Immunological challenges during pregnancy : preeclampsia and egg donation

Hoorn, M.L. van der

Changes in cytokine production and composition of peripheral blood leukocytes during pregnancy are not associated with a difference in the proliferative immune response to the fetus

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Lisa Lashley
Marie-Louise van der Hoorn
Barbara van der Mast
Tamara Tilburgs
Nadine van der Lee
Carin van der Keur
Els van Beelen
Dave Roelen
Frans Claas
Sicco Scherjon

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Abstract

Objective: We analyzed peripheral blood from women at term pregnancy for leukocyte composition, *in vitro* proliferative responses and cytokine production after non- and fetus-specific stimulation.

Methods: Maternal PBMCs were collected and stimulated with umbilical cord blood (UCB) of own child, 3rd-party UCB, non-specific stimulus PHA and anti-CD3 antibody, with non-pregnant females (cPBMC) as control. Nine combinations of patient-child-3rd-party child and control were selected on basis of sharing one HLA-DR antigen. The response of mPBMC upon specific stimulation with fetal antigens was similar to cPBMC.

Results: No differences were found when comparing the maternal response upon stimulation to her own child with stimulation to a control child. Non-specific stimulation with PHA and anti-CD3 antibody did not reveal a difference in proliferation rate between mPBMC and cPBMC. However, mPBMC contained a higher percentage of CD14+ cells ($p=0.001$) and activated T cells (CD25dim, $p<0.0001$), but a lower percentage CD16-CD56bright NK-cells ($p=0.001$) and CD16+CD56+ NK-cells ($p=0.003$). mPBMC produced more IL-6, IL-10 and IL-17 compared to cPBMC ($p<0.05$).

Conclusions: We found differences in lymphocyte composition and cytokine production between mPBMC and cPBMC. These differences did not result in quantitative changes in proliferative responses during pregnancy compared to non-pregnant controls.

Introduction

During pregnancy, semi-allogeneic fetal tissue is directly exposed to the maternal blood since it invades the maternal decidua. This implies a possible attack of fetal tissue by the immune system of the mother. However, the fetus escapes from maternal rejection and is tolerated by the induction of several maternal and fetal mechanisms. In 1953, Medawar suggested several mechanisms to explain this 'immunological paradox of pregnancy' [1]. One of his explanations is based on a diminished maternal responsiveness to pregnancy, leading to acceptance of the foreign fetus. Indeed, the cellular immune response seems to be decreased during pregnancy, reflected by the increased susceptibility to viral infections and specific intracellular pathogens, such as *Listeria monocytogenes* and by the remission of some T-cell mediated autoimmune diseases in pregnancy [2,3]. Other clinical observations including flare-ups of humoral autoimmune diseases in pregnancy like systemic lupus erythematosus [4], suggest a paradoxical activation of other arms of the immune system, including B cells and innate immunity [5].

In fact, there is direct evidence for fetus-specific antigen recognition by the maternal adaptive immune system even during the first trimester exemplified by local lymph node swelling in mice in first pregnancy, a recall flare in the second pregnancy [6] and the formation of anti-paternal antibodies [7]. These antibodies are developed in 10-30% of women against paternal inherited human leukocyte antigens (HLA) of the fetus and can persist for more than 10 years [7]. In pregnancy, there are two ways of maternal sensitization: one locally in the fetal-maternal interface via processing of major histocompatibility complex (MHC) alloantigens by antigen-presenting cells and the second via fetal cell entry in the maternal circulation. This entry can consist of fetal whole cells (microchimerism), syncytiothrophoblast fragments, fetal DNA, and debris from apoptotic cells. The (long-term) consequence of the HLA antibodies is unclear; e.g. the presence of anti-paternal antibodies in patients with recurrent spontaneous abortion is associated with a higher [8] as well as with a reduced success rate [9] on live birth. T-cell allo-reactivity is observed in pregnancy. Primed T cells to paternal HLA antigens and fetus-specific minor histocompatibility complexes, like HY, have been demonstrated in the peripheral blood of pregnant women [10-12]. In addition, recent studies by our group show that the CD4+CD25dim (activated) T-cell population increases in maternal peripheral blood during pregnancy [13].

Pregnancy has long been suggested as a balance of the maternal immune system with a predominance of T helper 2 immunity [4,14,15]. Nowadays, little consensus on this Th1/Th2 shift in peripheral blood in normal human pregnancy exists [14,16,17] and more candidate mechanisms have been proposed to describe immunostimulation and immunoregulation during pregnancy. Saito et al. [18] state that while the Th1/Th2 balance is shifted, Th3 and Tr1 cells, which produce immunosuppressive cytokines TGF-β and interleukin (IL)-10 respectively, regulate the Th1 cell-induced rejection. A specialized subset of T cells, CD4+CD25bright regulatory T cells, regulate overstimulation of either type 1 or type 2 responses [18] and are therefore able to suppress autoimmunity [19]. In addition, recently a regulatory NK cell subset and NKr1 cells, producing IL-10, have been demonstrated which might play an important role in the maternal immune response [18,20,21].

These mechanisms (non-specific or specific for fetal antigens) have been described for complicated pregnancies in which human placental tissue damage was suggested to occur after immune activation [5,22,23]. However, so far specific and non-specific maternal immune responses during normal pregnancy have not been compared to non-pregnant controls. Therefore, we determined the phenotype of different subsets of leukocytes in the peripheral blood of pregnant and non-pregnant women using flow cytometry. We also studied the proliferation capacity and cytokine production of maternal peripheral blood mononuclear cells (mPBMC) in a mixed lymphocyte reaction (MLR) after stimulation with umbilical cord blood (UCB) derived lymphocytes of the own child and lymphocytes of another child (3rd-party UCB). A significant positive correlation was

found between the number of HLA-DR mismatches and the alloreactivity in transplant recipients [24]. Therefore, in this study we used 3rd-party UCB controls with an equal number of HLA class II mismatches compared to the own child.

Material and Methods

Blood samples

Heparinized maternal peripheral blood and UCB was obtained from healthy women after uncomplicated term pregnancy (with a minimal gestational age of 37 weeks, n=50). UCB was obtained directly after cord clamping from the umbilical cord veins. Patients tested in the proliferation experiments were 9 women who delivered by a cesarean section and 2 women who delivered spontaneously. Control PBMC (cPBMC) samples were obtained from age-matched healthy non-pregnant female volunteers (n=30). For each patient-child combination a control was selected on the basis of sharing one HLA-DR antigen with the child. We screened for maternal HLA antibodies and excluded combinations with HLA-DR antibodies. Table 1 shows the HLA-DR typing. Informed consent was obtained from all women. The study was approved by the Ethics Committee of the Leiden University Medical Center.

Blood was layered on a Ficoll Hypaque (LUMC pharmacy; Leiden, The Netherlands) gradient for density gradient centrifugation at room temperature (20min/800g). After centrifugation PBMCs were collected from the interface, washed twice and counted. Part of the cells were fixed with 1% paraformaldehyde and stored at 4°C until time of cell staining for flow cytometry analysis. For proliferation studies the remaining cells were frozen in liquid nitrogen.

Couple	Mother	UCB	3rd-party UCB	Control
1	DR17, DR4	**DR17**, DR15	**DR4**, DR13	DR17, DR4
2*	DR17, DR4	**DR4**, DR13	**DR4**, DR13	DR4 DR11
3	DR1, DR17	DR8, **DR17**	**DR1**, DR15	DR1, DR17
4	DR15, DR16	DR17, **DR16**	DR17, **DR15**	DR15, DR16
5	DR1, DR17	**DR17**, **DR15**	**DR17, DR7**	DR7 DR15
6	DR10, DR13	**DR4, DR13**	**DR7, DR10**	DR4, DR7
7	DR10, DR13	**DR7, DR10**	**DR4, DR13**	DR4, DR7
8	DR15, DR16	**DR17, DR15**	**DR1, DR15**	DR1, DR17
9*	DR4, DR9	**DR4**, DR13	**DR4**, DR13	DR4, DR11
10		**DR4**, DR13	**DR4**, DR13	DR4 DR17
11		**DR17**, DR15	**DR17**, DR7	DR1 DR17

Table 1 HLA-DR typing of mother, own child (UCB), control child (3rd party UCB) and control. Shared antigens are depicted in bold font. Combination 2 and 9 were omitted from the MLR results, since the HLA-DR antigens were similar between own and control child. Therefore, two extra control-child combinations were added with one shared HLA-DR antigen.

Flow cytometry

The following directly conjugated mouse-anti-human monoclonal antibodies were used for four-color immunofluorescence surface staining of the PBMCs: CD45-APC, CD14-FITC, CD19-PE, CD3-PerCP, CD4-APC, CD8-PE, CD16-FITC, CD25-PE, CD28-APC, CD56-PE, CD69-FITC and HLA-DR-FITC (Becton Dickinson, Franklin Lakes, NJ, USA), used in concentrations according to manufactures instructions. Flow cytometry was performed on a FACS Calibur using Cellquest-Pro software (Becton Dickinson). Percentages were calculated within gates set around the lymphocytes (in FCS/SSC dotplot) and the CD45+, CD45+CD3+, CD45+CD3+CD4+, or CD45+CD3+CD8+ fraction. %CD14+ cells were calculated within the CD45+ fraction without a lymphogate. Gating strategies were performed on basis of previous research [13].

Non-specific stimulation

Cultures were established in triplicate in flat-bottomed 96-well plates (Costar, Cambridge, MA, USA). One well contained $1x10^5$ PBMC's as responder cells in 100 µl of culture medium. Culture medium contained RPMI 1640 with 10% human serum and 3 mM L-glutamine. For mitogen stimulation, 100 µl of purified phytohemagglutinin (0.4 mg/ml, PHA) (Welcome, Dartford, UK) was added. For stimulation with CD3 antibody (Ab) the plates were incubated with 50 µl of anti-CD3 (OKT3, Ortho Biotec, Bridgewater, NJ, USA), diluted in PBS at 1 µg/ml concentration per well for 90 minutes at 37°C in a humidified atmosphere of 5% CO_2. Plates were washed twice with PBS before cells were added. Culture medium alone was used as a negative control. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 days. Cultures were pulsed with 20 µCi/well ^{3}H-thymidine diluted in RPMI 1640 medium for the last 8 hours of incubation. Just before pulsing, 100 µl of supernatant was removed from each well and stored at -20°C until further analysis. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy using a betaplate counter (Perkin Elmer, Waltham, MA, USA). The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Specific stimulation in one-way mixed lymphocyte reaction

Mixed lymphocyte cultures (MLR) were set up with 100 µl of $1x10^5$ mPBMC or cPBMC in culture medium added in triplicate wells in a round-bottom 96-well plate (Costar) to 100 µl of (a) $1x10^5$ irradiated (30 Gy) fetal leukocytes of her own child; (b) $1x10^5$ irradiated fetal leukocytes of a third party child or (c) culture medium. Proliferation was measured on day 5 and day 7 by incorporation of ^{3}H-thymidine added during the last 16 hours of culture. Just before pulsing, 100 µl of supernatant was removed from each well and stored at -20°C until further analysis. The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Cytokine Analysis

Harvested supernatants were tested for the following cytokines: IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IFN-γ, TNF-α, GM-CSF, using a Bio-Plex assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) following manufacturers instructions. Samples were analyzed using a Bio-Plex^{tm} Array Reader with Bio-Plex software.

Statistical Analysis

To determine differences between more than 2 groups an ANOVA was performed. If $p<0.05$, the Mann-Whitney test was performed to compare the phenotype of the different cell-subsets,

the proliferative responses and cytokine production of maternal lymphocytes and control lymphocytes. To compare the proliferative responses of maternal lymphocytes after specific stimulation with lymphocytes of own child and control child, the Wilcoxon signed rank test was performed. For all tests the value of $p<0.05$ was defined as significant.

Results

Phenotypic analysis

To compare the different subsets of leukocytes in the peripheral blood between pregnant and non-pregnant women, we performed a phenotypic analysis using flow-cytometry. No difference was observed in %CD3+ T-cells and %CD19+ B-cells. However, mPBMC contained a significantly lower percentage of CD16-CD56bright NK-cells (p=0.001) and CD16+CD56+ NK-cells (p=0.003) compared to non-pregnant cPBMC (Figure 1a). The %CD14+ monocytes were significantly higher in mPBMC (p=0.001, Figure 1b). Analysis of the different subsets of (CD3+) T-cells revealed no difference in %CD4+ or %CD8+ T-cells (Figure 1c). The activation state of CD3+ T-cells was studied by measuring CD69 expression (early marker of activation), IL-2R expression (CD25) and

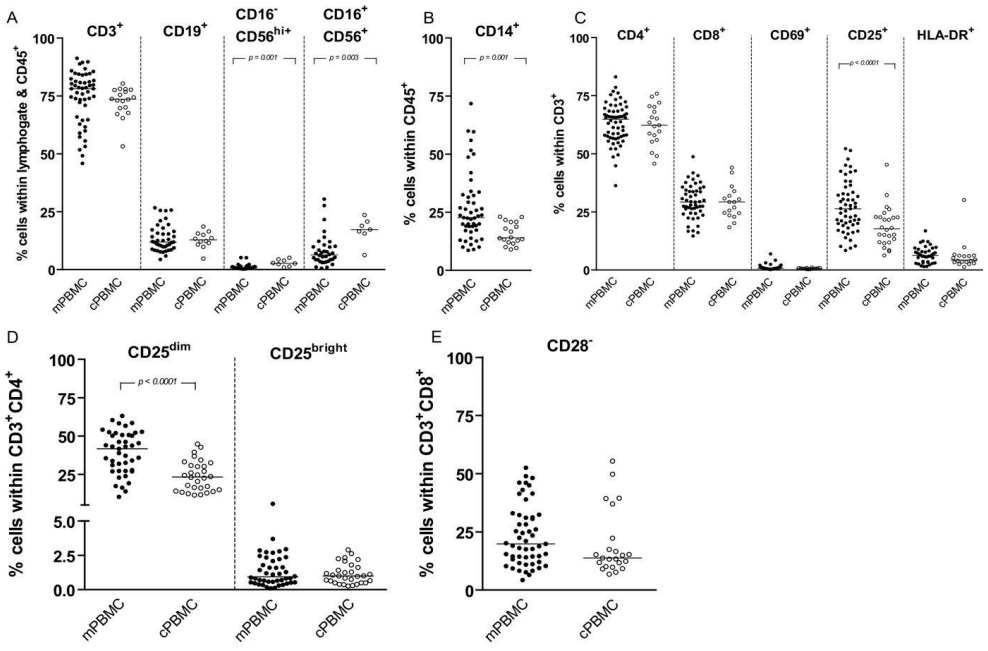

Figure 1 Distribution of different subsets of leukocytes in peripheral blood between pregnant (n=50) and non-pregnant (n=30) women. All lines are median percentages. A. Percentage of CD3+ within lymphogate and CD45+ cells in mPBMC (78.1%) and cPBMC (73.6%), percentage of CD19+ in mPBMC (11.9%) and cPBMC (12.8%), percentage of CD16-CD56hi+ in mPBMC (0.7%) and cPBMC (2.7%), and percentage of CD16+CD56+ in mPBMC (6.3%) and cPBMC (17.3%). B. Percentage of CD14+ within CD45+ cells in mPBMC (22.7%) and cPBMC (14.0%). C. Percentage of CD4+ within CD3+ cells in mPBMC (64.9%) and cPBMC (62.3%), percentage of CD8+ in mPBMC (29.2%) and cPBMC (29.3%), percentage of CD69+ in mPBMC (0.7%) and cPBMC (0.61%), percentage of CD25+ in mPBMC (26.3%) and cPBMC (17.7%), and percentage of HLA-DR+ in mPBMC (6.2%) and cPBMC (4.3%). D. Percentage of CD25dim within CD3+CD4+ cells in mPBMC (41.7%) and cPBMC (23.4%), percentage of CD25bright in mPBMC (0.9%) and cPBMC (1.0%). E. Percentage of CD28- within CD3+CD8+ cells in mPBMC (19.8%) and cPBMC (13.7%).

HLA-DR expression (late marker of activation). mPBMC contained a significant higher percentage of CD3+CD25+ T-cells compared to cPBMC (p<0.0001), no difference in percentage of CD69+, and a slightly higher but not significant increase in percentage HLA-DR+ T cells (p=0.11, Figure 1c). CD4+ T cells which express CD25 can be divided into a CD25dim population (activated phenotype) and a CD25bright population (regulatory phenotype). mPBMC contained a significantly higher percentage of CD4+CD25dim T-cells compared to cPBMC (p<0.0001, Figure 1d). However, there was no difference in percentage of CD4+CD25bright (regulatory) T-cells. The percentage of CD8+CD28- T-cells, another cell population with possible suppressive capacity, was not different from non-pregnant controls (Figure 1e).

Non-specific proliferative response to PHA and anti-CD3

In order to determine the proliferation capacity of mPBMC and cPBMC, cells were stimulated with PHA and anti-CD3 Ab for 3 days. There was no significant difference in proliferation to PHA or anti-CD3 Ab between maternal and control PBMC (p=0.55 vs. p=0.90, Figure 2).

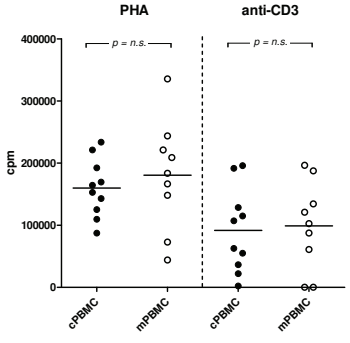

Figure 2 Proliferative response. Proliferative response of maternal PBMC (mPBMC, ○) and non-pregnant control PBMC (cPBMC, ●) upon stimulation with PHA or anti-CD3 antibody at day 3. Median values are depicted by a horizontal line.

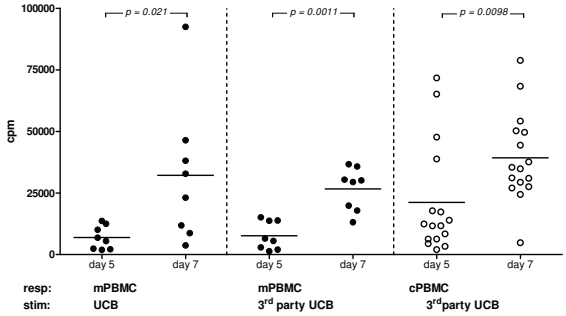

Figure 3 Proliferation of mPBMC to own child or a control child. Proliferation of mPBMC (●) to own child (UCB, left panel) or to a control child (3rd-party UCB, middle panel) measured at day 5 and 7. Proliferation of cPBMC (○) 3rd-party UCB (right panel) measured on day 5 or day 7. Median values are depicted by a horizontal line.

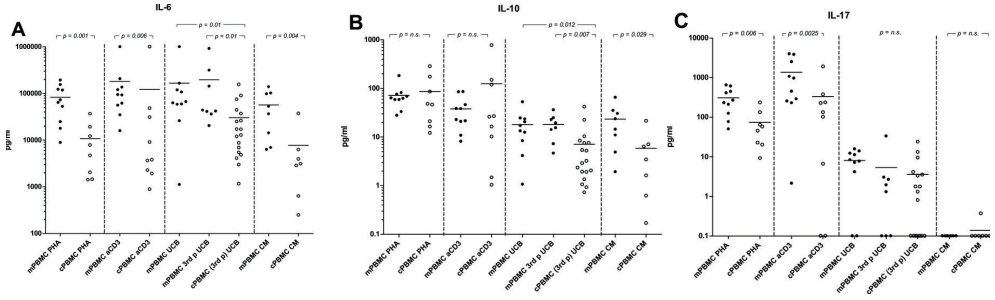

Figure 4 Cytokines in supernatant (pg/ml). IL-6 (**A.**), IL-10 (**B.**) and IL-17 (**C.**) levels in supernatants of mPBMC (●) vs. cPBMC (○) stimulated with PHA, anti-CD3 antibodies, own child (UCB), control child (3rd-party UCB) or culture medium (CM).

Fetus-specific immune response

To determine differences in the maternal immune response to UCB of her own child compared to a 3rd-party UCB, we analyzed proliferative capacity of mPBMC in a MLR. The response of mPBMC after stimulation with cells from the own child (UCB), with a control child (3rd-party UCB), and the response of cPBMC was significantly higher on day 7 compared to day 5 (p=0.021, p=0.001 and p=0.009 respectively), as expected with a normal mixed lymphocyte reaction. A non-parametric one-way ANOVA showed no significant differences between the responses of mPBMC, after stimulation with cells from her own child or control child, and cPBMC, both on day 5 (p=0.11) and on day 7 (p=0.34, Figure 3).

Cytokine production

The cytokine production by mPBMC and cPBMC was measured in the supernatant after co-culture of PBMC with the different stimuli on the fifth day. Only IL-6, IL-10 and IL-17 showed a significant difference between mPBMC and cPBMC responses with stimulation anti-CD3, UCB, or 3rd-party UCB (Table 2). We analyzed the amount of these cytokines (pg/ml) after mixed lymphocyte reaction daily to determine the day of maximum production. For IL-6, IL-10 and IL-17 this maximum was on day 5 (data not shown).

There was no difference in cytokine production by mPBMC when stimulated with the own child (UCB) compared to control child (3rd-party UCB). However many differences were found between mPBMC and cPBMC. mPBMC produced significantly more IL-6 after stimulation with all the non-specific and fetus specific stimuli (Figure 4a). The IL-10 production after allogeneic stimulation was significantly higher in mPBMC compared to cPBMC cultures (Figure 4b). mPBMC produced significantly more IL-17 compared to controls after PHA and aCD3 stimulation (Figure 4c), no differences were observed in IL-17 production after UCB stimulation.

Furthermore in control cultures with control medium alone a significantly higher production of IL-6 and IL-10 was observed in mPBMC compared to cPBMC.

	IL2	IL4	IL5	IL6[a]	IL10	IL12[b]	IL13	IL15	IL17	GM-CSF	IFNγ	TNFα
PHA	-	↓*	↓*	↑**	-	↓	-	-	↑**	-	-	-
aCD3	-	-	-	↑**	-	-	-	-	↑*	-	-	-
UCB	-	-	-	↑*	↑*	-	-	-	-	-	-	-
3p UCB	-	-	-	↑**	↑*	-	-	-	-	-	-	-
CM	-	-	-	↑*	↑*	-	-	-	-	↑*	-	↑*

Table 2 Cytokine production in supernatants of mPBMC versus cPBMC. Cells stimulated with PHA, anti-CD3, own child (UCB), control child (3p UCB) or culture medium (CM). [a] production very high, [b] production very low, - = similar levels, ↓ = decreased in mPBMC, ↑ = increased in mPBMC, *p<0.05, **p<0.01.

Discussion

In this study we examined leukocyte composition, proliferative responses and cytokine production in mPBMC and cPBMC. We observed a significant increased percentage of monocytes and activated T cells (CD3+CD25+) in mPBMC compared to cPBMC. In contrast we observed a decreased percentage of both NK-cell subsets (CD16+CD56+ and CD16-CD56bright) in mPBMC.

No differences between mPBMC and cPBMC were observed in the proliferative responses to anti-CD3, PHA, fetus specific UCB and 3rd-party UCB. However, a significant increase in IL-6, IL-10 and IL-17 was observed in mPBMC compared to cPBMC. No differences between fetus specific and 3rd-party UCB were observed. These data indicate that the maternal peripheral immune response is altered during pregnancy, though these differences do not result in quantitative changes in proliferative responses during pregnancy compared to non-pregnant controls.

The increase in percentage of CD14+ monocytes in pregnant woman versus non-pregnant women confirms an increased production of monocytes or an increased trafficking of the monocytes. Macrophages and monocytes have been reported to be more activated with cell surface marker expression similar to those during systemic sepsis [25,26]. Absolute numbers of circulating NK-cells (CD16+CD56+) have been described to increase in early pregnancy and decrease in late pregnancy when compared to non-pregnant healthy controls [27,28]. We confirm these data by showing a decreased percentage of both NK-cell subsets (CD16+CD56+ and CD16-CD56bright) at term pregnancy in pregnant versus non-pregnant women.

With respect to the acquired immunesystem we found no difference in percentage of CD8+ T-cells, CD4+ T-cells or B-cells in pregnant versus non-pregnant women. Large contradictions between the results of different studies have been described; for CD8+ T-cells an increase [29], no change [27] and even a decrease [30] were found in pregnant women compared to non-pregnant controls. During labor an increase of CD8+ T-cells has been reported [31]. Discrepancies also exist for the CD4 (helper) T-cell subset. Some studies show no change [27,32] whereas others found a decreased percentage in pregnant women [28]. Frequency and counts of B-cells seem to be unaltered during pregnancy [2,28]. These inconsistent findings may be caused by difference in analyzing methods or most likely by differences between patient groups.

We did find a higher percentage of activated T-cells (CD4+CD25dim) in pregnant women compared to non-pregnant controls, and a slightly higher percentage of HLA-DR+ T-cells (p=0.11), confirming earlier studies by our group [13]. These findings provide evidence for activation of the adaptive immune system during pregnancy.

Alterations in the distributions of T cells may lead to pregnancy complications. Decreased numbers of regulatory T cells in peripheral blood have been found in preeclampsia and recurrent spontaneous abortions [23,33]. These results postulate that a sufficient number of regulatory T cells is necessary to maintain an uncomplicated pregnancy. The exact mechanism how regulatory T cells are activated and induce tolerance during pregnancy remains to be elucidated. We found a significantly higher percentage of activated T cells (CD4+CD25dim), but no significant difference between the percentage of CD4+CD25bright in mPBMC compared to cPBMC. Previous studies found a significantly increased CD4+CD25bright T cells fraction in peripheral blood samples of pregnant women [34,35]. This discrepancy might be explained by different time points of maternal blood sampling or due to differences in gating strategies of CD25 expression. We earlier showed that differences in gating strategies might be responsible for different results [13].

HLA-mismatching between maternal and fetal antigens is a possible source of immune activation during pregnancy. The responsiveness to fetal antigens is probably a key factor controlling the activity of the maternal immune system in pregnancy and may influence pregnancy outcome [36]. In this study we do not demonstrate a difference between the maternal peripheral response to

own child UCB and 3rd-party UCB. In contrast to other studies, we used 3rd-party UCB controls with an equal number of HLA-DR mismatches compared to the own child. Since we performed HLA typing before proliferation, we had to use frozen cells, which is a drawback of this study. Our results confirm an earlier study where reactivity of mPBMC to own and unrelated newborn lymphocytes was not different [37]. Steinborn et al. showed reduced responses in MLR to own child compared to control donors [38]. In this study, the cells were obtained from adult volunteers instead from UCB. The observed difference can be explained because fetal antigen presenting cells are less efficient than adult antigen presenting cells.

Our data show that the mother's peripheral immune system has an equal proliferation capacity to cells from her own child as to those from an unrelated control child.

We observed also no differences in cytokine response between stimulation with own child and an unrelated child. However, significant differences in IL-6, IL-10 and IL-17 production between mPBMC and non-pregnant cPBMC were observed. Recently, Visser et al. reviewed the literature on cytokine and chemokine mapping in pre-eclampsia [39], including a few studies on normal pregnancies compared to non-pregnant women. One study described increased serum/plasma levels of IL-6 and TNF-α in pregnant women compared to non-pregnant controls [40]. In cultured PBMC (monocytes stimulated with LPS) no difference was found in IL-1β, IL-6 or TNF-α production [26]. We found an increase in IL-6 production by PBMC from pregnant women compared to non-pregnant controls, either spontaneously, but also after non-specific and allo-specific stimulation. TNF-α production was only higher in supernatant from cells with culture medium alone, which was also seen for IL-6 and IL-10 production. Probably these cytokines are produced by activated monocytes from the maternal peripheral blood. Again this suggests a more activated innate immune system in pregnancy.

We found no difference in IFN-γ levels and a slight decrease in IL-4 after mitogen stimulation. Other studies observed a decrease in numbers of maternal lymphocytes producing IFN-γ [14,41,42] and no difference in producing IL-4 [41,42]. A significantly increased number of PBMC producing IL-4 and unchanged number of cells secreting IFN-γ in the second and third trimester was found by Ekerfelt et al. [43]. These discrepancies in the outcomes of IL-4 and IFN-γ production are possibly due to different methods of stimulation or different methods of measuring cytokine production. In addition, we used PBMCs while other studies analyzed different cell populations.

Furthermore, we found hardly any IL-12 in our supernatants, which may be due to the fact that we used non-separated leukocytes in one culture well (about 20% of CD45+ cells were CD14+) or that the percentage of CD14+ macrophages was too low to be able to detect any IL-12 produced. On IL-12 also contradictory results have been described; Sakai et al. found a decreased production in cultured PBMC (no stimulus) [44] whereas an enhanced production of IL-12 by monocytes was seen (stimulation with endotoxin and IFN-γ) by Sacks et al. [41]. It seems that an increased or decreased production of IL-12 is dependent on the method applied.

In our patients, IL-10 production was increased especially after stimulation with allo-antigens, but also spontaneously. IL-10 is a major T helper cell type 2 or regulatory cytokine produced by T regulatory cells or NK cells. It inhibits T cell activation and production of cytotoxic cytokines (IL-12 and IFN-γ) but stimulates induction of regulatory T cells [3]. Hereby, the Th1 response is suppressed [18]. It is tempting to speculate that Th2 cells do play a role in allo-responses during pregnancy, but IL-10 can also be produced by Th1 cells, macrophages and B cells, not only by Th2 cells. Populations of peripheral blood IL-10-producing NK cells in early pregnancy were increased [45]. Veenstra van Nieuwenhoven et al. also reported a mild increase in the IL-10 production of pregnant peripheral blood NK in the third trimester of pregnancy compared to non-pregnant women [42]; however this increase was not significant. The same group found no change in IL-10 producing T cells after stimulation with PMA and ionomycin (unpublished data).

We observed more IL-17 production after non-specific stimulation, but no difference after allo-specific stimulation. Nakashima et al. also showed no difference in IL-17 production after non-specific stimulation (PMA and ionomycin) of PBMC [46]. Th17 cells, the CD4+ cells that produce pro-inflammatory IL-17, is a recently discovered population involved in the maternal immunomodulation [47,48]. These cells are closely related to regulatory T cells and differentiate upon inflammatory signals whereas conditions that promote tolerance favor generation of Treg [49]. A balance between Th17 and Treg might be correlated with successful pregnancy; however the role of Th17 in human pregnancy remains to be investigated more substantially.

In conclusion, our results demonstrate that in the peripheral circulation, the innate and the acquired immune system are enhanced during pregnancy compared to non-pregnant controls reflected by phenotype of PBMC and *in vitro* cytokine production. However, there is no changed immune response when measuring proliferation capacity. The mother is capable of creating a fine-tuned environment optimal for the fetus to grow but also optimal to maintain adequate immune responses to diseases.

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