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Immunological challenges during pregnancy : preeclampsia and egg donation

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Citation

Hoorn, M. L. van der. (2012, January 11). *Immunological challenges during pregnancy : preeclampsia and egg donation*. Retrieved from <https://hdl.handle.net/1887/18330>

Version: Corrected Publisher's Version

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Differential immunoregulation in successful oocyte
donation pregnancies compared with naturally
conceived pregnancies



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Abstract

Background: In oocyte donation (OD) pregnancies, there is a higher level of antigenic dissimilarity between mother and fetus compared to naturally conceived (NC) pregnancies. This might lead to a higher degree and/or a different type of immunoregulation to maintain an uncomplicated pregnancy.

Methods: To test the hypothesis, different immunological aspects of OD (n=28) were compared with those of NC (n=51), and as an additional control non-donor IVF (n=20) pregnancies. The expression of IL-10, IL-6, galectin-1, pSMAD2 and Flt-1 was studied immunohistochemically in decidua and in maternal serum. Maternal peripheral blood mononuclear cells (mPBMCs) were characterized by flowcytometry and correlated with the number of HLA mismatches. mPBMCs were stimulated with umbilical cord blood or control PBMCs in a mixed lymphocyte culture.

Results: Compared to NC, OD pregnancies expressed less IL-10, IL-6, galectin-1, pSMAD2 and Flt-1 in the decidua and more IL-10 and IL-6 in serum. The percentages of CD4+CD25^{bright} and CD4+CD25^{dim} cells were higher in mPBMCs of OD and IVF pregnancies compared to NC. The number of HLA mismatches was positively correlated with the percentage of activated CD4+CD25^{dim} cells in mPBMCs of OD. Functional studies showed a lower proliferative response in OD pregnancies.

Conclusion: Immunoregulation in OD is different than in NC pregnancies. A higher degree of peripheral immunoregulation and a different cytokine profile in the decidua was found in OD and IVF pregnancies compared to NC pregnancies. More HLA mismatches in OD pregnancies lead to higher percentages of activated T cells in peripheral blood, but their reactivity is effectively compensated by regulatory T cells.

Introduction

In pregnancy, genetically different or semi-allogeneic fetal tissue invades the maternal decidua and is directly exposed to the maternal blood. This causes a risk of being attacked by components of the immune system of the mother. However, the fetus not only escapes maternal immune rejection, but induces tolerance, creating an immune privileged site at the fetal-maternal interface [1,2]. The most accepted view is that a successful pregnancy depends on an appropriate balance of the maternal immune system with a predominance of T helper 2 immunity [3-5]. In oocyte donation (OD) pregnancies, there is a higher degree of antigenic dissimilarity compared to naturally conceived (NC) pregnancies. In OD pregnancies, adaptation of the maternal immune system is probably even more necessary to maintain uncomplicated acceptance of the allogeneic fetus [6]. Indeed, an increased percentage of intracellular IFN- γ (Th1) and IL-4 (Th2) positive CD4⁺ T lymphocytes was found in the peripheral blood of pregnant women who conceived by OD compared with pregnant women after natural conception [7]. This hyperactivation of Th1 and Th2 by the allogeneic fetus is specific for OD pregnancies [7]. Histological findings in OD placentas often show a host versus graft rejection phenomenon similar to that seen with solid organ transplantation [8]. Severe chronic deciduitis admixed with fibrinoid deposition has been observed in OD placentas compared with non-donor in vitro fertilization (IVF) placentas [8]. Chronic deciduitis is found in the basal plate of the placenta, the site where extravillous cytotrophoblast interfaces with the maternal decidua. This pathology is thought to have an immune basis. Although OD pregnancies represent a very interesting model to investigate immunological interactions, most research has focused on the medical maternal and fetal complications rather than on the basic immunology. Although much research has been performed on the immunology of normal pregnancy, the immunology of OD pregnancies has not been studied intensively. The current study focused on immunological aspects of OD pregnancies compared to NC pregnancies. We hypothesized that there are differential immunoregulatory mechanisms that govern OD pregnancies compared to NC and IVF pregnancies at both local (fetal-maternal interface) and peripheral (peripheral blood) levels.

Maternal immune adaptations to the developing embryo are necessary in order to guarantee pregnancy success. Cytokines play an important role in promoting immune tolerance. Interleukin-(IL)-10 is seen as a facilitator of successful pregnancy; alterations of its levels may be related to adverse pregnancy conditions [9,10]. In addition, increased concentrations of IL-6 and other pro-inflammatory (IL-1, TNF- α , and IL-8) cytokines are found in the placentas of pregnancies complicated by pre-term premature rupture of the membranes [11]. During pregnancy, the expression of gal-1 is upregulated during implantation. Supplemental administration of gal-1 rescues the pregnancy in a mouse model of spontaneous abortion by inducing expansion of regulatory T cells that produce IL-10 [12]. Decreased expression of gal-1 in trophoblasts may partly explain disturbed differentiation during early placentation that leads to early pregnancy loss [13]. Another important immunoregulatory molecule is TGF- β , which is involved in blastocyst implantation by inducing apoptosis of endometrial cells within the uterus [14]. Decidual TGF- β is proposed to act on uterine natural killer (NK) cells to downregulate their cytotoxicity, resulting in the uterine-specific phenotype [15]. Binding of TGF- β to its receptor leads to activation of the TGF- β /ALK5 signaling pathway, which results in a cascade of reactions that eventually lead to the phosphorylation of SMAD2 (pSMAD2). TGF- β is a repressor of cytotrophoblast outgrowth [15], and it plays a role in angiogenesis. Angiogenesis also forms part of the maternal adaptation during embryo implantation. Regulation of vascular endothelial growth factor (VEGF) levels is a highly regulated process. Flt-1 is a receptor for VEGF and placental growth factor (PLGF). A splice variant of Flt-1 is sFlt1 (also known as sVEGFR-1), which antagonizes the VEGF and PLGF receptors. This soluble form prevents interactions of VEGF and PLGF with the functional membrane bound Flt-1, which thereby leads to endothelial dysfunction [16]. In the peripheral blood of preeclampsia patients, soluble Flt-1 (sFlt-1) is expressed in excessive amounts [17]. Since OD pregnancies are

associated with a higher incidence of preeclampsia, the levels of sFlt-1 in serum and decidua were tested as well.

The maternal blood is present in the intervillous space and is in direct contact with the outer syncytiotrophoblast layer of the placenta. This layer undergoes turnover, which precipitates shedding of microparticles into maternal blood. Therefore, in addition to the analysis of cytokine expression in the decidua, we also studied cytokine levels in maternal serum and the phenotype of peripheral blood mononuclear cells (PBMCs) by flow cytometry. CD4⁺CD25^{bright} regulatory T cells are believed to have a crucial role in maintaining pregnancy [18]. Transfer of CD4⁺CD25⁺ T cells from normal pregnant mice in to abortion prone mice prevents fetal rejection in the abortion prone mice [19]. In human an increased percentage of CD4⁺CD25⁺ T cells is present during pregnancy [20], however this study does not distinguish between CD4⁺CD25^{bright} and CD4⁺CD25^{dim} cells. Previously, it has been shown that the CD4⁺CD25⁺ cells can be divided in three fractions [18]. The percentage of FoxP3⁺ cells within the CD25^{bright} T cells in peripheral blood of pregnant women is around 53% [21]. FoxP3 is an additional marker which may help to distinguish between effector and regulatory cells. However, this phenotypic distinction remains controversial [22] and therefore functional tests remains to be established until a specific marker for regulatory T cells is found. Since OD pregnancies are characterized by a higher number of HLA mismatches compared with NC pregnancies, the role of the number HLA mismatches was studied by correlating phenotypic results with the degree of antigenic dissimilarity. Furthermore, functional assays were performed to demonstrate the immune reaction of maternal PBMCs against fetal cells. We hypothesized that differences in the immunoregulatory mechanisms are present between OD, non-donor IVF, and NC pregnancies.

Material and Methods

Patient selection

Pregnancies conceived by OD (n=28), non-donor IVF (n=20), and NC (n=51) were studied. The non donor IVF group consisted of cases that conceived by IVF with the woman's own oocytes. Medical records were reviewed and clinical data were summarized. Placentas, peripheral blood and umbilical cord blood (UCB) samples were collected at delivery from women after uncomplicated pregnancies at 37-42 weeks' gestational age. Exclusion criteria were complications such as preeclampsia, preterm birth, immunological diseases, and infections. Placental tissue samples were collected within five hours after delivery. The study protocol was approved by the ethics committee of the Leiden University Medical Center (LUMC), and informed consent of every patient was obtained.

Blood samples

Peripheral blood samples from the mothers and the UCB were obtained at term and collected in heparinized tubes. 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. The cells were frozen in fetal calf serum with 10% dimethyl sulfoxide and stored in liquid nitrogen until proliferation studies and flow cytometry analyses were performed.

HLA typing

For every mother and child the HLA-types were determined in the typing laboratory of the Leiden University Medical Center. DNA was typed for the loci HLA-A, -B, -C, -DRB1 and -DQB1 using sequence specific oligonucleotides (SSO) PCR. The number and types of HLA matches and mismatches for every mother and child combination was calculated (Calculation by Microsoft Access 2003).

Cytokine determination in serum and supernatant

The levels of IL-10 and IL-6 in maternal serum were tested using a Th1/Th2 Bio-Plex Luminex™ system assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) following the manufacturer's instructions. TGF-β1 was tested with the Milliplex™MAP single plex assay (Millipore Corporation, Billerica, MA, USA). Samples were analyzed using a Bio-Plex™ Array Reader with Bio-Plex software. An enzyme-linked immunosorbent assay (ELISA) for the presence of galectin-1 was performed as described [23] and sFlt-1 in maternal serum was performed by following the manufacturer's operating instructions (R&D Systems, Minneapolis, Minnesota).

Immunohistochemistry

For every study group (OD, NC and non donor IVF pregnancies) ten cases were selected randomly for immunohistochemical staining. These selected cases were representative of the patient characteristics as shown for the whole cohort in Table I. The mode of delivery was not significantly different between the three groups. Tissue samples of the placenta and rolls of fetal membranes were fixed in 4% formalin and processed for immunohistochemistry as previously described [24]. Briefly, sequential serial sections (4µm-thick) were cut. Tissue sections were deparaffinized and endogenous peroxidase was blocked. Antigen retrieval was performed by boiling the sections for 10 minutes in citrate buffer (pH 6.0). The optimal dilution for each primary antibody was determined in positive decidual tissue selected on the basis of maximal specific reactivity and minimal background staining; IL-10 1:100 (HP9016, Hycult Biotech Inc, Plymouth meeting, PA), IL-6 1:20 (AF-206-NA R&D Systems Europe Ltd.), pSMAD2 1:1000, Flt-1 1:250 (SC-316, Santa Cruz Biotechnology, Inc, Heidelberg, Germany), gal-1 1:500 (sc-28248, Santa Cruz Biotechnology). The primary antibodies were incubated for one hour (IL-10 and IL-6 overnight) at room temperature at the appropriate dilutions in PBS with 1% BSA. As a negative control the primary antibody was replaced with PBS with 1% BSA. Slides were incubated for 30 minutes with Envision (DAKO, North America Inc, USA) or for IL-10 with Powervision (Immunologic, Duiven, the Netherlands). For IL-6, a secondary goat antibody was labeled with HRP, (DAKO, North America Inc, USA, 1:200), and for gal-1 a secondary goat anti-rabbit antibody was labeled with HRP, followed by incubation with diaminobenzidine (DAB, DAKO Cytomation). The tissue sections were subsequently counterstained with haematoxylin (SIGMA, Switzerland, Steinheim), except for the pSMAD2 slides, since this staining is positive in the nuclei. The slides were mounted in mounting medium (Surgipath Medical Ind., Inc. Richmond) and covered. Images of all immunohistochemical staining results were captured using a microscope (Carl Zeiss Inc., Oberkochen, Germany) and digitally analyzed (Zeiss Axioskop 40, magnification 200x, Zeiss Axiocam MRC 5 camera, 150x150dpi). For every staining of one placenta a total of 5 pictures of the decidua parietalis and 5 of the decidua basalis was taken, blinded for the study group. Only the decidua was selected; blood vessels and shadows were digitally removed. Using Image-J software [25], the number of positive pixels per area was measured, indicating the level of expression for each immunohistochemical staining. This program is able to identify and measure positive cells by setting a threshold. For every staining experiment, an automatically running function was made, predefining the threshold of a positive cell. This threshold was independently defined by two observers.

Phenotypic characterization of maternal peripheral blood cells

Flow cytometric analysis was performed with a standardized protocol, using gating strategies as previously described [18]. In short, a four-color immunofluorescence staining was performed with directly conjugated mouse-anti-human monoclonal antibodies. CD45-APC, CD14-PE, CD25-PE, CD3-PerCP and CD4-APC were used in concentrations according to manufacturer's instructions (Becton Dickinson). The maternal PBMCs used for the functional assays were phenotyped. Only spontaneously deliveries were included for this analysis. These values were compared with a previously run control panel of 39 NC spontaneously delivered pregnancies that were obtained using the same standardized protocol [26]. Flow cytometry was performed on a Calibur flow cytometer using Cellquest Pro software (Becton Dickinson) and all samples were analyzed using the same template. Percentages were calculated within the lymphocyte set around the viable lymphocytes based on the expression of CD45, CD14 and CD3. The percentages of CD4+CD25dim and CD4+CD25bright cells were calculated within the CD3+CD4+ cell population.

Functional analysis

Functional assays were performed to demonstrate the reaction of mPBMCs against mother's paired umbilical cord blood (UCB), a third party UCB (3p UCB) and third party peripheral blood leukocytes (3p PBL). Responders (mPBMCs) and stimulators (UCB, 3p UCB and 3p PBL) for the mixed lymphocyte cultures (MLC) were selected on the basis of the number of HLA-DR mismatches. As a reflection of the normal genetics in pregnancy, responders of the OD, non donor IVF and NC pregnancies were selected to have 1 HLA-DR mismatch with their own UCB (as is usually the situation in non donor pregnancies). In addition, a fully allogeneic OD group was studied, which had 2 HLA-DR mismatches with own UCB (indicated in the figures as OD*). As controls, the maternal responder cells were stimulated with 2 HLA-DR mismatched 3p UCB and 3p PBL. In each group, a total of five mother-UCB combinations were tested. MLCs were set up with 50 μ l of 1×10^6 mPBMCs in culture medium added in triplicate wells in a round-bottom 96-well plate (Greiner Bio-one) to 50 μ l with 1×10^6 irradiated (30 Gy/3000 Rad) stimulators or culture medium. Proliferation was measured on day 7 by incorporation of ^3H -thymidine added during the last 16 hours of culture. The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Statistical analysis

Descriptive statistical analyses were performed using Graph Pad Prism (Graph Pad Software Inc.) and SPSS (SPSS Inc 17). The non parametric one-way ANOVA Kruskal-Wallis test was performed, and when significant, the post test Dunns was used to analyze between more than two independent groups. The non-parametric Mann Whitney test was used to identify differences between two independent groups. Linear data was analyzed with the linear regression analysis. Data were considered significant at $p < 0.05$.

Results

Clinical data

The patient characteristics are shown in Table I. No significant differences between the three groups were present with respect to gestational age. Maternal age was significantly higher and

gravidity was significantly lower in the OD group compared with the NC pregnancies. The mode of delivery differed significantly between the three groups. Therefore the influence of the mode of delivery (primary section, secondary section and spontaneous) and the pregnancy group was analyzed in a logistic regression model backward-stepwise (Wald). Pregnancy group and mode of delivery were used as a predictor for high (above median) or low (below median) serum levels. Using the backward model, pregnancy groups seemed to be the predictor for the levels of IL-10, IL-6, TGF- β , and the mode of delivery had no influence. For Gal-1 the mode of delivery

	Oocyte donation (OD) N=26	Naturally conceived (NC) N=51	Non donor IVF (IVF) N=20	P value ANOVA	Post test
Gestational age (days)	278.0 (257-293)	275.5 (264-293)	273.0 (257-294)	ns	
Maternal age (years)	37.5 (30-45)	33.0 (28-40)	36.0 (28-41)	0.0003	ED vs NC: *** ED vs IVF: ns IVF vs NC: ns
Gravidity (number)	2 (1-5)	3 (1-7)	2 (1-5)	0.023	ED vs NC: * ED vs IVF: ns IVF vs NC: ns
Total HLA mismatches (A, B, C, DR, DQ)	6 (2-10)	4 (0-5)	3 (0-4)	<0.0001	ED vs NC: *** ED vs IVF: *** IVF vs NC: ns
Mode of delivery				0.0012	ED vs NC: ns ED vs IVF: * IVF vs NC: **
- Primary section	7	35	4		
- Secondary section	5	1	2		
- Spontaneous	14	15	14		
- Vacuum extraction	2	1	1		
HLA class I mismatches	4.0 (1-6)	2.5 (0-3)	2.0 (0-3)	<0.0001	ED vs NC: *** ED vs IVF: *** IVF vs NC: ns
HLA class II mismatches	2 (0-4)	1 (0-2)	1 (0-2)	0.0001	ED vs NC: *** ED vs IVF: * IVF vs NC: ns

Table 1 Patient characteristics. Values are medians with the minimum and maximum. The one-way ANOVA Kruskal-Wallis test was performed. When significant, the post test Dunns was used to analyze differences between groups.

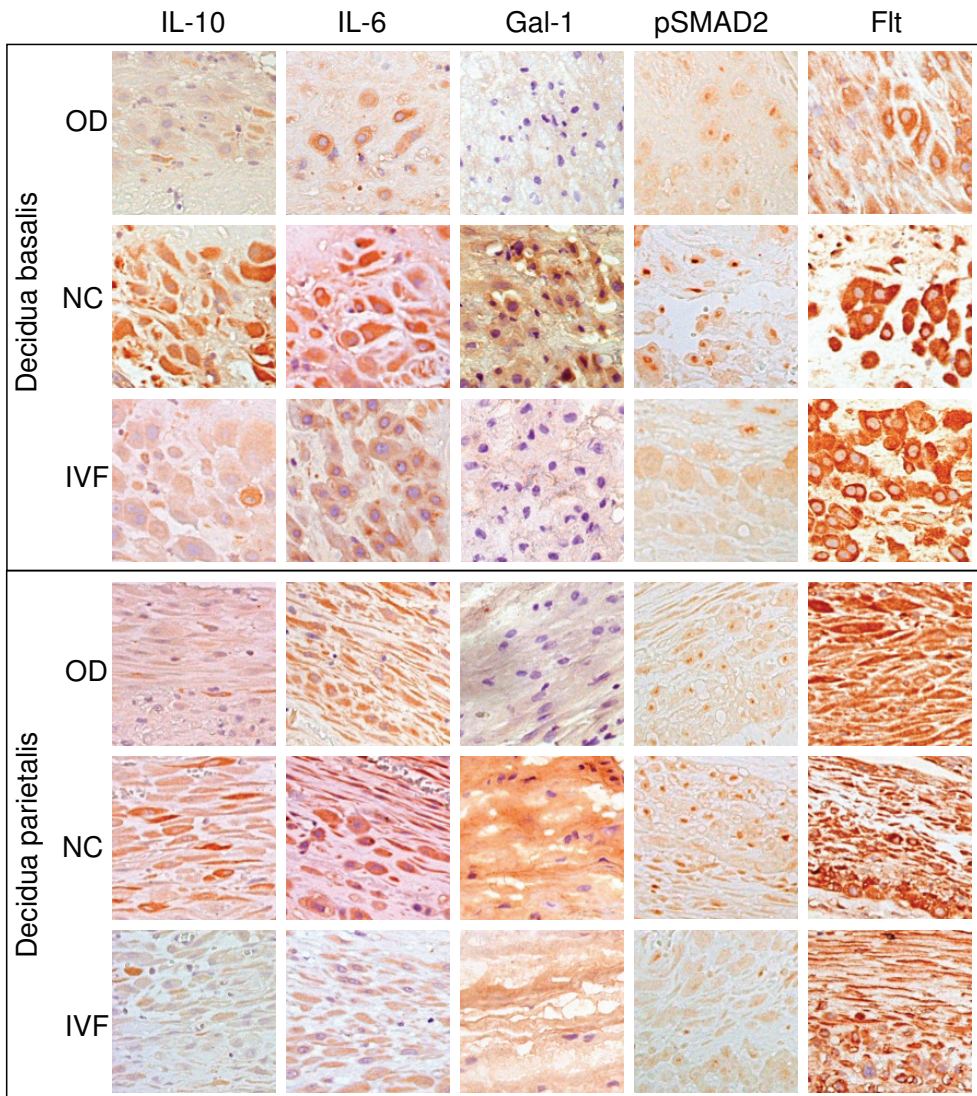


Figure 1 Immunohistochemical analysis. Photomicrographs of sections stained for IL-10, IL-6, galectin-1, pSMAD2 and Flt of the decidua basalis (upper panel) and decidua parietalis (lower panel). Original magnification x200. For every group, oocyte donation (OD), naturally conceived (NC) and non donor IVF, a representative example per staining is given. Positive cells are stained brown. Nuclei are stained blue (except for pSMAD2). The results of the digital image analysis of the immunohistochemical staining are depicted in Figure 2.

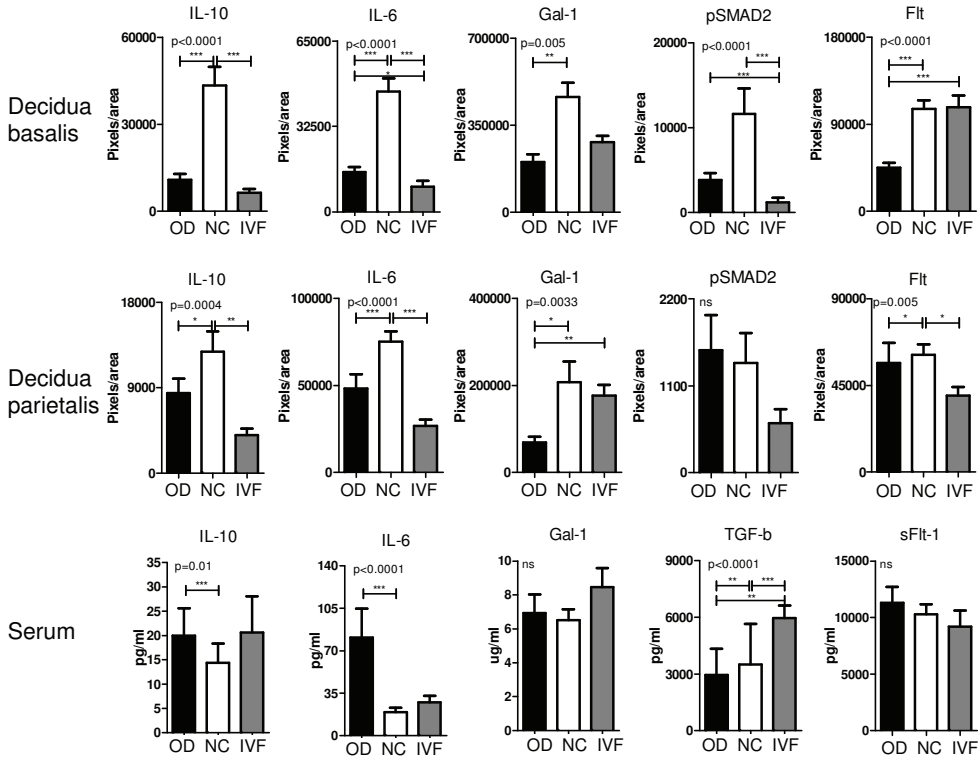


Figure 2 Placental and serum cytokine levels. Upper two rows show graphs illustrating the amount of positive pixels per standardized area in the decidua basalis and decidua parietalis of oocyte donation (OD, n=10), naturally conceived (NC, n=10) and non donor IVF pregnancies (IVF, n=10) tested by immunohistochemical staining. Lowest row shows results of serum cytokine levels defined by ELISA compared between OD (n=26), NC (n=51) and IVF (n=20). Level of significance indicated with asterisk, *** = $p < 0.001$, ** = $p = 0.001-0.01$, * = $p = 0.01-0.05$, ns = not significant. Values presented as means, error bars indicate the SEM.

seemed to be a predictor for higher serum levels. For sFlt, neither mode of delivery nor pregnancy group had an influence on the serum levels. This statistical test was not performed for the immunohistochemistry analysis since in those selected cases there was no significant difference between the groups in mode of delivery. For the flow cytometry analysis only spontaneously delivered pregnancies were used. Inherent to OD pregnancies the number of HLA mismatches was significantly higher compared to NC and non donor IVF pregnancies (6, 4, 3 respectively, $p < 0.0001$). When analyzed separately, both the number of HLA class I and class II mismatches were significantly higher in OD compared to NC and non donor IVF pregnancies ($p < 0.0001$ and $p = 0.0001$ respectively).

Immunohistochemical studies in placenta

The decidua of OD, NC and IVF pregnancies all showed cells that were positively stained by the antibodies used (Figure 1). Figure 1 shows representative pictures of the immunohistochemical staining by location for every antibody used. The results of the digital image analysis of the immunohistochemical staining are depicted in Figure 2. Significant differences in the decidua basalis and decidua parietalis of OD, NC and IVF pregnancies were found for all the tested

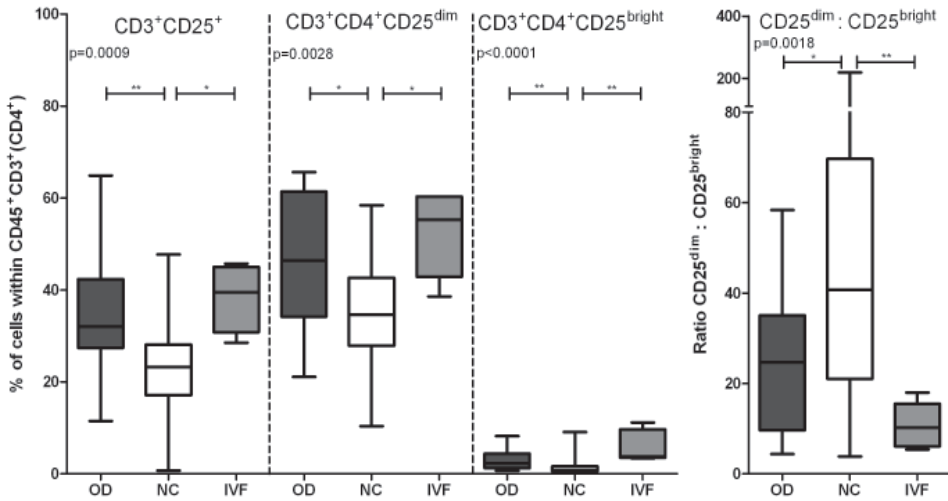


Figure 3 CD25+ cells in peripheral blood samples of oocyte donation (OD, n=13), naturally conceived (NC, n=39) and non donor IVF pregnancies (IVF, n=5). Level of significance indicated with asterisk, *** = p<0.001, **= p=0.001-0.01, * = p=0.01-0.05, ns = not significant. All values are of spontaneously delivered pregnancies. Values presented as means, error bars indicate the SEM. The horizontal line within the box indicates the median, the ends of the box correspond to the upper and lower quartiles of the data and the whiskers indicate minimum and maximum values.

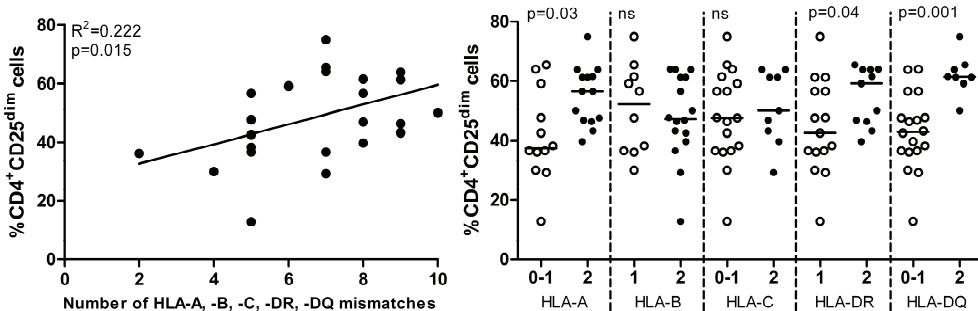


Figure 4 Correlation between the number of HLA mismatches and the percentage of CD4+CD25^{dim} cells in oocyte donation (OD) pregnancies (n=26) in peripheral blood. A. Considering HLA-A, -B, -C, -DR and -DQ the maximal number of HLA mismatches is 10 in OD pregnancies. A positive correlation between the number of mismatches and more CD4+CD25^{dim} cells in peripheral blood of OD pregnancies is found. B. The number of mismatches of the HLA-A, HLA-DR and HLA-DQ antigens relates with more CD4+CD25^{dim} cells in peripheral blood of OD pregnancies (p=0.03, p=0.04 and p=0.001 respectively). The horizontal lines represent the median of the data. The non parametric Mann-Whitney test was performed to analyze values between the groups (ns = non significant).

cytokines except for pSMAD2 in the decidua parietalis. The level of expression in the decidua basalis and parietalis was significantly lower in OD pregnancies compared with NC pregnancies for IL-10, IL-6, gal-1 and Flt (Figure 2). IVF showed similar results at both locations compared to OD for IL-10, IL-6 and Flt (only in the decidua parietalis, Figure 2). The level of expression of IL-6 and pSMAD2 in the decidua basalis was significantly higher in OD pregnancies compared with IVF pregnancies (Figure 2). The level of expression of Flt in the decidua basalis and gal-1 in the decidua parietalis is significantly lower in OD pregnancies compared with IVF pregnancies (Figure 2).

Maternal serum analysis

The ANOVA test showed significant differences between OD, NC and IVF pregnancies with respect to serum levels of IL-10, IL-6 and TGF- β 1 ($p=0.01$, $p<0.0001$ and $p<0.0001$ respectively, Figure 2). The levels of sFlt-1 and galectin-1 did not significantly differ between the three groups. Serum levels of IL-6 and IL-10 are significantly higher in OD pregnancies compared with NC pregnancies. TGF- β 1 serum levels were statistically significantly lowest in OD pregnancies, followed by NC pregnancies, and highest in IVF pregnancies.

Correlation between the number of HLA mismatches and the percentage of CD4CD25dim cells in the peripheral blood

The percentage of CD3+CD25+ cells was higher in OD and IVF pregnancies compared to NC pregnancies ($p=0.0009$, Figure 3). Within the CD25 fraction the percentage of CD25dim cells was also higher in OD and IVF pregnancies compared to NC pregnancies ($p=0.0028$, Figure 3). The same was found for CD4+CD25bright cells; the percentage of CD4+CD25bright cells was higher in OD and IVF pregnancies compared to NC pregnancies ($p<0.0001$, Figure 3). The ratio of CD4+CD25dim:CD4+CD25bright cells was lower in OD and IVF pregnancies compared to NC

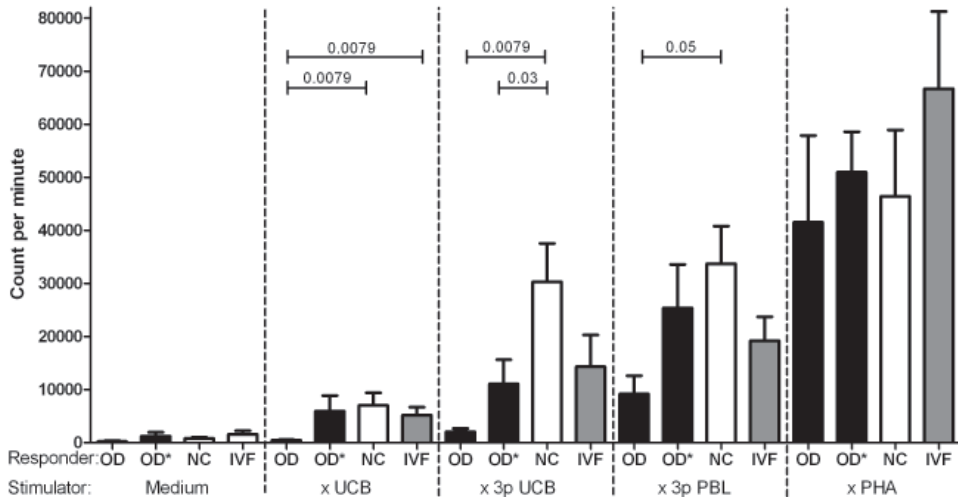


Figure 5 Mixed lymphocyte culture. Results in counts per minute. The responders were the maternal PBMCs of OD with one HLA-DR mismatch ($n=5$), OD with two HLA-DR mismatch (OD*, $n=5$), normal conceived (NC, $n=5$) and non donor IVF pregnancies (IVF, $n=5$). They were stimulated with their own UCB, 3p UCB or 3p PBL. Medium and PHA were used as a control. Values are presented as means, with error bars indicating the SEM. The non parametric T test was performed to analyze values between the groups. A p value of <0.05 was considered significant.

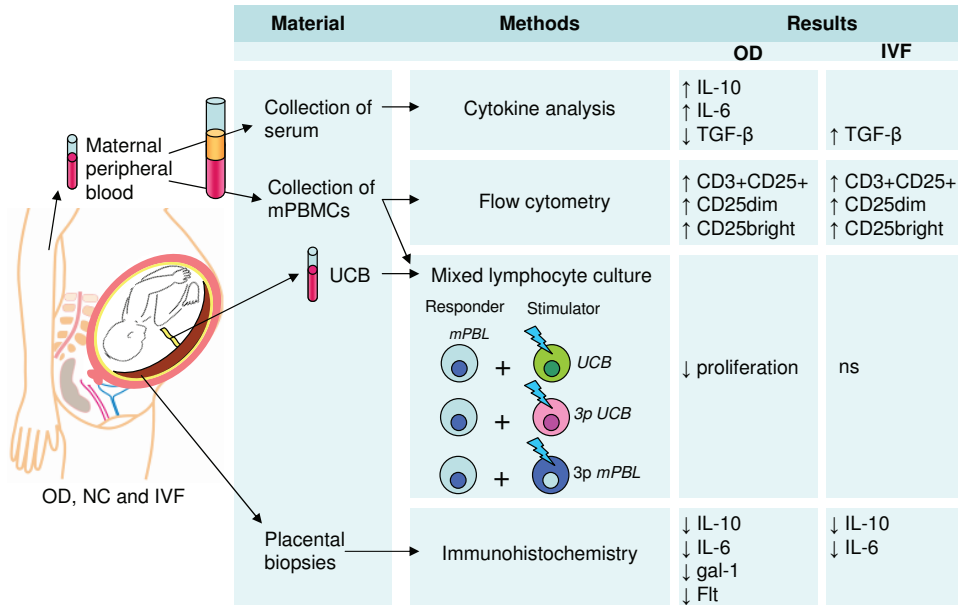


Figure 6 Overview of materials, methods and results. Maternal blood was used to collect serum for the performance of cytokine analysis by ELISA. mPBMCs were collected and analyzed by flow cytometry. Together with irradiated UCB, 3p UCB and 3p mPBL mPBMCs were cultured. The placenta and fetal membranes were collected after delivery to perform immunohistochemical staining. The main results for OD and IVF compared to NC are shown in the last two rows. Abbreviations: oocyte donation (OD), naturally conceived (NC) in vitro fertilization (IVF), maternal peripheral blood mononuclear cells (mPBMCs), maternal peripheral blood lymphocytes (mPBL) umbilical cord blood (UCB), third party (3p), non significant (ns).

pregnancies ($p=0.0018$, Figure 3). A significant positive correlation between the percentage of CD4+CD25dim in peripheral blood and the number of HLA mismatches was found in OD pregnancies ($R^2:0.022$ $p=0.015$, Figure 4). No correlation was found between the number of HLA mismatches and CD4+CD25bright or CD4+CD25dim cells in NC pregnancies (data not shown). However, the number of HLA mismatches did not affect the ratio of CD4+CD25dim:CD4+CD25bright cells in OD and NC pregnancies (data not shown). To determine whether mismatches of a specific HLA locus were responsible for the increased percentage of CD4+CD25dim cells, the HLA mismatches were analyzed separately by locus. HLA-A, HLA-DR and HLA-DQ mismatches significantly correlated with the increased percentage of CD4+CD25dim in peripheral OD blood ($p=0.03$, $p=0.04$ and $p=0.001$, respectively, Figure 4).

Mixed lymphocyte cultures

To investigate the fetus-specific immune response of mPBMCs collected after OD, NC, and non donor IVF pregnancies, these cells were stimulated with their own UCB, control UCB, and PBL. The OD group was subdivided in a group with one HLA-DR mismatch and a group with two HLA-DR mismatches (OD*). To determine the proliferative capacity of mPBMCs, the cells were stimulated with PHA. No significant differences between the groups were observed (Figure 5). As a negative control, cells were cultured with medium for 6 days. No significant differences were found here either between the groups (Figure 5). In the case of one HLA-DR mismatch, the mPBMCs of the OD pregnancy group showed significantly lower proliferation against their own UCB compared with

NC pregnancies ($p=0.0079$, Figure 5), and also compared with IVF pregnancies ($p=0.0079$, Figure 5). Also the response to 3p UCB and 3p PBL by OD pregnancies showed a significantly lower proliferation compared with NC pregnancies ($p=0.0079$ and $p=0.05$ respectively, Figure 5). Even when OD with two HLA mismatches were compared to NC, with a single HLA-DR mismatch, the response by OD pregnancies was still significantly lower compared with NC pregnancies ($p=0.03$, Figure 5).

An overview of the major differences observed between the different types of pregnancies is given in Figure 6.

Discussion

OD pregnancies are a result of in vitro fertilization of a donated oocyte by a relative, or more commonly an unrelated donor. In the latter case, neither of the fetal haplotypes matches with the gestational carrier. This creates a unique immunological situation to study the immune mechanisms that underlie these pregnancies. A better understanding of the maternal immune adaptations during OD pregnancy could increase the chance of successful gestation in patients with a history of infertility. In this work, we found strong peripheral regulation in OD and IVF pregnancies compared to NC pregnancies, and a differential maternal adaptation is in the decidua of OD and IVF pregnancies (Figure 6).

The results of this study showed that several immunological aspects are different in OD pregnancies compared with NC pregnancies. OD pregnancies express less IL-10, IL-6, gal-1, pSMAD2 and Flt-1 in the decidua (except for pSMAD2 in the decidua parietalis) compared to NC pregnancies. Although less immunoregulation in the decidua of OD and IVF pregnancies was found, we found more immunoregulation in the peripheral blood compared with NC pregnancies. In serum, OD pregnancies express more IL-10 and IL-6 but less TGF- β compared with NC pregnancies. Although these results suggests that IL-10 is plays an important role in uncomplicated pregnancy, recent data in mice show that a defect in IL-10 is not harmful for the pregnancy [27]. Immunohistochemical staining of pSMAD2 was used in this study to define TGF- β signaling. TGF- β is a repressor of cytotrophoblast outgrowth [15], possibly resulting in a proper balance for optimal placental growth. An imbalance in TGF- β signaling may lead to placental pathologies. We found less pSMAD2 placental expression in uncomplicated OD and IVF pregnancies, without placental pathology. Generally, in IVF pregnancies, placenta accreta occurs more frequently [28]. It is possible that the lower amount of pSMAD2 is counterbalanced by an altered mechanism, preventing placental pathologies. Gal-1 can be present intracellular and extracellular and thereby may elicit different functions [29]. Immunohistochemical staining of Gal-1 in our study shows that it is expressed in cytoplasm compartment in decidua basalis and extracellularly in decidua parietalis. It is possible that this different location is a result of trophoblast invasion. In the decidua parietalis non-invading trophoblast contacts the chorion and in the decidua basalis there is interaction between decidual cells and invading villous trophoblast.

The cytokines analyzed in this study have an immunological regulatory role during pregnancy. We therefore hypothesized that we would find increased IL-10, IL-6 and gal-1 expression in the decidua of OD pregnancies compared with NC. However, we found the opposite. Overall, we found that these cytokines were lower in OD and IVF pregnancies compared to NC pregnancies at the fetal-maternal interface. It is possible that maternal adaptation of the fetal allograft is more prominent during the first trimester. Therefore, it would be worthwhile investigating the immunological alterations of cytokines in decidua of first trimester samples. There are, however, limitations to the access of human first trimester samples. Furthermore, it is possible that immunoregulation in OD pregnancies also takes place on the fetal side of the placenta, as

suggested by preliminary observations which shows an OD-specific lesion at the fetal side of the placenta, containing cells involved in the immunomodulation (Schonkeren et al, submitted). More studies are necessary to unravel the underlying mechanisms in the immunologic differences between OD and NC pregnancies.

The percentages of CD4+CD25bright and CD4+CD25dim cells were higher in mPBMCs of OD and IVF pregnancies compared to NC. In OD, the number of HLA mismatches was positively correlated with the percentage of CD4+CD25dim cells (activated T cells) in mPBMCs of OD. However, there was no correlation with the number of HLA mismatches and the ratio CD4+CD25dim:CD4+CD25bright in OD pregnancies, suggesting that a higher number of CD4+CD25bright cells is necessary to regulate the immune system peripherally in OD pregnancies. That seems to be the case, as we demonstrated by our functional analyses. In OD pregnancies, more peripheral immunoregulation is present. The ratio of CD4+CD25dim:CD4+CD25bright was significantly lower in OD and IVF peripheral blood samples compared to NC. This suggests that relatively more CD4+CD25bright regulatory cells are present compared to NC pregnancies with the same number of CD4+CD25dim cells. A previous study showed hyperactivation of T helper 1 and T helper 2 cells in peripheral blood of OD compared with NC pregnancies [7]. The authors stated that the activation of T helper 2 cells and the relative suppression of T helper 1 chemokine expression reflected an additional regulatory counteractive mechanism. In agreement with this finding, we found a higher percentage of blood CD4+CD25dim activated T cells in OD compared with NC pregnancies, and the percentage of blood CD4+CD25bright regulatory T cells was higher compared to the activated T cells, suggesting a counteractive response in OD pregnancies.

The number of HLA mismatches also appears to play a crucial role, as a positive correlation was found between with the percentage of CD4+CD25dim cells in peripheral blood of OD pregnancies. This correlation was not present in the peripheral blood of NC pregnancies. A higher number of HLA-A, -DR and -DQ mismatches leads to more CD4+CD25dim cells in maternal peripheral blood in OD pregnancy. No correlation was found between the ratio CD4+CD25dim:CD4+CD25bright and number of HLA mismatches for OD and NC pregnancies (data not shown), suggesting that the higher number of activated cells is controlled by a higher number of regulatory cells. The percentage of CD4+CD25bright and CD4+CD25dim cells in peripheral blood of term NC pregnancies is comparable with previous studies [18]. Previously, we showed a central role of HLA-C mismatches in the induction of the decidual lymphocyte response to fetal cells by CD4+CD25dim cells [26]. In contrast, the number of CD4+CD25dim cells in the peripheral blood of OD pregnancies was not mediated by the number of HLA-C mismatches, or by the presence of HLA-C1 or HLA-C2 mismatches (data not shown). This shows that for decidual regulation HLA-C, the only classical HLA antigen expressed on trophoblast, plays an important role. In contrast, for peripheral responses, HLA-A, -DR and -DQ but not HLA-C are essential, since the presence of more CD4+CD25dim cells in the periphery of OD pregnancies was associated with a higher number of mismatches on the HLA-A, HLA-DR and HLA-DQ antigens. To explain these results, we postulate that the impact of fetal microchimerism in maternal blood plays a role in the immunological response, as seen in the OD pregnancies. Antigens on fetal cells migrate in to the maternal blood, and fetal (and thus partly paternal) antigens may be able to modulate the maternal immune response during pregnancy, which persists in maternal circulation for decades after delivery [30]. The presence of fully allogeneic fetal cells in maternal circulation has been demonstrated in after OD [31]. Strong peripheral immunoregulation in OD might therefore be beneficial for the persistence of microchimerism.

Our functional assays confirm the presence of a stronger peripheral immunoregulation in OD and IVF pregnancies. mPBMCs of OD pregnancies with one HLA-DR mismatch showed less proliferation upon stimulation with own UCB compared to NC and IVF pregnancies. This indicates that in IVF and NC pregnancies less peripheral regulation of immunological response towards the

woman's infant is present. Even in OD pregnancies with two HLA-DR mismatches the alloimmune response to the UCB is lower than in NC pregnancies. The mechanisms behind the differences in response to fetus-specific stimulation in an OD pregnancy remain to be established. In a previous study in NC pregnancies, we found no significant differences between the responses of mPBMCs to her own child or a control child compared to non-pregnant controls [32]. This indicates that peripheral immune regulation in NC pregnant women is not very different from that of non-pregnant controls. Here, we show that in OD pregnancies the situation is different. The peripheral immune response is significantly altered compared to NC pregnant controls.

A limitation of this study is that it is difficult to define a proper control group for OD pregnancies. Woman who undergo non donor IVF receive two hormonal treatments; one to retrieve the oocytes and a second one for the induction of a proper milieu before embryo transfer. Woman who undergo OD only receive the hormonal treatment necessary for embryo transfer. Although upon implantation the embryo consists of only a few cells, and alterations in the immune system during decidualization by hormonal treatment used in assisted reproductive techniques may affect those cells in the first trimester, it is unlikely that this treatment is responsible for immunological disturbances in the last trimester. However, vulnerability to changes in the hormonal surroundings of the blastocyst in the periconceptual period might result in peri-implantation programming and explains long term effects via changing of phenotype of fetal cells. This remains to be further elucidated and indeed it has been shown that in vitro culture of embryos is associated with changes in fetal outcomes [33]. It even has been proposed that the composition of culture medium is of more influence than the procedure of in vitro culture itself [34].

In conclusion, in this study we provide evidence that the immunoregulation in OD pregnancies is different compared with NC pregnancies, both with regard to the peripheral and the local immune responses. The number of HLA mismatches in OD pregnancies affects the number of activated T cells in the periphery. The mechanisms by which this altered immune response is evoked remain to be established. Future studies are necessary to investigate the immunoregulatory mechanisms involved in successful, but also in pathological, OD pregnancies.

Acknowledgements

We kindly thank the midwives and Dorrith Schonkeren for help with collecting material, Geert Haasnoot for the statistical help and Luuk Hawinkels for help with the pSMAD2 staining. The authors thank P. Moschansky, for technical assistance in generating this work.

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