAMPLES USED IN PAPERS I AND II

## Separation of PBMCs

The sodium-heparinized peripheral whole blood, as used in papers I and II, was separated on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) according to Bøyum (1968). The peripheral blood mononuclear cells (PBMC) were removed from the interphase and washed three times with Hanks' balanced salt solution (HBSS, Life Technologies, Paisley, UK). PBMCs thus obtained were then resuspended in culture medium (CM) consisting of Iscove's modification of Dulbecco's medium (IMDM, Gibco BRL, Paisley, UK) supplemented with (given as final concentrations in the medium): L-glutamine 292 mg/L (Sigma, St. Louis, MO, USA), sodium bicarbonate 3.024 g/L, penicillin 50 U/ml and streptomycin 50 µg/ml (Flow Laboratories, Irvine, UK), 100× non-essential amino acids 10 ml/L (Flow Laboratories) and 5% heat-inactivated fetal calf serum (FCS, Sigma). Cells were then counted in a phase contrast microscope and categorized into lymphocytes and monocytes/macrophages. The lymphocyte density of the whole sample was then adjusted to $1 \times 10^6$ lymphocytes/ml.

When handling cells *in vitro*, it is possible to study the specific response to different types of antigens. In papers I and II, paraformaldehyde fixated cells, antigens and a mitogen were used to stimulate cytokine secretion from the maternal responder cells.

## The principle of mixed leukocyte culture

PBMCs can respond to a variety of stimuli. By using inactivated, paraformaldehyde (PFA) fixated cells as a stimuli for the active responder cells (Dubey, et al. 1986, Ekerfelt, et al. 1997), it is possible to measure the one-way cytokine response of one cell type to another, as described previously (Ekerfelt, et al. 1997), see also Figure IV. Of special interest when studying reproductive immunology is the maternal PBMC response toward fetal antigens. As a model for the fetus, PFA-treated paternal PBMCs can be used to stimulate the maternal responder cells, since it is not ethically justified or practically possible to take fetal samples during the pregnancy. The fetus is a maternal/paternal genetic mix, so from the point of view of the maternal immune system, any foreign antigens on the fetus come from the father to be. Therefore, it is possible to use inactivated PBMCs from the expecting father as a stimulus, mirroring the fetal antigens. A mixed pool of inactivated leukocytes was also used to determine the maternal response toward pregnancy-unrelated HLA antigens. To be sure that the maternal response was specific toward the paternal antigens, an MLC reaction with autologous cells was also used as a methodological control. The response toward autologous cells was subtracted from the response toward the MLC pool and paternal MLC reaction in order to calculate the net specific response against these antigens.

The treatment of the stimulator cells with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, was chosen because it has been shown to fixate the cells, thereby rendering them inactive, in addition to having a high degree of retained antigenicity (Andersson, et al. 1988). Furthermore, the paraformaldehyde preserves the membrane stability of leukocytes and cell morphology (Hallden, et al. 1989).

### Mixed leukocyte culture reactions as used in papers I and II

As discussed above, three kinds of stimulator cells were used in the mixed leukocyte culture reactions in papers I and II: paternal PBMCs, autologous PBMCs, and pooled PBMCs from 18 unrelated blood donors. PBMCs from the pregnant woman were used as responder cells. The cell density for all responder and stimulator cells was adjusted to $1 \times 10^6$ lymphocytes/ml, respectively. Maternal PBMCs in culture medium were mixed with PFA-treated PBMCs from the expecting father, in order to be able to study the maternal "fetus-specific" response. To study the response toward unrelated alloantigens, pooled PBMCs

from a pool of 18 blood donors were used as stimulator cells. Autologous maternal cells were also PFA-treated and stimulation with these cells was used as a background control.

To achieve net MLC values, the value of the response to autologous cells was subtracted from the values obtained in response to allogenic stimulation with paternal and pooled PBMCs. All different types of MLC reactions, i.e., responder-paternal, responder-autologous and responder-pooled unrelated donors, were assayed in quadruplicate for detection of IFN-$\gamma$, IL-4, IL-10 or IL-12 secretion, respectively. In addition, the spontaneous secretion was determined by incubating the responder cells only in CM. For paper I, the MLC cultures were incubated in an ELISPOT-plate for 14–19 hours at 37°C in a humid atmosphere with 5% $CO_2$ added. For paper II, the MLC cultures were incubated in cell culture tubes for 2, 3, 4, 5, 6 and 7 days, respectively.

To test that inhibition of cytokine secretion was successful, all PFA-treated stimulator cells were separately stimulated with phytohemagglutinin (PHA, 20 µg/ml, Sigma-Aldrich) and assayed for cytokine secretion using the sensitive ELISPOT assay, as described in paper I. Only a few spots were occasionally found in the control wells for the PFA-treated cells, showing that the inactivation was successful.
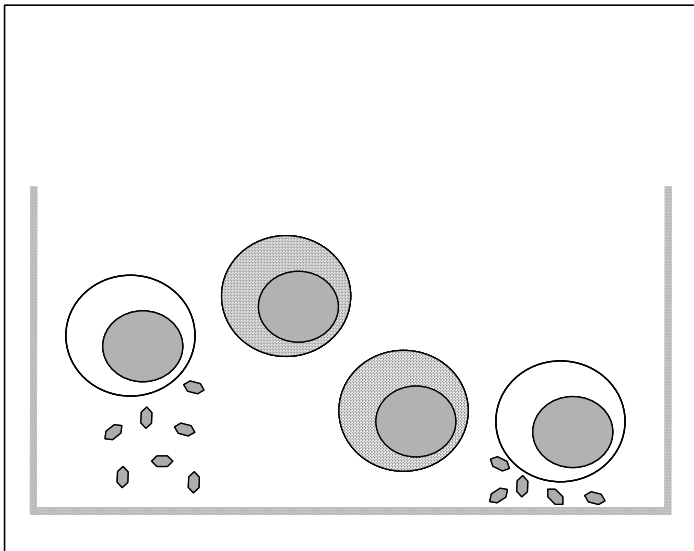


Figure IV Principle of the MLC reaction

The responder cells (white) are mixed with PFA-treated cells (gray shadow), and this one-way cell reaction can be monitored by measurement of the produced cytokines.

## Action of antigens unrelated to pregnancy and mitogen used in papers I and II

For both paper I and paper II, the maternal responder cells were also incubated with different types of antigens, chosen as antigens unrelated to pregnancy with different actions on the maternal PBMC response. The antigens and mitogen used are as follows.

Purified protein derivative of *Mycobacterium tuberculosis* (PPD) is known to induce secretion of IL-2 and IFN-γ and, in contrast, limited or no levels of IL-4, in previously immunized healthy individuals (Del Prete, et al. 1991). Therefore, 10 µg/ml of PPD was used in papers I and II as a Th1-specific stimulus for T-lymphocytes.

Tetanus toxoid (TT; SBL Vaccin, Stockholm, Sweden) has been shown to induce secretion of both IFN-γ and IL-4 from T-cell clones in a previously immunized healthy individual (Parronchi, et al. 1991). TT at the concentration of 5 Lf/ml was therefore used in papers I and II as an immunostimulatory agent for PBMCs. Furthermore, the IFN-γ response of PBMCs toward TT has also been shown to be relatively constant in individuals during a 2-year follow-up period, when measured by ELISPOT (Mayer, et al. 2002). This indicates that the tetanus toxoid is a suitable immunostimulatory agent, and that the ELISPOT method produces reliable results and is not subject to much variation.

Lipopolysaccharide (LPS) is a product of the cell wall of Gram-negative bacteria and, as reviewed by Emödy et al. (2003), has known effects on the cytokine synthesis of IL-1 and TNF-α, as well as enhancing the inflammatory response. It also functions as a polyclonal B-cell mitogen, since the LPS molecule exerts an immunoadjuvant effect that promotes the humoral immune response to other antigens of the pathogen. LPS from *Escherichia coli* 026:B6 (Sigma, St. Louis, MO, USA) was used in paper I.

Every antigen culture (PPD, TT and LPS) in paper I was assayed in triplicate for detection of the number of IFN-γ, IL-4, IL-10 and IL-12 secreting cells after 38–42 hours of incubation at 37°C in a humid atmosphere with 5% $CO_2$ added. For paper II, the antigen cultures were incubated for 2, 3, 4, 5, 6 and 7 days, respectively. In addition, spontaneous secretion in papers I and II was determined by culturing the responder cells in only CM. To achieve net antigen values, the value of the spontaneous secretion, produced on the antigen stimulation plate, was subtracted from the values obtained in response to the antigens (PPD, TT, LPS).

Phytohemagglutinin A (PHA; Sigma-Aldrich) is a plant lectin that binds to the T-cell receptor (TCR) in an antigen-mimicking way, thereby causing a powerful

activation of T-lymphocytes, i.e., having a mitogenic effect (Licastro, et al. 1993). Therefore, 20 µg/ml PHA per well was used as a positive control in papers I and II.

The MLC reaction was used both for paper I, where the number of cytokine-secreting cells was enumerated by ELISPOT and for paper II, where ELISA was used to measure the levels of the cytokines in the cell culture supernatants. Likewise, the antigen/mitogen stimulation was used for both papers I and II, with the exception of LPS, which was not included in the studies of cell culture supernatants in paper II.

## LABORATORY TECHNIQUES

# ELISPOT

The enzyme-linked immuno-spot assay (ELISPOT) is a method designed to measure the number of cytokine secreting cells *in vitro* (Czerkinsky, et al. 1988, 1984). It is a sensitive method, able to detect single cytokine secreting cells, and about 10-200 times more sensitive than a conventional ELISA performed on cell culture supernatants (Tanguay and Killion 1994) By using this method it is possible to estimate the fraction of activated cells responding to a certain type of stimuli, but it does not give any clue about the biological levels represented in the human body. It can be used to monitor differences between different states, e.g., sick/healthy and before/after treatment. This method is especially good if one is interested in monitoring cytokines that are difficult to detect, e.g., IL-4. For IL-4 ELISPOT is the absolute superior method of detection, since IL-4 itself is known to be difficult to detect (Bullens, et al. 1998, Ekerfelt, et al. 2002). The drawback of this method is that it is labor intensive and that the manual counting of spots can be subject to bias, therefore the same person, blinded to the diagnosis, should perform all the counting throughout a study.

## *Principle of the ELISPOT method as used in paper I*
The principle of the ELISPOT used in paper I is shown in Figure V. The cytokine produced by a specific cell is captured on primary antibodies precoated on the nitrocellulose membrane at the bottom of the ELISPOT wells. After incubation, the cells are washed away and a secondary biotinylated antibody, recognizing a different epitope than the primary antibody, is added to the wells and allowed to bind to the bound cytokine. Excess antibodies are then washed away before streptavidin conjugated to alkaline phosphatase is added and allowed to bind to the biotin molecule on the secondary antibody. After an additional wash, the alkaline phosphatase substrate is added to the wells and when it reacts with the enzyme, an insoluble blue-colored product is formed and

precipitates at the site of the binding area of the secondary antibody. After a final wash, to remove excess substrate, the plates are allowed to dry and the sites of the enzymatic reaction, i.e., the location of the cytokine-producing cells, can be analyzed using a light microscope or an ELISPOT reader. The cytokines studied in paper I were IFN-γ, IL-4, IL-10 and IL-12, which were chosen as markers for the Th1/Th2 balance and anti-inflammatory status.

As a negative control, one well per cytokine and plate was incubated with CM alone, without cells, otherwise treated as the other wells. No spots were found in any of these wells. A very low number of spots was occasionally found in the control wells for the PFA-treated cells (PHA autologous, PHA father and PHA MLC pool), but the numbers were not considered to have any influence. PHA stimulation of responder cells always elicited strong responses for all cytokines.
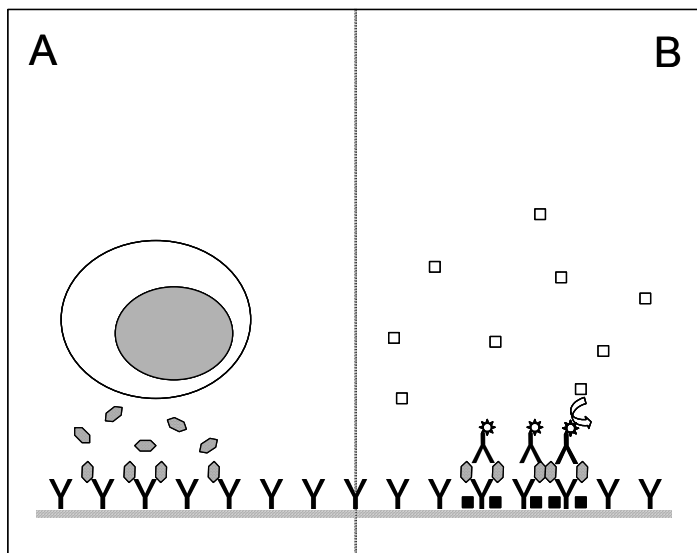


Figure V The principle of ELISPOT

(A) The cytokine produced from a cell is captured onto the primary antibodies. (B) After removal of the cells, the secondary antibodies, enzyme and color substrate are added. The reaction between the enzyme and substrate forms an insoluble product that stains the membrane of the well, thus creating an imprint of the cell.

Because the MLC reactions sometimes generated some cellular debris in the ELISPOT well, these spots were not suitable for counting in the ELISPOT reader, since the machine had trouble distinguishing a genuine spot from an artifact. Therefore, the MLC spots and the accompanying spontaneous spots were counted manually, by the same person, blinded to the diagnosis. On the other hand, it was possible to use the reader for the PPD-, TT- and LPS-

stimulated spots and their accompanying spontaneous spots. Since it is quite cumbersome to count spots manually, the antigen-stimulated spots were counted with the aid of an ELISPOT reader. This difference in how the spots were counted will not affect the results, since the results from the MLC and antigen stimulation were counted, treated and further analyzed separately. It is the spontaneously formed spots from the MLC plates that were used for comparisons between the women with preeclampsia and those with normal pregnancies. The spontaneously formed spots on the antigen stimulation plates were only used to obtain the net secretion toward each antigen.

# ELISA

Another way to study the cytokine production is to use the enzyme-linked immunosorbent assay (ELISA). The ELISA technique was originally developed by Engvall and colleagues (1971, 1971) to quantify the amounts of IgG and has since undergone a number of modifications to allow detection of both antibodies and antigens (Kemeny 1992). With the sandwich type of ELISA, as used in papers II and III, it is possible to detect the levels of cytokines in fluids, in this case supernatants and serum. The drawback of this method is that it, like ELISPOT, shows relatively high inter-assay variation and is sensitive to changes, whether this is change of antibodies, pH of the coating buffer, or temperature in the laboratory. By including standardized controls and calculating the cytokine values from a standard curve included on each plate, it is possible to obtain reliable cytokine values that can be used in a comparison between patient groups or between before and after treatment.

## *Principle of the ELISA method as used in papers II and III*

In paper II the cytokines IL-5, IL-6, IL-10, IL-12, IL-13 and TNF-α were analyzed in cell culture medium (CM). These cytokines were chosen as markers for the Th1/Th2 and pro- and anti-inflammatory balance. In paper III, the ELISA method was also used for detecting the levels of the soluble IL-4 receptor (sIL4R) and soluble CD14 (sCD14). As suggested from studies in mice by Chilton and Fernandez-Botran (1993), the level of the soluble IL-4 receptor was originally thought to reflect the IL-4 level, therefore the receptor level was chosen as a Th2 marker in paper III. The level of soluble CD14 can be used as a marker for macrophage activation (Scherberich and Nockher 2000), since this receptor is shed from the macrophages upon activation.

Basically, the principle for the ELISAs used in paper II was as follows. Primary antibodies were coated onto the walls of the polystyrene microtiter wells before portions of the cell culture medium were added and incubated in the wells. In parallel, a standard curve of recombinant cytokines was also incubated on the

plate. Next, the wells were washed and a secondary biotinylated antibody, recognizing a different epitope than that of the primary antibody, was added to the wells and incubated. Excess antibody was washed away, and streptavidin poly-horseradish-peroxidase (HRP) was added and allowed to bind to the biotin on the secondary antibody. After washing away excess streptavidin-HRP, the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to the wells. When it reacts with the enzyme the substrate forms a yellow, soluble product, in proportion to the amount of cytokine bound to the primary antibody. The color intensity is then detected with a spectrophotometer and the amount of cytokine in the sample is determined by comparison with the standard curve. For details, see Figure VI.
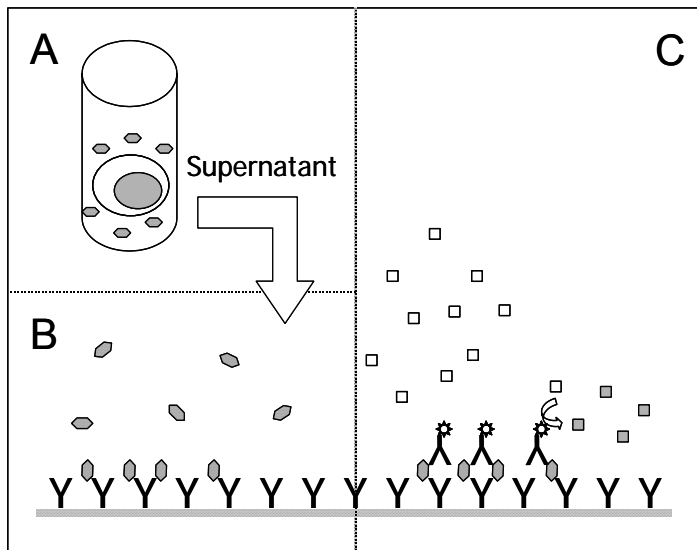


Figure VI The principle of ELISA

(A) The cytokines produced in a cell culture are transferred to a microtiter well, (B) pre-coated with capture antibodies. (C) Secondary antibodies conjugated with enzyme react with the substrate to form a colored soluble product that can be detected by spectrophotometry. The amount of colored product is in proportion to the captured cytokine.

The mean value of duplicates was used and the OD from the CM alone was subtracted from the samples and the standard curve before the concentration was calculated. The lower detection limits were 3.9 pg/ml for IL-5, 1.3 pg/ml for IL-6, 3.1 pg/ml for IL-10, 7.8 pg/ml for IL-12, 63 pg/ml for IL-13, and 3.9 pg/ml for TNF-α. When necessary, samples with known high cytokine levels were diluted in CM before the levels were determined by ELISA.

Net spontaneous secretion was obtained by subtracting the CM values from the spontaneous secretion. Net paternal antigen-induced secretion was calculated by subtracting the values obtained from stimulation with inactivated autologous PBMCs from the values obtained from stimulation with inactivated paternal PBMCs. The spontaneous secretion was subtracted from TT-, PPD- and PHA-stimulated secretion to obtain antigen/mitogen-induced secretion.

In paper III, the levels of sCD14 and sIL-4 R in serum were determined by Quantikine ELISA kits (R & D Systems, Minneapolis, MN, USA). The principle is basically the same as described above, with the exception of the plates, which were pre-coated, and the reagents, which were supplied by R & D Systems.

All measurements of cytokines, sIL-4R and sCD14 were made on freshly thawed samples, i.e., re-frozen and re-thawed material was never used in these studies.

## Blood status and allergy screening

In paper II, additional analysis of blood status and allergy screening was performed at the Department of Clinical Chemistry, University Hospital Linköping, Sweden. The blood cell counting and distribution of leukocyte populations were analyzed using a cell-counter instrument (Cell-Dyn 4000; Abbott Diagnostic, Santa Clara, CA, USA). Allergy screening of serum samples was done using the Phadiatop system (Pharmacia, Uppsala, Sweden), which detects IgE antibodies against common air-borne allergens (*Dermatophagoides farinae*, *D. pteronyssinus*, *Cladosporium herbarum* and pollen from timothy grass, mug worth and birch tree, as well as dandruff from dog, horse and cat).

## Multiplex bead array analysis

Multiplex bead array systems are a development of the ELISA technique with the advantage of multiple analyses simultaneously using a very small sample volume (50 μl). It has in general the same advantages and disadvantages as the ELISA, but the multiple detection system can give a more accurate and broader picture (due to the large number of analytes analyzed at the same time) of what is happening in a biological system, whether it is an organism or cell culture. The method is also faster to work with than the ELISA and since it requires less sample volume, it is of benefit to the patient, who needs to donate less volume of, e.g., blood, than when the same tests are performed with the traditional ELISA.

*Principle of the multiplex bead array analysis*

Instead of using the wells of the microtiter-plate, as in the ELISA technique, fluorescently labeled microspheres are used as the solid support for the antibodies. The mixture of two fluorescent dyes in the beads allows up to 100 different types of individually labeled microspheres. The primary antibodies, specific for the protein of interest, are covalently attached to a unique dyed sphere before the bead/primary antibody complex is added to the sample of interest. The secondary antibody, coupled to a fluorochrome, reports the amount of cytokine attached to the bead/primary antibody complex. In this way, the combination of spheres and antibodies allows for simultaneous detection of, e.g., several cytokines. In paper III the immunological markers IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p40, IL-13, IL-15, IL-17, IFN-α, IFN-γ, TNF-α, GM-CSF, MIP-1α, MIP-1β, MCP-1, eotaxin and RANTES were analyzed in serum by using multiplex bead array kits from Biosource (Camarillo, CA, USA). The analyses of the levels of cytokines were then made using the Luminex 100 instrument (Luminex Corp., Austin, TX, USA) and STarStation software (V1.1, Applied Cytometry Systems, Sheffield, UK).

In short, the method is as follows. Fifty microliters of sample or standard solution is added to 25 µl of the bead mixture in a well on the filter plate. The analyte of interest or recombinant standard is then allowed to bind to its respective primary antibody on the spheres during the 2 hours of incubation. After washing, a mixture of biotinylated secondary antibodies is added to each well and allowed to bind to the captured analyte on the beads. After removal of excess antibodies, streptavidin-RPE (fluorochrome) is added and allowed to bind to the biotin on the secondary antibodies during the third incubation step. The excess streptavidin-RPE is washed away and the fluorescence of the bead (which identifies the immunological marker) and the RPE fluorescence are then quantitated. The RPE fluorescence is directly proportional to the concentration of each analyte present in the original specimen. Thus it is possible to detect the levels of each specific cytokine. The principle is also shown in Figure VII.

The whole procedure was performed according to the manufacturer, except that nine standard points, instead of the usual seven, were used. Extra serial dilutions of the standard curve were performed to obtain a more sensitive standard curve that reached further down to lower detection levels. This proved helpful, since most of the samples showed quite low levels of analytes. All multiplex bead array measurements of cytokines and chemokines, were made on freshly thawed samples, i.e., re-frozen and re-thawed material was never used in these studies.
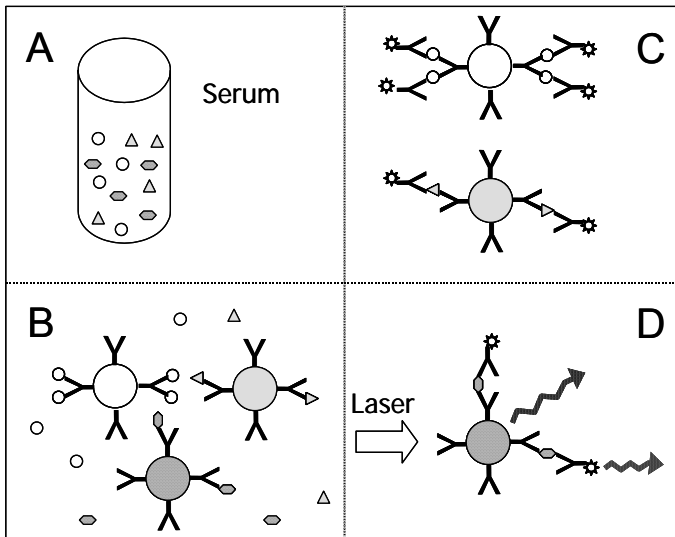
Figure VII The principle of the multiplex bead array technique

The serum sample containing cytokines (A) is mixed with the detection beads (B).
After incubation, the fluorochrome-labeled secondary antibodies bind to the captured
cytokines (C) and the bead region, which is cytokine specific, and amount of cytokine
can be detected by laser analysis (D).

## Immunohistochemistry

Immunohistochemistry is a good method when analyzing where in the body or
tissue specimen a particular cytokine is expressed. It gives a good picture of
where a cytokine is located and thus can provide information about the action of
the cytokines. Unfortunately, this method does not discriminate between locally
produced cytokine and cytokine uptake, but only shows where the cytokine is
located. Moreover, this is a laborious method and, as with all antibody-based
methods, its drawback lies in the specificity of the antibodies used and in this
case the fixation process, general sample treatment, and epitope retrieval may
alter the antigenicity of the cytokine.

*Principle of the immunohistochemistry used in paper IV*

Immunohistochemical studies were performed in paper IV to analyze the
presence of IL-4 and TNF-α, CD45^{+} infiltrating cells, and villous morphology of
the placenta. Formalin-fixed and paraffin-embedded placental biopsies were
sectioned (4 μm thick) and placed on silianized glass microscope slides. After
deparaffinization with xylene preparation and rehydration in alcohol of
decreasing concentration, the tissue sections were cooked for heat-induced
epitope retrieval. Citrate buffer (pH 6) was used for the TNF-α epitope retrieval
and Tris–EDTA buffer (pH 9.0) for the IL-4 and CD45 epitope retrieval.

To detect cytokines and the presence of CD45^{+} infiltrating cells the primary antibodies, mouse monoclonal $IgG_1$ antihuman TNF-$\alpha$ (CY-014, clone 35G10, diluted 1:100) (Innogenetics, Gent, Belgium), mouse monoclonal $IgG_1$ IL-4 (MAB 304, clone 3007, diluted 1:50) and mouse monoclonal $IgG_1$ antihuman CD45 (2B11+ PD7/26, DakoCytomation, Glostrup, Denmark) were added to the sections and allowed to incubate. As negative control, the irrelevant mouse $IgG_1$ was used (X0931, DakoCytomation). After removal of excess primary antibodies, the secondary peroxidase coupled goat antimouse/rabbit antibody (DAKO EnVision™ HRP) was added to the sections. Following an additional wash to remove excess secondary antibody, the substrate diaminobenzidine (DAB) (K 5007, DakoCytomation) was added to the sections. When the substrate reacts with the peroxidase a brown insolvable product is formed that stains the cells with bound cytokine/antibody complex. This reaction is similar to the ELISPOT reaction; for more details see Figure VIII. In addition, after the DAB-antibody staining, the sections were also counterstained with Mayer's hematoxylin.

Formalin-fixed and paraffin-embedded human tonsil sections, from tonsillectomized patients, were used as positive staining controls for the IL-4 and TNF-$\alpha$ antibodies. The CD45 antibody is routinely used for clinical analysis at the Department of Pathology, thus the function of the antibody is regularly tested. The primary mouse $IgG_1$ isotype control gave, as expected, no staining, thus the antibodies used in this study were functioning properly.

A section of each placental biopsy was also stained with hematoxylin and eosin to determine the placental morphology regarding chorangiosis, trophoblastic knots, perivillous fibrin deposits, and villitis. These four parameters can be associated with circulatory insufficiency in the placenta, but they can also be an imprecise phenomenon of the mature placenta. In placentas from normal pregnancies, the distribution is 2–6 fetal capillaries per villus. The classification of chorangiosis, as seen for example in preeclampsia, was used in our study when more than 10 terminal villi showed 10 or more capillaries in several areas of the placenta (Kraus 2004). The presence of syncytiotrophoblastic knots is believed to indicate circulatory problems in the placenta, though it may also be a phenomenon of the mature placenta. Perivillous fibrin deposits are normally present in the placenta toward the end of gestation and for this study, the normal distribution was classified as 1–10% (grade 1) of the total area of the section, and the abnormal distribution as 10–50% (grade 2) and 50–100% (grade 3) of the total section area. The sections were also classified as having villitis or not, based on the morphology seen in the hematoxylin and eosin-stained sections in combination with the sections stained for CD45^{+} cells (Kraus 2004).

Examination of cytokine distribution was done under a light microscope with the scorer blinded to the clinical diagnosis at the time of assessment. The specimens were assessed for staining in villous trophoblasts, villous stroma and fetal endothelial cells within villi. The staining intensity was assessed in the grades of no staining = 0, weak staining = 1, intermediate staining = 2 and strong staining = 3, and the percentage of stained cells versus non-stained cells in each cell category was given the grades 25 (0–25%), 50 (26–50%), 75 (51–75%) and 100 (76–100%), for any staining.
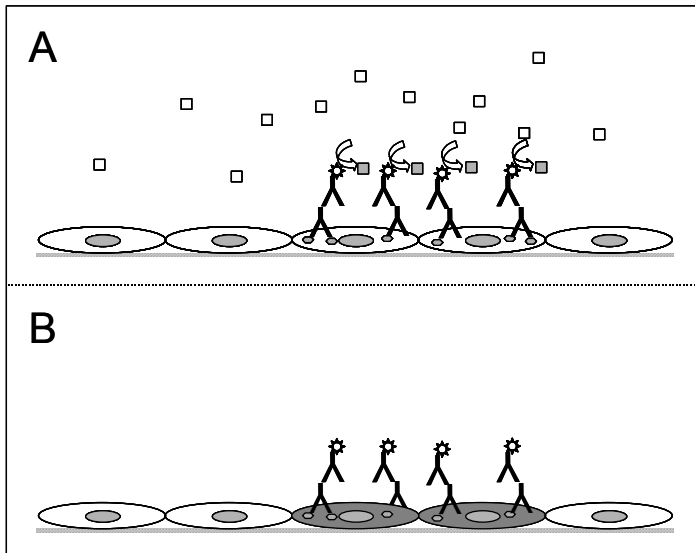


Figure VIII The principle of immunohistochemistry

(A) Primary antibodies bind to intracellular cytokine. The secondary antibody is then added and the enzyme coupled to the antibody reacts with the substrate, thus staining the location of the intracellular cytokine.

## DATA HANDLING AND STATISTICS

Non-parametric statistical tests were used in all papers, due to some outliers and the uneven distribution of parameters obtained from the small number of women recruited for the studies.

The Mann–Whitney $U$-test was used for unpaired comparisons in all papers. The Spearman rank correlation test was used for correlations in papers II and IV. Fisher's exact test was used for examining the distribution of measured variables between the groups in papers II and IV.

Bonferroni's correction was carried out to avoid mass significance.

Calculations were performed using StatView version 5.0.1 (SAS Institute Inc., Cary, NC, USA).

## ETHICS

All studies were approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping.

# RESULTS AND DISCUSSION

## TH1/TH2 BALANCE

In this section, the cytokine findings related to the Th1/Th2 balance will be reported and discussed. As mentioned earlier in Table I, the cytokine IFN-γ is the signature cytokine for Th1 responses, and IL-4 and IL-5 are the signature cytokines for Th2 responses. In addition, IFN-α and IL-12 are Th1-inducing, and could thus be considered as Th1-associated. IL-13, which in man is not a true Th2 cytokine, but still produced more by Th2 than Th1 responses, will therefore be discussed here as one of the Th2 cytokines.

## Th1/Th2 results and discussion

No differences between preeclampsia and normal pregnancy in the number of IFN-γ, IL-12 or IL-4 secreting cells were detected in PBMC by ELISPOT, neither for spontaneous secretion or antigen-induced secretion (paper I).

In paper II, when measuring the concentration of cytokines in cell culture supernatants by ELISA, preeclamptic women showed decreased levels of spontaneously secreted IL-5 compared with those of normal pregnancy, as Figure IX. No other differences were found for IL-5 or spontaneous or antigen stimulated secretion of IL-13. IL-12 was below the detection limit in all stimulations. However, the number of basophils in peripheral blood was decreased in preeclampsia compared with normal pregnancy, which supports the finding of decreased IL-5, since IL-5 stimulates (Miura, et al. 2001, Norman 2004) and promotes survival of basophils (Miura, et al. 2001).

To better understand the ongoing *in vivo* situation, the cytokine levels in serum were determined in paper III. The levels of IFN-γ, IL-4, IL-5 and IL-12 p40 were detected in the serum; however, no significant differences were found

between women with preeclampsia and those with normal pregnancies. IL-13 was below detection limits in all samples.

In addition to the cytokine measurements, the levels of the soluble IL-4 receptor were also determined in paper III. The levels of the soluble IL-4 receptor, but not the IL-4 levels, were increased in serum from women with preeclampsia compared to women with normal pregnancies. See also Figure X.

In paper IV, IL-4 cytokine staining was observed in placental tissue. The cytokine was either completely missing in a sample, or simultaneously detected in the trophoblastic layer, fetal endothelium and fetal stroma, see Figure XIII. However, there were no statistical differences in the staining intensity for IL-4 between placental sections from women with preeclampsia and from women with normal pregnancies, nor was there any statistical difference in the number of stained cells in the placental sections.

Overall, in this thesis the findings of decreased spontaneous secretion of IL-5 from PBMC, combined with decreased numbers of basophils—both of which are associated with Th2 responses—indicates a decrease in Th2 responses in preeclampsia compared with normal pregnancy. However, this could not be confirmed when measuring IL-5 in serum. This discrepancy in the IL-5 results may be explained by the fact that T-cell cytokines act in a paracrine manner, i.e., cytokines are secreted in the close vicinity of receptor-expressing cells, which bind the cytokine shortly after it has been secreted. In line with this, a local alteration of IL-5 secretion would not be reflected in serum. Spontaneous secretion from PBMC *in vitro*, on the other hand, may better reflect local secretion since the cells more likely will show some grade of activation, as they have been taken out of the circulation and handled *in vitro*. No other signs of decreased Th2 responses in preeclampsia, i.e., the secretion of IL-4 or IL-13 from PBMC, the levels of IL-13 in serum and the local expression of IL-4 in placenta, was found, which makes the interpretation of the results in terms of Th1/Th2 balance difficult. Furthermore, the serum level of the soluble IL-4 receptor (sIL-4R), which was chosen as a marker thought to mirror IL-4 levels, was increased in preeclampsia. It has to be noted that this finding only fulfilled the lower demand on the significance level that was used for the screening of novel immunomarkers, and thus has to be interpreted with caution.

In paper II, decreased numbers of basophils were found during preeclampsia, while previous studies have shown increased (Barden, et al. 1999) or unchanged levels (Lurie, et al. 1998) during preeclampsia. IL-5 activates B-cells, as well as stimulating differentiation and activation of eosinophils and basophils (Curfs, et al. 1997). Furthermore, IL-5 affects basophil actions and has, for example, been shown to induce faster adhesion of basophils to bovine serum albumin *in vitro* (Quan, et al. 2002). Thus, our present data could indicate that effector responses

mediated by B-cells, as well as by eosinophils and basophils, may be lowered in cases of preeclampsia compared with normal pregnancies. Our finding of decreased numbers of basophils in cases of preeclampsia is contrary to previous studies but in line with the decreased secretion of IL-5, strengthening the biological relevance of this finding. The significance of basophils in the maintenance of pregnancy is unknown today.
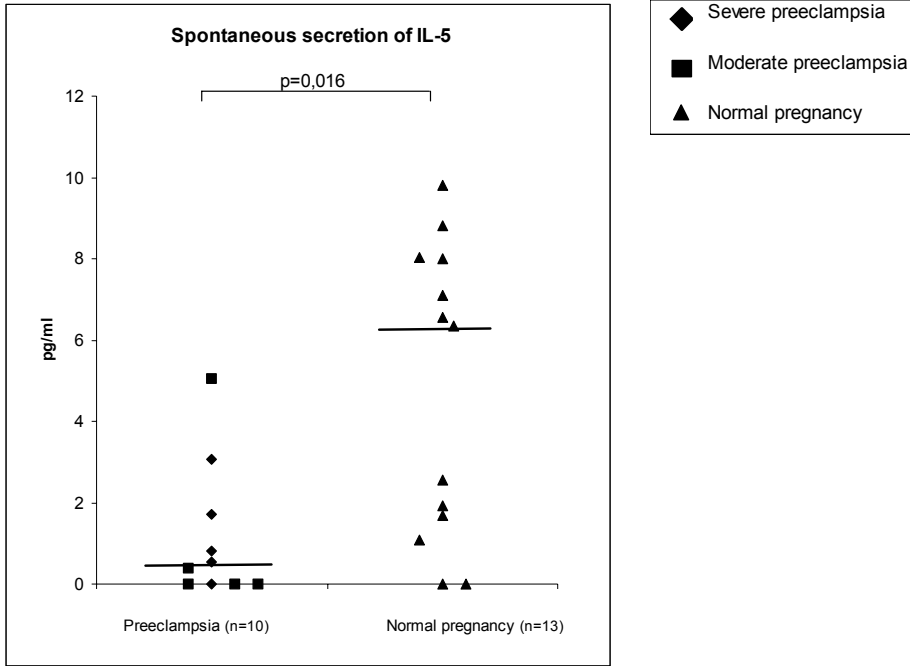


Figure IX  Spontaneous secretion of IL-5 in cell culture supernatant

Serum concentration of the Th2 cytokine IL-5. *p*-values from the comparison with the Mann–Whitney *U*-test is shown. Median is indicated

Our findings may reflect a decreased ability to generate allergic inflammation in preeclampsia, which in turn indicates an altered Th1/Th2 balance compared with normal pregnancy. To examine whether the differences in Th2-like immunity were related to different incidences of atopic allergy in the groups, allergy screening of all women was performed. The allergy screening indicated atopic allergy in 2 of 15 preeclamptic women and in 7 of 15 women with normal pregnancies. This difference was, however, not significant and would thus not explain the differences in basophil numbers.

The indication of increased sIL-4R levels in serum from preeclampsia was surprising, and could be considered as contradictory to the other findings of this

thesis. In mice spleen cells, sIL-4R has been suggested to be a substitute marker for the levels of IL-4, since the soluble receptor levels increased in response to increasing levels of IL-4 (Chilton and Fernandez-Botran 1993). Since IL-4 is known to be difficult to detect (Bullens, et al. 1998, Ekerfelt, et al. 2002) and it has been observed that the proteolytic cleavage of the sIL-4R is different from IL-4 (Jung, et al. 1999), allowing sIL-4R to remain longer in the circulation, it was an appealing idea to use the receptor levels as a marker for the Th2 status in serum from women with preeclampsia. However, as shown in paper III, divergent results between the cytokine measurements and the levels of the sIL-4R were obtained. In serum there were no differences in the levels of the IL-4 cytokine between women with preeclampsia and those with normal pregnancies, but women with preeclampsia showed higher serum levels of the soluble IL-4 receptor ($p = 0.037$).

The current finding of increased sIL-4R levels in preeclampsia may have several possible explanations. First, if the receptor levels reflect the cytokine levels this means that the hypothesis of decreased Th2 responses in preeclampsia could be questioned.

Second, the sIL-4R might not reflect IL-4 levels. As discussed in Chilton et al. (1993), murine sIL-4R can compete with membrane bound IL-4R for the cytokine in a negative regulatory way, thus inhibiting the actions of IL-4, or acts as a carrier of the cytokine, i.e., delaying clearance of the cytokine and prolonging the half-life of the cytokine, thus prolonging the actions of IL-4 *in vivo*.

Third, there might exist a negative correlation between IL-4 levels and sIL-4R levels as reported by Protonotariou et al. (2003). Previous studies in human *in vitro* models (Garrone, et al. 1991), and *in vitro* and *in vivo* murine models (Chilton and Fernandez-Botran 1993, Gessner, et al. 1994) have shown that sIL-4R can neutralize unbound IL-4, suggesting that sIL-4R plays a role in IL-4 regulation. Speculatively, since sIL-4R is believed to persist longer in the circulation than IL-4 (Jung, et al. 1999), the increased level of sIL-4R in preeclampsia may reflect a current decrease of IL-4 secretion, i.e., sIL-4R was shed from Th2 cells upon IL-4 secretion earlier in pregnancy and, after neutralization of all circulating IL-4, only the excessive amount of sIL-4R could still be detected.

Fourth, the receptor levels might be increased to suppress the Th2 reactions. In studies on IL-12-treated cancer patients, Haicheur et al. (2000) found elevated systemic levels of IL-4 mRNA, but no detectable serum levels of the cytokine IL-4, although the sIL-4R was increased in plasma from these patients. These findings were interpreted as that the induction of sIL-4R might be an additional

mechanism by which IL-12 inhibits the development of the Th2 response *in vivo*. Although no differences in the serum levels of IL-12 were detected between women with preeclampsia and normal pregnancies in paper III, the divergence in the findings of IL-4 and sIL-4R in that study could also be explained by the findings of Haicheur et al. (2000). The levels measured in paper III were those of IL-12 p40, which is not the bioactive form of the cytokine. Therefore, it is possible that the levels in serum of the bioactive form, IL-12 p70, which was not measured, could differ between women with preeclampsia and those with normal pregnancies. In paper I, however, which measured the number of IL-12 p70 secreting cells, there were no differences between women with preeclampsia and normal pregnant women. Unfortunately, the levels of IL-12 p70 were below the detection limit of the standard ELISA used in paper II. The use of a high-sensitivity ELISA might enable the finding of differences in IL-12 p70 between women with preeclampsia and those with normal pregnancies in future studies, which when taken together with the increased levels of sIL-4R would support the suggestion of sIL-4R as a marker for suppression of Th2.

Considering sIL-4R as a possible marker for Th2 suppression makes the indication of an increase of this marker in preeclampsia a finding in line with the decreased levels of IL-5 and of basophil numbers. Hence, although no differences in the expression of IL-4 and IL-13 were detected, the present data on decreased Th2 responses, in some aspects, would partly support the hypothesis of a deviation toward Th1 in preeclampsia compared with normal pregnancy. On the other hand, no hallmarks of a corresponding increase of Th1 responses in preeclampsia were found, which also would have been expected in line with the hypothesis. The Th1/Th2 concept has, however, been repeatedly questioned (Allen and Maizels 1997, Borish and Rosenwasser 1997) since most immune responses do not reflect an absolute Th1 or Th2 pattern (Kelso 1995). Thus, the Th1/Th2 concept is likely an oversimplification, although immune responses generally seem to be functionally dominated by Th1 or Th2 cytokines, which exert antagonistic effects (Kelso 1995, Parronchi, et al. 1992b) and the cytokine patterns are not strictly restricted to the classical Th1/Th2, as illustrated by the data of this thesis. It also has to be kept in mind that the number of samples analyzed in these studies is quite small and type II errors cannot be excluded, i.e., differences could exist in the study material that were not detected due to the relatively small number of samples.
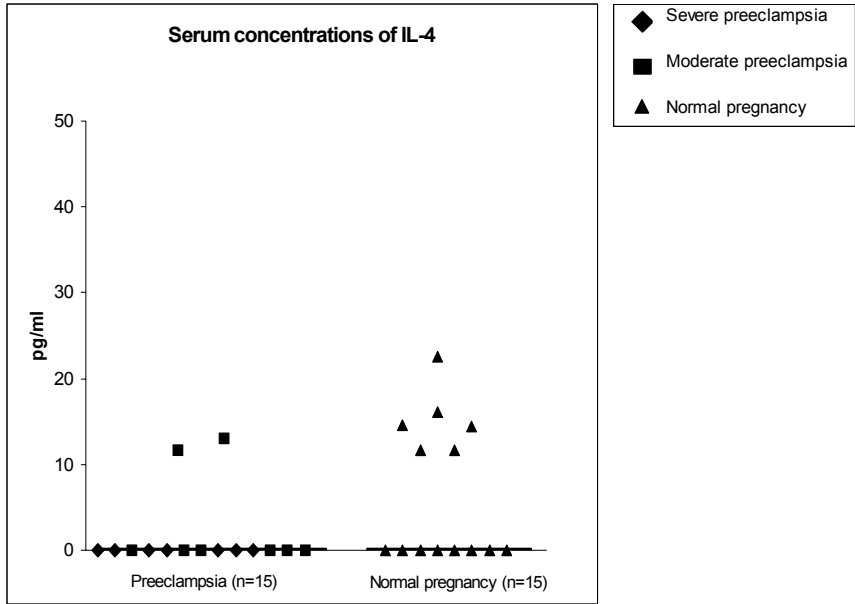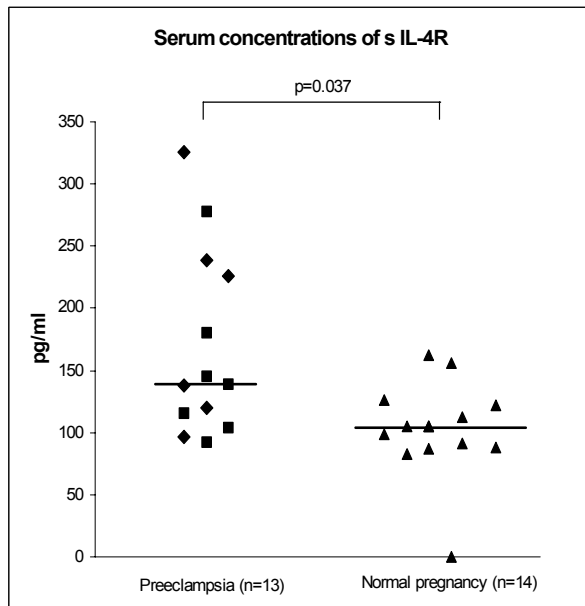
Figure X Serum levels of IL-4 and sIL-4R.

Serum concentrations of the Th2 cytokine IL-4 (A) and the soluble IL-4 receptor (B). *p*-values from the comparisons with the Mann–Whitney *U*-test are shown. Median is indicated

To our knowledge, the report made in paper II of decreased spontaneous secretion of IL-5 in PBMC cultures is the first publication concerning decreased levels of IL-5 during preeclampsia. Recently, another report was published dealing with the levels of IL-5 and other cytokines during preeclampsia (Azizieh, et al. 2005). Azizieh et al. examined the Th1/Th2 balance in PHA-stimulated PBMCs and also found decreased levels of IL-5 in women with preeclampsia compared to normal pregnant women. Furthermore, they found increased levels of IFN-γ together with decreased levels of IL-4, suggesting that there is an increase in Th1 responses and a decrease in Th2 responses during preeclampsia, the latter corroborating our finding.

Sakai et al. (2002) found, in contrast to us, increased spontaneous levels of IL-12 p70 in PBMC cultures from severely preeclamptic women compared to normal pregnant women, but were unable to detect differences between women with moderate preeclampsia and those with normal pregnancy. Although few samples showed detectable levels of IL-12 p70, Daniel et al. (1998) also found increased plasma levels of IL-12 during preeclampsia compared to normal pregnancy.

The functional IL-12 p70 molecule, which binds and signals through the IL-12 receptor, is a heterodimer, consisting of two chains, p35 and p40. The p40 chain can be secreted solely, and binds to the IL-12-receptor, but induces no signal in the cell. Consequently, p40 can be considered as an inhibitor of IL-12 responses, competing with the functional p70 heterodimer in the binding to the IL-12 receptor (Borish and Steinke 2003). The IL-12 p70 findings in paper I are in line with the findings of Sacks et al. (Sacks, et al. 1997) and Dudley et al. (Dudley, et al. 1996), who found no evidence that the heterodimer of IL-12 is increased in serum or plasma in preeclampsia. In contrast, an elevated production of p40 IL-12 was reported in preeclamptic pregnancies compared with normal pregnancies (Dudley, et al. 1996),which we could not confirm in paper III. Taken together, the results on IL-12 in preeclampsia are contradictory and the role of IL-12 in preeclampsia needs to be further investigated.

Rein et al. (2002) were not able to detect differences between women with preeclampsia and women with normal pregnancies regarding the phorbol-12 myristate-13 acetate (PMA)-induced intracellular expression of IFN-γ and IL-4 in T-cells. On the other hand, by using flow cytometry analysis, Satio et al. (1999a) found a dominance of the number of Th1 cells over Th2 cells and IFN-γ dominance over IL-4 in preeclampsia compared to normal pregnancy. These findings are further supported by Sakai et al. (2002), who also found a dominance of Th1 over Th2 cells in preeclampsia.

Saito et al. (1999b) detected increased spontaneous and PHA-stimulated levels of IFN-γ from PBMC cultures from women with preeclampsia compared to normal pregnant women. Spontaneous levels of IL-4 did not differ between the two groups, but PHA stimulation led to increased levels of IL-4 from women with normal pregnancies. This was interpreted as an increased Th1 response and a non-functioning Th2 response during preeclampsia.

Darmochwal-Kolarz et al. (1999), found increased levels IFN-γ in PHA-stimulated PBMC cultures. However, they were not able to detect any differences in the expression of IL-4 in stimulated PBMCs from women with preeclampsia and those with normal pregnancies (Darmochwal-Kolarz, et al. 2002). On the other hand, they found significantly elevated levels of IFN-γ in NK cells from women with preeclampsia.

Omu et al. (1996) found increased levels of IL-4 in serum from women with preeclampsia when compared with normal pregnant women. In addition, they were able to show that antihypertensive treatment has no effects on the serum levels of IL-4. That antihypertensive drugs do not affect the production of IL-4 is valuable knowledge, especially since some preeclamptic women receive this treatment to relieve the symptoms of preeclampsia.

Henriques et al. (1998) was also able to detect IL-4, but in placental tissues. However, they were unable to detect any difference between placentas from preeclamptic pregnancies and those from normal pregnancies.

Taken together, most reports suggest a Th1/Th2 imbalance during preeclampsia with a Th1 shift coupled to preeclampsia. Indeed, some of the reports suggested defective Th2 responses, which is in line with our findings, while others did not detect any difference between women with preeclampsia and normal pregnancies. These divergent results could reflect the complexity of the disease, different sampling times during gestation, or different immune responses systemically compared to the local reactions in the uterus. However, in these reports different laboratory methods are used, different compartments have been analyzed, and both stimulated and unstimulated cells have been used, which to some degree can explain the differences in the results.

## Moderate versus severe preeclampsia

Additional comparisons regarding the number of cytokine-secreting cells were made between women with moderate (no. 8) and severe preeclampsia (no. 7) in paper I. These analyses showed no statistical differences between the two groups.

In paper II no difference in the spontaneous secretion of IL-5 between moderate and severe preeclampsia was detected. Furthermore, in paper III comparisons were made between women with moderate and severe preeclampsia, although no differences were found. In paper IV, the study material consisted of only severely preeclamptic women.

Taken together, these results suggest that our findings are related to the syndrome of preeclampsia in general, and are not affected by the severity of the disease.

INNATE AND PRO- AND ANTI-INFLAMMATORY MEDIATORS

In this section the markers related to the innate immunity (sCD 14), cytokines, and chemokines, and the pro- and anti-inflammatory balance is discussed.

As described in Table I, the cytokines IL-1β, IL-2, IL-15, IL-17 and TNF-α are pro-inflammatory cytokines. The actions of these cytokines can be counterbalanced by IL-10, which is an anti-inflammatory cytokine. The IL-6 cytokine is a pleiotropic cytokine with both pro- and anti-inflammatory properties, but generally this cytokine is regarded as pro-inflammatory.

Chemokines induces chemotaxis in a variety of cells, including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes, reviewed in Borish and Steinke (2003). Thus, the chemokines are considered pro-inflammatory since they promote inflammation by attracting immune cells to the site of inflammation. The chemokines studied in this thesis are IL-8, GM-CSF, MCP-1, MIP-1α, MIP-1β, RANTES and eotaxin.

## Innate, pro- and anti-inflammatory results and discussion

The anti-inflammatory cytokine IL-10 was studied in paper I, but no differences were found in the number of cytokine-secreting cells between women with preeclampsia and those with normal pregnancies.

However, as shown in paper II, the paternal antigen-induced IL-10 secretion was lowered in PBMC cultures from preeclamptic women compared with cultures from women with normal pregnancies ($p = 0.012$), as shown in Figure XI. No other differences were found for the other cultures where IL-10 was measured, nor was there any difference in the spontaneous or antigen-stimulated secretion of IL-6 and TNF-α.

In paper III, women with preeclampsia showed significantly higher serum concentrations of the innate cytokine IL-6 ($p = 0.002$) and elevated levels of the pro-inflammatory chemokine IL-8 ($p = 0.003$) compared to women with normal

pregnancies, see Figure XII. For all the other pro- and anti-inflammatory factors tested, i.e., IL-1β, IL-2, IL-10, IL-15, IL-17, TNF-α, GM-CSF, MIP-1α, MIP-1β, MCP-1, RANTES and eotaxin, there were no significant differences between women with preeclampsia and those with normal pregnancies. The levels of sCD14 also showed no difference between the two groups of women.

In paper IV, TNF-α staining was seen in the trophoblastic layer of the placental biopsies, as shown in Figure XIII. However, there were no statistical differences in the staining intensity for TNF-α between placental sections from women with preeclampsia and from women with normal pregnancies. Nor was there any statistical difference between the two groups regarding the number of stained cells in the placental sections.

Overall, the decreased anti-inflammatory IL-10 secretion in response to paternal antigen suggests a decrease in the anti-inflammatory response during preeclampsia. The decrease of paternal antigen-stimulated secretion of IL-10 could not be confirmed when measuring the number of IL-10 secreting cells in paper I or in the serum levels in paper III. This may be explained by the fact that it is not the number of cytokine-producing cells that is important but rather the cytokine-producing ability, and that this differs between women with preeclampsia and women with normal pregnancies. Furthermore, the finding of paper II suggests a "fetus"-specific response toward antigens and does not seem to have general systemic implications.

The serum levels of IL-6 and IL-8 were increased during preeclampsia in paper III. The IL-6 findings could not be confirmed by the PBMC cell culture studies in paper II, but this may be explained by the fact that other cells than PBMCs can be involved in the production of IL-6, which contributes to the increased systemic levels. This finding may suggest a general shift toward pro-inflammatory reactivity. IL-6 is also known to down-regulate the production of TNF-α and IL-1β, which can, to some degree, explain why we were unable to detect any differences regarding these in serum between women with preeclampsia and women with normal pregnancies.

For the group of not so well defined immunomarkers during preeclampsia, i.e., IL-15, IL-17, MIP-1α, MIP-1β, MCP-1, eotaxin and RANTES, there exist scarce, if any reports dealing with the levels of these during preeclampsia. Despite their documented pro-inflammatory properties, their actions during preeclampsia are relatively unknown and it may well be true that they do not differ in serum between women with preeclampsia and those with normal pregnancies, as indicated in paper III. Further studies are needed to fully exclude their contribution to preeclampsia, since the amount of material studied in paper III was quite small.
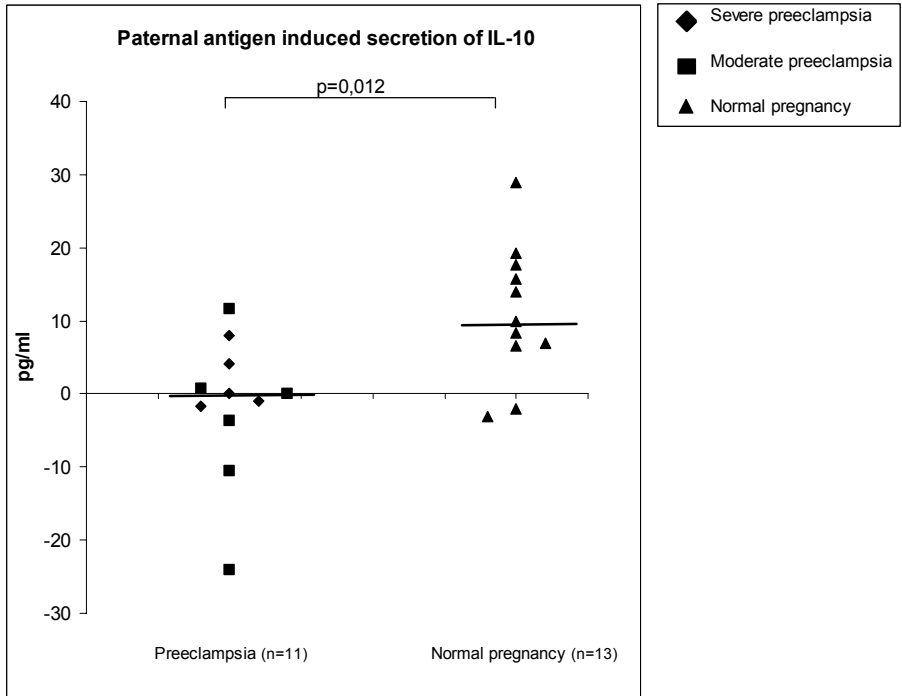
Figure XI Net secretion of IL-10 in response to paternal antigens.

Net secretion of IL-10 in response to inactivated paternal PMBCs in cell culture supernatants from women with preeclampsia and women with normal pregnancies. *p*-value from the comparison with the Mann–Whitney *U*-test is shown. Median is indicated
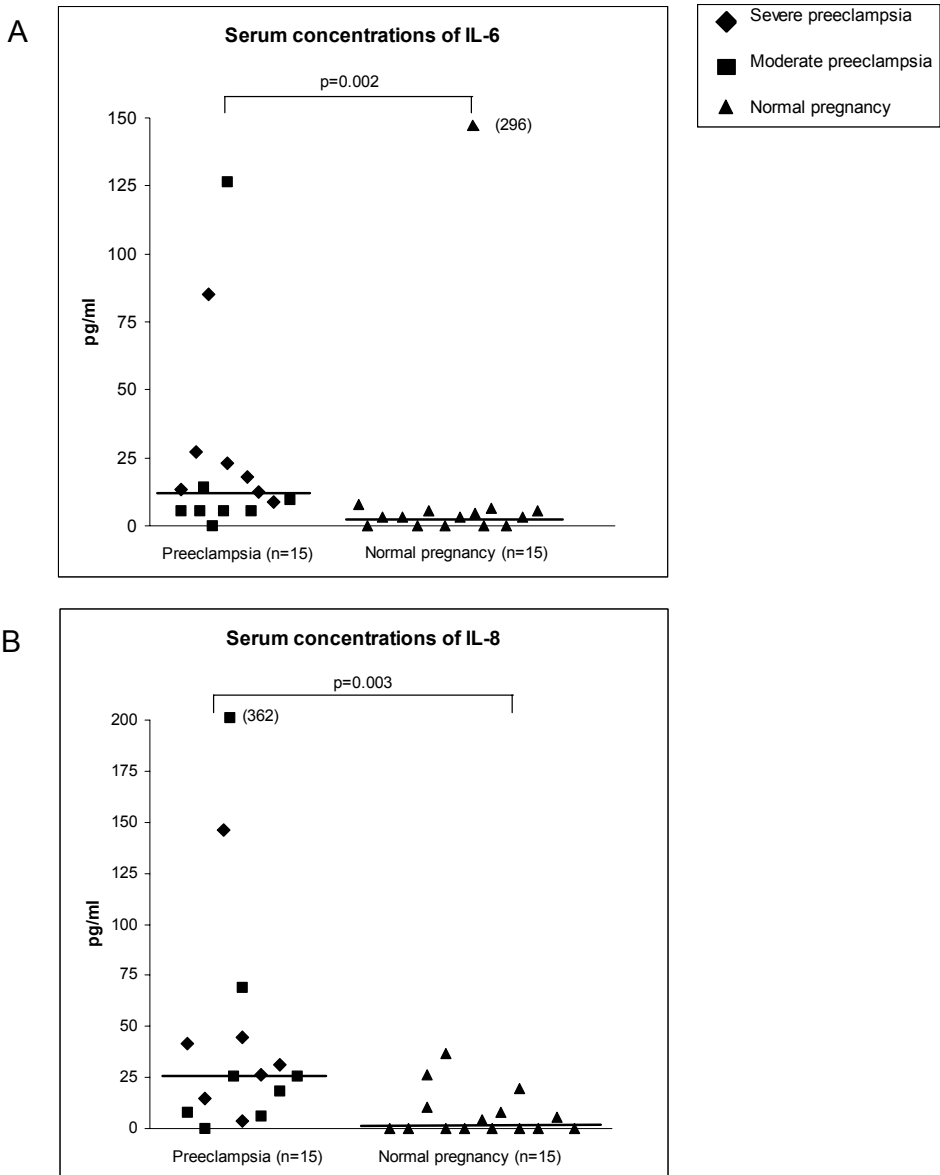
Figure XII Serum concentrations of IL-6 and IL-8.

Serum concentrations of the pro-inflammatory cytokine IL-6 (A) and the chemokine IL-8 (B) from women with preeclampsia and normal pregnancies. *p*-values from the comparisons with the Mann–Whitney *U*-test are shown. Median is indicated.

The levels of IL-2 did not differ in serum from women with preeclampsia compared to women with normal pregnancies. Darmochwal-Kolarz et al. (1999) found increased levels of IL-2 in PHA-stimulated PBMC cultures from women with preeclampsia compared to normal pregnant women. Furthermore, Darmochwal-Kolarz et al. (2002) and Rein et al. (2002) found increased expression of IL-2 in PMA-stimulated T-lymphocytes in women with preeclampsia compared to women with normal pregnancy. Increased levels of IL-2 were found for both spontaneous and stimulated secretion in PBMC cultures from women with preeclampsia compared to those with normal pregnancies (Saito, et al. 1999b). IL-2 is mainly produced by activated T-cells and, as suggested by the IL-6 results, the main source of the pro-inflammatory cytokines studied in this thesis may not be immune cells. This may explain the co-existing findings of increased levels of IL-6 and IL-8 and the absence of any difference in IL-2 levels in serum from women with preeclampsia and those with normal pregnancies.

Increased levels of IL-6 in plasma and serum during preeclampsia have been reported earlier (Conrad, et al. 1998, Ellis, et al. 2001, Kocyigit, et al. 2004, Madazli, et al. 2003), which support the present findings. In contrast, Al-Othman et al. (2001) found no differences in serum levels of IL-6 between women with preeclampsia and normal pregnant women, while Azizieh et al. (2005) found decreased levels of IL-6 in PHA-stimulated PBMC cultures from women with preeclampsia compared to women with normal pregnancy.

When studying placental homogenates Benyo et al. (2001) were not able to detect any differences in the levels of IL-6 between women with preeclampsia and those with normal pregnancies.

IL-8 has been shown to have multiple effects on neutrophils *in vitro*, including shape change, induction of respiratory burst, and increase in the number of adhesion molecules (Mukaida, et al. 1998). Furthermore, it has been shown to induce *in vitro* chemotaxis of human CD4^{+} and CD8^{+} peripheral blood T-lymphocytes and cause rapid concentration-dependent neutrophil infiltration *in vivo*, i.e., IL-8 has pro-inflammatory properties. Increased serum levels of IL-8 during preeclampsia have been reported previously (Kocyigit, et al. 2004, Velzing-Aarts, et al. 2002), while Ellis et al. (2001) found increased plasma IL-8 levels only when comparing severe preeclamptic women with normal pregnant women. Taken together, these results are in line with our findings in paper IV.

Darmochwal-Kolarz et al. (1999, 2002) found decreased levels of IL-10 in PHA-stimulated PBMC cultures and lowered expression of IL-10 in PMA-stimulated T-lymphocytes from women with preeclampsia compared to women with normal pregnancy. In line with this, Azizieh et al. (2005) found decreased

levels of IL-10 from PHA-stimulated PBMC cultures from women with preeclampsia compared to normal pregnant women. In contrast, Benian et al. (2002) and Madazli et al. (2003) found increased levels of IL-10 in plasma from women with preeclampsia compared to women with normal pregnancies. On the other hand, others have reported no differences in serum and plasma levels of IL-10 (Conrad, et al. 1998, Ellis, et al. 2001, Gratacos, et al. 1998). The present finding of decreased levels of IL-10 only in response to paternal antigen is interesting, and indicates that the lowering of the anti-inflammatory responses may be partly selective for the fetus and the placenta.

Increased levels of TNF-α in plasma and serum have been reported earlier (Conrad, et al. 1998, Hayashi, et al. 2005, Kocyigit, et al. 2004, Velzing-Aarts, et al. 2002). In contrast, Ellis, et al. (2001) were not able to detect differences in plasma TNF-α between preeclampsia and normal pregnancy. Increased levels of TNF-α have also been detected both for spontaneous (Saito, et al. 1999b) and PHA-stimulated secretion in PBMC cultures from women with preeclampsia compared to those with normal pregnancies (Azizieh, et al. 2005, Saito, et al. 1999b).

Wang and Walsh (1996) have previously found increased levels of TNF-α in placental tissue homogenates, and similarly also found increased levels of peroxides secreted from the placentas from preeclamptic pregnancies as compared to normal pregnancies. The findings of Wang and Walsh (1996) must, however, be interpreted with caution, since they used a mixture of placentas delivered both vaginally and by caesarian section, and it is known that the process of labor influences the cytokine levels (Keelan, et al. 2003, Opsjon, et al. 1993). Nevertheless, Wang et al. were able to detect increased levels of TNF-α in placentas from preeclampsia compared to normal pregnancy. Hennessy et al. (1999) performed immunohistochemical studies on placenta TNF-α and found a tendency toward increased levels during preeclampsia compared to normal controls. However, when studying placental homogenates, Benyo et al. (2001),Rein et al. (2003) and Munno et al (1999) were not able to detect any differences in the levels TNF-α between women with preeclampsia and those with normal pregnancies.

Interestingly, when TNF-α levels were compared between plasma samples taken from a uterine vein and a systemic vein, there existed a difference in the TNF-α levels between women with preeclampsia and normal pregnant women, but there was no difference between the local and systemic levels of TNF-α (Benyo, et al. 2001).

As reviewed by Conrad and Benyo (1997) most, but not all investigators found increased plasma levels of TNF-α from preeclamptic women, when measured by

ELISA. The lack of complete consistency, including the fact that paper III failed to detect such an increase, may be due to the relatively short half-life of the cytokine as well as possible transient and episodic release, which causes the plasma levels to vary considerably. Alternatively, the endothelium in some preeclamptic women might be more sensitive to activation by cytokines, so that "normal" levels become injurious.

Kocyigit et al. (2004) found that serum levels of IL-1β were significantly increased in preeclamptic women compared to normal pregnant women. In contrast, Conrad et al. (1998) were not able to detect any differences in plasma levels of IL-1β, in line with paper III, and Benyo et al. (2001) were not able to detect any differences in placental homogenate levels between women with preeclampsia and normal pregnant women.

Gratacos et al. (1998) were not able to detect any differences in serum GM-CSF between women with preeclampsia and those with normal pregnancies. This finding is in line with our results from paper III.

As reviewed by Redman et al. (1999), the endothelial cells can be activated, and present damage similar to the damage seen in preeclampsia, by free fatty acids, lipoproteins, oxidized lipoproteins or lipid peroxides, TNF-α, fibronectin degradation products, and deported syncytiotrophoblastic microvillous fragments. In line with this, a generalized maternal inflammatory response has been shown to occur during preeclampsia together with increased levels of TNF-α, IL-6 and IL-8, as reviewed by Redman and Sargent (2003).

Vince et al. (1995) found increased levels of IL-6 and TNF-α in plasma from women with preeclampsia compared to women with normal pregnancies. As further discussed by Vince et al. (1995), TNF-α has a very short half-life in circulation, and this can to some extent explain the differences in reported observations on TNF-α levels. In addition, IL-6 is induced by TNF-α (Borish and Steinke 2003), which can explain the simultaneous detection of both these cytokines. However, IL-6 also down-regulates the ongoing TNF-α and IL-1 responses, which can explain the finding of increased levels of IL-6, but not TNF-α, in paper II. In addition, Meekins et al. (1994) did not find any differences in blood levels of TNF-α between women with preeclampsia and those with normal pregnancies.

In a recent study, Xu et al. (2005) used placental biopsies from women with normal as well as preeclamptic pregnancies, and stimulated the biopsies in culture with *E. coli* LPS. In the villous placental explants, also co-cultured with glucocorticoids, the levels of cytokines TNF-α and IL-6 were decreased compared to untreated explants. However, the there was no significant

difference in the cytokine levels between women with normal or preeclamptic pregnancies. In contrast, the culture supernatant levels of IL-10 seemed unaffected by the glucocorticoid treatment. Both the TNF-α-positive immunostaining in placentas and the absence of difference between placentas from preeclamptic pregnancies and normal pregnancies are in line with our results from paper IV. In addition, we detected a decrease in the intensity of the TNF-α staining in placentas from preeclamptic pregnancies treated with glucocorticoids compared to placentas obtained from preeclamptic pregnancies without steroid treatment.

Most researchers have found increased levels of IL-6 and IL-8 during preeclampsia, corroborating the findings in paper III, while the results for IL-10 are not as consistent. In addition, the systemic levels of TNF-α seem to be mostly increased during preeclampsia, while the local expression in the placenta has divergent reported levels. The divergent results for TNF-α could be explained by the relatively short half-life for this cytokine as well as transient and episodic release, which makes the levels vary over time.

In conclusion, in this thesis the decreased levels of IL-10 in response to paternal antigens and the systemically increased levels of IL-6 and IL-8 suggest a specific decrease of anti-inflammatory responses toward fetal antigens together with a systemic activation of pro-inflammatory mediators during preeclampsia. The findings of no difference in the levels of IL-1β and TNF-α may be explained by the negative effect of IL-6 on these cytokines.

Interestingly, as reviewed by Mukaida et al. (1998), IL-8 has chemotactic activities in basophils and eosinophils. Thus, the increased levels of IL-8 in serum from preeclamptic women may suggest that the basophils from preeclamptic women have migrated out of the circulation toward the origin of IL-8, which also may explain the decrease in the number of basophils in preeclampsia.

Finally, since the amount of material studied in these papers is rather small, type II errors cannot be fully excluded, and other differences in pro- and anti-inflammatory markers than those reported may exist.


## Moderate versus severe preeclampsia
Additional comparisons regarding the number of cytokine-secreting cells were made between women with moderate (no. 8) and severe preeclampsia (no. 7) in paper I. These analyses showed no differences between the two groups.

No difference in the paternal antigen-stimulated secretion of IL-10 between moderate and severe preeclampsia was detected in paper II. Furthermore, in paper III comparisons were made between women with moderate and severe preeclampsia, although no differences were found. In paper IV, the study material consisted of only severely preeclamptic women. Thus, the severity of the disease does not seem to affect the pro- and anti-inflammatory immune balance during preeclampsia.

## PLACENTAL MORPHOLOGY

## Villous morphology

The hematoxylin and eosin-stained sections revealed no statistical differences between women with preeclampsia and women with normal pregnancies regarding the prevalence of chorangiosis, trophoblastic knots, perivillous fibrin deposits and villitis, see Figure XIV. However, one woman with normal pregnancy had a placental section that showed signs of chorangiosis, when classified according to Kraus (2004), although this pregnancy ended with the birth of a healthy baby weighing 4200 grams. Trophoblastic knots were found in 8 out of 11 placental sections from preeclamptic pregnancies and from 10 out of 12 normal pregnancies. Perivillous fibrin deposits were found in all placental sections regardless of diagnosis, and most were of normal levels (0–10%). However, four sections taken from severely preeclamptic pregnancies together with one section from a normal pregnancy showed a higher distribution of perivillous fibrin deposits (10–50%). IUGR was diagnosed in three of these four severely preeclamptic pregnancies, although there were no significant differences regarding perivillous fibrin deposits between the placental sections from women with preeclampsia or women with normal pregnancies, see Table V. Salafia et al. (1995) have reported severely increased syncytiotrophoblastic knots, severely increased perivillous fibrin deposits and increased villitis in preeclampsia compared to premature labor. In contrast, Kos et al. (2005) were not able to detect differences in villitis, syncytiotrophoblastic knots or intervillous thrombosis between preeclamptic and normal placentas.

In response to tissue injury, the inflammatory process begins and is characterized by movement of leukocytes and fluid from the blood into extravascular tissues. Therefore, the leukocyte infiltration is the hallmark of inflammation (Mukaida, et al. 1998) and thus, the low frequency of detected CD45^{+} cells in the placental sections further support the finding of absent villitis in paper IV. Occasional CD45^{+} cells were found in the placental sections but were not close to abnormal infiltration, regardless of whether the pregnancy was normal or complicated by preeclampsia.

today there are no reports examining the effect of atopy on preeclampsia, so further studies are warranted.

## EFFECTS OF GLUCOCORTICOIDS

When the preeclamptic symptoms become too severe, delivery of the fetus is required (Working Group on High Blood Pressure in Pregnancy 2000, ACOG Committee on Obstetric Practice 2002). In these cases glucocorticoids need to be used in order to aid the maturation of the fetal lung development prior to birth (Gojnic and Pervulov 2005, Grier and Halliday 2004). However, the treatment can hamper our results, since glucocorticoids have a proven effect on cytokines (Barnes 2001, Reddy, et al. 2004, Xu, et al. 2005).

In a review by Barnes (2001) it is reported that corticosteroids inhibit the transcription of IL-4, IL-5 and IL-13, and presumably also suppress IFN-$\gamma$ and IL-12 production. Corticoids might therefore be expected to help polarize the immune response toward the Th2 pattern, if it were not for the inhibitory effects the IL-4, IL-5 and IL-13 as well. The findings of Reddy et al. (2004) are in line with this previous report, since they found that dexamethasone (corticoid) inhibits the production of IL-2, IL-4, IL-5, IL-10, IFN-$\gamma$ and TNF-$\alpha$ from PBMC cells stimulated with PHA. In addition, corticosteroids decrease the survival of T-cells and eosinophils by increasing apoptosis, which is a positive effect in allergy treatment (Barnes 2001), but the apoptotic and cytokine suppressive effects may affect the immunological studies performed in this thesis.

In papers I–III, glucocorticoids were administered to four women with preeclampsia, although, there were no differences in cytokine expression between preeclamptic women with or without treatment. In paper IV, seven of the eleven preeclamptic women received glucocorticoid treatment and an effect on the placental cytokine expression was detected. The intensity of the TNF-$\alpha$ staining ($p = 0.04$), but not the number of stained cells, was decreased in the placental sections from preeclamptic women who received glucocorticoids compared to sections from untreated preeclamptic women. However, the IL-4 intensity and number of cells stained did not differ between treated or untreated preeclamptic women. These findings may suggest that administration of glucocorticoids is advantageous to the local immune balance during preeclampsia, due to the down-regulation of TNF-$\alpha$, but unaltered expression of IL-4. However, the amount of material studied in paper IV was rather small, thus further studies are needed to establish the effects of glucocorticoids on the local immune balance.

Although glucocorticoids are known modulators of the immune responses (Barnes 2001, Reddy, et al. 2004), we allowed inclusion of women undergoing glucocorticoid treatment since (1) it is not ethically justified to deny any patient treatment because of participation in a study; (2) the everyday situation at the clinic involves a mixture of patients receiving treatment or not, all due to their individual situation; (3) there were limitations on the number of suitable patients for the studies (due to exclusion of patients with severely complicating factors, such as chronic hypertension); and (4) there are limits to the possible number of patients attending the obstetric clinics at the hospitals involved in the patient recruitment for these studies. As shown in another study (Matthiesen, et al. 1999), glucocorticoids did, at least, not affect the proliferative responses of PBMCs from women with preeclampsia treated with glucocorticoids compared to untreated preeclamptic women. In addition, glucocorticoids have previously been suggested to induce secretion of anti-inflammatory cytokines (Ossege, et al. 1998). Thus, the finding of decreased levels of "fetus-specific" IL-10 in preeclampsia would not be due to the administration of glucocorticoids in this group, since the glucocorticoids rather increase the levels of the anti-inflammatory cytokine IL-10.

However, the results in this thesis may need to be interpreted with caution regarding this point. The use of glucocorticoids in the treatment of patients is justified and furthermore, it is unethical to deny treatment to patients due to their participation in a study. But the presence of glucocorticoids needs to be taken into consideration when taking samples for study. A previous report have, however, shown significant immunological differences between normal pregnant and preeclamptic women regardless of the use of glucocorticoids (Sacks, et al. 1998).

It is also sometimes necessary to administer antihypertensive pharmaceuticals to preeclamptic women in order to prevent dangerously high blood pressure (Working Group on High Blood Pressure in Pregnancy 2000). However, these agents should not have any effect on the immune system, which is also shown by Omu et al. (1996) who reported no difference in the serum levels of IL-4 in preeclamptic women with or without hypertensive treatment.


GENERAL DISCUSSION

It is highly interesting to study the peripheral immune response during preeclampsia, especially since peripheral blood is relatively easy to obtain and after future thorough research, a blood sample might further aid the clinician in the monitoring and treatment of those patients with preeclampsia. Furthermore, the maternal immune system has been suggested to be involved in the maternal endothelial dysfunction and systemic immune reactions seen in preeclampsia

(Roberts, et al. 1989, Saito and Sakai 2003). In addition, fetal trophoblasts have been detected in the maternal circulation(Dekker and Sibai 1998, Sargent, et al. 2003), which also can partly explain the cause of the maternal immune reactions seen in preeclampsia.

Preeclampsia probably originates from the placenta (Loke and King 1995, Piering, et al. 1993, Redman and Sargent 2005, Robillard 2002, Soto-Wright, et al. 1995), so although measurements of systemic variables are of interest, it is also of interest to study the local immune environment at the placenta to (1) obtain an idea of how the preeclamptic syndrome originates and (2) observe what happens immunologically when the syndrome has already started. Immunohistochemical studies, such as the one carried out in paper IV, have the advantage of revealing ongoing immune reactions at the site of the placenta in well-established preeclampsia and normal pregnancy. However, since it is not ethically justified to take placental samples during ongoing pregnancy, we have to be content with the samples taken at delivery. For severe preeclampsia the delivery might have to be premature, due to maternal and fetal medical indications and, no matter how interesting these samples are, it is very difficult, if not impossible, to obtain gestationally matched normal pregnant controls for this type of patient material. The placental material available for this type of research therefore must come from the time of delivery and, preferentially, before the onset of labor. Placental material from elective abortions could possibly supply the material needed for these types of studies; however, elective abortions are performed up until gestational week 18. As the preeclamptic symptoms develop in the second half of gestation, i.e., after gestational week 20, placental samples from elective abortions perhaps do not contribute many clues as to the development of preeclampsia, since the pregnancy is terminated before the maternal onset of symptoms. In addition, these types of placental samples will not be suitable as early normal controls either, since it is difficult to say whether these pregnancies would have developed complications or not.

On the other hand, placentas from tubal pregnancies can give rise to preeclamptic symptoms quite early in gestation and thus, theoretically, could reveal clues about the preeclamptic development. These tubal pregnancies have to be terminated on maternal indications, but the trophoblasts and maternal cells at the site of implantation do not behave as those same cell types do in intrauterine implantation (Loke and King 1995). Therefore it is difficult to imply results from tubal pregnancies to normal or preeclamptic pregnancies.

It is possible that the absence of differences between women with preeclampsia and normal pregnancies in paper I could be due to the fact that the ELISPOT technique only enumerates the number of cytokine-secreting cells instead of the actual levels of cytokines. Cytokine levels may vary with the degree of

activation although the number of cells remains constant. Indeed, in paper II, which was based on the same patient material, women with preeclampsia showed decreased levels of paternal antigen-induced secretion of IL-10, whereas in paper I, similar numbers of IL-10 secreting cells were found in response to paternal antigens between both groups. As paternal leukocytes express the same foreign antigens as the fetus, such stimulation likely mirrors responses against the fetus. The decreased levels of IL-10 in response to this "fetal" antigen during preeclampsia suggest a fetus-specific down-regulation of the anti-inflammatory response compared to normal pregnancy, which could offer one explanation for the immunopathology seen in preeclampsia. This finding of IL-10 was detected only at the level of concentration and not regarding the number of IL-10 secreting cells, pointing out the importance of using different techniques when analyzing cytokine responses.

PPD, TT and LPS are antigens that are unrelated to pregnancy and, as such, they can be used to stimulate immune responses *in vitro*. Since the responses toward these antigens are dependent on the general immune balance, the actual responses during pregnancy and preeclampsia can be useful indicators of any ongoing general shifts of the immune system. However, we were not able to detect any differences between women with preeclampsia and normal pregnancies regarding the response toward these antigens, which suggests that the immune system is selectively altered during pregnancy, but maintains reactivity toward infectious antigens.

An up-regulation of the number of IL-4 secreting cells in response to paternal antigens has previously been reported during normal pregnancy (Ekerfelt, et al. 1997). In paper I, we failed to detect any differences between preeclampsia and normal pregnancy regarding "fetus"-specific IL-4 secreting cells, indicating that fetus-specific Th2 responses are not decreased during preeclampsia. The similar findings of Th1 and Th2 cytokines in paper I, in combination with the results from paper II, where a decrease in the Th2 cytokine IL-5 was found, demanded further analysis of the Th1 and Th2 balance during preeclampsia. In paper III, the aim was to gain more information about the actual *in vivo* levels in serum, since taking cells out of the circulation and stimulating them with different antigens does have an *in vitro* effect, so an examination of the *in vivo* levels was warranted. In addition, besides blood cells other cells can contribute to the systemic cytokine levels, which are captured by measuring the levels in serum. Also, the use of serum levels resembles more the setting available for clinical use, although it can have its disadvantages, as cytokines circulate in low levels due to their local mode of action (Bienvenu, et al. 2000). Although, when considering cytokine analyses in preeclampsia and normal pregnancy it still could be effective to measure serum levels because (1) due to different factors the endothelial damage is systemic (Roberts, et al. 1989) and (2) peripheral T-

lymphocytes are able to respond specifically to paternal leukocytes, as shown by Ekerfelt et al. (1997) and paper II, which means that "fetus-specific" T-lymphocytes are occurring in the circulation. Furthermore, trophoblasts have also been shown to occur in the maternal circulation, which can contribute to the maternal symptoms (Sargent, et al. 2003). It is therefore likely to assume that the maternal immune system sometimes reacts wrongly with systemic consequences, as seen for instance in preeclampsia. However, we were unable to detect any differences in the Th1 and Th2 balance in paper III, except for increased serum levels of sIL-4R in women with preeclampsia. This was an elusive finding, since the receptor was originally thought to reflect the levels of IL-4, but has recently been shown to have both agonistic and antagonistic properties on the IL-4 levels.

The divergence among reports regarding immunological markers can be partly explained by the use of different analysis methods, with different sensitivity levels, different analysis techniques, and the use of different compartments of measurements. It is difficult to compare results from a stimulated PBMC culture with, for example, a serum sample. One result is obtained *in vitro* and often after stimulation, while serum/plasma levels provide a momentary picture of the situation at the time of sampling. Furthermore, as discussed in Bienvenu et al. (2000), cytokines exert pleiotropic activities in a complex network and normally circulate at low systemic concentrations. Therefore it is generally difficult to specify the precise role of a specific cytokine in an ongoing process. Furthermore, which results should be considered as more likely to reflect the ongoing situation in preeclampsia: the result from spontaneously cytokine-producing cells, which could to some degree have become exhausted in vivo, and thus are unable to produce more cytokines *in vitro*, or the results from cells stimulated with strong and non-physiological stimuli such as PMA and PHA?

Nevertheless, all analyses give clues to the pathogenesis of preeclampsia; the difficulty lies in achieving a complete image of the pieces of the puzzle obtained through these analyses.

## Other possible causes of the development of preeclampsia

Other reports suggest that there also can be factors other than immunological ones that are also involved in the development of preeclampsia. There exist reports about increased incidence of protein S deficiency, activated protein C resistance, hyperhomocysteinemia, and anti-cardiolipin antibody in preeclampsia (Dekker, et al. 1995, van Pampus, et al. 1999). Furthermore, the cytokine TNF-α has been shown to down-regulate part of the production of protein S (Hooper, et al. 1994). In addition, recent reports suggest that the development of preeclampsia might have a genetic component, as women with

preeclampsia are more likely to have a sister who had preeclampsia (Carr, et al. 2005), or daughters of women who had preeclampsia are more likely to develop the disease themselves (Esplin, et al. 2001) compared to the normal population. While Page et al. (2000, 2001) have found increased levels of the vasoconstrictor peptide neurokinin B in preeclampsia.

In summary, preeclampsia is a very complex disease of multifactorial origin with no effective treatment at hand today. It is therefore of interest to further study the development and symptoms of this disease and immunological studies are still of interest.


## FURTHER STUDIES

Further measurements of the bioactive form of IL-12 to determine its levels during preeclampsia are needed, partly because the cytokine in itself is Th1-promoting, but also because of its effects on sIL-4R levels. It is possible that bioactive IL-12 could increase the levels of sIL-4R, thus inhibiting Th2 responses.

It would also be of interest to study the levels of IL-16, IL-18, IL-20, IL-22, IL-23 and IL-25 during preeclampsia. These cytokines are of interest since the Th1 differentiation is also mediated by IL-18 and IL-23 (Borish and Steinke 2003), while IL-16, IL-20 and IL-22 are involved in pro-inflammatory reactions (Mathy, et al. 2000, Pestka, et al. 2004). In mice, IL-25 has been shown to induce Th2 responses (Fort, et al. 2001, Kawaguchi, et al. 2004) and further studies of these cytokines may provide further information about the Th1 and Th2 balance and pro-inflammatory reactions during preeclampsia.

Larger study groups would be preferable, to obtain more statistically reliable material. However, some of the laboratory methods used in this thesis are quite laborious and thus are not suited for large study groups. ELISPOT and ELISA on cell culture supernatants are two such examples; these methods cannot be automated and require a lot of time for each sample; the limit in these studies lies in the work effort required for each and every sample. The multiplex bead array analysis of serum is quite easy to perform compared to conventional ELISA, but has the drawback of a high price per analysis kit. But with this method it is easier to compare several analytes per sample, for instance a cytokine network, both in serum and cell culture samples. It also has the advantage of requiring a small amount of sample for each analysis; in paper III, only 50 μl of serum was used each time a multiplex bead array analysis was performed. This saves time and most of all, spares the patient from large blood sample collections.

Immunohistochemistry is also a laborious method, although it has become automated over the years. It still needs, as does any laboratory analysis, some initial calibrations, but after that the sectioning of the samples and the process of labeling have become automated. It would therefore be advantageous to develop the immunohistochemical studies further, to include more samples and immunological markers for analysis. Although the analysis of each slide is performed manually and although the fact that this method also uses quite expensive antibodies, it is of merit to continue with these studies, because it is at the local site of the placenta that the preeclamptic syndrome starts and therefore, it is of vital interest to further study the local environment to obtain clues to understanding this cumbersome disease.

It would also be of interest to study the correlation of the levels of the cytokines in combination with, for example, the levels of progesterone. This hormone is produced by the corpus luteum and the placenta, and is one of the hormones essential for pregnancy. As reviewed by Druckmann and Druckmann (2005) and Piccinni et al (2001), progesterone and the progesterone inhibitory factor (PIBF) are involved in the establishment of the Th2 shift required for pregnancy. Therefore, it would be interesting to study the combination of progesterone, PIBF and cytokine levels during preeclampsia.

Neurokinin B is also a substance worth studying more extensively during preeclampsia, due to its vasoconstrictive properties and abundant secretion from preeclamptic placentas.

The studies described here in papers I–IV are made on material from women already affected by preeclamptic disease, so it is difficult to separate cause from consequence. Therefore it would be suitable to collect a large amount of material from women in the first trimester of the pregnancy and look for markers (novel or less defined) that might be involved in the development of preeclampsia.

Since regulatory T-cells play such an important part in the acceptance of foreign antigens, whether it is self antigens or antigens derived from transplantation (Akl, et al. 2005), it would be very interesting to study the prevalence and status of regulatory T-cells during normal pregnancy and preeclampsia. What is it in the female immune system that makes the pregnancy function normally in the majority of cases and what is it that goes wrong with the immune system in preeclampsia? Could it be that the regulatory T-cells function properly in normal pregnancy, but are absent or dysfunctional during preeclampsia? It would be of great interest to study placental biopsies in order to look for TGF-β and the cell markers for regulatory T-cells and furthermore, to examine the levels of regulatory T-cells in peripheral blood and their cytokine-producing ability, to

ascertain whether any differences exist between women with preeclampsia and women with normal pregnancies.

## CONCLUSION

The maternal immune system is involved throughout the maintenance of pregnancy. It gets involved from the beginning of placentation and stays active and modified throughout the whole pregnancy. Optimally, the maternal immune system maintains protective responses toward harmful pathogens, but selectively decreases harmful Th1 and pro-inflammatory reactions, and increases the beneficiary Th2 and anti-inflammatory responses directed toward the fetus.

However, sometimes the regulation of the normal immune reactions seems to malfunction, as seen in preeclampsia. For the studies performed for this thesis, decreased levels of the Th2 cytokine IL-5 and the anti-inflammatory cytokine IL-10 were detected during preeclampsia. Furthermore, increased levels of the pro-inflammatory cytokine IL-6 and chemokine IL-8 were found during preeclampsia. These findings indicate that a decrease of Th2 response and up-regulation of pro-inflammatory reactions, in combination with a lack of anti-inflammatory response, is involved in established preeclampsia.

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