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Research article

Different immunoregulatory components at the decidua basalis of oocyte donation pregnancies

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ABSTRACT

Oocyte donation (OD) pregnancies are characterized by more fetal-maternal human leukocyte antigen (HLA) mismatches compared with naturally conceived (NC) and *in vitro* fertilization (IVF) pregnancies. The maternal immune system has to cope with greater immunogenetic dissimilarity, but involved immunoregulation remains poorly understood. We examined whether the amount of regulatory T cells (Tregs) and immunoregulatory cytokines in decidua basalis of OD pregnancies differs from NC and IVF pregnancies. The cohort included 25 OD, 11 IVF and 16 NC placentas, maternal peripheral blood, and umbilical cord blood of uncomplicated pregnancies. Placenta slides were stained for FOXP3, IL-10, IL-6, gal-1, TGF-β and Flt-1. Semi-quantitative (FOXP3+ Tregs) and computerized analysis (cytokines) were executed. The blood samples were typed for HLA class I and II to calculate fetal-maternal HLA mismatches. The percentage of Tregs was significantly higher in pregnancies with 4–6 HLA class I mismatches (n = 17), compared to 0–3 mismatches (n = 35; p = 0.04). Cytokine analysis showed significant differences between OD, IVF and NC pregnancies. Flt-1 was significantly lower in pregnancies with 4–6 HLA class I mismatches (p = 0.004), and in pregnancies with 6–10 HLA mismatches in total (p = 0.024). This study suggests that immunoregulation at the fetal-maternal interface in OD pregnancies with more fetal-maternal HLA mismatches is altered.

1. Introduction

It has been argued that a well-balanced ratio between a pro- and anti-inflammatory immune response is essential for successful pregnancy [1–3]. In naturally conceived (NC) and *in vitro* fertilization (IVF) pregnancy, a semi-allogeneic fetus is tolerated by the maternal immune system for a period of nine months. Oocyte donation (OD) pregnancies are characterized by a higher degree of immunogenetic dissimilarity, reflected by fetal-maternal human leukocyte antigen (HLA) mismatches, since the fetus obtains paternal and donor-derived genes (Fig. 1). In OD pregnancies, the maternal immune system has to cope with greater immunogenetic dissimilarity, the involved immunoregulatory mechanisms however remain poorly understood.

Possibly, fetal cells at the fetal-maternal interface in the placenta are not attacked by the maternal immune system because of a tolerogenic microenvironment caused by regulatory T cells (Tregs) and other immunoregulatory components. T cells represent 10–20% of all leukocytes in the fetal-maternal interface [4–6]. About 30–45% of T cells in the decidua are CD4+ T cells, of which roughly 5% include FOXP3+ Tregs [6–8]. The forkhead box P3 (FOXP3) is an important transcription factor for Treg development and function, and a reliable marker to detect FOXP3+ Tregs [12]. Tregs play a key role in maintaining tolerance at the fetal-maternal interface, and thus have been found to be key players of fetal survival [8–12]. It has been shown that the amount of peripheral blood and decidual FOXP3+ Tregs is decreased in

Abbreviations: Tregs, regulatory T cells; OD, oocyte donation; HLA, human leukocyte antigen; NC, naturally conceived; IVF, *in vitro* fertilization; FOXP3, forkhead box P3; TGF-β, transforming growth factor β; IL, interleukin; gal-1, galectin-1; Flt-1, Fms-like tyrosine kinase 1; sFlt-1, soluble Fms-like tyrosine kinase 1; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; pSMAD2, phosphorylated gene SMAD2.

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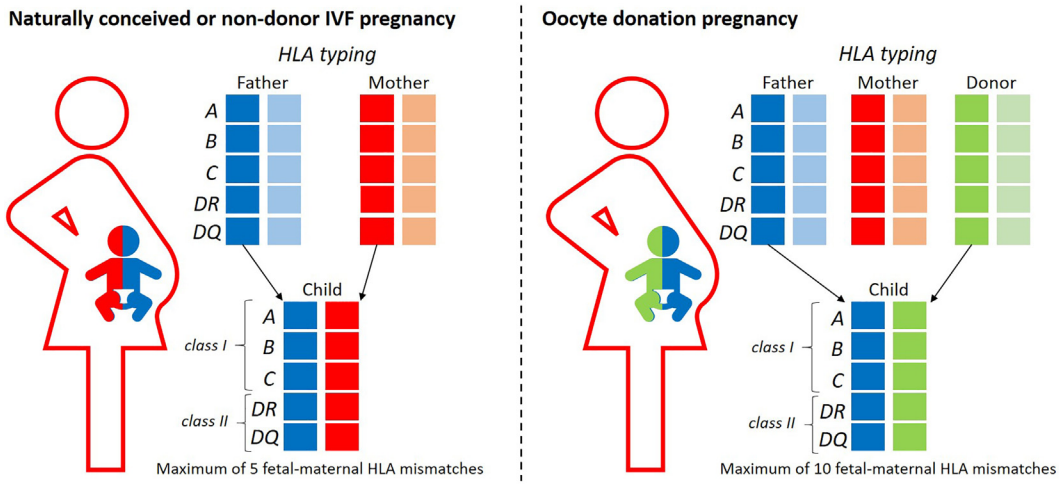

Fig. 1. Fetal-maternal human leukocyte antigen (HLA) mismatches in naturally conceived (NC) and non-donor *in vitro* fertilization (IVF) pregnancies versus oocyte donation (OD) pregnancies. In OD pregnancy, the child inherits paternal and donor-derived genes, and could therefore be totally allogeneic to the mother. In NC and IVF pregnancies, only one mismatch of each HLA locus can occur. Hence, a maximum of five HLA mismatches is possible (maximum of three HLA class I and two HLA class II mismatches). In OD pregnancies, it is possible to have two mismatches of each HLA locus. Hence, a maximum of ten HLA mismatches can occur (six HLA class I and four HLA class II mismatches).

pregnancy complications, such as preeclampsia [13–17], (recurrent) spontaneous miscarriage [18], and preterm labor [19,20].

Various cells in the decidua, such as Tregs, trophoblasts, uNK cells, and macrophages, are involved in the secretion of important immunoregulatory cytokines, including TGF-β, IL-10, gal-1, IL-6 and Flt-1. Transforming growth factor beta (TGF-β) and interleukin-10 (IL-10) suppress effector lymphocytes and promote angiogenesis as part of maternal adaptation during embryo implantation [21–23]. IL-10 could also be activated by IL-6, a cytokine that can have both pro-inflammatory and anti-inflammatory effects and that is important for hematopoiesis and acute phase reactions [10]. In addition, the immunoregulatory molecule galectin-1 (gal-1) promotes apoptosis of activated T cells and the expansion of Tregs [24–26]. Another potentially important factor in maternal immune tolerance during embryo implantation is Fms-like tyrosine kinase 1 (Flt-1), a receptor for vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which are both regulators of angiogenesis [27]. Altogether, Tregs and these regulatory TGF-β, IL-10, gal-1, IL-6, and Flt-1 have a pivotal role in the maintenance of a healthy pregnancy [10].

Compared to NC and IVF pregnancies, OD pregnancies are associated with a higher incidence of pregnancy complications, especially with hypertensive complications [28–30]. Little is known about the pathophysiology of these complications in OD pregnancies. Thus, to increase our understanding we should first study immunoregulatory components in the decidua of healthy OD pregnancies. Possibly, an altered immunoregulation is needed in order to prevent pregnancies with a greater immunogenetic dissimilarity from complications. This study therefore focused on the quantification of Tregs and the aforementioned immunoregulatory cytokines in the decidua basalis of uncomplicated OD pregnancies compared to NC and IVF pregnancies.

2. Materials and methods

2.1. Patient selection

This cohort study included patients with pregnancies achieved by OD (n = 23), IVF (n = 11) and NC (n = 16). The IVF group consisted of women that conceived by *in vitro* fertilization with their own oocytes. The NC group consisted of women that conceived spontaneously, without artificial reproductive technology. Placentas were collected at delivery after uncomplicated pregnancies at 37–42 weeks of gestational age between 2005 and 2013. Pregnancies complicated by preeclampsia, growth restriction or gestational diabetes were excluded. In the OD group, two dizygotic twins were born, resulting in a total of 25 placentas. Twin pregnancies were analyzed separately. In the IVF and NC groups only singletons were born, resulting in 11 and 16 placentas respectively. Placental tissue samples were collected within five hours after delivery. Furthermore, maternal peripheral blood and umbilical cord blood were collected in heparinized tubes for HLA typing. Clinical data was obtained from medical records. The indication for OD was unknown to our laboratory. The study was approved by the medical ethics committee of the Leiden University Medical Center (P08.229/228; P10.009), and informed consent of every patient was obtained.

2.2. Immunohistochemical staining of FOXP3

The placentas were processed into microscopic sections of four μm. The sections were deparaffinized and antigen retrieval was performed. The slides were incubated with a mouse monoclonal anti-FOXP3 antibody (Table 1) for one hour. Thereafter, slides were incubated with the secondary antibody, EnVision mouse (DAKO Cytomation). FOXP3+ Tregs were visualized by adding diaminobenzidine (DAKO Cytomation). Hematoxylin was used to counterstain. The slides were mounted and covered. All slides were scanned by a Pannoramic Midi scanner (3DHISTECH, Budapest, Hungary), and the decidua basalis was annotated using the digital microscopic program CaseViewer (3DHISTECH). The PatternQuant tool (3DHISTECH) was used to calculate the total area

Table 1
Antibody characteristics.

Antibody	Dilution	Source
FOXP3	1:150	Abcam, Cambridge, UK
IL-10	1:100	Hycult Biotech Inc, Plymouth Meeting, USA
IL-6	1:20	R&D Systems Europe Ltd, Oxon, UK
pSMAD2	1:1000	Santa Cruz Biotechnology Inc, Heidelberg, Germany
Gal-1	1:500	Santa Cruz Biotechnology Inc, Heidelberg, Germany
Flt-1	1:250	Santa Cruz Biotechnology Inc, Heidelberg, Germany

of decidua basalis of every slide. The annotated slides were scored semi-quantitatively by counting the total number of FOXP3+ Tregs. Fifteen slides were randomly selected to determine the inter-observer and intra-observer variability of this scoring method, respectively 0.553 and 0.678. Consensus of the scoring was obtained in a meeting. The counted number of FOXP3+ Tregs was divided by the total area of the decidua basalis to correct for the differences in area size of decidua basalis between the slides. All the analyses and scoring were performed blinded for study group (OD, IVF and NC).

2.3. Immunohistochemical staining of cytokines

Out of every study group (OD, IVF and NC), ten cases were selected randomly for immunohistochemical staining of IL-10, IL-6, gal-1, Flt-1, and the activity of TGF-β. Binding of TGF-β to its receptor leads to a cascade of reactions with phosphorylation of gene SMAD2 (pSMAD2) as a result [21], so immunohistochemical staining of pSMAD2 was used to define activity of TGF-β. The primary antibodies and optimal dilutions are presented in Table 1. The antibodies for pSMAD2, gal-1 and Flt-1 were incubated for one hour, and IL-10 and IL-6 overnight at room temperature. Incubation with EnVision was done, except for IL-10 which was incubated with PowerVision (Immunologic, Duiven, the Netherlands). For IL-6, a secondary goat antibody was labelled with horseradish peroxidase, followed by incubation. For gal-1, a secondary goat anti-rabbit antibody was labelled with horseradish peroxidase, followed by incubation with DAB. Counterstaining was done with hematoxylin, except for the pSMAD2 slides. The slides were mounted and covered. Images of all slides 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, five pictures were taken of the decidua basalis, blinded for study group. Only the decidua basalis was selected; blood vessels and shadows were digitally removed. Using Image-J software [31], the number of positive pixels per decidua basalis was measured, indicating the level of expression for each immunohistochemical staining without restriction to particular cell types. This program is able to identify and measure positive cells by setting a threshold. For every slide, an automatically running function was made, predefining the threshold of a positive cell.

2.4. HLA typing

To relate the amount of Tregs and cytokines to the allogeneic nature of the fetus, paired samples of peripheral maternal and umbilical cord blood were collected after delivery for HLA typing. Both blood samples were typed for HLA class I (HLA-A, -B, -C), and class II (HLA-DRB1, -DQB1) at the Dutch National Reference Laboratory for Histocompatibility Testing in the Leiden University Medical Center (LUMC) using the Sequence Specific Oligonucleotides PCR technique [32]. All HLA antigens were evaluated at split level, and the number of fetal-maternal HLA mismatches was calculated. Regarding the five HLA genes typed for, the maximum number of fetal-maternal HLA mismatches in NC and IVF pregnancies is five, in contrast to a maximum of ten in OD pregnancies (Fig. 1).

2.5. Statistical analysis

Descriptive statistical analysis was performed using SPSS statistics 25 (IBM SPSS Software) and GraphPad Prism 8.4.2 (GraphPad Software Inc.). To analyze differences between two independent groups, the Mann-Whitney *U* test was used for continuous data, and the Chi-square test for categorical data. To analyze differences between three independent groups, the Kruskall-Wallis test was used for continuous data, and the Chi-square test was used for categorical data. For all tests, a value of $p < 0.05$ was defined as significant.

3. Results

3.1. Clinical characteristics

The clinical characteristics of the women and pregnancies are shown in Table 2. Significant differences were found in maternal age, gravidity, and parity between the groups. Post test analysis showed a significantly higher maternal age in the NC group (39.3 years) compared to the IVF group (35.0 years). In the OD group, the median gravidity (2; 1–5) was significantly lower compared to the NC group (3; 1–7). The median parity was significantly higher in the NC group (2; 0–5) compared to both the OD (0; 0–2) and IVF group (0; 0–2). In the OD group, two dizygotic twins were born, though this did not appear as a clear difference between the groups. Mode of delivery (vaginally or caesarean section) did not significantly differ between the three groups. The mean gestational age at birth was 279 days (259–296) in the OD, 277 days (259–294) in the IVF, and 275 days (265–294) in the NC group, not showing significant differences. Likewise, mean birthweight did not differ significantly between the OD (3642 grams), IVF (3323 grams), and NC group (3680 grams).

3.2. HLA mismatches

In NC and IVF pregnancies, only one mismatch of each HLA locus can occur. Hence, a maximum of five HLA mismatches is possible (maximum of three HLA class I and two HLA class II mismatches). In OD pregnancies, it is possible to have two mismatches of each HLA locus. Hence, a maximum of ten HLA mismatches can occur (six HLA class I and four HLA class II mismatches; Fig. 1). The number of HLA class I, HLA class II, and total HLA mismatches is depicted in Table 3. In the OD group, the number of fetal-maternal mismatches for all HLA loci was significantly higher compared to the IVF and NC group. The HLA class I mismatches had a median of four (1–6) in the OD, two (1–3) in the IVF, and three (0–3) in the NC group. The HLA class II mismatches had a median of two (0–4) in the OD, two (1–2) in the IVF, and two (0–2) in the NC group. As for the HLA mismatches in total, the OD group showed a median of six (3–10), whereas both the IVF (3–5) and NC group (0–5) showed a median of four mismatches.

3.3. Semi-quantitative analysis FOXP3

All the deciduae basalis of OD, IVF and NC pregnancies showed presence of FOXP3+ Tregs. Fig. 2A shows a representative picture of FOXP3+ Tregs in the decidua basalis. No significant differences were found when comparing the three groups for the mean percentage of FOXP3+ Tregs (Fig. 2B).

The mean percentage of Tregs was significantly higher in pregnancies with 4–6 HLA class I mismatches (n = 17), compared to those with 0–3 HLA class I mismatches (n = 35; p = 0.04; Fig. 2C). Analysis of the HLA class I genes separately (HLA-A, -B, -C) did not show significant differences. The number of Tregs did not significantly differ in relation to the number of HLA class II mismatches or the total number of HLA mismatches (Fig. 2C). Likewise, when only taking OD pregnancies into account, no significant differences were found in the number of Tregs between pregnancies with low and high class I, class II, and total HLA mismatches.

Table 2
Clinical characteristics of the included pregnancies.

		OD N = 25		IVF N = 11		NC N = 16			
		percentage	N	percentage	N	percentage	N	p-value[#]	
Mode of delivery	Vaginally	54.5	12	72.7	8	31.2	5	0.201	
	CS	45.5	10	27.3	3	68.8	11		
	missing	12.0	3		0		0		
Twin gestation		8.7	2	0.0	0	0.0	0	-	
		mean	min-max	mean	min-max	mean	min-max	p-value[$]	Post test[&]
Maternal age (years)		37.7	27-48	35.0	30-40	39.3	37-43	0.021	OD vs IVF: p = 0.125
missing		0		0		0			OD vs NC: p = 0.290
Gestational age (days)		279	259-296	277	259-294	275	265-294	0.662	IVF vs NC: p = 0.001
missing		1		0		0			
Birthweight (gram)		3642	2820-4260	3323	2550-3820	3680	2975-4600	0.158	
missing		2		0		0			
		median	min-max	median	min-max	median	min-max	p-value[$]	Post test[&]
Gravidity (N)		2	1-5	2	1-4	3	1-7	0.009	OD vs IVF: p = 0.267
missing		1		0		0			OD vs NC: p = 0.003
Parity (N)		0	0-2	0	0-2	2	0-5	0.000	IVF vs NC: p = 0.100
missing		1		0		0			OD vs IVF: p = 0.371
									OD vs NC: p = 0.000
									IVF vs NC: p = 0.008

N = number; OD = oocyte donation; IVF = *in vitro* fertilization; NC = naturally conceived; vs = versus; CS = caesarean section.
All p-values are 2-sided.
[#] p-values calculated with Chi-square test.
[$] p-values calculated with Kruskall-Wallis test.
[&] p-values calculated with Mann-Whitney U test.

Table 3
Number of HLA mismatches of the included pregnancies.

	OD N = 23		IVF N = 11		NC N = 16			
	median	min–max	median	min–max	median	min–max	p-value[$]	Post test[&]
HLA class I mm	4	1–6	2	1–3	3	0–3	0.000	OD vs IVF: p = 0.000
								OD vs NC: p = 0.000
								IVF vs NC: p = 0.680
HLA class II mm	2	0–4	2	1–2	2	0–2	0.005	OD vs IVF: p = 0.100
								OD vs NC: p = 0.004
								IVF vs NC: p = 0.212
Total HLA mm	6	3–10	4	3–5	4	0–5	0.000	OD vs IVF: p = 0.000
								OD vs NC: p = 0.000
								IVF vs NC: p = 0.716

N = number; OD = oocyte donation; IVF = *in vitro* fertilization; NC = naturally conceived; HLA = Human Leukocyte Antigen; mm = mismatches; vs = versus.
[$] p-values calculated with Kruskall-Wallis test.
[&] p-values calculated with Mann-Whitney *U* test.

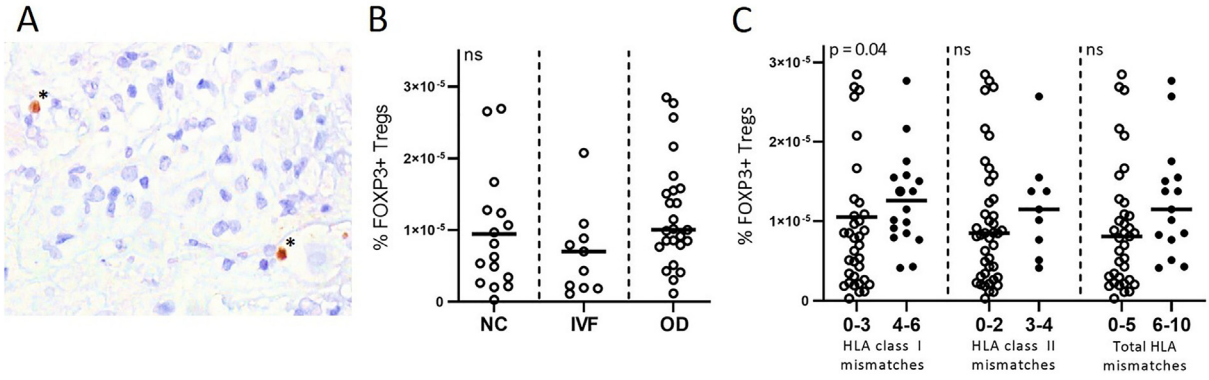

Fig. 2. Immunohistochemical staining of FOXP3+ regulatory T cells (Tregs) in placental tissue and results of the semi-quantitative analysis. A. Screenshot made with CaseViewer (3DHISTECH). Nuclei are stained blue. FOXP3+ Tregs in the decidua basalis are stained brown, indicated with the asterisks* (magnification x45). B. No significant difference was found in the percentage of FOXP3+ Tregs between the naturally conceived (NC), *in vitro* fertilization (IVF) and oocyte donation (OD) group. C. The percentage of FOXP3+ Tregs is significantly higher in pregnancies with 4–6 HLA class I mismatches than in pregnancies with 0–3 HLA class I mismatches (p = 0.04). Analysis of the HLA class I genes separately (HLA-A, -B, -C) did not show significant differences. The bars represent the mean percentages. The Mann-Whitney *U* test was performed to analyze two groups. The Kruskall-Wallis test was performed to analyze data between three groups.

3.4. Cytokine analysis

The deciduae basalis of OD, IVF and NC pregnancies all showed positive cells for the used antibodies (Fig. 3). The results of the Image-J analysis are depicted in Fig. 4. OD pregnancies express less IL-10, IL-6, gal-1, TGF-β, and Flt-1 in the decidua basalis compared to NC pregnancies. Compared with IVF pregnancies, the level of Flt-1 was significantly lower, and the level of IL-10, IL-6, and TGF-β was significantly higher in the decidua basalis of OD pregnancies. The decidua basalis of NC pregnancies showed significantly higher levels of IL-10, IL-6, and TGF-β compared to IVF pregnancies, whereas the level of gal-1 and Flt-1 did not differ.

In addition, we compared the amount of cytokines between pregnancies with a low and high allogeneic nature. The amount of Flt-1 is significantly lower in pregnancies with 4–6 fetal-maternal HLA class I mismatches (n = 23), compared to those with 0–3 fetal-maternal HLA class I mismatches (n = 7; p = 0.004; Fig. 5A). When analyzing the HLA class I genes separately, the amount of Flt-1 was significantly lower in pregnancies with 2 HLA-A (n = 5), -B (n = 6), or -C (n = 3) mismatches compared to pregnancies with 0–1 HLA-A (n = 25; p = 0.021), -B (n = 24; p = 0.003), or -C (n = 27; p = 0.025) mismatches (Fig. 5B). Furthermore, the amount of Flt-1 is significantly lower in pregnancies with 6–10 HLA mismatches in total (n = 5) than in pregnancies with 0–5 HLA mismatches in total (n = 25; p = 0.024; Fig. 5A). When only taking the OD pregnancies into account, no significant differences were found in the amount of Flt-1 between pregnancies with low and high class I, class II, and total HLA mismatches. With regard to the other immunoregulatory cytokines, no correlation was found with the number of fetal-maternal HLA mismatches.

4. Discussion

In this cohort study we focused on the quantification of Tregs and several immunoregulatory cytokines in the decidua basalis of OD pregnancies compared to NC and IVF pregnancies. The OD pregnancies showed a high level of HLA mismatching between fetus and mother, including 68% (17/25) of pregnancies with 4–6 fetal-maternal HLA class I mismatches. The percentage of FOXP3+ Tregs was significantly higher in these pregnancies with a higher than natural occurring number of HLA class I mismatches compared to pregnancies with 0–3 HLA class I mismatches. In addition, the level of several immunoregulatory cytokines was altered in OD pregnancies compared to IVF and NC pregnancies. Most striking is the significant lower amount of Flt-1 in pregnancies with 4–6 fetal-maternal HLA class I mismatches, and in pregnancies with a total of 6–10 HLA mismatches.

Comparable to our study, Nakabayashi et al. [33] semi-quantitatively analyzed the decidua basalis of OD, NC, and IVF pregnancies for immunohistochemical staining of FOXP3+ Tregs. In contrast, they found a decrease of decidual FOXP3+ Tregs in OD pregnancies, possibly explaining why OD pregnancies have a higher risk to develop preeclampsia [28–30]. Likewise, other studies found that decidual FOXP3+ Tregs are decreased in autologous pregnancies complicated by preeclampsia [14–16]. More recently, Rudenko et al. [34] found a decrease in the number of CD4 + CD25+ Tregs in the decidua basalis of OD and surrogate pregnancies compared to non-donor IVF controls. However, the women in the OD and surrogate group significantly differed from the IVF group with regard to risk factors for developing pregnancy complications, as gestational hypertension, preeclampsia and preterm birth occurred more often. In the current study, we only investigated uncomplicated OD pregnancies, and our results did not show significant differences in the amount of FOXP3+ Tregs when comparing the decidua basalis of OD, NC, and IVF pregnancies.

Previous studies have acknowledged the immunogenetic dissimilarity in OD pregnancies [35–39]. However, the number of mismatches in OD pregnancies does not necessarily deviate from the situation in NC and IVF pregnancies, in which a maximum of five mismatches is possible. This could be more prevalent in our cohort, since commercial and anonymous donation in the Netherlands is forbidden by law [40]. This might lead to more family-related donations, and consequently less fetal-maternal HLA mismatches. Furthermore, comparing the OD, NC, and IVF groups introduces several confounding factors. Therefore, we took the number of fetal-maternal HLA mismatches into account when studying several immunoregulatory components. We showed an increase of FOXP3+ Tregs related to more fetal-maternal HLA class I mismatches. This could imply that pregnancies with greater fetal-maternal immunogenetic dissimilarity need a higher extent of immunoregulation by Tregs in order to prevent complications,

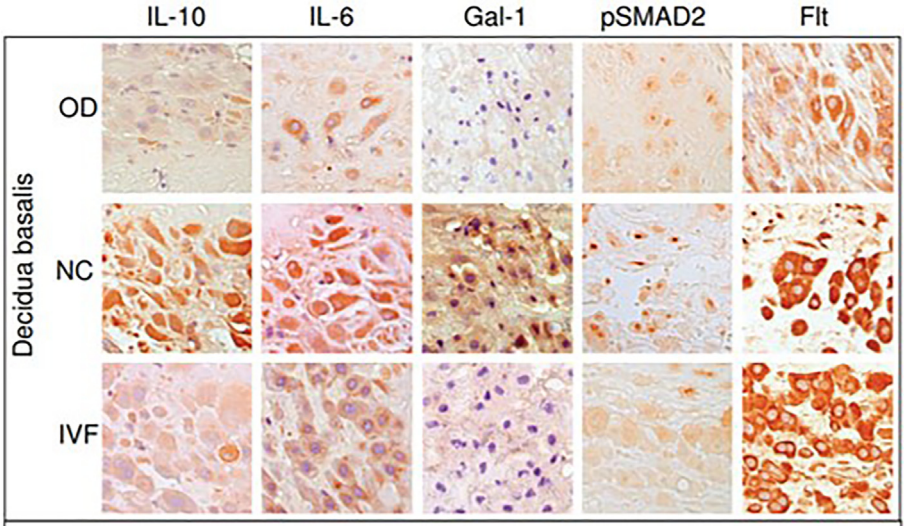

Fig. 3. Immunohistochemical staining of cytokines. Photomicrographs of sections stained for IL-10, IL-6, gal-1, pSMAD2 (TGF-β activity), and Flt-1 in the decidua basalis. Original magnification x200. For every group, oocyte donation (OD), naturally conceived (NC) and non-donor *in vitro* fertilization (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 Fig. 4.

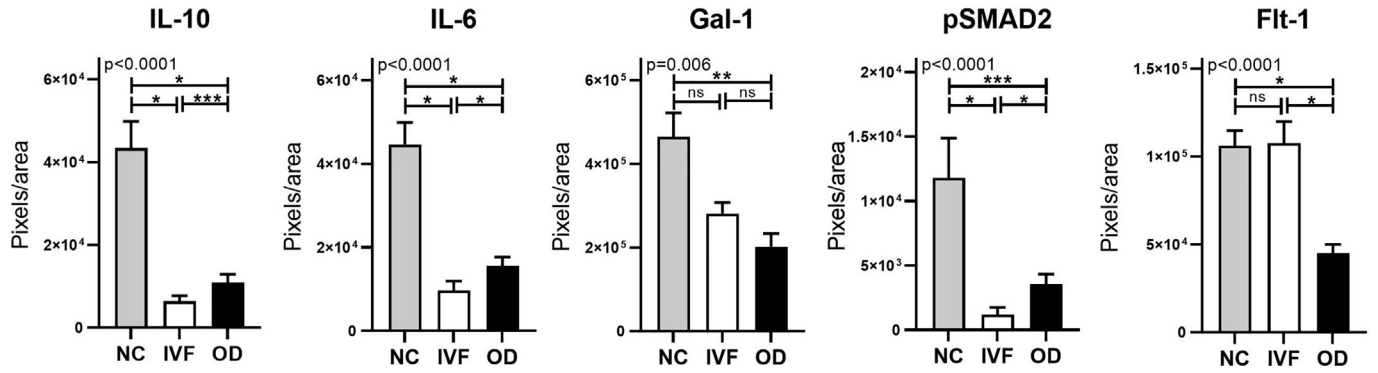

Fig. 4. Decidual cytokine levels. The graphs illustrate the amount of positive pixels per standardized area in the decidua basalis of ten oocyte donation (OD), ten naturally conceived (NC), and ten non-donor *in vitro* fertilization (IVF) pregnancies tested by immunohistochemical staining. Level of significance indicated with asterisks: *p < 0.001, **p = 0.001–0.01, ***p = 0.01–0.05, ns = not significant. Values presented as means, error bars indicate the standard error of the mean. The Mann-Whitney *U* test was performed to analyze two groups. The Kruskall-Wallis test was performed to analyze data between three groups.

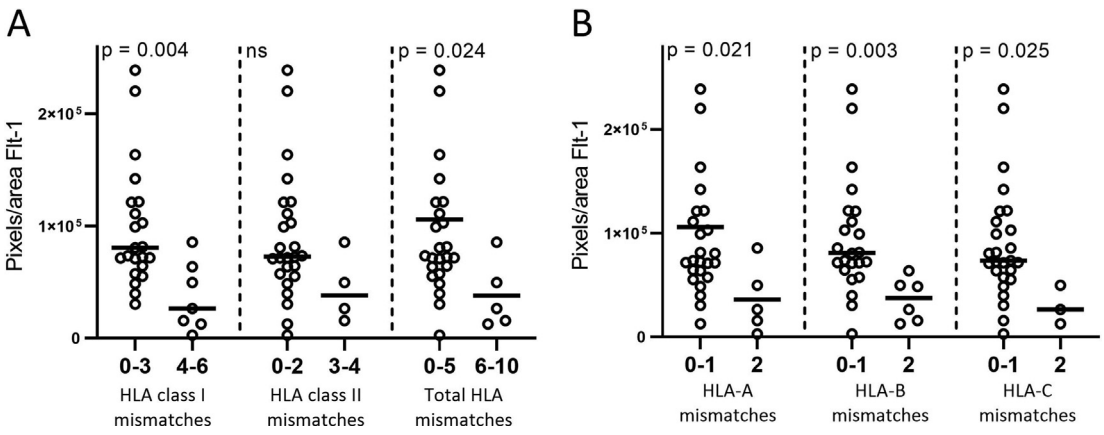

Fig. 5. Separate analysis of Flt-1 in relation to the number of fetal-maternal HLA mismatches. A. The amount of Flt-1 is significantly lower in decidua basalis of pregnancies with 4–6 HLA class I mismatches than in pregnancies with 0–3 HLA class I mismatches (p = 0.004). Likewise, the amount of Flt-1 is significantly lower in decidua basalis of pregnancies with 6–10 HLA mismatches in total than in pregnancies with 0–5 HLA mismatches in total (p = 0.024). B. Analysis of the HLA class I genes separately. For HLA-A, -B, and -C applies that the amount of Flt-1 in the decidua basalis is lower in pregnancies with a higher number of mismatches. The bars indicate the means. The Mann-Whitney *U* test was performed to analyze data between two groups.

such as preeclampsia. Unfortunately, Nakabayashi et al. [33] and Rudenko et al. [34] did not report on the number of fetal-maternal HLA mismatches that could have supported this assumption. Still, a positive correlation between the number of HLA-DR and -DQ mismatches and the amount of activated T cells in the peripheral blood of women pregnant after OD was found [41], suggesting that these T cells could have a regulatory character. Moreover, we previously demonstrated a significant influence of HLA class II mismatches on the development of preeclampsia in OD pregnancies [39]. The results of these studies might suggest a decreased amount of Tregs in complicated OD pregnancies with a higher number of fetal-maternal HLA mismatches.

In addition to Tregs, immunoregulatory cytokines were analyzed. We found decreased levels of IL-10, IL-6, gal-1, TGF-β (pSMAD2), and Flt-1 expression in the decidua basalis of uncomplicated OD pregnancies compared to NC pregnancies, suggesting that OD pregnancies are susceptible to develop complications. We only analyzed uncomplicated pregnancies and hypothesized to find increased IL-10, IL-6, gal-1, TGF-β, and Flt-1 expression. Indeed, the level of IL-10, IL-6, and TGF-β was significantly higher in the decidua basalis of OD pregnancies compared to IVF pregnancies. However, both the decidua basalis from OD and IVF pregnancies showed significantly lower levels of IL-10, IL-6, and TGF-β compared to NC pregnancies. This suggests that, next to greater fetal-maternal immunogenetic dissimilarity in OD pregnancies, the maternal immune response might also be influenced by the difference in hormonal therapies. Though data from mice studies illustrate that a decrease in IL-10 is not harmful for pregnancy [42], decreased decidual IL-10 has been linked to preeclampsia in human pregnancy [23,43,44], and (recurrent) spontaneous miscarriage [45,46]. Recently, low placental gal-1 has been associated with fetal growth restriction [47], while other studies showed that gal-1 is upregulated in preeclamptic placentas [48,49]. Decreased TGF-β levels have been associated with spontaneous miscarriage [50], whereas elevated placental TGF-β has been associated with preeclampsia [22,51,52]. Furthermore, less TGF-β has been linked to placental pathologies, as it is a repressor of cytotrophoblast outgrowth [21]. In this study, less TGF-β was found in uncomplicated OD and IVF pregnancies compared to NC pregnancies, without placental pathologies. IVF pregnancies are associated with a higher occurrence of placenta accreta [53], but the decreased TGF-β could possibly be counterbalanced another mechanism. Higher levels of IL-6 are often found in preeclampsia and preterm labor [54]. Decreased IL-6 in OD pregnancies could be an advantage for other immunoregulatory components, since elevated IL-6 may be related to abnormal functioning of Tregs in recurrent spontaneous miscarriage [55]. Hence, a decrease of decidual gal-1, TGF-β, and IL-6 in OD pregnancy, as shown in this study, could potentially be beneficial in the prevention of complications.

Decreased levels of Flt-1 were found in pregnancies with 4–6 fetal-maternal HLA class I mismatches, and in pregnancies with 6–10 HLA mismatches in total. The analysis of the HLA class I genes separately showed no significant influence of one HLA gene in particular, all three genes (HLA-A, -B, -C) showed equal results with regard to the level of Flt-1. The splice variant soluble Flt-1 (sFlt-1), a circulating antiangiogenic protein, is able to compete with Flt-1 by binding with VEGF and PlGF, removing them from the circulation, and weakening their angiogenic effects [27]. In healthy pregnancy, these factors are balanced to maintain normal angiogenesis, but increased sFlt-1 and decreased PlGF are found in the placenta of preeclamptic pregnancies [27,56,57]. This suggests that low Flt-1, as found in this study, makes high allogeneic pregnancies more susceptible for preeclampsia.

In OD research it is a challenge to define a proper control group. To strengthen this study, we included non-donor IVF pregnancies and NC pregnancies, which has been described in prior research [38,41] and is aimed at minimizing confounding by factors associated with the mode of conception. Indeed, maternal age, ethnicity, mode of conception, and plurality need to be taken into account as possible confounders [58]. The NC group consisted of women with a significantly higher maternal age compared to the IVF group (Table 2). Increasing age affects the immune system to protect against new pathogens, although this is more dominantly seen in CD8+ T cells than in CD4+ T cells. Moreover, ageing is associated with increased production of inflammatory cytokines [59]. The NC group showed significantly higher amounts of IL-10, IL-6, and TGF-β compared to the younger IVF group, possibly affected by older age. As for plurality, maternal serum cytokine levels are found to be lower in twin compared to singleton pregnancies, possibly to create a favorable environment for greater placentation [60]. Only in the OD group two dizygotic twins were born, though this did not appear as a clear difference between the groups. The unknown ethnicity of the included women could introduce bias, as expression frequencies of HLA alleles and T-cell responses differ across ethnicities [61,62].

Our study is limited by small sample size and adjusting for these factors was not possible. Furthermore, our data was only obtained from third trimester deciduae basalis, but maternal adaptation of the fetal allograft could be more prominent early in pregnancy or in complicated pregnancy. To account for these limitations, the DONOR (DONation of Oocytes in Reproduction) study is set up in the LUMC, in which maternal peripheral blood samples, products of conception, placentas, and umbilical cord blood are collected at various time points during and after OD pregnancy [58]. For the DONOR study, we calculated a sample size of 146 OD pregnancies and 146 non-donor pregnancies to demonstrate a significant difference in the development of hypertensive complications, adjusted for important confounding factors. Finally, we only examined FOXP3+ Tregs, while other studies acknowledged that pregnancy is associated with distinct Treg subsets [63,64]. Trophoblasts express other immunoregulatory cell surface molecules crucial for Treg induction, such as CTLA-4 and PD-L1 [65]. Previous research has shown that placental PD-L1 is decreased in uncomplicated OD pregnancies compared to uncomplicated NC pregnancies [66]. In contrast, a higher percentage of CTLA-4+ Tregs in maternal peripheral blood of OD pregnancies compared to NC pregnancies was found, possibly to achieve a tolerant state [8]. Since the mechanisms of suppression and the cellular effects by FOXP3+ Tregs, CTLA-4+ Tregs and PD-L1^{HI} Tregs could be different, it would be worthwhile investigating these immunoregulatory components using other methods next to immunohistochemistry, such as immunofluorescence and suppression assays.

In conclusion, OD pregnancies are a greater challenge to the maternal immune system in comparison to NC and IVF pregnancies, since a high level of fetal-maternal dissimilarity can occur. This study shows alterations in immunoregulation at the fetal-maternal interface in OD pregnancies with a higher number of fetal-maternal HLA mismatches. Despite these alterations, there still seems to be a sufficient extent of immunoregulation in the studied placentas to prevent from pregnancy complications. Unravelling mechanisms of immunomodulation during OD pregnancy could help to understand the mechanisms that contribute to the development of complications in OD pregnancies. Though, for future research a broader spectrum and larger sample size of materials needs to be investigated to obtain a full perspective on the differences in immunoregulation throughout OD, NC, and IVF pregnancies.

Author contributions

KvB, MB, ME, and MLvdH developed the idea for this study. KvB and MB executed the immunohistochemical staining and semi-quantitative analysis of the FOXP3+ Tregs. MLvdH executed the immunohistochemical staining and computerized analysis for the cytokines. CvdK and HK assisted with laboratory work and arranged the HLA typing. KvB analyzed the amount of Tregs and cytokines in relation with HLA mismatches, and drafted the manuscript. All authors contributed to the writing and reviewing of this article, and gave final approval of the version to be published.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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