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ORIGINAL ARTICLE

Peripheral monocyte subsets are altered during gestation in oocyte donation pregnancy complicated with pre-eclampsia

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Abstract

Oocyte donation (OD) pregnancies show a higher fetal-maternal incompatibility and a higher risk of developing pre-eclampsia (PE) than autologous pregnancies. As maternal monocytes play a role in the tolerization of the allogeneic fetus, the aim of this study was to analyse monocyte phenotypes in healthy and PE OD pregnancies. We collected maternal peripheral blood at different gestational time points in healthy ($n = 10$) and PE ($n = 5$) OD pregnancies. Fetal-maternal human leukocyte antigen (HLA) mismatches were calculated. We used a 35-colour antibody panel for Aurora spectral flow cytometry to analyse the composition and surface marker expression of monocyte subsets. Expression of CD38 on intermediate monocytes significantly increased throughout gestation in healthy OD pregnancies. Compared with the healthy group, the PE group exhibited even higher CD38 expression on monocyte subsets, with statistical significance. Immune inhibiting receptors CD85j (LILRB1) and CD85d (LILRB2), as well as monocyte recruitment regulating molecules CCR2 and CD91, also showed significantly enhanced expression on monocyte subsets during PE. When comparing healthy and PE OD only in pregnancies with high HLA mismatches, the different CD38 and CD85j expression in monocyte subsets was still significant. In conclusion, in healthy OD pregnancies, the upregulated CD38 expression might reflect a proinflammatory condition specifically at the third trimester. In PE OD pregnancies, expression of both inflammatory and immune regulatory markers is increased in maternal peripheral monocyte subsets. The elevated expression of CCR2 and CD91 on these subsets might reflect monocyte chemotaxis and the effect from systemic vascular dysfunction at the late stage of PE.

KEYWORDS
monocytes, oocyte donation, pre-eclampsia

Michael Eikmans and Marie-Louise P. van der Hoorn Shared senior authorship.

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1 | INTRODUCTION

During pregnancy the maternal immune system encounters the challenge of tolerating an allogeneic fetus while simultaneously defending against potential pathogens. Oocyte donation (OD) pregnancy introduces a unique condition wherein the fetus inherits not only paternal genes but also genetic material from the oocyte donor, resulting in a higher dissimilarity between fetal-maternal genes compared to naturally conceived pregnancies. This may necessitate an even stronger demand for tolerance by the maternal immune system compared to autologous pregnancies.

Studies have reported a higher incidence of pregnancy complications, including hypertensive complications such as pre-eclampsia (PE), in OD pregnancies compared to natural conception or other assisted reproductive technologies.[1,2] Possibly the gene dissimilarity, reflected by the fetal-maternal human leukocyte antigen (HLA) mismatches, in OD pregnancy is related to the higher incidence of PE. Furthermore, an association was found between PE development and the number of fetal-maternal HLA class II mismatches.[3,4] Therefore, the pathogenesis of PE in OD pregnancies might be partially immune-related, whereby a high extent of fetal-maternal HLA mismatching leads to alteration in the immune response during gestation.[5]

Monocytes play a role in the maternal immune response by supporting the tolerization of an allogeneic fetus. This notion was initially supported by the increasing number of circulating monocytes during pregnancy.[6] Furthermore, the activation of circulating monocytes is increased by soluble factors such as syncytiotrophoblast membrane microparticles in the intervillous space, resulting in the secretion of interleukin (IL), such as IL-6.[7,8] Studies have demonstrated an even greater activation of monocytes in pregnancies complicated by PE, leading to elevated reactive oxygen species and cytokine production.[6,9]

Regulation of maternal monocytes in OD pregnancy may persist throughout the whole gestation to achieve a successful pregnancy until term. Monocyte subsets, identified by CD14 and CD16 markers, include classical ($CD14^{high}CD16^{neg}$), intermediate ($CD14^{high}CD16^{pos}$), and non-classical ($CD14^{pos}CD16^{high}$) monocytes.[10] Each subset serves distinct functions. Classical monocytes act as phagocytes responsive to pathogens and inflammation and play crucial roles in tissue repair. On the other hand, intermediate monocytes contribute to antigen presentation and cytokine secretion. As for non-classical monocytes, they are highly specialized cells engaging in Fc receptor-mediated phagocytosis.[11–13] A recent study analysing circulating monocytes from the first to the third trimester reported that there was a significant shift in the cell frequency from classical toward intermediate monocytes, and the surface antigens on these cells also changed during gestation.[14] Our previous study focusing on healthy OD pregnancy at term found that OD pregnancies had a higher proportion of M2 macrophage (with an anti-inflammatory function and regulate wound healing[15]) percentage in total decidual macrophages compared to autologous pregnancies.[16] As collecting placenta tissue before term is relatively challenging, studying circulating monocytes provides insight into changes in the myeloid population throughout gestation in OD pregnancies. Meanwhile, studying three monocyte subsets separately will give more detailed information regarding their functions.

Therefore, in this study, we analysed the peripheral monocyte subsets in healthy/PE OD pregnancies and their alterations throughout gestation. We hypothesize that the composition and the surface marker expression of monocytes vary across different trimesters and different pregnancy conditions (healthy or PE) and that these differences relate to the extreme fetal-maternal HLA mismatches in OD pregnancies.

2 | MATERIALS AND METHODS

2.1 | Patient selection and sample collection

This nested case–control study included 10 uncomplicated and five PE OD pregnancies, which matched for age and BMI, from the DONOR cohort.[17] Medical records from the Leiden University Medical Center (LUMC) were reviewed, and clinical data were summarized (Table 1). Exclusion criteria comprised multiplets, multiparity, maternal age > 40 years, maternal body mass index (BMI) $\geq 30\,kg/m^2$, maternal chronic morbidities such as diabetes and hypertension, and gestational complications other than PE. PE was defined as newly onset hypertension (≥140/90 mmHg) and proteinuria after 20 weeks of gestational age, or other maternal organ dysfunction, or uteroplacental dysfunction resulting in fetal growth restriction. Informed consent was obtained, and the study was approved by the Medical Ethics Committee of the LUMC (LUMC-LDD; P16.048).

Maternal peripheral blood samples were collected in sodium heparinized and ethylenediaminetetraacetic acid (EDTA) tubes at various time points during gestation: before pregnancy, in the first (7–11 weeks), second (18–21 weeks), third trimester (35–39 weeks), and 6 weeks postpartum. The blood samples were layered on a Ficoll Hypaque gradient for density gradient centrifugation. Peripheral blood mononuclear cells (PBMCs) were collected from the interface, washed twice, counted, frozen

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TABLE 1 Samples characteristics.

Clinical parameters		OD-healthy ($n=10$)	OD-PE ($n=5$)	*p*-value
Mother	Age (years)	38.5 (25–43)	39.0 (34–42)	0.85
	GA (weeks)	39.2 (37.1–41.4)	38.3 (32.1–38.7)	0.05
	BMI (kg/m^2)	25.7 (18.94–27.68)	22.9 (19.94–26.20)	1.00
	Gravidity	1.5 (1–4)	1.0 (1–1)	0.83
Fetal-maternal HLA mismatches		7 (3–10)	7 (5–9)	0.17
Mode of delivery (caesarean section) (number [percentage])		5 (50%)	2 (40%)	1.00
Child	Gender (male) (number [percentage])	3 (30%)	2 (40%)	1.00
	Birth weight (grams)	3275 (2445—3995)	3275 (2142—3735)	0.54

Note: Mode of delivery and Gender are described using numbers and percentages. Other data are described by median with the minimum and maximum. To determine *p*-value, Fisher's exact tests were performed on categorical variables (BMI, Gravidity, Fetal-maternal HLA mismatches, Mode of delivery and Gender), Mann–Whitney *U* tests were performed on non-normally distributed numerical variables (Age, GA, and Birth weight).

Abbreviations: BMI, body mass index; GA, gestational age; HLA, human leukocyte antigens; OD, oocyte donation; n, number; PE, pre-eclampsia.

in fetal calf serum (FCS) with 10% dimethyl sulfoxide, and stored in liquid nitrogen until flow cytometry staining.

2.2 | HLA typing and mismatches calculation

Umbilical cord blood (UCB) samples were collected at delivery. In the Immunology laboratory of the LUMC HLA typing was performed. For UCB samples and their corresponding maternal peripheral blood samples were typed for HLA-A, B, C, DRB1, and DQB1 at the split level (2 digit), using Luminex technology (Lifecodes, Werfen). The number of fetal-maternal HLA mismatches was manually calculated from these results.

The OD group was divided into two subgroups based on fetal-maternal HLA mismatches: one with mismatches lower than half of the tested HLA locus (0–5 HLA mm) and another with mm higher than half of the HLA locus (6–10 HLA mm). As there was only one sample in the PE group with 0–5 HLA mm, this sample was excluded from the comparison.

2.3 | Immunophenotyping of PBMCs

A 35-colour panel was developed for the 5-laser Aurora spectral flow cytometer (Cytek Biosciences®) (Table 2). PBMCs were thawed in medium (RPMI with 50% FCS) and washed in FACS buffer (PBS with 1% FCS). Subsequently, PBMCs were stained with the viability marker, followed by incubation with the first extracellular staining mix (FACS buffer with antibodies in the right dilution [Table 2]). The second extracellular antibody mix was added after two washes. Each incubation lasted 30 min at 4°C in the dark. After fixation, all samples were measured on the same day to minimize inter-assay variation. Spectral unmixing was calculated with prepared single-stained reference controls (Table 2). To account for autofluorescence, an unstained PBMC control was incorporated. We then utilized the OMIQ.AI flow cytometry software (Dotmatics) for thorough analysis, which involved cleaning the data, compensating for variations, setting gates, and extracting numerical data for statistical evaluation.

2.4 | Statistical analysis

Statistical analysis utilized SPSS Statistics 29 (IBM SPSS Software) and GraphPad Prism 9.3.1 (GraphPad Software Inc.). The Mann–Whitney U test was employed for continuous data, and Fisher's exact test for categorical data when analysing differences between two independent groups. The Friedman test was applied for paired, non-parametric data over the course of pregnancy, followed by the Wilcoxon signed-ranks test for post-hoc analysis in case of statistical significance. A significant level of $p<0.05$ was defined for all tests.

3 | RESULTS

3.1 | Monocytes throughout gestation

To obtain an overview of circulating monocytes in healthy OD pregnancy, we assessed the composition of monocytes

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TABLE 2 Antibodies panel.

Channel	Fluorochrome	Antibody	Clone	Dilution	Controls	Supplier
UV laser						
UV2	BUV395	CD15	HI98	50	Beads	BD Bioscience
UV6	Live/Dead Blue	Live/Dead		1000	PBMCs	
UV7	BUV496	CD16	3G8	25	Lymphocytes	BD Bioscience
UV9	BUV563	CD163	GHI/61	25	Monocytes	BD Bioscience
UV10	BUV615	CD206	15–2	25	Beads	BD Bioscience
UV11	BUV661	CD123	7G3	25	Beads	BD Bioscience
UV16	BUV805	CD7	M-T701	50	Lymphocytes	BD Bioscience
Violet laser						
V1	BV421	CX3CR1	2A9-1	50	Monocytes	BD Bioscience
V2	Super Bright 436	CD56	TULY56	100	Lymphocytes	eBioscience
V3	VioBlue	TCRgd	11F2	100	Lymphocytes	Miltenyi Biotec
V5	BV480	HLA-DR	G46-6	200	Lymphocytes	BD Bioscience
V6	BV510	CD19	HIB19	50	Lymphocytes	BioLegend
V8	BV570	CD45RO	UCHL1	200	Lymphocytes	BioLegend
V11	BV650	CD39	TU66	25	Monocytes	BD Bioscience
V13	BV711	CD68	Y1/82A	50	Beads	BD Bioscience
V14	BV750	CD11b	M1/70	200	Monocytes	BioLegend
V15	BV785	CCR5	J418F1	25	Lymphocytes	BioLegend
Blue laser						
B1	Vio®Bright515	CD86	REA968	25	Beads	Miltenyi Biotec
B3	Alexa Fluor®532	CD4	SK3	50	Lymphocytes	eBioscience
B3	Spark Blue™550	CD8	SK1	200	Lymphocytes	BioLegend
B6	NovaFluor Blue610	CD14	MEM-15	25	Monocytes	eBioscience
B8	PerCP	CD45	HI30	50	Monocytes	BioLegend
B9	BB700	CD91	A2MR	25	Monocytes	BD Bioscience
B10	PerCP/eFluo®710	CD1c	L161	50	Lymphocytes	eBioscience
Yellow/Green laser						
YG1	PE	CD85j	GHI/75	10	Monocytes	BioLegend
YG3	PE/CF594	CD244	2–69	25	Beads	BD Bioscience
YG4	PE/Fire640	CD66b	6/40c	25	Beads	BioLegend
YG5	PE/Cy™5	CD64	LS-C812115	50	Monocytes	LifeSpan Bioscience
YG7	PE/Fire™700	CD45RA	HI100	25	Lymphocytes	BioLegend
YG9	PE/Cy™7	CD85d	42D1	50	Monocytes	BioLegend
Red laser						
R1	APC	CD33	WM53	200	Monocytes	BioLegend
R2	NovaFluor Blue660	CD3	UCHT1	50	Lymphocytes	eBioscience
R4	R718	CCR2	LS132.1D9	10	Beads	BD Bioscience
R7	APC/Fire™750	CD172a	P84	25	Monocytes	BioLegend
R8	APC/Fire™810	CD38	HIT2	25	Lymphocytes	BioLegend

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and their subsets in maternal PBMCs at different gestational time points. Five healthy samples with a complete set of blood collections during gestation (first trimester, second trimester, and third trimester) were used to perform the analyses.

To identify subsets within the monocyte compartment and study the alterations of their phenotypes, we developed and optimized a 35-colour antibody panel for spectral flow cytometry. Markers were selected based on reported differential expression on monocyte subsets (Table 2).

After gating single, live CD45+ cells, monocytes were separated based on their FCS/SSC characteristics. CD14 and/or CD16 positive cells were defined as monocytes. Further gating excluded cells positive for other lineage markers (CD66b, CD19, CD123, CD3, CD8, TCRgd, CD7). Three monocyte subsets were identified: $CD14^{high}CD16^{neg}$ (classical), $CD14^{high}CD16^{pos}$ (intermediate), and $CD14^{low}CD16^{high}$ (non-classical) monocytes (Gating strategy in Figure S1).[10,18,19]

The frequencies of monocytes or their subsets did not show differences with significance among different trimesters (Figure 1).

3.2 | Monocytes marker expression during gestation

We further studied whether cell surface markers on the three monocyte subsets were changed during gestation among these five healthy pregnancy samples. Unsupervised clustering revealed diverse expression patterns among the three monocyte subsets (Figure S2). Median fluorescence intensity (MFI) of functional markers (CD38, CD39, CD91, CD85d, CD85j, CD172a, CD33, HLA-DR) and chemokine receptor markers (CCR2, CCR5, CX3CR1) was statistically analysed during gestation, and it demonstrated varieties among different monocyte subsets and different trimesters (Figure 2).

In these healthy OD samples, CD38 expression on intermediate monocytes showed a significant increase throughout gestation ($p = 0.022$). A significantly higher expression of CD38 on intermediate monocytes was found in third trimester compared with first trimester ($p = 0.034$, Figure 2).

3.3 | Monocyte marker expression between healthy and PE OD pregnancies

To assess function and kinetics of monocyte characteristics in OD pregnancy, we compared surface marker expression (functional and chemokine receptor markers) on three monocyte subsets in each time point of gestation respectively. Besides the five healthy pregnancies, another five healthy pregnancies without complete peripheral blood collection from first to third trimester were added to the analyses (giving in total 10 healthy pregnancies), together with five PE OD pregnancies. The PE group had a lower gestational age compared to the healthy group ($p = 0.05$), although most reached term.

For classical monocytes, the PE group showed significantly higher expression of CD85j ($p = 0.024$), CD91 ($p = 0.042$), and CCR2 ($p = 0.042$) (Figure 3 first column) than the healthy group in the second trimester. In the third trimester, the PE group exhibited significantly higher expressions of CD38 ($p = 0.028$), CD85j ($p = 0.048$), and CD91 ($p = 0.028$) (Figure 3 first column) than the healthy group. Postpartum, the PE group displayed significantly higher expression of CD85d than the healthy group ($p = 0.030$, Figure 3 first column). For intermediate monocytes, the PE group had significantly higher expression of CD38 ($p = 0.024$) and CCR2 ($p = 0.012$) in second trimester, and higher expression of CD38 ($p = 0.016$) and CCR2 ($p = 0.016$) in third trimester than the healthy group (Figure 3 second column). Non-classical monocytes had significantly higher CD85d expression in the third trimester in the PE group compared to the healthy group ($p = 0.048$, Figure 3 third column).

There were no significant correlations between gestational age and the monocyte phenotypes described above (data not shown). No significant differences were found in other clinical parameters (Table 1).

3.4 | Monocytes marker expression in OD pregnancies with different extent in HLA mismatches

Given the higher fetal-maternal HLA mismatches in OD pregnancies and their association with increased PE incidence, we investigated whether the marker expressions showed differences among healthy and PE groups with different fetal-maternal HLA mismatches.

Within healthy OD pregnancies, we did not find significant differences in monocyte surface marker expression between 0 and 5 HLA mismatches (mm) healthy OD pregnancies and 6–10 HLA mm healthy OD pregnancies (Figure 4), suggesting that in healthy OD pregnancies the extent of fetal-maternal HLA mismatching is not related to alterations in monocyte surface marker expression.

Next, we explored the association between fetal-maternal HLA mismatches and monocyte phenotypes in PE OD pregnancies. The significantly higher CD38

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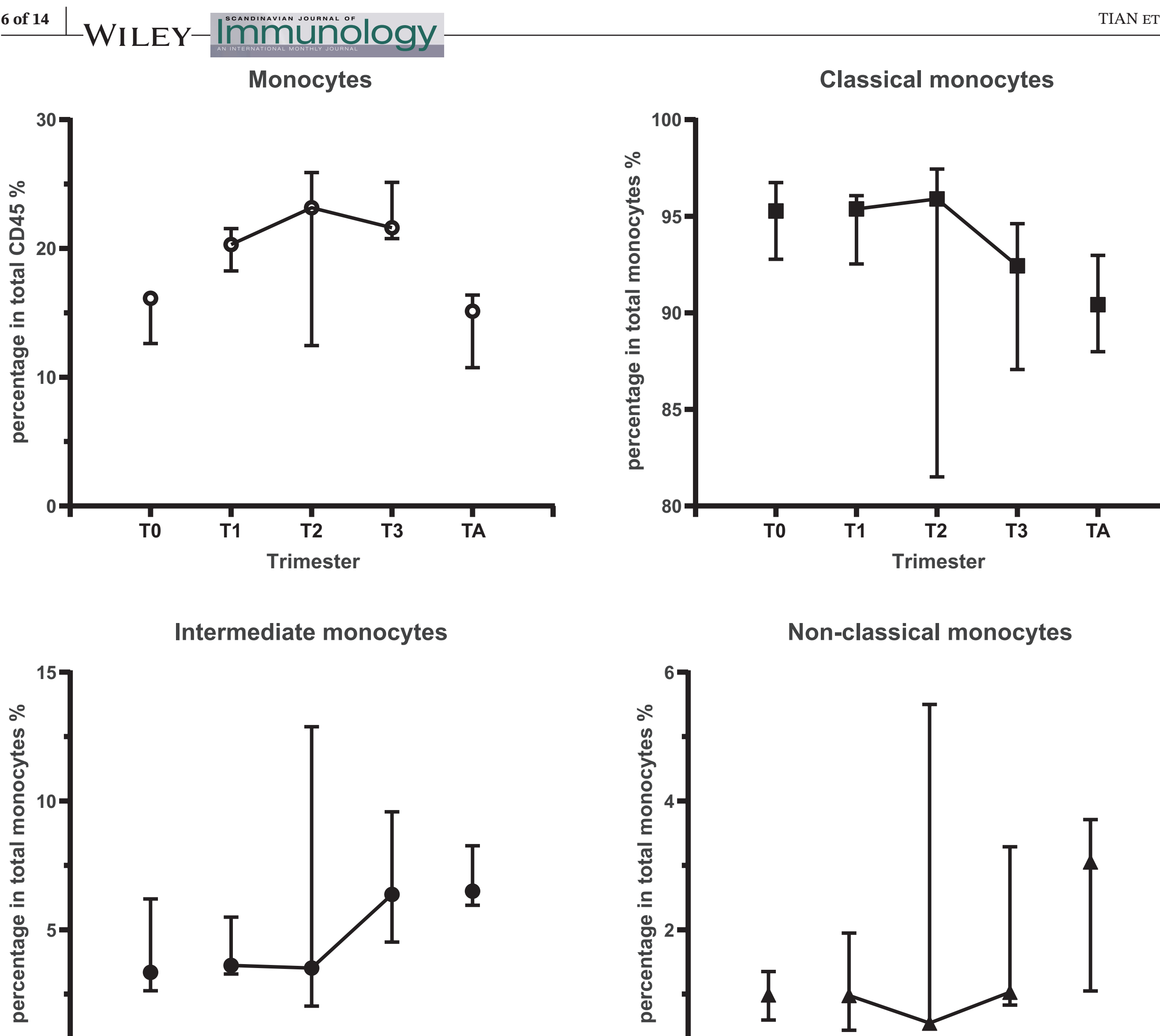

FIGURE 1 The frequency of monocytes and their subsets over gestation. Paired series of samples from five healthy OD pregnancies were used. Three of these pregnancies also had before- and after- pregnancy samples, illustrated by the separated dots. Data is presented with medians and range. The Friedman test was used to observe significant changes in the paired, non-parametric data. The Wilcoxon signed rank test was used for post-hoc analyses between two time points. T0, before pregnancy; T1, first trimester; T2, second trimester; T3, third trimester; TA, after pregnancy.

and CD85j expression on classical monocytes in the third trimester was still present when comparing 6–10 HLA mm PE OD group to 6–10 HLA mm healthy OD group ($p=0.036$, Figure 4), while there was no significant difference in the expression of these markers between 6 and 10 HLA mm PE OD group and 0–5 HLA mm healthy OD group. The significantly higher expression was also observed for CD38 on intermediate monocytes in the third

FIGURE 2 The marker expression on monocyte subsets over gestation. Complete paired series of five healthy OD pregnancies were used. Three of these pregnancies also had before- and after-pregnancy samples, illustrated by the separated dots. Classical monocytes are shown with orange squares, intermediate monocytes are shown with green dots, non-classical monocytes are shown with blue triangles. MFI values lower than 0 indicating negative expression are not shown in the figure. The Friedman test was used to observe significant changes in the paired, non-parametric pregnancy data. The Wilcoxon signed rank test was used for post-hoc analyses between two time points. Results with significant differences are shown with * at the top of the graphs. T0, before pregnancy; T1, first trimester; T2, second trimester; T3, third trimester; TA, after pregnancy.

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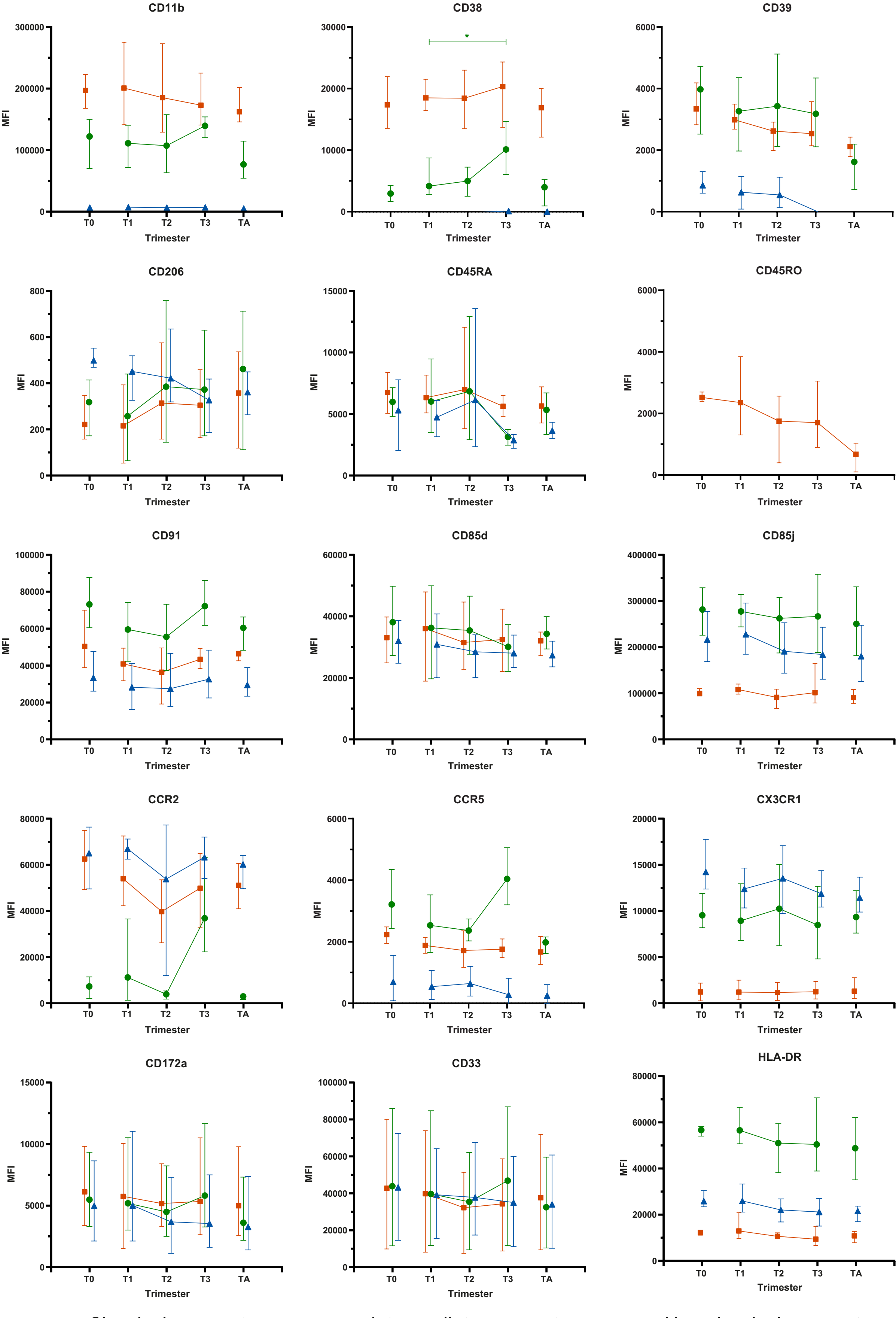

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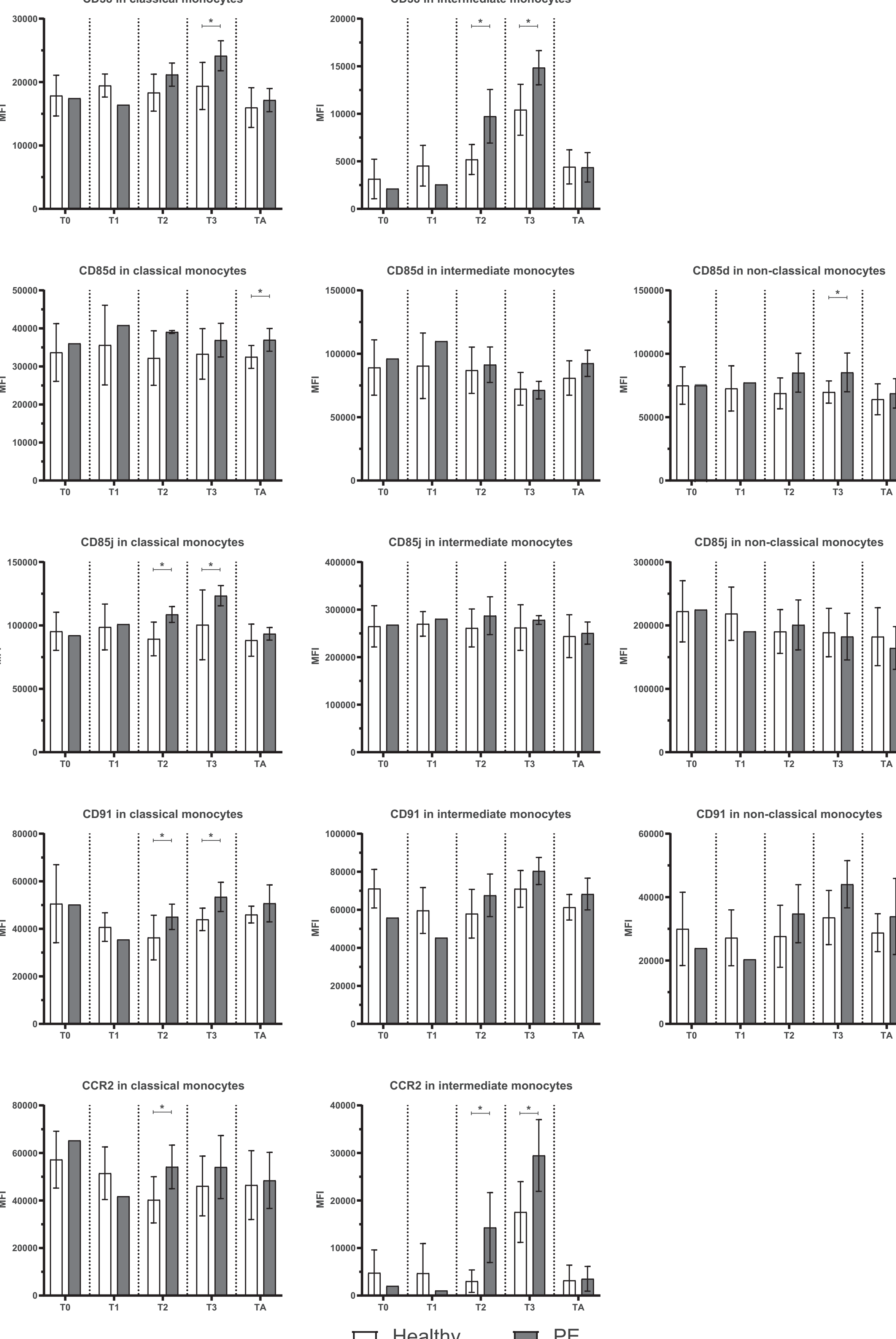

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FIGURE 3 The difference in marker expression on monocyte subsets between healthy and PE OD pregnancies at different gestational time points. MFI are shown as means and standard deviation. CD38 and CCR2 on non-classical monocytes are not shown here for their negative expression. Mann–Whitney *U* tests were performed to identify differences between two groups. Results for statistical analyses results between groups are shown with * at the top of the graphs. T0, before pregnancy; T1, first trimester; T2, second trimester; T3, third trimester; TA, after pregnancy. OD, Oocyte donation; PE, Pre-eclampsia.

trimester when comparing 6–10 HLA mm PE OD group to 6–10 HLA mm healthy OD group ($p=0.036$, Figure 4), but not between 6 and 10 HLA mm PE OD group and 0–5 HLA mm healthy OD group. Since there was only one patient in 0–5 HLA mm PE OD group, we were not able to compare monocyte marker expression between 0 and 5 HLA mm PE OD group and 6–10 HLA mm PE OD group.

4 | DISCUSSION

To reveal the role of peripheral monocytes during the development of PE in OD pregnancy, we analysed the composition and marker expression of monocyte subsets throughout healthy and PE OD pregnancy. We have found that surface markers exhibited divergent expressions on monocyte subsets at different trimesters. Activation marker CD38 on intermediate monocytes showed a significantly increasing expression throughout gestational age in healthy OD pregnancy, suggesting an increasing proinflammatory condition from first to third trimester. The expression of CD38 on monocyte subsets was significantly higher in the PE group compared with the healthy group. The expression of immune inhibiting receptors CD85j, CD85d and monocyte recruitment regulating molecules CCR2 and CD91 on monocyte subsets were also significantly higher in the PE group than in the healthy group. When comparing healthy and PE OD pregnancies with high fetal-maternal HLA mismatches, the different CD38 and CD85j expression in monocyte subsets still showed significance. These findings suggest that expression of both immune activation and regulatory markers is increased in maternal peripheral monocyte subsets of PE OD pregnancies, as well as markers related with monocyte recruitment for damage repair and systemic vascular dysfunction.

In our study, the upregulated CD38 expression on monocyte subsets might reflect a condition of proinflammatory activation of peripheral monocyte subsets in third trimester compared to first trimester, and in PE pregnancy compared to healthy pregnancy. CD38 has been identified as a surface activation marker in T cells and it was first described as an enzyme capable of catalysing the conversion of nicotinamide-adenine dinucleotide (NAD). For monocytes, evidence also showed that in humans high CD38 activity contributes to inflammatory cytokine release from monocytes.[20] Recent studies have shown that blockade of CD38 leads to suppressed proinflammatory transcriptional reprogramming.[21] Additionally, in mice CD38 on monocytes is identified as an inflammatory mediator, significantly depleting NAD+ in cells. And as NAD+ is essential for creating cellular energy, this depletion might be related with monocyte exhaustion.[22] Significantly higher CD38 expression on monocyte subsets was also found in PE pregnancy with high fetal-maternal HLA mismatches compared to healthy OD pregnancy with high HLA mismatches, but not to healthy OD pregnancy with low HLA mismatches. This might suggest that for OD pregnancy with extreme fetal-maternal HLA dissimilarity, a higher extent of immune tolerization is needed to maintain healthy pregnancy during gestation.

CD85j is an inhibiting receptor that can inhibit cytotoxicity and the stimulation of the immune response. The ligands for CD85j (LILRB1) and CD85d (LILRB2) include class I HLA molecules and HLA-G, the latter being highly expressed by fetal trophoblast cells in both membrane-bound and soluble form. The fetal HLA molecules may deliver regulatory signals to maternal monocytes via these receptors.[23] Our study showed that classical monocytes from PE pregnancy had higher CD85j expression than healthy OD pregnancy, and this higher CD85j expression still showed significance when comparing high HLA mismatched PE pregnancy to high HLA mismatched healthy pregnancy. However, there were no significant differences when comparing highs HLA mismatched PE pregnancy to low HLA mismatched healthy pregnancy. This suggests that fetal HLA molecules highly mismatched with maternal HLA molecules might be related to the high expression of this tolerogenic marker on classical monocytes in PE pregnancy. In addition, a previous study showed that peripheral monocytes after coculturing with media from decidualized cells also display higher expression of the tolerogenic marker CD85j,[24] suggesting that maternal decidual cells participate in triggering CD85j expression on monocytes as well.

The myeloid cell receptor CD91/LRP1 has been shown to regulate monocyte recruitment and angiogenesis in tumours.[25] The deficiency of CD91 on monocytes may contribute to an increased amount of vascular endothelial growth factor (VEGF) in the microenvironment.[25] It was reported that for PE pregnancies, the circulating concentration of VEGF is lower in late gestational age or the time close to the onset of PE, compared to that in healthy

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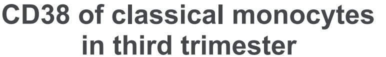

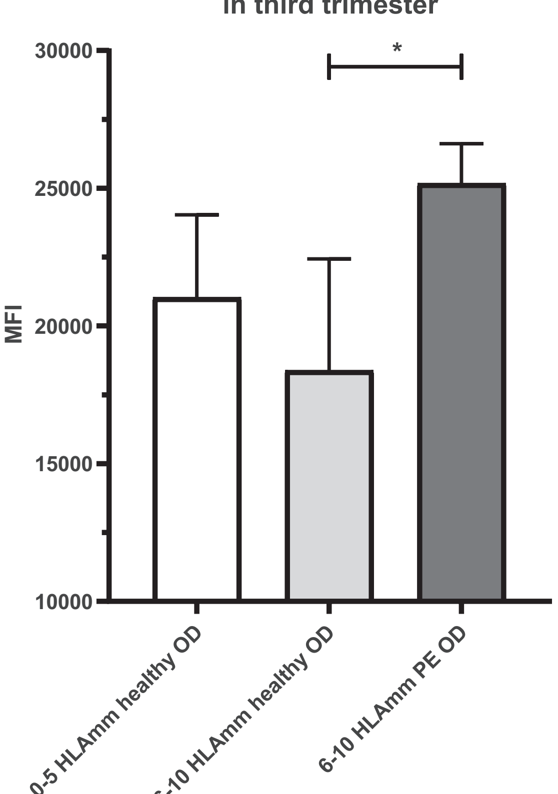

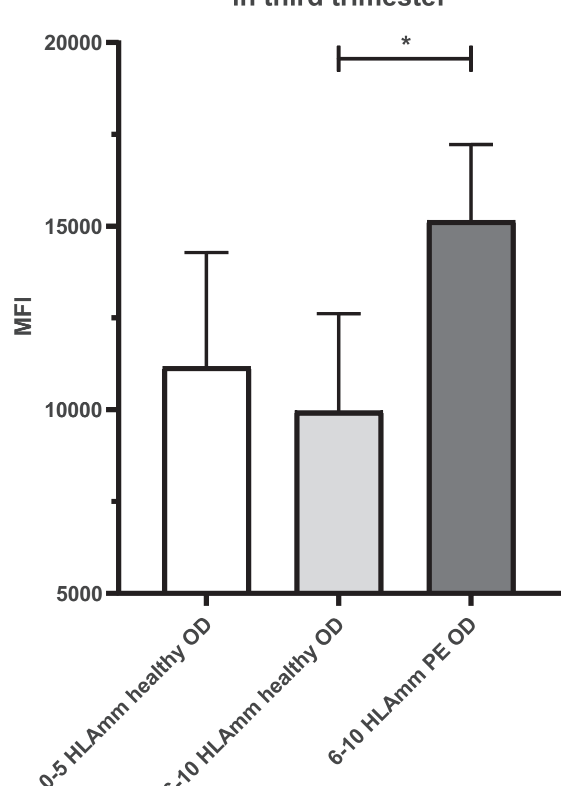

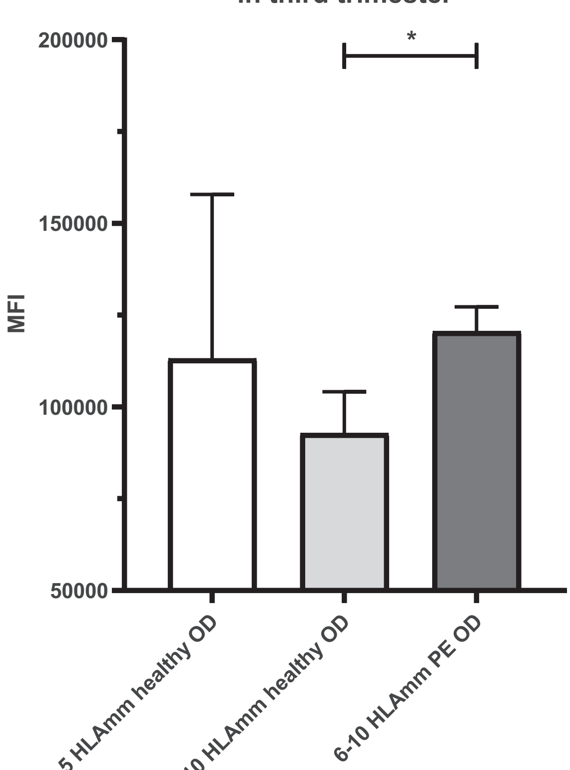

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FIGURE 4 The association between marker expression on monocyte subsets and fetal-maternal HLA mismatches. MFI are shown as means and standard deviation. Mann–Whitney U tests were performed to identify differences between two groups. Results for statistical analyses results between groups are shown with *on the top. T0, before pregnancy; T1, first trimester; T2, second trimester; T3, third trimester; TA, after pregnancy. OD, Oocyte donation; PE, Pre-eclampsia.

pregnancy.[26] The higher CD91 expression on classical monocytes in the PE group at second and third trimester in our study might reflect the systemic vascular dysfunction in the maternal circulation in the late stage of PE.[27,28] However, how these peripheral monocytes would contribute or react to the impaired angiogenesis in PE pregnancy is still unclear.

Our study also showed that the expression of CCR2 on classical and intermediate monocytes was significantly higher in PE pregnancy compared to healthy OD pregnancy in second and third trimester. CCR2 acts as a chemokine receptor binding to CC-chemokine ligands 2 (CCL2), and it plays a crucial role in orchestrating monocyte dynamics and chemotaxis, which facilitates their exit from the bone marrow into the circulation and peripheral tissues, both in homeostatic conditions and in response to inflammatory stimuli.[29,30] The functional specialization of monocytes into macrophages, dependent on CCR2, establishes an autocrine positive feedback loop that amplifies inflammation.[31] In response to infection, newly recruited CCR2$^+$ monocytes give rise to tissue macrophages, emphasizing the critical role of de novo differentiated monocyte-derived macrophages in host defence against infections.[32] The involvement of CCR2$^+$ monocytes in acute post-ischemic inflammation would also contribute to functional recovery.[33] Evidence supports the notion that inflammatory CCR2$^+$ monocytes, reaching the damaged organ shortly after tissue injury, are responsible for the observed fibrotic patterns at later stages.[34] They play a role in promoting fibrosis by inhibiting collagen degradation.[35] However, it was also reported that CCR2 is crucial for monocyte migration from the bone marrow to the circulation, but that it does not play a role for monocyte migration from the circulation into the tissues.[36] On the other hand, tissue-resident CCR2$^+$ macrophages play a pivotal role in promoting monocyte recruitment and mobilization.[37] The higher expression of CCR2 on monocytes in the circulation of PE compared to healthy OD pregnancy in our study might reflect monocytes recruitment from bone marrow into circulation and even into the decidua, potentially supporting damage tissue repair locally. The exact function of these monocytes and their functional pathway in OD pregnancy with PE still needs to be studied.

Furthermore, our study did not find significant differences in the composition of total circulating monocytes or monocyte subsets across different trimesters in healthy OD pregnancy. Other studies demonstrated an elevation in total monocyte count during pregnancy, which was primarily driven by an increasing proportion of the intermediate subset and a reduction in the classical subset.[14,38] Our findings reflected a comparable trend but without statistical significance, which may be ascribed to the relatively small sample size.

The aetiology of PE in OD pregnancy with extreme fetal-maternal HLA mismatches is complex and heterogeneous. A confluence of genetic, maternal, and immunological factors may precipitate placental dysfunction, triggering the release of antiangiogenic factors and inflammatory mediators into the circulation, ultimately culminating in the onset of PE. A study on uterine artery Doppler findings demonstrated that, compared to natural conceptions, OD pregnancies had a lower pulsatility index in the uterine arteries despite a higher risk of PE; this contrasted with naturally conceived PE pregnancies, where the pulsatility index is elevated due to inadequate spiral artery remodelling.[39] Similarly, our previous study on placental pathology revealed that PE OD pregnancies, on average, exhibited lower maternal vascular malperfusion pathology scores compared to naturally conceived PE pregnancies.[40] These findings collectively suggest that the aetiology of PE in OD pregnancies might not be only from vascular dysfunction. The dynamic alterations observed in peripheral monocytes in our study may reflect a complex interplay, encompassing tolerization from the maternal immune cells toward the fetus, reflections of fetal-maternal interactions within the intervillous space, and responses to antiangiogenic factors. We hypothesize that a dysbalanced immune regulation at the fetal-maternal interface, combined with systemic vascular dysfunction in the maternal circulation during late gestation, contributes to the development of PE in OD pregnancies. Still, this intricate network calls for further exploration and comprehensive understanding. Cultured trophoblasts and maternal decidual cells or organoid placenta models might be helpful to study how peripheral monocytes can reflect or be affected by the immune response in/from the placenta.

Given that OD remains a relatively novel technique, with limited research studying immune response in this kind of pregnancies, especially the changes in monocyte subsets, this study addresses a significant gap in our understanding. By simultaneously measuring multiple functional markers, we provide a comprehensive overview of the alterations occurring in monocyte subsets

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during healthy and PE OD pregnancies and highlight the key changes. However, it is important to acknowledge the limitations of this exploratory study. The sample size is relatively small in this study, which might increase the risk of confounding. PE OD group exhibited significantly lower gestational age compared with healthy OD group, reflecting a characteristic feature of PE. We further performed correlation analysis and did not find significant correlations between the observed differences in surface marker expression and gestational age. Moreover, as in our study most markers on monocyte populations exhibited a gradual shift from dim to positive without distinct peaks, it was challenging to categorize monocyte subsets into phenotypically distinct subclusters based on marker expression patterns. Therefore, MFI of all functional markers was assessed in this study. Additionally, the actual functional changes in maternal peripheral monocytes influenced by placenta factors need to be test in future functional assays.

In conclusion, the composition and the surface marker expression of monocytes dynamically vary across trimesters and OD pregnancy conditions (healthy or PE). In healthy OD pregnancies, the evaluated CD38 expression on intermediate monocytes throughout gestational age suggests an increasing pro-inflammatory condition in maternal peripheral blood. In PE OD pregnancies, the expressions of immune activation marker CD38 and immune regulatory markers CD85j and CD85d are both increased on monocyte subsets. In addition, the expression of CCR2 and CD91, reflecting monocyte chemotaxis, were significantly increased on monocyte subsets of PE OD pregnancies, suggesting possible recruitment of monocytes for damage repair and systemic vascular dysfunction which happens at the late stage of PE. These findings pointed out potential biomarkers in maternal peripheral blood to predict PE in OD pregnancies during gestation, offering the potential for improved monitoring of such pregnancies. Further research with functional assays and a larger sample size is needed.

AUTHOR CONTRIBUTIONS

Xuezi Tian: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; writing—original draft; writing—review and editing. **Jia Li:** formal analysis; investigation; methodology; writing—review and editing. **Kim van Bentem:** Data curation; methodology; writing—review and editing. **Ciska Lindelauf:** Investigation; methodology; writing—review and editing. **Johanna M. Kapsenberg:** Methodology; writing—review and editing. **Carin van der Keur:** Methodology; writing—review and editing. **Lisa E.E.L.O. Lashley:** Conceptualization; resources; writing—review and editing. **Vincent van Unen:** Formal analysis; investigation; writing—review and editing. **Dave L. Roelen:** Methodology; writing—review and editing. **Frits Koning:** Formal analysis; investigation; writing—review and editing. **Michael Eikmans:** Conceptualization; formal analysis; funding acquisition; project administration; resources; supervision; writing—review and editing. **Marie-Louise P. van der Hoorn:** Conceptualization; formal analysis; funding acquisition; project administration; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

All authors have no conflicts of interest to declare relevant to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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