

Systemic Th1/Th2 Cytokine Responses to Paternal and Vaccination Antigens in Preeclampsia: No Differences Compared with Normal Pregnancy

Jonsson Y, Ekerfelt C, Berg G, Nieminen K, Sharma S, Ernerudh J, Matthiesen L. Systemic Th1/Th2 cytokine responses to paternal and vaccination antigens in preeclampsia: no differences compared with normal pregnancy. AJRI 2004; 51:302–310 © Blackwell Munksgaard, 2004

PROBLEM: A Th1-shift has been suggested to be involved in the pathogenesis of preeclampsia. This study was designed to compare Th1/Th2 related cytokine secretion in blood between women with preeclampsia ($n = 15$) and normal pregnancies ($n = 15$), using a high-sensitivity technique for cytokine detection.

METHODS OF STUDY: Spontaneous as well as 'fetus-specific' and recall antigen-specific (purified protein derivative of *Mycobacterium tuberculosis*, tetanus toxoid and lipopolysaccharide) secretion of interferon- γ , interleukin (IL)-4, IL-10 and IL-12 in peripheral blood mononuclear cells (PBMC) was detected by enzyme-linked immunosorbent spot-forming cell assay (ELISPOT). Fetus-specific secretion was induced by stimulation with paternal PBMC in a mixed leukocyte culture assay.

RESULTS: All cytokines were secreted by PBMCs both from women with preeclampsia and women with normal pregnancies. No differences in the number of cytokine-secreting cells were found between the two groups.

CONCLUSIONS: No evidence was found for a shift in the systemic Th1/Th2 responses, in preeclampsia compared with normal pregnancy. This does, however, not exclude differences in the local immune responses related to the fetoplacental unit.

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Key words: ELISPOT, interferon- γ , interleukin-10, interleukin-12, interleukin-4, preeclampsia

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Submitted November 15, 2002;
accepted August 12, 2003

INTRODUCTION

It is commonly believed that a T helper type 2 (Th2) shift in the maternal immune responses is required in order to maintain a normal pregnancy,¹ although this has been questioned.^{2,3} Furthermore, it has been suggested that an imbalance of the Th1/Th2 type responses, with a shift towards a Th1 response, may be involved in the etiology of preeclampsia.⁴ Previous studies examining the Th1/Th2 balance in preeclampsia focused on cytokine levels in serum and plasma,^{5–7} whereas some have determined *in vitro* cytokine secretion in cell culture supernatants.^{4,8} Saito et al. found elevated *in vitro* production of the Th1-cytokine

interferon- γ (IFN- γ), the proinflammatory cytokine tumor necrosis factor- α (TNF- α) and interleukin-2 (IL-2) in preeclampsia compared with normal pregnancies.⁴ In contrast, Omu et al.⁵ found an elevated production of the Th2-cytokine IL-4 in plasma from preeclamptic patients compared with controls, whereas Sacks et al.⁶ and Dudley et al.⁷ found no differences in the production of the bioactive p70 heterodimer of the Th1-inducing cytokine IL-12 between preeclamptic patients and normal pregnancies. Thus, there are contradictory reports on the Th1/Th2 balance in preeclampsia. This may partly be a result of difficulties associated with detection of cytokines. T-cell cytokines are secreted in a paracrine manner, and are rapidly

bound to receptors on neighboring cells, making detection in body fluids and cell supernatants not representative of the secretion.^{9,10} The enzyme-linked immunosorbent spot-forming cell assay (ELISPOT) provides a strategy to overcome this problem. In this assay, the secreted cytokines are immediately captured by high affinity anti-cytokine antibodies, making ELISPOT outstanding in sensitivity.¹¹

The aim of this study was to compare Th1/Th2 related cytokine secretion in blood between women with preeclampsia ($n = 15$) and gestational age-matched normal pregnancies ($n = 15$), using the sensitive ELISPOT assay. In addition, the results gave an opportunity to confirm our previous data on normal pregnancy. The spontaneous cytokine secretion from peripheral blood mononuclear cells (PBMC) was determined *ex vivo*. In addition, *in vitro* stimulated fetus-specific cytokine secretion was analyzed, using a previously developed mixed leukocyte culture assay¹² and in parallel, recall antigen-induced secretion [purified protein derivate of *Mycobacterium tuberculosis* (PPD), tetanus toxoid (TT) and lipopolysaccharide (LPS)] was measured. We chose to study secretion of IFN- γ and IL-4, which are the best markers of Th1 and Th2 responses, respectively,¹³ IL-12 the most prominent inducer of Th1 responses, and IL-10, which in man is produced by both Th1 and Th2 cells, exerting potent anti-inflammatory effects,¹⁴ which are believed to be important in pregnancy.^{15,16}

MATERIALS AND METHODS

Material

Peripheral venous blood samples were drawn from 15 women with moderate or severe preeclampsia (see Table I) and 15 women with normal pregnancies (controls) and their respective male partner (father-to-be). The controls were matched for parity and age with the preeclamptic women. All blood samples were

immediately transported to the laboratory and prepared as described below.

In the preeclamptic women, the blood samples were taken when they were diagnosed and hospitalized, whereas blood samples from the healthy women were taken at the Antenatal Care Clinic in the pregnancy week decided by their matching. Data on the participating women are shown in Table I.

Separation of Peripheral Blood Mononuclear Cells

Sodium-heparinized peripheral whole blood was separated on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) according to Bøyum,¹⁷ followed by washing three times with Hank's balanced salt solution (HBSS) (Life Technologies, Paisley, Scotland). The mononuclear cells were resuspended in a tissue culture medium (TCM) consisting of Iscove's Modification of Dulbecco's Medium (IMDM) (Gibco BRL, Paisley, Scotland) supplemented with (given as final concentrations in the medium): L-glutamine 292 mg/L (Sigma, St Louis, MO, USA), sodium bicarbonate 3024 g/L, penicillin 50 U/ml and streptomycin 50 μ g/mL (Flow Laboratories, Irvine, Scotland), 100x non-essential amino acids 10 mL/L (Flow Laboratories) and 5% heat-inactivated fetal calf serum (FCS) (Sigma). Cells were counted under a phase contrast microscopy, and the maternal cell (responder) density was then adjusted to 1×10^6 lymphocytes/mL.

Inhibition of Stimulator Cell Cytokine Secretion

Allo-reactivity of lymphocytes was determined with a one-way mixed leukocyte culture (MLC) assay, i.e. cytokine secretion was determined exclusively from the maternal cells, here called responder cells, as described earlier.¹² Three kinds of stimulator cells were used; paternal PBMCs, autologous PBMCs and pooled PBMCs from 18 unrelated blood donors. The inhibition of cytokine secretion from the stimulator cells was achieved by incubation with 4% paraformaldehyde (PFA) (Merck, Hohenbrunn, Germany) dissolved in

TABLE I. Data on the Participating Women. Moderate and Severe Preeclampsia are Defined According to WHO Criteria. (Moderate Preeclampsia = BP > 140/90 and Proteinuria Less Than 5 g/24 hr. Severe Preeclampsia = BP > 160/110 and/or Proteinuria of More Than 5 g/24 hr and/or Subjective Symptoms Such As Visual Impairment, Headache, Abdominal Pain.)

Diagnosis	Mean maternal age (years) at delivery (range)	Gestational age (completed weeks) at blood sampling (range)	Allergy, number of women	Number of parous women
Controls $n = 15$	30 (25–42)	33.4 (29–37)	6	2
Moderate preeclampsia $n = 8$	29 (22–32)	36.6 (33–40)	2	1
Severe preeclampsia $n = 7$	29 (26–32)	31.7 (29–34)	0	1
All preeclamptic women $n = 15$	29 (22–32)	34.4 (29–40)	2	2

phosphate buffered saline pH 7.4 (PBS) for 10 min. The solution was sterile-filtered and stored in 10 mL vials at 70 °C for 3 months. After incubation, the cells were washed with 10 mL sterile PBS and centrifuged for 10 min at 400 g. The washing was repeated and the cells were counted and resuspended in TCM at a density of 1×10^6 lymphocytes/mL. To test that inhibition of cytokine secretion was successful, all PFA-treated responder cells were separately stimulated with phytohemagglutinin (PHA, 20 µg/mL) (Sigma) and assayed for cytokine secretion. We have previously shown that PHA causes vigorous secretion of IFN- γ and IL-4 as measured by ELISPOT.¹²

Mixed Leukocyte Culture (MLC)

Aliquots of 100-µL cell suspension containing 100×10^3 responder lymphocytes was added to the wells of coated microtiter plates (see below). A quantity of 100-µL cell suspension containing 100×10^3 PFA-treated stimulator lymphocytes was then added to each well containing responder cells. All different types of MLCs, responder-paternal, responder-autologous and responder-pooled unrelated donors, were assayed in quadruplicates for detection of IFN- γ , IL-4, IL-10 or IL-12 secretion, respectively. In addition, spontaneous secretion was determined by adding 100 µL of TCM only to the responder cells. The number of cytokine-secreting cells was determined after 14–19 hr of incubation.

Antigenic Stimulation

The responder cells were also stimulated with the following agents (final concentrations are given); PPD of tuberculin, 10 µg/mL (Statens seruminstitut, Copenhagen, Denmark), TT, 5 LF/mL (SBL Vaccin, Stockholm, Sweden) and LPS from *Escherichia coli* 026:B6, 0.05 ng/mL (Sigma). Every antigen culture (PPD, TT and LPS) were assayed in triplicates for detection of IFN- γ , IL-4, IL-10 and IL-12 secretion. In addition, spontaneous secretion was determined by adding 100 µL of TCM only to the responder cells. The number of cytokine-secreting cells was determined after 38–42-hr of incubation.

Enumeration of IFN- γ , IL-4, IL-10 and IL-12

Secreting Cells with ELISPOT

The ELISPOT was performed essentially as described by Czerkinsky et al.,¹⁸ slightly modified for the detection of cytokine secreting cells as described previously.¹⁹ Nitrocellulose-bottomed 96-well microtiter plates (Multiscreen HA; Millipore, Bedford, MA, USA) were coated over night at +4 °C with 100 µL per well of mouse anti-human IFN- γ (mAb 1-D1K), mouse anti-human IL-4 (mAb IL-4-I), rat anti-human IL-10 (mAb 9D7) and mouse anti-human IL-12 (mAb

IL-12-I) (all antibodies purchased from Mabtech, Stockholm, Sweden) in sterile PBS, pH 7.4 at a concentration of 15 µg/mL. The plates were then emptied by suction, using a multiscreen vacuum manifold (Millipore) and washed eight times with 100 µL per well of sterile PBS. Non-specific binding sites on the nitrocellulose were blocked by incubation with 100 µL TCM for at least 30 min at +37 °C. After emptying, MLC and antigen cultures were applied as described above. In addition, as positive control to each set of culture and cytokine, one well with responder cells were separately stimulated with PHA, 20 µg/mL (Sigma). The cells were then cultured undisturbed at +37 °C in a humidified atmosphere with 5% CO₂. After 14–19 or 38–42 hr of incubation, depending on the type of culture, the plates were emptied and washed twice with PBS and twice with PBS containing 0.05% Tween 20 (PBS-Tween). Subsequently, 100 µL of biotinylated mouse IFN- γ (mAb 7-B6-1-biotin), mouse IL-4 (mAb IL4-II- biotin), rat IL-10 (mAb 12G8-biotin) and mouse p70 IL-12 (mAb IL-12-III-biotin) antibodies, respectively, diluted to 1 µg/mL in PBS-Tween, was added to the wells. The plates were then incubated 2–4 hr in the dark at room temperature. After additional four washes with PBS-Tween, 100 µL of streptavidin conjugated to alkaline phosphatase (Mabtech), diluted 1/1000 in PBS-Tween, was added. The plates were then incubated for 60–90 min in the dark at room temperature prior to washing four times with PBS. The spots were developed using an 'AP conjugate substrate kit' (Bio-Rad Laboratories, Hercules, CA, USA). Spots were seen after 5–10 min, but the reaction was allowed to proceed for totally 15 min, before the wells were rinsed with excessive amounts of tap water. The wells were then emptied and dried over night at room temperature.

The spots from the MLC cultures were counted in a dissection microscope, while the spots from the antigen culture was counted by an ELISPOT reader (AID ELISPOT reader system, version 2.9, AID, Strassberg, Germany). The median value of the quadruplicates (MLC) and triplicates (antigen culture) was calculated. To achieve net MLC-values, the value of the response to autologous cells was subtracted from the values obtained in response to allogenic stimulation (paternal PBMCs and pooled PBMCs from unrelated donors). To achieve net antigen-values, the value of the spontaneous secretion was subtracted from the values obtained in response to the antigens (PPD, TT, LPS). As a negative control, one well per cytokine and plate was incubated with TCM alone, without cells, otherwise treated as the other wells. No spots were found in any of these wells. 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 considered neglectable. PHA stimulation of responder cells always elicited strong responses for all cytokines. Samples were generally analyzed in quadruplicates for the MLC cultures except for six samples with triplicates and two samples with duplicates. Also, samples were generally analyzed in triplicates for the antigen cultures, except for four samples, which were analyzed in duplicates. As a result of laboratory technical problems, data are not available on all cytokines from all women.

Statistics

The non-parametric Mann-Whitney *U*-test was used for comparison between the groups. Correction for multiple comparisons was carried out with Bonferroni correction.

Ethics

This study was approved by The Regional Ethics Committee for Human Research at the University Hospital of Linköping. Informed consent was obtained from all individuals.

RESULTS

Spontaneous Cytokine Secretion

All cytokines analyzed (IFN- γ , IL-4, IL-10 and IL-12) were spontaneously secreted by PBMCs from women with preeclampsia as well as from women with normal pregnancies, (Fig. 1). No differences in the number of cells spontaneously secreting any of the cytokines were found between women with preeclampsia and women with normal pregnancies (Fig. 1). Regarding IL-12 secretion it should, however, be noted that IL-12 was analyzed in a limited number of women (preeclampsia $n = 6$; normal pregnancies $n = 8$), because of technical reasons. Neither were there any differences in the ratios between the numbers of cells secreting IFN- γ /IL-4, IFN- γ /IL-10 or IL-4/IL-10 (data not shown).

Fetus-specific Cytokine Secretion

The fetus-specific cytokine secretion, induced by stimulation with paternal PBMC (expressing the same foreign HLA as the fetus), is shown in Fig. 2. In general for all cytokines, stimulation with paternal PBMC generated similarly increased numbers of cytokine secreting cells

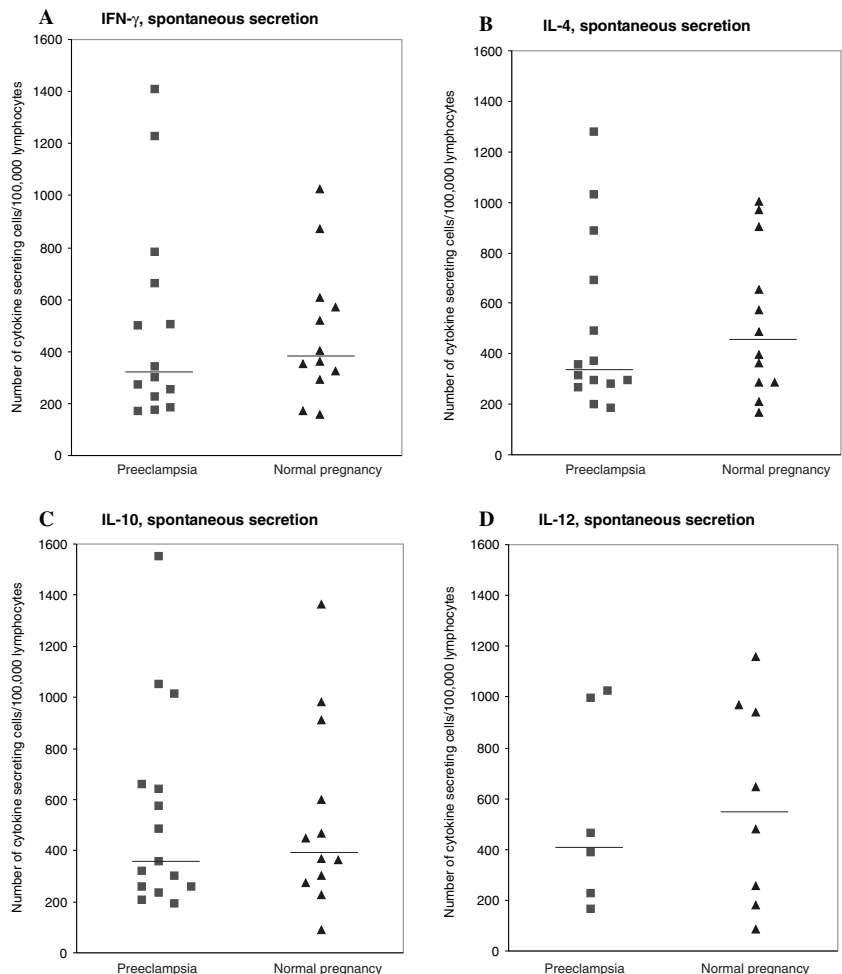


Fig. 1. Number of cells per 100,000 lymphocytes from women with preeclampsia and normal pregnancies, spontaneously secreting interferon (IFN)- γ A, interleukin (IL)-4 B, IL-10 C and IL-12 D. Median is indicated.

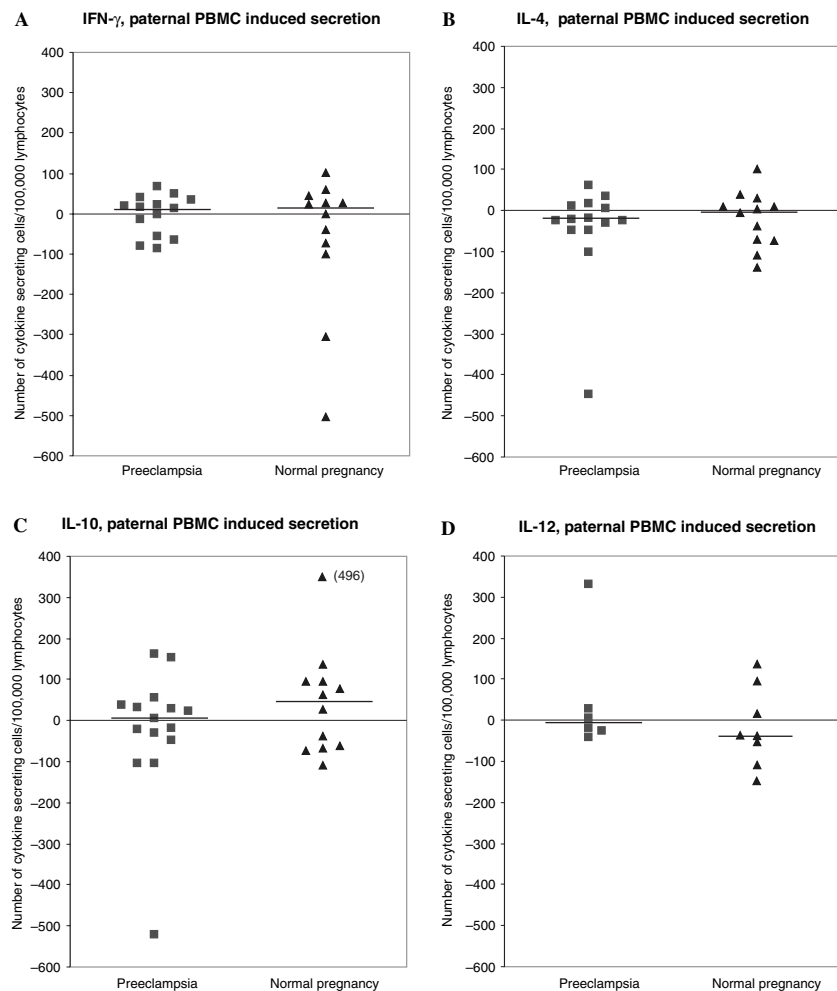


Fig. 2. Number of cells per 100,000 lymphocytes from women with preeclampsia and normal pregnancies, secreting interferon (IFN)- γ **A**, interleukin (IL)-4 **B**, IL-10 **C** and IL-12 **D** in response to inactivated paternal peripheral blood mononuclear cells. All values are net values, where the number of autologous IFN- γ , IL-4, IL-10 and IL-12 responding cells, respectively, are subtracted. Median is indicated.

in approximately half of the pregnant women, in preeclampsia as well as in normal pregnancies. The other half of the pregnant women showed decreased number of cytokine secreting cells in response to paternal PBMC. No differences were found when comparing the fetus-specific number of cells secreting IFN- γ , IL-4, IL-10 or IL-12 between women with preeclampsia and women with normal pregnancies.

Recall Antigen (PPD and TT)-induced Secretion of IFN- γ , IL-4 and IL-10

The PPD-induced secretion of IFN- γ , IL-4 and IL-10 is shown in Fig. 3A–C. A majority of the pregnant women responded to PPD-stimulation with increased numbers of cells secreting IFN- γ as well as cells secreting IL-4 and they also showed increased numbers of cells secreting IL-10. The number of cells secreting IL-10 in response to PPD was generally larger compared with the number of IFN- γ or IL-4 secreting cells in both preeclampsia and normal pregnancies. There were no differences between preeclampsia and normal pregnancies in the numbers of PPD-specific cells

secreting IFN- γ , IL-4 or IL-10. Similar patterns of cytokine secretion was seen in response to TT (Fig. 3D–F), with no differences found between women with preeclampsia and women with normal pregnancies.

LPS-induced Secretion of IL-12

As a result of technical limitations, only five women with preeclampsia and 11 women with normal pregnancies were analyzed for LPS-induced secretion of IL-12. Only a few women responded to LPS-stimulation with increased secretion of IL-12 and there were no differences between preeclampsia and normal pregnancy (Fig. 4).

Comparison of Results from Moderate and Severe Preeclampsia

There were no significant differences in any of the cytokine responses between patients with moderate and severe preeclampsia data not shown). When comparing the paternal antigen induced secretion of IFN- γ , Mann–Whitney *U*-test showed lower number of IFN- γ secreting cells in severe compared

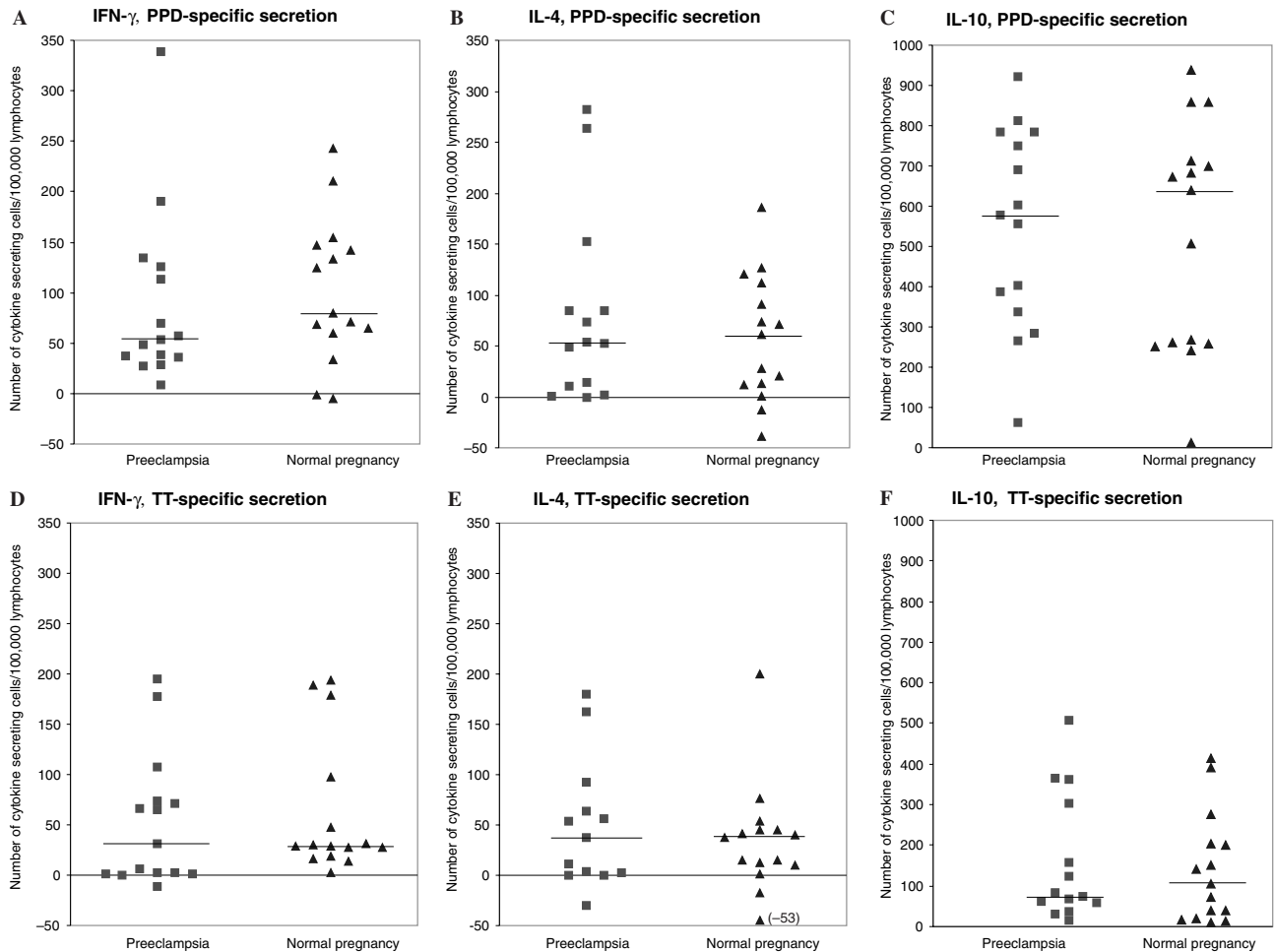


Fig. 3. Number of cells per 100,000 lymphocytes from women with preeclampsia and normal pregnancies, secreting interferon (IFN)- γ **A**, interleukin (IL)-4 **B** and IL-10 **C** in response to purified protein derivate of tuberculin (PPD). Also the figure shows the secretion of IFN- γ **D**, IL-4 **E** and IL-10 **F** in response and tetanus toxoid (TT). All values are net values, where the number of spontaneously IFN- γ , IL-4, IL-10 and IL-12 secreting cells, respectively, is subtracted. Median is indicated. Note the different scales in the diagrams.

with moderate preeclampsia ($P = 0.039$), but this was not significant when correcting for multiple comparisons.

DISCUSSION

The main finding of this study is the lack of differences between preeclampsia and normal pregnancy regarding spontaneous and antigen-induced secretion of IFN- γ , IL-4, IL-10 or IL-12 from PBMC. The finding of no difference in the spontaneous secretion of IL-4 between preeclamptic patients and women with normal pregnancies is in line with previous reports.^{4,20} In contrast, another group found elevated levels of IL-4 in sera from preeclamptic patients compared with controls.⁵ The spontaneous production of IFN- γ in preeclamptic patients has previously been reported to be increased, when compared with normal pregnancy,⁴

a finding that we could not confirm, possibly because of methodological differences.

We found similar secretion patterns of IFN- γ and IL-4 in preeclampsia and normal pregnancies, including spontaneous as well as fetus-specific and recall antigen induced secretion, detected by the ELISPOT assay, proven to be highly sensitive.²¹ Our finding strongly suggests that preeclampsia is not associated with a shift in the systemic immune balance towards Th1. This does, however, not rule out a role for a shift in the Th1/Th2 balance in preeclampsia. There could still be differences in the local cytokine responses in the close vicinity of the fetoplacental unit. We have previously shown that cytokine secretion in blood does not reflect local cytokine secretion in the decidua during the first trimester of normal pregnancy.²² Whether this holds true also for the third trimester is, however, still uncertain. The formation of spiral arteries during the second trimester and maybe also the

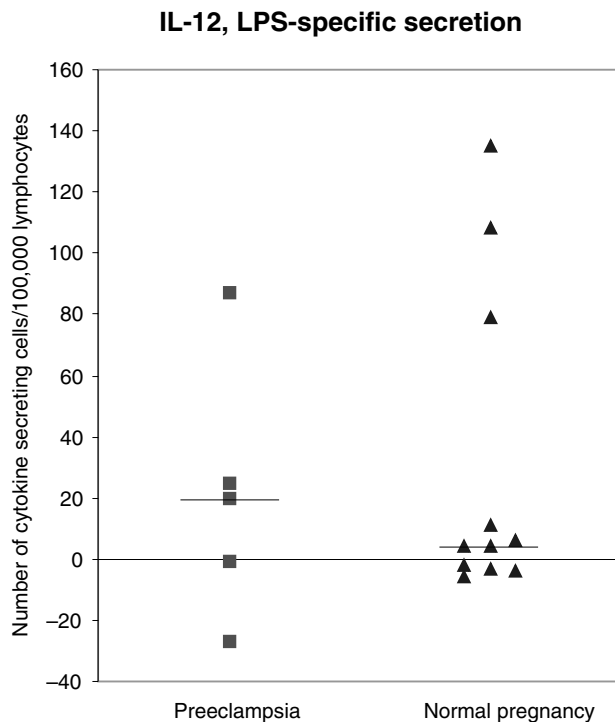


Fig. 4. Number of cells per 100,000 lymphocytes from women with preeclampsia and normal pregnancies, secreting interleukin (IL)-12 in response to lipopolysaccharides (LPS). All values are net values, where the number of spontaneously IL-12 secreting cells is subtracted. Median is indicated.

occurrence of fetomaternal hemorrhage during the third trimester,²³ could, speculatively, contribute to a mirroring of local immune responses in blood during late stage pregnancy.

The Th2-bias in pregnancy has been questioned.^{2,3} Assuming that pregnancy is not associated with a shift in the Th1/Th2 balance would obviously provide an alternative explanation to our present findings. Shimaoka et al.,² found no differences in systemic Th1/Th2 cytokine production when comparing normal pregnancy with non-pregnant women or postpartum women. There was, however, a slight increase in the IL-2/IL-10 ratio in the third trimester. It was suggested that pregnancy is associated with decreased systemic activity of the maternal immune system, including both Th1- and Th2-type responses. This is in conflict with our previous findings in normal pregnancies, showing increased numbers of both IFN- γ and IL-4 secreting cells, respectively, in PBMC,²⁴ mirrored by similarly increased PBMC expression of IFN- γ mRNA and IL-4 mRNA.²⁵ Furthermore, we have previously reported increased secretion of IL-4 in response to paternal allo-antigen in PBMC from normal pregnancies compared with age-matched non-pregnant controls.¹² Strikingly, non-pregnant women in fertile age showed IFN- γ secretion exclusively (lack of IL-4), in response to

allo-antigens.²⁶ Interestingly, no decrease in the fetus-specific IFN- γ response was seen in normal pregnancies compared with non-pregnant controls.¹² Thus, in sharp contrast to the report by Shimaoka and co-workers,² our previous findings indicate an increase of both Th1 and Th2 responses in blood during normal pregnancy. This may be interpreted as a Th2-situation, as the effects of IL-4 have been shown to predominate over the effects of IFN- γ .²⁷⁻²⁹ The present study confirms and extends our previous observations. First, all women, including both preeclampsia and normal pregnancies, showed high numbers of both IFN- γ and IL-4 secreting cells in the blood. Secondly, paternal PBMC induced increased secretion of both IFN- γ and IL-4 in preeclampsia as well as in normal pregnancy in a (substantial) number of cases. Thirdly, and most interestingly, PPD-stimulation similarly induced secretion of both IFN- γ and IL-4. PPD is known to induce secretion of IFN- γ , but no IL-4, in previously immunized healthy individuals.³⁰ PBMC from patients with atopic allergy have, however, been shown to secrete IL-4, in addition to IFN- γ , in response to PPD.³¹ This finding probably reflects the Th2-bias that is evident in atopic individuals.³² Consequently, the present finding of PPD-specific IL-4 secretion in pregnant women could be interpreted as reflecting a Th2-bias in the systemic immune responses during pregnancy and thus, strengthens the hypothesis of a Th2-bias being involved in normal pregnancy. Furthermore, as both the preeclamptic women and normal pregnancies showed an identical IFN- γ /IL-4 pattern in response to PPD, this strongly speaks against a systemic Th1-shift in preeclampsia.

The functional IL-12 molecule (p70), 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. Consequently, p40 is considered as an inhibitor of IL-12 responses, competing with the functional p70 heterodimer in the binding to the IL-12 receptor. Our p70 IL-12 findings are in line with the findings of Sacks et al.⁶ and Dudley et al.,⁷ 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.⁷ Taken together, these findings suggest that there is no increase in the induction of Th1-responses in preeclampsia, but rather the latter finding may suggest a decrease.

Interestingly, there are indications that the severity of the preeclampsia may influence the levels of IL-12 in blood. Sakai et al.⁸ found significantly higher levels of p70 IL-12 in cell culture supernatants when comparing severe preeclampsia with normal controls, but no

elevated levels in milder disease. In the present study, however, no differences in any of the cytokine responses were found between moderate and severe preeclampsia.

Another plausible explanation may be that there are less striking differences in innate immune responses (which include secretion of IL-12) between normal pregnancies and preeclampsia, than between normal pregnancy and the non-pregnant state.³³ This would be due to that normal pregnancy is associated with a dramatic increase in systemic innate responses, compatible with the picture in sepsis, and in preeclampsia a further increase of innate responses has been suggested.³³

In humans, in contrast to mice, the potent anti-inflammatory cytokine IL-10 is not a pure Th2-cytokine, but could be produced by both Th1 and Th2 cells¹⁴ as well as by non-T-cells, the main sources of IL-10 in peripheral blood being B-cells and macrophages.³⁴ There are strong indications that IL-10 is of great importance in the maintenance of pregnancy.¹⁶ Furthermore, IL-10 has been shown to restore pregnancy in mice programmed to acquire inflammatory immunity as a result of either Leishmaniasis major infection or intrinsic immune polarization.³⁵ The increased inflammatory response seen in preeclampsia,³³ would speculatively suit well with decreased levels of IL-10. However, we found no differences in IL-10 secretion between preeclampsia and normal pregnancy. This is in line with Gratacos et al.,²⁰ who found no differences in serum, but in contradiction to Benian et al.³⁶ who found elevated levels of IL-10 in plasma from preeclamptic women.

In conclusion, using the sensitive ELISPOT assay we found no evidence for a shift in the systemic Th1/Th2 responses, or alterations in the levels of the immunosuppressive cytokine IL-10, in preeclampsia compared with normal pregnancy. This does, however, not exclude differences in the local immune responses.

Acknowledgements

We are most grateful to all mid-wives of the clinic of Obstetrics and Antenatal Care in Linköping and Motala, who helped us with taking samples. This study was supported by financial grants from The County Council of Östergötland and The Health Research Council in the South-east of Sweden.

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