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Maternal-fetal HLA compatibility and trophoblast-immune interactions in healthy and preeclamptic pregnancy: elegance in complexity

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Decreased expression of ligands of placental immune checkpoint inhibitors in uncomplicated and preeclamptic oocyte donation pregnancies

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ABSTRACT

Oocyte donation (OD) pregnancies are characterized by a complete immunogenetic dissimilarity between mother and fetus, which requires enhanced immunoregulation compared to naturally conceived (NC) pregnancies. The trophoblast expresses co-inhibitory ligands crucial for regulation of the maternal T cell response. Therefore, we studied the role of placental immune checkpoint inhibitors for the establishment of fetal tolerance and their relation to the development of preeclampsia in OD compared to NC pregnancies. Placental tissue from uncomplicated OD (n =21) and NC (n =21) pregnancies, and OD (n =9) and NC (n =15) pregnancies complicated with preeclampsia were studied. Protein expression of co-inhibitory ligands PD-L1 and CD200 was double blind semi-quantitatively determined by immunohistochemistry. Messenger RNA expression of PD-L1, CD200 and indoleamine 2,3-dioxygenase (IDO) was determined using qPCR. Decreased PD-L1 and CD200 protein expression and increased IDO mRNA expression was observed in uncomplicated OD versus NC pregnancies (all p <0.05). CD200 protein expression was positively correlated with PD-L1 expression in all groups, with the number of HLA total mismatches and with HLA class I mismatches in uncomplicated OD cases (all p <0.05). Preeclamptic cases showed lower PD-L1 protein and CD200 protein and mRNA expression in OD compared to NC pregnancies (all p <0.05). This study shows that signaling by co-inhibitory PD-L1 and CD200 and by immunosuppressive IDO is altered in the placenta of OD pregnancies, suggesting a contribution to the higher risk for preeclampsia. These insights provide future prospects in unraveling the immune paradox of oocyte pregnancy, which are applicable for better risk management and treatment of uncomplicated and preeclamptic pregnancies.

INTRODUCTION

The semi-allograft setting of a pregnancy requires balanced immunoregulation in order to establish maternal-fetal tolerance. (1-3) Oocyte donation (OD) pregnancies provide a unique insight for understanding fetal protection from the maternal immune response, since there is greater immunogenic dissimilarity between mother and fetus compared to naturally conceived (NC) pregnancies. The extra challenge for the maternal immune system to ascertain tolerance in OD pregnancies compels even better regulation of leukocytes at the fetal-maternal interface. (4) Interestingly, preeclampsia has a higher incidence in OD pregnancies independently from OD specific maternal risk factors for pre-eclampsia, such as older age and primiparity. The increased incidence of pre-eclampsia in OD pregnancies shows the indispensability of immunoregulation, since the etiology of pre-eclampsia is partly immune mediated. (4-6) Previous studies showed that immunoregulation in OD pregnancies contains other characteristics when compared to NC pregnancies. (7-9) Most importantly, van der Hoorn, et al. showed that a higher number of HLA mismatches in OD pregnancies is correlated with an increase in activated T cells in peripheral blood, but not a higher alloreactivity to the fetus. (7) Hence, the OD pregnancy offers an exceptional setting to study the effect of immunogenetic dissimilarities between mother and fetus on the placenta.

Trophoblasts express a set of immunoregulatory cell surface molecules, co-inhibitory pathways serving as immune checkpoints, which are critical for tolerance in organ transplantation and tumour immune modulation studies. (10-16) For example, activation of and negative feedback by the PD-1 receptor is critical for immune regulation through promoting regulatory T cell (Treg) development and inhibiting effector T cell responses. (17) Also, the immunosuppressive receptor CD200R directly inhibits NK cells, promotes Treg generation and induces production of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). (18) Not surprisingly, as maternal immune alteration is essential for placental and fetal development, a selection of co-inhibitory ligands seem to be of crucial importance for maintaining maternal-fetal tolerance in pregnancy as well. (19-21) In a murine pregnancy model, blockade of the PD-1/PD-L1 pathway resulted in increased inflammation, reduction of Tregs, a higher spontaneous abortion rate and less fetal survival. (22-24) Further studies showed decreased expression of PD-L1 with a correlation to the amount of Tregs, but also decreased co-inhibitory ligand CD200 expression in human placenta tissue of preeclamptic patients. (18, 25) Likewise, in an abortion-prone setting, immune checkpoint receptor CTLA-4 is downregulated on decidual T-lymphocytes. (26) Subsequently, a lower expression of immune checkpoint ligands CD80, CD86 and immunosuppressive IDO was found in the peripheral blood of preeclamptic women. (27)

As both the uncomplicated as well as the preeclamptic situation of OD are linked to altered immune regulation, we hypothesized that the co-inhibitory ligand expression on trophoblasts, which regulates T cells is of essential importance in OD pregnancies. Hence, we studied co-inhibitory ligands in OD pregnancies and expected to find a decrease in placental protein and mRNA expression in both uncomplicated and preeclamptic cases, which reflects the greater immunogenic dissimilarity compared to NC pregnancies.

MATERIAL AND METHODS

Subjects

Placentas from 66 pregnancy cases were collected of which 21 uncomplicated OD, 21 uncomplicated NC, 9 OD complicated with preeclampsia and 15 NC complicated with preeclampsia. All included subjects delivered at the Leiden University Medical Center (LUMC) from 2007 up until 2018 and their placental samples were collected within 24 h of delivery. Placenta tissue was collected and subsequently paraffin-embedded or frozen by -80°C . Clinical information about maternal age, gestational age, fetal gender, birth weight, gravidity, parity, pre-existing diastolic blood pressure, highest gestational diastolic blood pressure, urine protein level, body mass index (BMI), medication use before and during pregnancy, and smoking was collected from the medical records. Subjects were included as uncomplicated cases if the pregnancy had no preeclampsia, HELLP, preterm birth, decreased birth weight, fetal growth restriction or infection.

Preeclampsia was defined according to the definition by the International Society for the study of Hypertension in Pregnancy (ISSHP). (28, 29) All selected cases had co-existing proteinuria (≥ 0.3 g/l/24 h) and gestational hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg detected after 20 weeks of gestation) or worsening of pre-existing hypertension.

Exclusion criteria were multiplet pregnancies, previous abortus provocatus lege artis (APLA), extra-uterine gravidity (EUG) or molar pregnancy, pre-existing high diastolic blood pressure, maternal autoimmune diseases, the presence of chromosomal abnormalities, receipt of blood transfusions, and organ transplantation in the medical history, BMI above 35 in the first trimester and infection during pregnancy. Cases with HELLP without preeclampsia were also excluded.

Informed consent was obtained from every patient. The study protocol was approved by the ethics committee of the LUMC with protocol number P16.048.

HLA typing

After delivery, paired samples of peripheral maternal blood and umbilical cord blood were collected. Sequence Specific Oligonucleotides PCR was used to determine the type of HLA-A, -B, -C, -DR, and -DQ within paired blood samples. The number of fetal-maternal HLA mismatches was calculated at the Dutch National Reference Laboratory for Histocompatibility Testing (LUMC). On the basis of the above mentioned five HLA genes, the maximal number of (mis)matches between mother and child is 10 for OD pregnancies and five for NC pregnancies.

Immunohistochemistry

Molecules were selected for analysis by immunohistochemistry based on their mRNA expression determined by qPCR. Tissue blocks of placental biopsies with villi and decidua basalis on comparable locations (halfway in between umbilical cord and lateral edge) were selected and embedded in paraffin according to standardized protocol.

Sequential serial sections of $4\ \mu\text{m}$ were placed on adhesive-coated glass slides and dried overnight at 37°C . Deparaffinisation and rehydration was done and antigen retrieval was performed by boiling the sections in citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. The primary antibody was diluted in 1% bovine serum albumin/PBS and incubated for 1 h at room temperature. The secondary antibody was incubated for 30 min at room temperature (further details in Supplementary Table 1). Tissue with validated protein expression as a positive control and an isotype matched negative control was used for every antibody. Visualisation was achieved by using diaminobenzidine (Envision + System HRP (DAB), K4011, Dako). Haematoxylin was used for counterstaining before slides were dehydrated and covered using mounting medium.

Semi-quantitative scoring for immunohistochemistry analysis

Philips IMS viewer was used for the imaging of the immunohistochemistry staining of PD-L1 and CD200. The maximum amount of decidua basalis was 10% of the slide. Per slide, six random annotations of fetal villi of $1\ \text{mm}^2$ were selected throughout the slide at a magnification of $0.5\times$. Areas with an excessive amount of erythrocytes were excluded. Every annotation was scored at magnifications of $20\times$ and $40\times$. The amount of stained circumference of the villi was scored as absent ($<10\%$ of villi circumference stained), low ($10\text{--}50\%$ of villi circumference stained), moderate ($50\text{--}90\%$ of villi circumference stained) or high ($>90\%$ of villi circumference stained). Two additional annotations, one with villi adjacent to the decidua and one with villi most distal from the decidua were scored according to the same four categories. All slides were scored by the same observer and with the same brightness settings. The observer was blinded for the cases, group, type of pregnancy and location of the annotations within the slide. One hundred sixty random annotations were

double scored by the initial observer and by a second observer. Overall, the intraobserver ICC was 0.988 (0.984-0.991) and the interobserver ICC was 0.854 (0.763-0.905).

RNA isolation and cDNA synthesis

Equivalent to the paraffin tissue, tissue blocks of placental biopsies with villi and (maximum 10% of) decidua basalis on comparable locations (halfway in between umbilical cord and lateral edge) were selected, embedded in TissueTek (Sakura) and frozen at -80°C according to standardized protocol.

Of every frozen placenta sample, 2-3 sections of 10 µm were cut and as much TissueTek as possible was removed before the immediate transfer to at least 1 ml Trizol (Lifetechnologies), directly followed by vortex. The vortexed Trizol samples were stored at -80°C till RNA isolation. RNA isolation was performed using chloroform, glycogen, 100% isopropanol and 75% ethanol subsequently according to manual instructions. All isolated RNA samples had a 260/280 absorbance ratio greater than 1.79. Complementary DNA (cDNA) synthesis was established using AMV reverse transcriptase (Roche).

Quantitative Polymerase Chain Reaction

Quantitative PCR was performed to analyse placental mRNA expression of PD-1, PDL-1, CD80, CD86, IDO, LGALS9, CD200, CD200R, PVR, CD3, and CD68. For each gene, two sets of primers were tested with the use of a cDNA titration. The optimal primer-set was chosen based on highest efficiency and least primer-dimer formation. H₂O was used as a negative control. Primer sequences are displayed in Supplementary Table 2.

Expression of every sample was normalized to the expression of housekeeping genes HPRT and GAPDH. Every sample was measured in duplicate. Verification of the specificity of amplification was based on a melting curve analysis for every sample. Samples were excluded as unspecific qPCR results based on the melting curve.

All SYBR green quantitative PCR experiments were performed with a CFX real-time system according to the manufacturer's protocol (Bio-Rad Laboratories). Of five cases (1 OD uncomplicated, 1 OD PE, 2 NC uncomplicated, 1 NC PE), there was no frozen placenta material available. Therefore, these cases were excluded for mRNA analysis by qPCR.

Statistical analysis

For statistical analysis the following groups were compared: uncomplicated OD pregnancies vs. uncomplicated NC pregnancies, OD pregnancies complicated with preeclampsia vs. uncomplicated OD pregnancies, NC pregnancies complicated with preeclampsia vs. uncomplicated NC pregnancies and OD pregnancies complicated with preeclampsia vs. NC

pregnancies complicated with preeclampsia. A p-value <0.05 was considered statistically significant.

Patient characteristics were analysed with the one-way analysis of variance for scale and ordinal values and with the Chi-squared test for nominal values. In the one-way analysis of variance, the LSD or Tamhane's T2 post hoc tests as correction for multiple testing were used based on the significance of the Levene's Homogeneity of Variances test.

Comparisons of results between separate groups were made with the one-way analysis of variance and the independent samples t-test for all continuous data. The Spearman's rho test was used to for nonparametric correlations followed by multiple regression and to explore potential confounding factors. Potential confounding factors that were examined in the analysis were maternal age, gestational age at time of delivery, fetal gender, gravidity, parity, BMI, smoking. Linear correlations of continuous data were computed to a Log10 scale and multiple regression analysis was performed to determine the Pearson-r of the correlation and interaction of the groups. To determine the intra- and interobserver reliability, a two-way mixed model with absolute agreement was used to calculate the intraclass correlation coefficient (ICC) of single measures with a 95% confidence interval. All statistical analyses were performed with the IBM SPSS statistics package (version; IBM).

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. Significant differences between the groups were found in maternal age, gravidity, parity, birthweight, highest diastole, proteinuria, caesarean section (see Table 1 for in between which groups). The mean highest diastole measured during pregnancy was significantly higher in women with uncomplicated OD pregnancies compared to women with uncomplicated NC pregnancies ($p < 0.05$). Although three women with uncomplicated OD pregnancies had proteinuria, none of them fulfilled the ISSHP criteria for preeclampsia. Three cases within the group of NC pregnancies complicated with preeclampsia and one in the group of OD pregnancies complicated with preeclampsia had the additional diagnosis of HELLP. No significant differences were present among the four groups with respect to BMI.

Table 1. Patient Characteristics.

	NC uncomplicated (n=21)	OD uncomplicated (n=21)	NC PE (n=15)	OD PE (n=9)
Maternal Age (y) , mean ± SD	32.5 ± 3.4	39.8 ± 6.6 ^a	31.0 ± 6.4 ^b	38.9 ± 6.5
Gravidity (range)	2.1 (1-3)	1.1 (1-2) ^a	1.0 (1-1) ^a	1.8 (1-3)
Parity (range)	0.8 (0-2)	0.1 (0-1) ^a	0.1 (0-1) ^a	0.2 (0-1)
Gestational Age (d) , mean ± SD	277.5 ± 6.7	279.9 ± 9.5	220.1 ± 17.0 ^{ab}	235.6 ± 22.3 ^{ab}
Fetal gender (male)	13 (61.9%)	10 (47.6%)	5 (33.3%)	4 (44.4%)
Birth weight (gr) , mean ± SD	3472 ± 477	3339 ± 483	1435 ± 510 ^{ab}	1892 ± 761 ^{ab}
Birth weight Percentile, mean (range, %)	51-80	51-80	21-50	21-50
Maternal BMI (kg/m²) , mean ± SD	26.9 ± 4.8	23.5 ± 3.8	24.0 ± 3.1	25.5 ± 1.8
Mode of Delivery				
Spontaneous	4 (19%)	10 (53%)	1 (7%)	3 (38%)
Caesarean section	17 (81%)	9 (47%) ^b	14 (93%) ^b	5 (63%)
Highest Diastole (mmHg) , mean ± SD	73.1 ± 9.4	85.0 ± 6.2 ^a	105.9 ± 8.0 ^{ab}	101.1 ± 7.8 ^{ab}
HELLP, in addition to PE	n.a.	n.a.	3 (20%)	1 (11%)
Proteinuria	0 (0%)	3 (21%)	15 (100%) ^{ab}	9 (100%) ^{ab}

NC = uncomplicated naturally conceived pregnancies; OD = uncomplicated oocyte donation pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD PE = oocyte donation pregnancies complicated with preeclampsia.

a. $p < 0.05$ compared to NC uncomplicated

b. $p < 0.05$ compared to OD uncomplicated

c. $p < 0.05$ compared to NC PE

Decrease of correlated PD-L1 and CD200 expression in placenta of OD cases

T cell and macrophage presence within the placental tissue did not differ between groups, reflected by comparable mRNA expression levels of CD3 and CD68, respectively (Supplementary Figure 1A and B). No mRNA expression of PD-1 receptor could be detected in the full thickness placental samples of any of the study groups. In control placentas, PD-1 receptor protein expression was absent in the decidua and present in a few cells in the intervillous space, while CD3 protein expression was observed in the decidua, villi and intervillous space. Protein expression of PD-L1 in the placenta was observed at the syncytiotrophoblast (Figure 1A and B). The PD-L1 protein expression was significantly lower in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies ($p < 0.05$, Figure 1E), suggesting a lesser extent of immune modulation through the PD-1/PD-L1 pathway in these OD pregnancies.

A gradient decrease in PD-L1 protein expression was observed moving away from the decidua, differing in a maximum of two scoring categories for the villi that were most distal from the decidua (Supplementary Figure 2). PD-L1 expression of villi most proximal to the decidua was significantly higher compared to villi most proximal to the fetal membranes ($p < 0.0001$). This gradient decrease was also significant within each type of pregnancy ($p < 0.05$ for all, Supplementary Figure 2C).

The syncytiotrophoblast showed protein expression of CD200 in different degrees (Figure 1C and D). Uncomplicated OD pregnancies showed a significant decrease of placental CD200 protein expression compared to uncomplicated NC pregnancies ($p < 0.05$, Figure 1G), suggesting that these uncomplicated OD pregnancies are involved with less immunoregulation through the CD200:CD200R axis. Preeclamptic cases of OD pregnancies showed a significantly lower level of CD200 protein expression compared to uncomplicated cases ($p < 0.05$), which was not present in NC pregnancies. A significantly lower expression of CD200 was also found in OD pregnancies complicated with preeclampsia when compared to NC pregnancies complicated with preeclampsia ($p < 0.05$). This difference was observed for both protein and mRNA expression of CD200. A significantly lower placental mRNA expression of CD200R mRNA was found in NC pregnancies complicated with preeclampsia compared to uncomplicated NC pregnancies ($p = 0.003$, Figure 1K). Similarly, OD pregnancies showed a lower placental CD200R expression in preeclamptic cases compared to uncomplicated cases ($p = 0.028$).

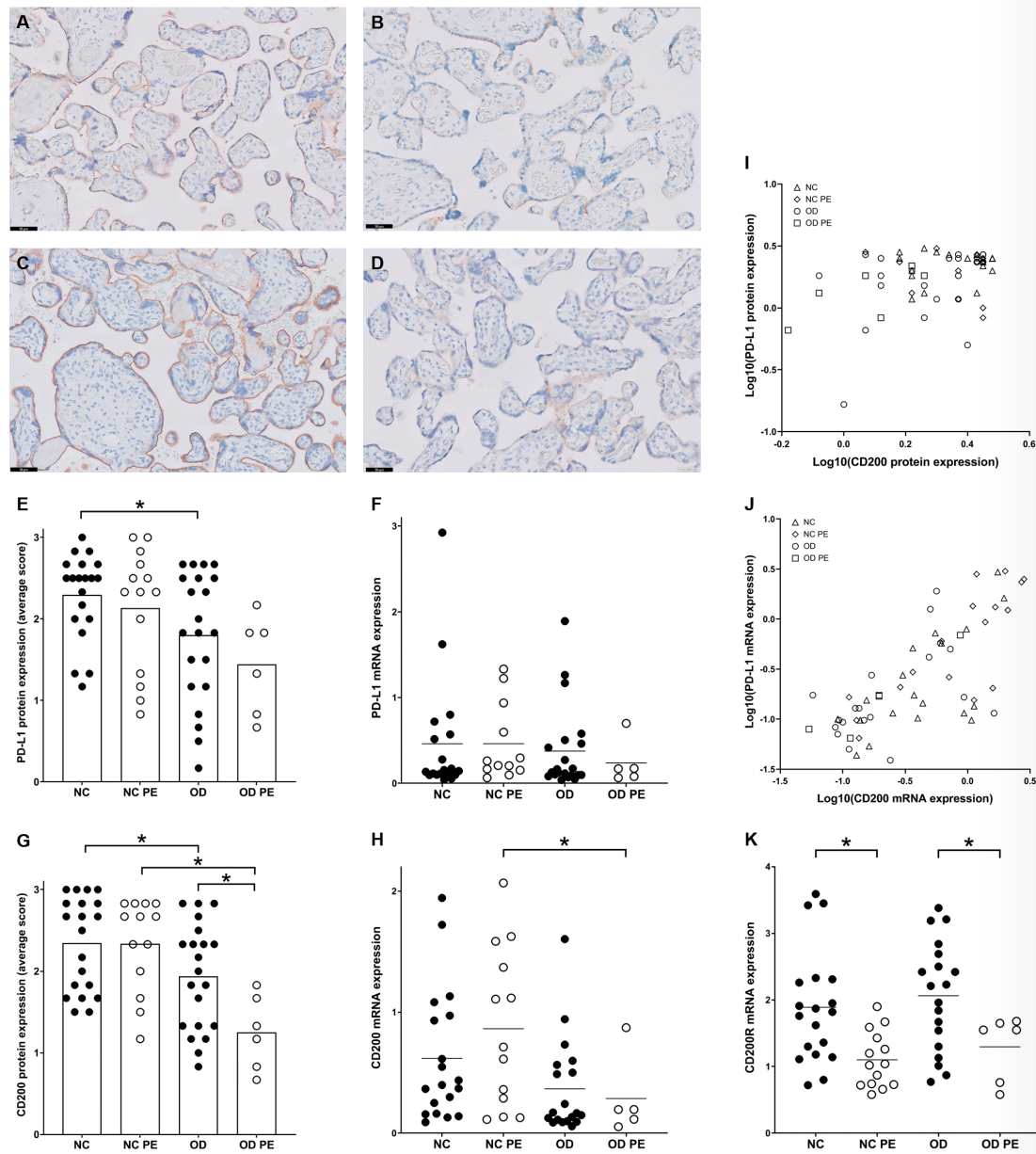


Figure 1. Placental PD-L1 and CD200 expression. A). Immunohistochemical staining of PD-L1, representative example of scoring category 3, high placental PD-L1 protein expression pattern; >90% of villi circumference stained. B). Immunohistochemistry staining of PD-L1, representative example of scoring category 1, low placental PD-L1 protein expression pattern; 10-50% of villi circumference stained. C). Immunohistochemistry staining of CD200, representative example of scoring category 3, high placental CD200 protein expression pattern; >90% of villi circumference stained. D). Immunohistochemistry staining of CD200, representative example of scoring category 1, low placental CD200 protein expression pattern; 10-50% of villi circumference stained. E). Placental PD-L1 protein expression, based on semi-quantified scoring analysis. F). Relative normalized placental PD-L1 mRNA expression. G). Placental CD200 protein expression, based on semi-quantified scoring analysis. H). Relative normalized placental CD200 mRNA expression. I). Positive correlation between log10 computed PD-L1 and CD200 protein expression levels. (Rho = 0.358*). J). Linear correlation between log10 computed PD-L1 and CD200 mRNA expression levels. (r² = 0.525**). K). Relative normalized placental CD200R mRNA expression. NC = uncomplicated naturally conceived pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD = uncomplicated oocyte donation pregnancies; OD PE = oocyte donation pregnancies complicated with preeclampsia. Scale bar = 50 μm. *p < 0.05, **p < 0.001.

A positive correlation was found between CD200 expression and PD-L1 expression on both protein (rho = 0.358, p=0.005, Figure 1I) and mRNA level (r² = 0.525, p<0.001, Figure 1J), which was independent of the type of pregnancy. Thus, the correlated expressions of PD-L1 and CD200 show a decrease on protein level in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies, whereas preeclamptic OD cases show even more decrease in CD200 protein expression.

Increased placental IDO mRNA expression in uncomplicated OD pregnancies

No significant differences between the types of pregnancy were found for the mRNA expression of CD80 and CD86. CD80 and CD86 mRNA expression showed a positive correlation (p<0.001, r² = 0.484, Figure 2C), which was consistent for every type of pregnancy. The mRNA expression of IDO was significantly increased in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies (p < 0.05, Figure 2D), which suggests that IDO is compensative in providing indicating an immunosuppressive environment in OD pregnancies. No significant differences of mRNA expression of immune checkpoint ligands LGALS9 or PVR were observed between the study groups (Supplementary Figure 1C and D). A positive correlation was observed for LGALS9 and CD200 mRNA expression levels (p<0.001, r² = 0.510, Figure 2E), which was consistent for every type of pregnancy. For LGALS9 and PVR mRNA expression, a positive correlation was observed independent of the type of pregnancy (p<0.002, r² = 0.406, Figure 2F). The positively correlated co-inhibitory ligands indicate a conjoined network of immune checkpoint inhibition in pregnancy. In NC pregnancies complicated with preeclampsia, IDO mRNA expression was positively correlated with birth weight (p < 0.05, r² = 0.360, Figure 2G). For the 15 samples with proteinuria, a positive correlation was found for LGALS9 and proteinuria level (p=0.011, r² = 0.404, Figure 2H), which was not different between NC and OD pregnancies. Thus, mRNA expressions of multiple co-inhibitory ligands are positively correlated in placental tissue. Furthermore, placental IDO mRNA expression is increased in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies.

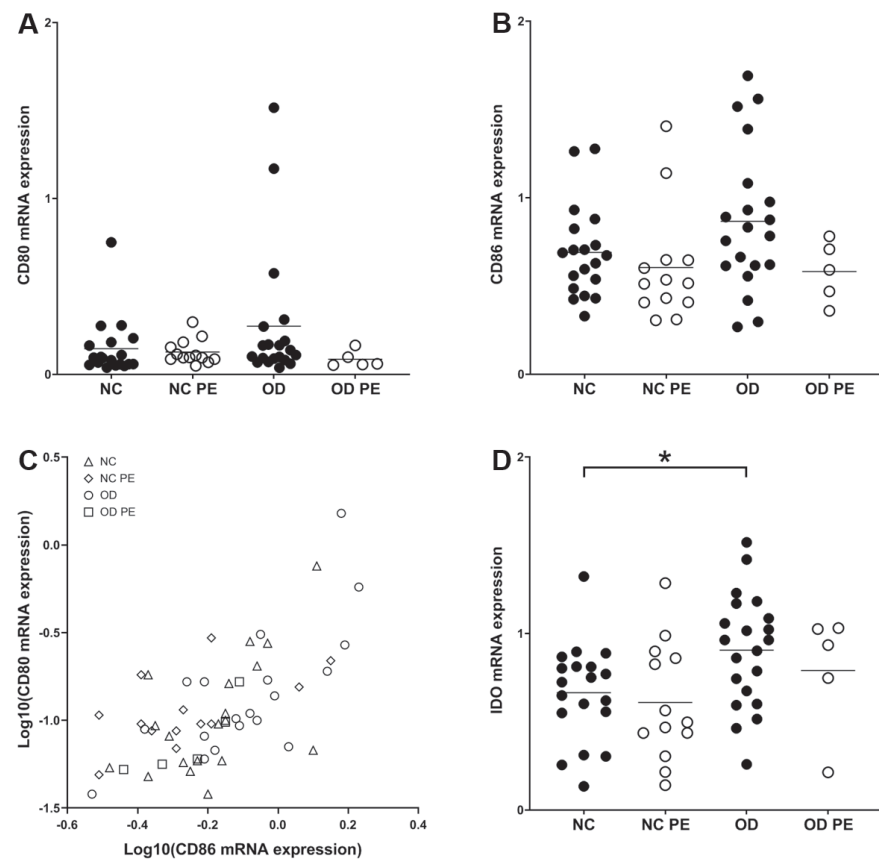
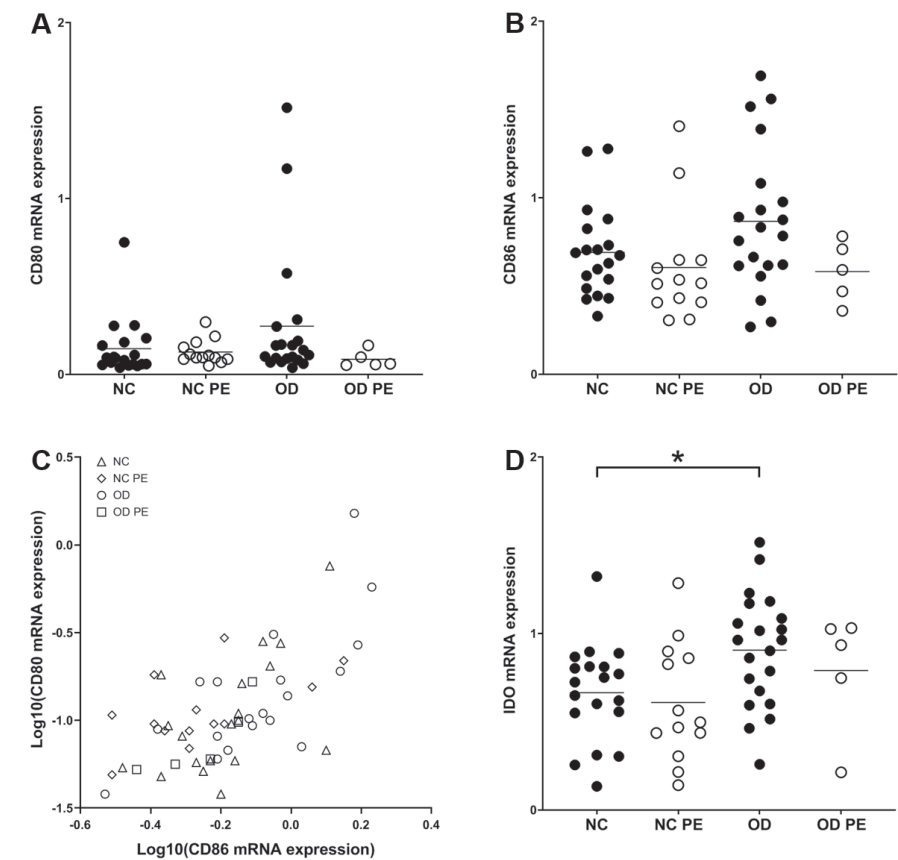


Figure 2. A). Relative normalized placental CD80 mRNA expression. B). Relative normalized placental CD86 mRNA expression. C). Linear correlation between log10 computed CD80 and CD86 mRNA expression levels ($r^2 = 0,484^{**}$). D). Relative normalized placental IDO mRNA expression. E). Positive correlation between log10 computed LGALS9 and CD200 mRNA expression levels. ($\rho = 0,510^{**}$). F). Positive correlation between log10 computed PVR and LGALS9 mRNA expression levels. ($\rho = 0,406^*$). G). Linear correlation between IDO mRNA expression and birth weight (gr) ($r^2 = 0,360^*$). H). Linear correlation between log10 computed LGALS9 and Proteinuria levels (mg/24h) ($r^2 = 0,404^*$). NC = uncomplicated naturally conceived pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD = uncomplicated oocyte donation pregnancies; OD PE = oocyte donation pregnancies complicated with preeclampsia; * $p < 0,05$, ** $p < 0,001$.



CD200 protein expression is correlated with the extent of HLA mismatching

OD pregnancies, both uncomplicated and preeclamptic cases, showed a significantly higher number of total HLA mismatches, as well as class I and class II mismatches separately, compared to NC pregnancies ($p < 0,001$, Supplementary Table 3). In uncomplicated OD pregnancies, there was a positive correlation between CD200 protein expression and both the amount of total mismatches ($p = 0,003$, $\rho = 0,636$, Figure 3A) as well as the amount of class I mismatches ($p = 0,003$, $\rho = 0,635$, Figure 3B). There was also a positive correlation between CD200 protein expression and the number of HLA-C mismatches in uncomplicated OD pregnancies ($p = 0,001$, $\rho = 0,704$, Figure 3E). Due to the nature of OD, two HLA-C mismatches were present in some of these cases. Furthermore, the correlation between CD200 protein expression and the number of HLA-C mismatches was negative in uncomplicated NC pregnancies ($p = 0,021$, $\rho = -0,499$, Figure 3C) and NC pregnancies complicated with preeclampsia ($p = 0,041$, $\rho = -0,622$, Figure 3D).

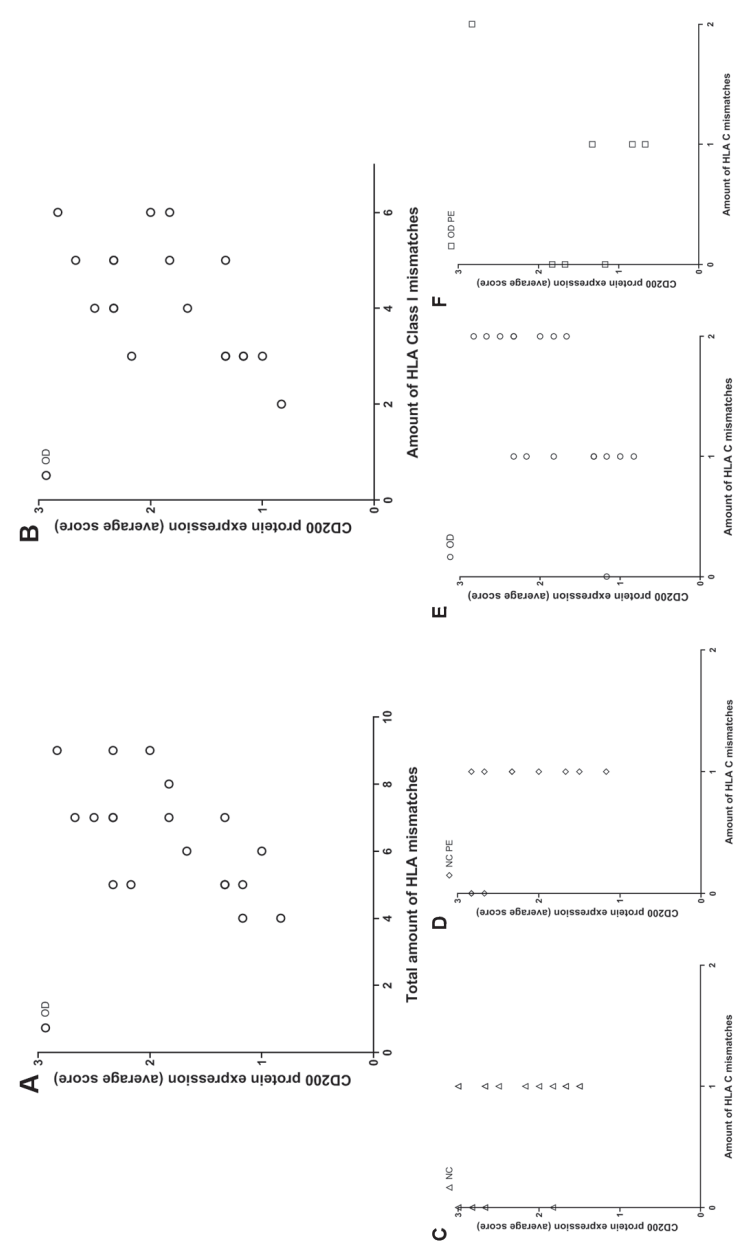


Figure 3. A). Correlation between CD200 protein expression and total amount of HLA mismatches in uncomplicated OD pregnancies (rho = 0.636*). B) Correlation between CD200 protein expression and HLA Class I mismatches in uncomplicated OD pregnancies (rho = 0.635*). C) Correlation between CD200 protein expression and HLA-C mismatches in uncomplicated NC pregnancies (rho = -0.499*). D) Correlation between CD200 protein expression and HLA-C mismatches in NC pregnancies complicated with preeclampsia (rho = -0.622*). E) Correlation between CD200 protein expression and HLA-C mismatches in uncomplicated OD pregnancies (rho = 0.704*). F) CD200 protein expression and HLA-C mismatches in OD pregnancies complicated with preeclampsia, no correlation. NC = uncomplicated naturally conceived pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD = uncomplicated oocyte donation pregnancies; OD PE = oocyte donation pregnancies complicated with preeclampsia; *p < 0.05.

DISCUSSION

Increased immunogenic dissimilarity due to the complete allograft status of the fetus in OD pregnancies poses an increased challenge for the establishment of immunoregulation in the placenta. The present study shows that the expression of immune checkpoint co-inhibitory ligands by trophoblasts in the placenta of OD pregnancies is altered in comparison to NC pregnancies. Protein expression of PD-L1 and CD200 is decreased in uncomplicated OD pregnancies, while mRNA expression of IDO downstream of co-inhibitory signaling is increased. In preeclamptic pregnancies, decreased CD200 protein expression in the placenta compared to uncomplicated pregnancies seems to support existing evidence of a disturbed immunosuppression and T cell regulation in preeclampsia. (6, 30) The altered signaling of co-inhibitory pathways in the placenta of OD pregnancies reflects the differential immunoregulatory processes in such pregnancies and might explain the increased risk of preeclampsia.

The linear correlation between the PD-L1 and CD200 both at the mRNA and the protein level strengthens the similar decrease of these ligands in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies. The correlation found for PD-L1 and CD200 expression is in line with a recent study showing a linkage of these molecules in acute myeloid leukaemia. (31) The pathways can act in tandem to produce a greater immunosuppressive effect on CD8⁺ T cells. Stimulation of the CD200:CD200R immune-axis can induce PD-1 expression on CD8⁺ T cells, resulting in a reduction of activated T effector cells and related TNF α production. In OD pregnancies, the higher extent of antigen recognition and the consecutive inflammatory factors might dampen the required response of trophoblasts to express PD-L1 and CD200 as essential stimulators of immune modulation in the placenta.

Interestingly, a significant increase of IDO mRNA expression in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies was observed. We suggest that increased immunoregulatory signaling through the CTLA-4 pathway, outcompeting CD28, is induced in the setting of higher tolerance necessity in OD pregnancies. (32, 33) A higher percentage of CTLA-4⁺ regulatory T cells in maternal PBMCs of OD pregnancies compared to NC pregnancies supports our results of increased IDO-mediated immune regulation in these women. (34) Furthermore, apart from degrading tryptophan for effector T cell inhibition, IDO also induces HLA-G cell surface expression, one of the principal molecules for inhibition of NK and CD8⁺ T cells in the placenta and fetal tolerance in general. (35) The detected increase in IDO mRNA expression in uncomplicated OD pregnancies may therefore reflect a compensating mechanism when other immunoregulatory pathways such as direct PD-1/PD-L1 and CD200R/CD200 mediated inhibition fails. On the contrary,

the number of HLA mismatches was previously shown to be associated with the amount of regulatory T cells in peripheral blood and parietal decidua of OD pregnancies. (7, 36) This suggests that increased fetal HLA recognition is countered by an inhibitory response, which eventually induces a sufficient immunoregulation in successful OD pregnancies. Whether the increased IDO expression is indeed explained by a well-functioning negative feedback mechanism causing enough suppression for an uncomplicated OD pregnancy is yet to be elucidated.

Decreased levels of PD-L1, CD80, CD86 and IDO in preeclamptic cases would support recent studies that show that decreased signaling of such co-inhibitory pathways plays an important role in the pathophysiological mechanism of preeclampsia. (19, 25, 27, 37) Dysfunctional co-inhibitory pathways might disrupt placental and possibly systemic regulatory and effector T cell balance, promoting preeclampsia development. (25) To illustrate, recent studies show that in peripheral blood of preeclamptic cases, PD-1 expression is downregulated on Th17 cells and clonally expanded CD8+effector memory T cells and upregulated on Tregs. (38, 39) This confirms there is a systemically lower level of co-inhibitory PD-L1 mediated immune regulation in preeclampsia. We found a lower placental CD200R mRNA expression in preeclamptic cases compared to uncomplicated cases in both OD and NC pregnancies. It is expected that a certain extent of the maternal immune cells that interact with the PD-L1 and CD200 ligands on trophoblasts pass by in the maternal blood without many cells residing in the tissue and that additionally, the blood is washed away from the intervillous space to a large extent during placenta preparation. Furthermore, CD200R expression by the syncytiotrophoblast has been described, which may explain the differential expression of PD-1 and CD200R in third trimester placenta tissue. (Xu et al., 2018) Interestingly, Xu J et al. did not find a difference in CD200R protein expression when comparing trophoblasts of normotensive and preeclamptic pregnancies. Thus, to fully investigate the significance of the difference in CD200R mRNA expression between preeclamptic and uncomplicated pregnancies, analysis of third trimester maternal immune cell composition and peripheral maternal blood is required.

The immune-mediated causality versus the endothelial dysfunction hypothesis remains a lively debate in preeclampsia research. However, several studies suggest that the two explanations go hand in hand. The PD-1/PD-L1 pathway for example mediates pro-inflammatory cell damage by CD8⁺ T cells of vascular endothelial cells. (17) Thus, in extension of our findings, an immunological dysregulation in the decidua basalis may induce impairment of spiral artery remodeling. (40) Additional support comes from our novel finding that shows a positive correlation of CD200 protein expression with the amount of total HLA mismatches, HLA class I mismatches and HLA-C mismatches in uncomplicated OD cases only. Of the classical HLA class I molecules, HLA-C is the only one

expressed by the extravillous trophoblast. HLA-C can thus stimulate protective immunity through peptide presentation while simultaneously, if mismatched, be a target for maternal leukocytes. (41) The found correlations show the importance of the immunosuppressive ligand CD200 in maternal-fetal tolerance, especially for a successful OD pregnancy, characterized by a high extent of HLA mismatching in general. In addition, such correlation with CD200 was not found for the presence of HLA class II mismatches. Therefore, HLA class I mismatch presence is suggested to be effectively counteracted by co-inhibitory pathways such as CD200, while similar compensation for HLA class II incompatibility is lacking and thus might contribute to the development of preeclampsia in OD pregnancies. This notion is supported by a recent study in which increased number of HLA class II mismatches is associated with preeclampsia in OD pregnancies. (42) Although the higher extent of HLA mismatching seems to be counterbalanced by enhanced CD200 expression, the decrease of CD200 expression in preeclamptic versus uncomplicated OD pregnancies suggests that it may not result in sufficient immunomodulation to prevent preeclampsia.

Interesting findings suggest the hypothesis of a differential pathophysiological mechanism of preeclampsia in OD compared to NC pregnancies. (7, 8, 40, 43-45) The lower PD-L1 protein expression and lower CD200 protein and mRNA expression in OD pregnancies complicated with preeclampsia compared to NC pregnancies complicated with preeclampsia found in this study are in support of the hypothesis. In addition, the correlation of IDO with birth weight in preeclamptic NC pregnancies, but not in OD cases may reflect absent protective immune regulatory activity and thus more vulnerability to the development of preeclampsia in OD pregnancies.

In conclusion, OD pregnancies, characterized by greater immunogenic dissimilarity, show an altered immunoregulation by co-inhibitory pathways. While PD-L1 and CD200 signaling appears suppressed by the increased antigen recognition, the CLTA-4 signaling and IDO mediated inhibition seem to be induced by the higher tolerance necessity in uncomplicated OD pregnancies. Insufficiency to overcome the increased challenge for immunoregulation may explain the higher risk for preeclampsia compared to NC pregnancies. The correlation with the extent of HLA mismatching further underlines the crucial role of CD200 tolerance induction. Our results provide the first insight in the role of co-inhibitory checkpoint signaling in the puzzling immunoregulatory mechanisms of both uncomplicated and preeclamptic OD pregnancies. Therefore, this study contributes to future prospects in OD pregnancy risk management. Ultimately, this includes optimizing donor conditions, preeclampsia screening and treatment options in both NC and OD cases and, in general, understanding reproductive immunology.

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APPENDIX

Supplementary Table 1. Overview of antibodies used for immunohistochemistry.

Target protein	Primary antibody	Dilution	Secondary antibody
PD-1	Human PD1 Polyclonal Goat IgG (AF 1086, R&Dsystems)	1:40	Rabbit Polyclonal anti-Goat Ig HRP (P0160, Dako)
PD-L1	Human PD-L1 monoclonal Mouse (M3663, Dako)	1:40	Anti-Mouse Envision + System HRP (K4011, Dako)
CD200	Human/Mouse/Rat CD200 polyclonal Goat IgG (AF2724, R&Dsystems)	1:200	Rabbit Polyclonal anti-Goat Ig HRP (P0160, Dako)

Supplementary Table 2. Primer sequences used for quantitative PCR.

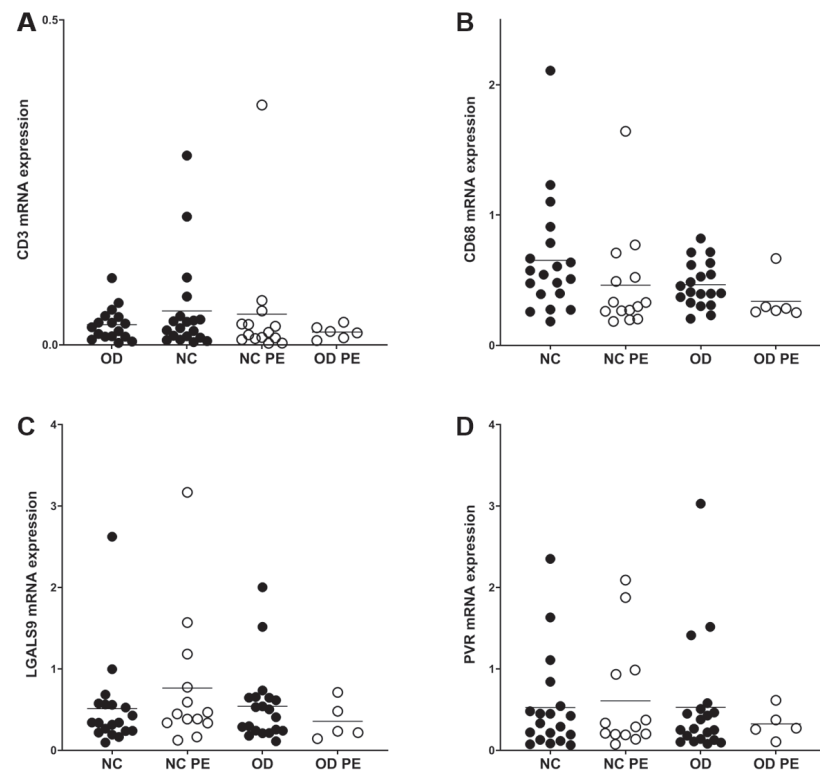
Gene	Forward	Reverse
PD-1	GCGGCCAGGATGGTTCTTAG	CACGAAGCTCTCCGATGTGT
PD-L1	AAATTGCAGGATGCAGGGGT	GGTGACTGGATCCACAACCA
CD80	GAAGCAAGGGGCTGAAAAG	GGAAGTTCCAGAAGAGGTCA
CD86	CGAGCAATATGACCATCTTCTG	CGCTTCTTCTTCTCCATTTC
IDO	CGGTCTGGTGTATGAAGG	CTAATGAGCACAGGAAGTTC
CD200	CCAGCTGGGACTCCAAAACCT	GTACATAGACGGTGAGGCAGG
CD200R	CGTGGGATTCATTTGGTTGT	TTCATCTCCTCAACAACCTGG
LGALS9	TCCAGCTGTCCCCTTTTCTG	GAGGGTTGAAGTGAAGGCA
PVR	TACACCTGCCTGTCGTAC	CCAGTGAGCTGGACCTTCTG
CD3e	CCGCCATCTTAGTAAAGTAACAG	AATACCACCCATTTCTTCATACC
CD68	TTCCCTATGGACACCTCAG	TTGTACTCCACCCGATGTA

Note: PD-1 = Programmed cell death protein 1; PD-L1 = Programmed cell death 1 ligand 1 (CD274); IDO = indoleamine 2,3-dioxygenase; LGALS9 = Galectin-9; PVR = PVR cell adhesion molecule.

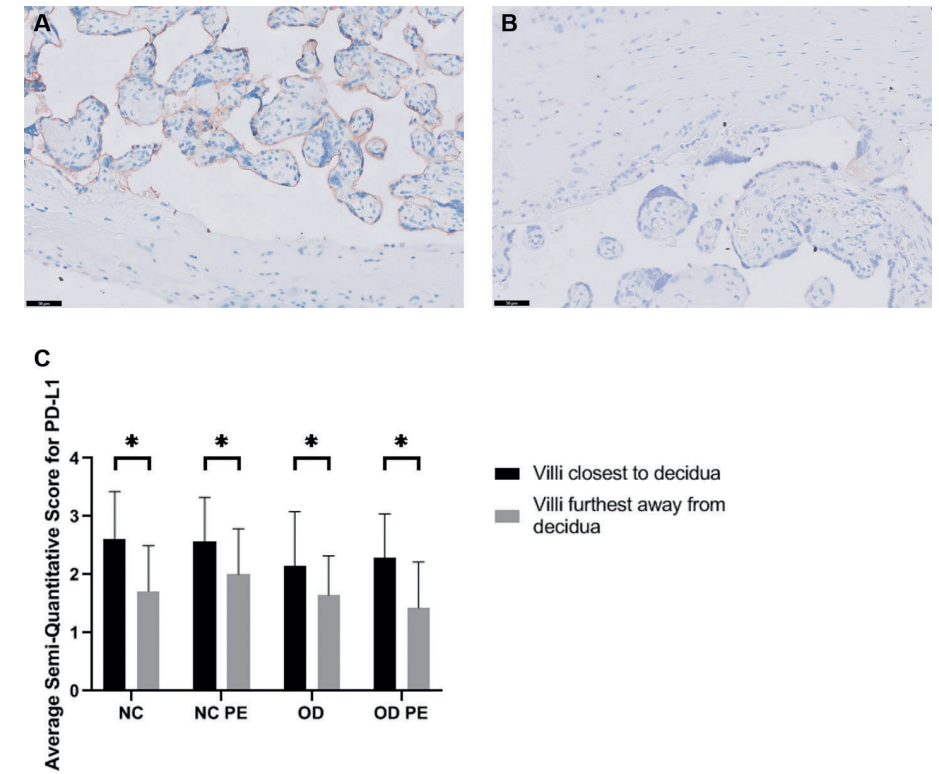
Supplementary Table 3. HLA mismatches presence per type of pregnancy.

	NC uncomplicated (n=21)	OD uncomplicated (n=19)	NC PE (n=12)	OD PE (n=8)
Total HLA mismatches, mean ± SD	3.4 ± 1.3	6.4 ± 1.6 ^a	3.5 ± 1.0 ^b	6.3 ± 1.7 ^{a,c}
HLA Class I mismatches, mean ± SD	2.2 ± 0.9	4.2 ± 1.2 ^a	1.9 ± 1.0 ^b	3.3 ± 1.6 ^{a,c}
HLA Class II mismatches, mean ± SD	1.2 ± 0.8	2.3 ± 0.7 ^a	1.6 ± 0.7 ^b	3.0 ± 0.9 ^{a,b,c}

Note: NC = uncomplicated naturally conceived pregnancies; OD = uncomplicated oocyte donation pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD PE = oocyte donation pregnancies complicated with preeclampsia. a = p < 0.05 compared to NC uncomplicated, b = p < 0.05 compared to OD uncomplicated, c = p < 0.05 compared to NC PE.



Supplementary Figure 1. A). Relative normalized placental CD3 mRNA expression. B). Relative normalized placental CD68 mRNA expression. C). Relative normalized placental LGALS9 mRNA expression. D). Relative normalized placental PVR mRNA expression. NC = uncomplicated naturally conceived pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD = uncomplicated oocyte donation pregnancies; OD PE = oocyte donation pregnancies complicated with preeclampsia.



Supplementary Figure 2. Gradient of PD-L1 protein expression from decidua towards fetal membranes. (A-B) representative example of gradient of PD-L1 within one sample. Causality with the experimental setup was eliminated due to random application of the tissue on the adhesive-coated glass slides. A). Villi most proximal to decidua (below). B). Villi most distal from decidua, proximal to fetal membranes (above). C). Average semi-quantitative PD-L1 protein score for villi most proximal to decidua and villi most distal from decidua per type of pregnancy. NC = uncomplicated naturally conceived pregnancies; NC PE = naturally conceived pregnancies complicated with preeclampsia; OD = uncomplicated oocyte donation pregnancies; OD PE = oocyte donation pregnancies complicated with preeclampsia; Scale bar = 50 μ m; *p < 0.05.