Decidual NK cells, extravillous trophoblast and their PD-1/PD-L1 interaction: New insights into recurrent pregnancy loss and placenta accreta spectrum disorders

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Decidual NK cells, extravillous trophoblast and their PD-1/PD-L1 interaction: New insights into recurrent pregnancy loss and placenta accreta spectrum disorders

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ABSTRACT

Objectives: Placentation is influenced by decidual Natural Killer (dNK)[1] cells. Defective placentation can lead to recurrent pregnancy loss (RPL)[2] or placenta accreta spectrum (PAS)[3] disorders. Interaction between Programmed Cell Death Protein 1 (PD-1)[4] on NK cells and Programmed Cell Death 1 Ligand (PD-L1)[5] can alter the cytokine expression and cytotoxic potential of NK cells. This study compares dNK and PD-1$^+$ dNK cell number, number of extravillous trophoblast (EVT)[6] and PD-L1$^+$ EVTs in two patient groups that experienced either RPL or PAS disorder to a control group.

Methods: For this retrospective case-control study tissue from in total 77 abortion samples (healthy women, women with RPL and PAS disorder) were immunohistochemically stained and the above-mentioned cells and receptors were counted and statistically compared between the three groups with either Kruskal Wallis or ANOVA and corresponding posthoc tests.

Results: The number of EVTs was reduced in the RPL group. There were more dNK and PD-1$^+$dNK cells as well as a higher ratio of dNK cells compared to EVTs in the RPL group compared to the control group. Furthermore, the proportion of PD-L1$^+$ cells in all EVT cells was higher in the RPL group compared to the control group.

Conclusion: Our findings show that both PD-1 and PD-L1 are differentially expressed on dNK cells and EVTs in the RPL group compared to the control group. Hereby suggesting that PD-1/PD-L1 interaction might influence NK cell function and therefore might be relevant for women experiencing RPL. This may be in terms of cytokine microenvironment or cell-cell-interaction.

1. Introduction

An adequately working placenta is one of the determinants for a healthy pregnancy [1]. Defective placental development during the first trimester of pregnancy can lead to several disorders, including recurrent pregnancy loss (RPL) and placenta accreta spectrum (PAS) disorders [2, 3]. Unexplained RPL affects 0.5–1 % of couples trying to conceive and is defined by at least two pregnancy losses below 20 weeks of gestation

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[1] dNK cells: Decidual Natural Killer cells
[2] RPL: Recurrent Pregnancy Loss
[3] PAS: Placenta Accreta Spectrum
[4] PD-1: Programmed Cell Death Protein 1
[5] PD-L1: Programmed Cell Death 1 Ligand 1
[6] EVT: Extravillous Trophoblast

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with no known cause [4,5]. As trophoblast invasion is known to be inadequate in some RPL cases [6,7], one hypothesis is that those unexplained losses are the result of defective placentation due to immunological dysregulation at the maternal-fetal interface [8]. In contrast, PAS disorders are characterized by excessive invasion of trophoblasts and are defined as abnormally adherent and invasive placenta. PAS disorders complicate around 0.2 % of pregnancies. There are three categories of PAS disorders which are defined by their invasiveness. Whereas a placenta accreta adheres to the myometrium of the uterus, a placenta increta penetrates the myometrium and a placenta percreta penetrates the uterine serosa or surrounding tissues [9]. Known risk factors include a history of caesarean section or other previous uterine surgery, placenta praevia and assisted reproductive surgery. However, the exact etiology of PAS disorders has not been unraveled yet and known risk factors for PAS disorders do not explain why primigravida without a risk factor develop PAS disorders [9]. In both RPL and PAS disorders, cell interactions at the maternal-fetal interface play a critical role and immune dysregulation could result in an aberrant crosstalk between maternal immune cells and invading trophoblast cells [8–11].

The maternal decidua is invaded by the semi-allogeneic fetal extravillous trophoblast (EVT) cells. This results in direct interaction of maternal immune cells with fetal antigens. Subsequently, there is a need to guide proper invasion of the trophoblast cells and inhibiting over-invasion [2]. One important player in establishing this guidance is the decidual Natural Killer (dNK) cell. dNK cells are the most abundant immune cells in the decidua, making up to 50–70 % of the total leukocyte population during early pregnancy [7]. They play an important role in maintaining the balance between inducing tolerance towards the semi-allogeneic fetus, supporting trophoblast invasion and spiral artery remodeling by secreting cytokines and growth factors such as VEGF, PLGF and interleukin 8, and still being able to respond to pathogens when needed [12]. To exert all these different functions, it is becoming clearer that different subsets reside in the decidua [13].

NK cells have an abundant receptor repertoire with unique properties that regulate their function by providing activating or inhibiting signals upon binding with a ligand. The balance of these signals determines whether NK cells become activated or not [14,15]. Although there has been considerable interest in phenotyping both activating and inhibitory receptors on dNK cells, comparability of results is limited by methodological heterogeneity, as different experimental techniques and analytical approaches have been employed to study receptor repertoires. In addition, ongoing studies on newly identified receptors highlight the existing gaps in fully elucidating a comprehensive receptor phenotype. A family of NK receptors that has recently gained a lot of interest due to their role as key regulators preventing overactivation of the immune system, is the family of the immune checkpoint receptors [14]. Especially the programmed cell death 1 (PD-1) receptor has shown to change the cytokine secretion of peripheral NK cells and their cytotoxic potential [16].

PD-1 expressing dNK cells have been compared to peripheral NK cells in mice, showing phenotypically increased PD-1 expression and functionally decreased cytotoxicity in the decidua [17]. Studies on PD-1 expression in human decidual immune cells and its association with inflammatory patterns in pregnancies complicated by preeclampsia suggest that PD-1 actively contributes to local immunoregulatory mechanisms in preeclamptic women [18]. PD-1 can bind to Programmed Cell Death 1 Ligand 1 (PD-L1) and PD-L1 has been shown to be expressed in murine decidua basalis [19] and on human extravillous trophoblast cells [20]. Blocking of PD-L1 has been shown to result in increased fetal rejection rates in mice [19] and a decreased expression of PD-L1 on trophoblast cells has been associated with decreased regulatory T cell differentiation and increased embryo loss in mice [21].

Together these previous studies suggest that both PD-1 and PD-L1 are involved in establishing proper immune regulation at the maternal-fetal interface supportive of pregnancy. Therefore, direct interaction between PD-1 and PD-L1 might regulate dNK function and ensure adequate trophoblast invasion preventing under-invasion possibly resulting in RPL and over-invasion resulting in PAS disorders.

To our knowledge, no study has yet focused on decidual PD-1 and PD-L1 expression in women with RPL or PAS disorders. Therefore, in this study, we investigated PD-1 and PD-L1 expression by comparing the numbers of NK cells, PD-1$^+$ NK cells, EVT cells and PD-L1$^+$ EVT cells in the human decidua basalis between women with a history of RPL, women with a PAS disorder and women with previous uncomplicated pregnancies (control group).

2. Methods

2.1. Participants and tissue selection

For this retrospective case-control study, clinical data and tissues were obtained from three different centers. For the group of patients with RPL, the pathology database of the Maastricht University Medical Centre (MUMC+), The Netherlands was used to identify tissue from patients who had undergone a curettage after a missed or incomplete abortion and who had a history of two or more spontaneous pregnancy losses. Exclusion criteria were the presence of a molar pregnancy or other trophoblastic disease like Placental Site Trophoblastic Tumor, Epithelioid Trophoblastic Tumor or choriocarcinoma. Clinical data were obtained using the electronic patient files that were filled in by an obstetrician at the MUMC+. The use of the tissue was approved by the Medical Ethical Committee of the MUMC+ (METC 16–4–047). Tissue from patients with PAS disorder was obtained from the Department of Pathology of the Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen University, Germany and the Department of Pathology of the Institut und Praxisgemeinschaft für Pathologie Düren, Germany. Tissue was sampled from women who underwent hysterectomies or curettages after abortion. Exclusion criteria for this group were tissue samples with the presence of inflammation and/or necrosis or signs of malignancy. Tissue from the control group was obtained from the abortion clinic CASA Maastricht, the Netherlands. Here women underwent elective abortion of healthy intrauterine pregnancies for social reasons. The included specimen derived from women who had at least one uncomplicated full-term pregnancy before and no history of spontaneous pregnancy loss. The tissue was obtained using vacuum aspiration. The use of the tissue for the control and the PAS group was approved by the Ethics Committee of the School of Medicine, University of Aachen. Written informed consent was obtained from each patient. In total, tissues and clinical data from 34 controls, 33 women with RPL and 10 women with PAS disorder were used for this study.

2.2. Immunohistochemistry (IHC) – single staining

The tissues from the control group and from the PAS group were fixed for four hours in 3.7 % buffered formalin, dehydrated, and embedded in paraffin. The tissue from the RPL group was fixed for at least 24 h in 4 % buffered formalin and processed in the Vacuum Infiltrating Processor Tissue-TEK VIP6 (Sakura Finetek USA Inc.). All tissues were cut in five serial 3 μm sections with a microtome and mounted on glass slides. The first tissue slice was stained with Hematoxylin-Eosin (H&E). In the second slice NK cells were identified using the surface maker CD56, and the third slice was stained for the NK cell receptor PD-1. In the fourth slice extravillous trophoblast (EVT) in the decidual basalis were identified by cytokeratin (CK) 7 staining and in the fifth slice PD-L1 as ligand expressed by EVT was labelled. Slices from nineteen samples [twelve from the RPL group and seven from the control group] were additionally stained with a CD68 marker for identifying macrophages. The H&E and CK7 stainings were used to identify the implantation site and depth of extravillous trophoblast invasion and distinguish between decidua basalis and parietalis.

All immunostainings, except for the CD68 staining, were performed using the Dako Autostainer Link 48 (Dako, Agilent Technologies, Santa

Clara, CA, USA) and recommended protocols (for details and antibody specifics see Table 1). Briefly, specimens were incubated in Target Retrieval Solution (Dako, Agilent Technologies, Santa Clara, CA, USA) at 97 °C for 10 min. For the PD-1 and PD-L1 staining, the low pH target retrieval solution was used while the high pH target retrieval solution was used for the CK7 and CD56 staining. After washing the specimens with washing buffer (Dako Wash Buffer + 0.1 % Tween20), they were put into the autostainer, rinsed with washing buffer and blocked with peroxidase blocking reagent (Dako, Agilent Technologies, Santa Clara, CA, USA) for 5 min. After washing the specimens, they were incubated in the respective primary antibody, diluted in antibody diluent (Dako, Agilent Technologies, Santa Clara, CA, USA) for 20 or 30 min at room temperature and washed with washing buffer. Subsequently, for the CD56 and PD-L1 immunostainings, specimens were incubated with EnVision FLEX/mouse linker (Dako, Agilent Technologies, Santa Clara, CA, USA) for 15 min or 30 min respectively at room temperature. For all immunostainings, specimens were incubated with EnVision FLEX/HRP (Dako, Agilent Technologies, Santa Clara, CA, USA) for 20 min or 30 min [PD-L1 staining] at room temperature and washed with washing buffer. Next, specimens were incubated with EnVision FLEX DAB+ (Dako, Agilent Technologies, Santa Clara, CA, USA) for 10 min and washed with washing buffer. For the PD-L1 immunostaining, slides were subsequently incubated in EnVision FLEX DAB enhancer (Dako, Agilent Technologies, Santa Clara, CA, USA) for 5 min and washed. Slides were then rinsed with distilled water, placed in the Automated Slide Stainer (Sakura Finetek USA Inc.) and counterstained with Haematoxylin Mayer (Diapath, Martinengo, Italy) for 90 s. Lastly, staining was stopped with tap water and the slides were dehydrated in ethanol and xylene.

For the CD68 staining 19 representative specimens were deparaffinized twice with xylene for 5 min. Afterwards, specimens were hydrated with a grated ethanol series from 100 to 50 percent and washed with demineralized water and phosphate buffered saline with Tween® (PBST) buffer. For antigen-retrieval specimens were boiled in sodium citrate (10 mM, pH 6.0) for 15 min and cooled down for 30 min at room temperature. After washing with PBST buffer endogenous peroxidases were blocked through incubating the specimens with 0.6 % H_2O_2 / dH_2O for 10 min and washed with demineralized water and PBST buffer. Subsequently, specimens were pre-incubated with Teng-T/ 10 % normal goat serum for 30 min and then incubated overnight with CD68 (Dako, PG-M1, MaH, M0876), diluted 1:50 with Teng-T/10 %NGS. Specimens were then washed with PBST 3 times for 5 min and incubated with the secondary goat-anti-mouse antibody (Bio-Rad Immun-Star Goat Anti-Mouse HRP Conjugate #1705,047) diluted 1:500 with Teng-T/10 % NGS for 30 min. After another wash with PBST for 5 min, specimens were incubated with HRP coupled ABComplex (Vectastain (PK-4000)) for 30 min. After washing the specimens with PBST for 5 min, they were developed with Novared for 5 to 15 min] and washed in dH_2O. Lastly, they were dipped in hematoxylin, washed with tapwater for 10 min and dehydrated in a grated ethanol series from 50 %−100 % and 4 times xylene.

Table 1
Specifications of the antibodies and immunostaining protocols.

	CK7	CD56	PD-1	PD-L1
Specific antibody	CK7 Clone OV-TL 12/ 30 [Dako]	CD56 Clone 123C3 [Dako]	PD-1 Clone MRQ22 [Cell Marque]	PD-L1 Clone 22C3 [Dako]
Antigen retrieval [Dako]	High [pH 9]	High [pH 9]	Low [pH 6]	Low [pH 6]
Endogenous peroxidase blocking reagent [Dako]	5 min	5 min	5 min	5 min
Dilution	Ready to use	Ready to use	1/50	Ready to use
Incubation primary antibody	20 min	20 min	20 min	30 min
Incubation secondary antibody	-	Mouse Linker 15 min	-	Mouse Linker 30 min
Labeled polymer HRP	20 min	20 min	20 min	30 min
DAB substrate	10 min	10 min	10 min	10 min

2.3. IHC – double staining

For the CD56/PD-1 double staining the above-described protocols for the CD56 and PD-1 single staining were used subsequently with the following slight adjustments. The PD-1 staining (PD-1 Clone nat105 (Cell Marque)) was performed first and the CD56 staining (CD56 Clone mrq-42(Cell Marque)) second. For the double staining antigen retrieval was performed for 30 min instead of 10 min and peroxidase blocking for 3 instead of 5 min. After blocking specimens were incubated with mouse linker for 10 min instead of 15 min and for the PD-1 staining the EnVision FLEX DAB+ was used for 5 min instead of 10 min. Lastly, for the CD56 staining Envision Flex Magenta was used for 10 min instead of EnVision FLEX DAB+, to get a red staining of the CD56.

2.4. Quantification

The stained slides were scanned using the Pannoramic 1000 (3D HISTECH, Budapest, Hungary). For the quantification of $CK7^+$, $CD56^+$, $PD\text{-}1^+$ and $PD\text{-}L1^+$ cells QuPath version 0.3.1 was used [22]. Cytokeratin 7 staining was used to identify areas of decidua basalis with EVTs in the tissue (see Fig. 2). Ten rectangular areas of the same size (400 μm x 272 μm) were chosen randomly from the decidua basalis tissue. For some tissues, the area of decidua basalis was too small, therefore a smaller number of rectangles were drawn. Cells were then manually counted in these areas and the mean number per area was calculated. For quantification of $PD\text{-}1^+$ NK cells, the CD56 staining and the PD-1 staining from sequential sections of the same patients were analyzed in parallel side by side. The cells that were positive for both stainings were counted manually (see Fig. 1a). The cell counts of four specimens derived from this approach were compared to the cell count derived from the double staining (see Fig. 1b). The percentage of $PD\text{-}1^+$ dNK cells of the total amount of NK cells and the percentage of $PD\text{-}1^+$ dNK cells of the total amount of $PD\text{-}1^+$cells were comparable for the double staining and the single staining for each patient.

We performed additional CD68 stainings from some specimens as macrophages can also be PD-1 and PD-L1 positive [23]. However, we analyze samples from the first trimester and very early second trimester, and there was only a limited number of macrophages present in the decidua basalis (data not shown). Therefore, the overall results should not be affected.

2.5. Statistics

All statistical analyses were performed using GraphPad PRISM, version 9.0 for Windows (GraphPad Software, San Diego, CA, USA). Data on baseline characteristics and cell counts were tested for normality using the Shapiro-Wilk test. Normally distributed data (age, number of EVT/area, percentage of $PD\text{-}L1^+$EVT/total EVT count) was analyzed using one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test for between-group differences. The Bonferroni correction was used to correct for multiple testing. Not normally distributed data (all data except the three mentioned above) was analyzed using Kruskal-Wallis H test and pairwise comparisons were performed using Dunn's test. A P-value below 0.05 was considered statistically significant.

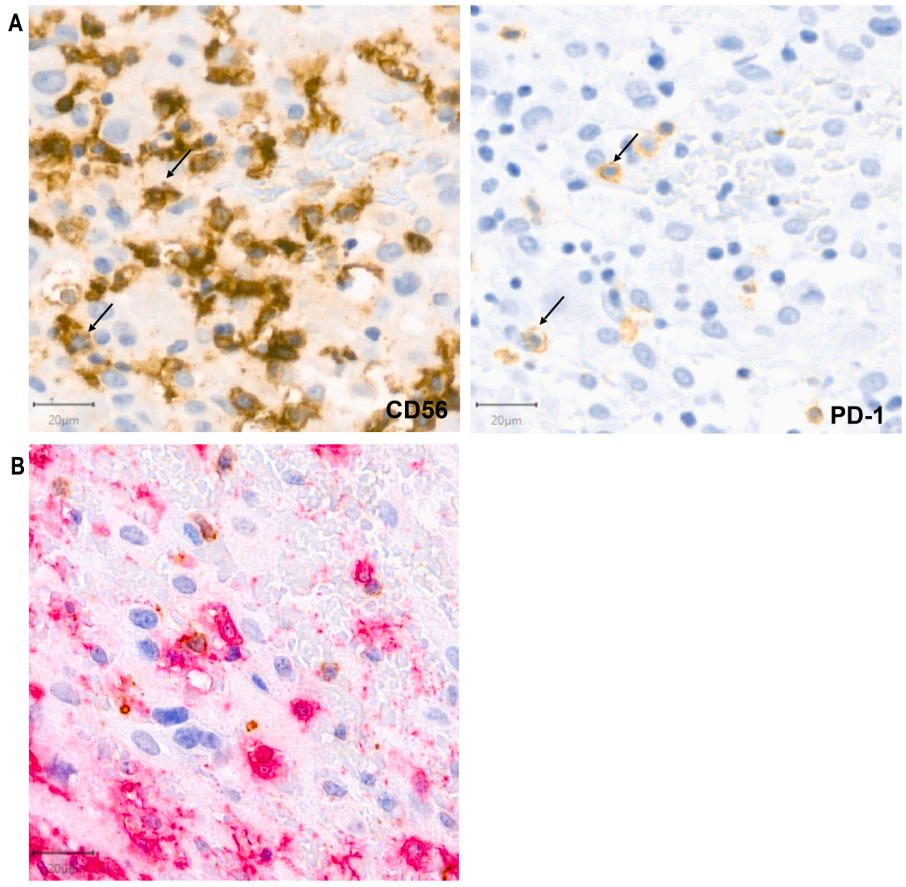


Fig. 1. A: Example of a sequential section of the CD56 and PD-1 staining. Both stainings were viewed simultaneously and CD56$^+$PD-1$^+$ cells were counted (see arrows for two representative double positive cells). B: Example of the CD56 (red) and /PD-1 (brown) double staining.

3. Results

3.1. Baseline characteristics of the study population

Clinical data and tissues from 34 controls, 33 women with RPL and 10 women with PAS disorder were included in the analysis. Tissues from three women in the PAS group, nine women in the control group and one woman in the RPL group were sampled in the second trimester. Women in the PAS group had a higher gestational age than women in the RPL group ($\chi2(3) = 6.078$, $P = 0.048$; pairwise comparison: $P = 0.041$). As expected, controls had a higher number of induced abortions and live births than women with RPL ($\chi2(3) = 45.46$, $P < 0.001$ and $\chi2(3) = 10.62$, $P = 0.049$; pairwise comparison: $P < 0.001$ and $P = 0.004$, respectively) and women with RPL had more pregnancy losses than the two other groups ($\chi2(3) = 63.61$, $P < 0.001$; pairwise comparison: $P < 0.001$ and $P = 0.016$) and more pregnancies than controls ($\chi2(3) = 7.288$, $P = 0.026$; pairwise comparison: $P = 0.002$). There was no difference between the groups regarding maternal age (Table 2).

Table 2
Baseline characteristics of the study population.

	Control group	RPL group	PAS disorder group	P
Age	33 ± 9	30 ± 8	34 ± 9	0.386
Gestational age in weeks	8 ± 5	8 ± 3	10 ± 4	0.048
Gravida	3 ± 2	4 ± 3	3 ± 3	0.026
Para	2 ± 1	1 ± 2	1 ± 2	0.005
Mater	2 ± 1	1 ± 2	1 ± 1	0.002
Miscarriages	0 ± 0	2 ± 1	1 ± 1	<0.001
Induced abortions	1 ± 0	0 ± 0	1 ± 1	<0.001

Data are presented as median and interquartile range.

3.2. Immunohistochemical stainings

All stainings demonstrated high specificity and allowed a clear distinction between positive and negative cells as shown in Fig. 1 and 2.

3.3. dNK cell and PD-1$^+$ dNK cell counts are significantly higher in the RPL group

dNK cell and PD-1$^+$ dNK cell counts were statistically different between the three groups ($\chi2(3) = 29.75$, $P < 0.001$ and $\chi2(3) = 14.42$, $P = 0.001$, respectively). Pairwise comparisons revealed a statistically significant higher number of dNK cells and PD-1$^+$dNK cells in the RPL groups compared to both other groups (CD56: $P < 0.001$; CD56$^+$PD-1$^+$: $P = 0.003$) (see Fig. 3A and 3B). There was however no difference between the percentage of PD-1$^+$ dNK cells of total dNK cell count between the three groups (see Fig. 3C). There was no difference in dNK cell and PD-1$^+$ dNK cell counts between the control and the PAS group.

If we only analyze the samples from the first trimester, the percentage of PD-1$^+$ dNK cell per total dNK cell count becomes significantly higher in the RPL group versus the control group ($\chi2(3) = 9.453$, $P = 0.009$; pairwise comparison: $P = 0.0088$; data not shown).

3.4. The invasion of EVTs is reduced in the RPL group, however, the percentage of PD-L1$^+$EVTs per total EVT count was higher in the RPL group versus controls

There were more EVTs per area in the control group versus the RPL

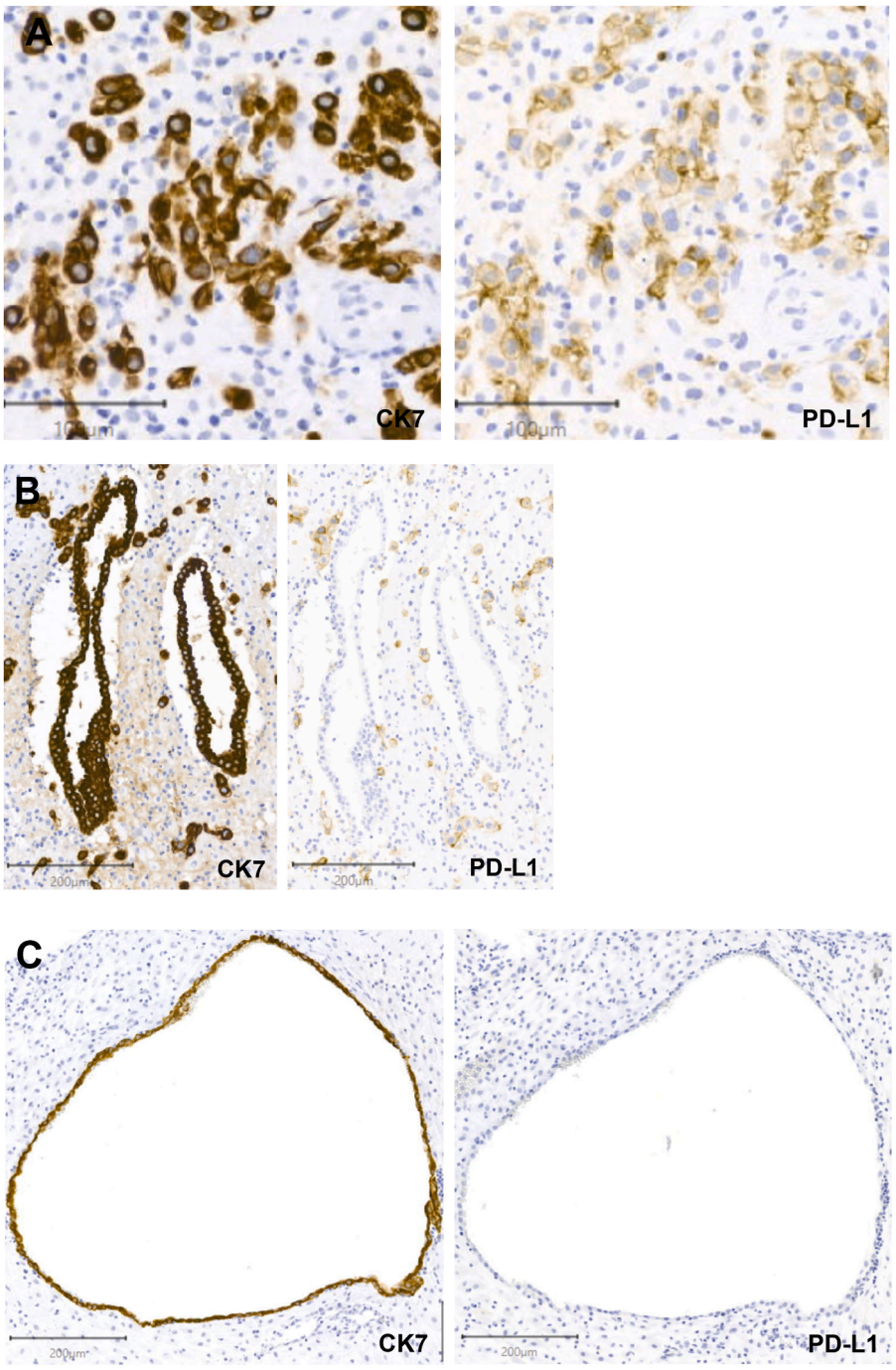


Fig. 2. Three examples of sequential sections of the CK7 and PD-L1 staining. A) CK7 staining was used to determine areas of decidua basalis with positive stained extravillous trophoblast cells. B) Decidua basalis containing endometrial glands. The endometrial glands are $CK7^{+}$ but do not stain for PD-L1. The PD-$L1^{+}$ single cells in the stroma are clearly EVT cells. Stroma cells were PD-$L1^{-}$. $CK7^{+}$epithelial cells of the glands were not counted. C) Parietal decidua showing endometrial glands but no single EVT cells. These areas were not counted.

group ($F = 3.944$, $P = 0.0236$; pairwise comparison: $P = 0.0215$) as seen in Fig. 4A. There was no significant difference found for the PD-$L1^{+}$EVT cell count per area between the three groups (see Fig. 4B). However, the percentage of PD-$L1^{+}$ EVT cells per total EVT count was statistically different between the three groups ($F = 7.306$, $P = 0.0013$). Pairwise comparisons revealed a significant higher percentage of PD-$L1^{+}$ EVT cells in the RPL groups compared to both other groups ($P = 0.032$) (see Fig. 4C). There was no difference in cell counts nor receptor expression between the control and the PAS group. Including only first trimester samples does not change the outcome of the data (data not shown).

3.5. There are more dNK cells per EVT in the RPL group

Lastly, there was a higher amount of dNK cells per EVT in the RPL group compared to both other groups ($\chi 2(3) = 45.49$, $P < 0.001$; pairwise comparison: $P < 0.001$) (see Fig. 5).

To sum up, the main findings of this study are:

1. There are more dNK cells and a higher ratio of NK cells compared to EVTs in the RPL group compared to the control group.

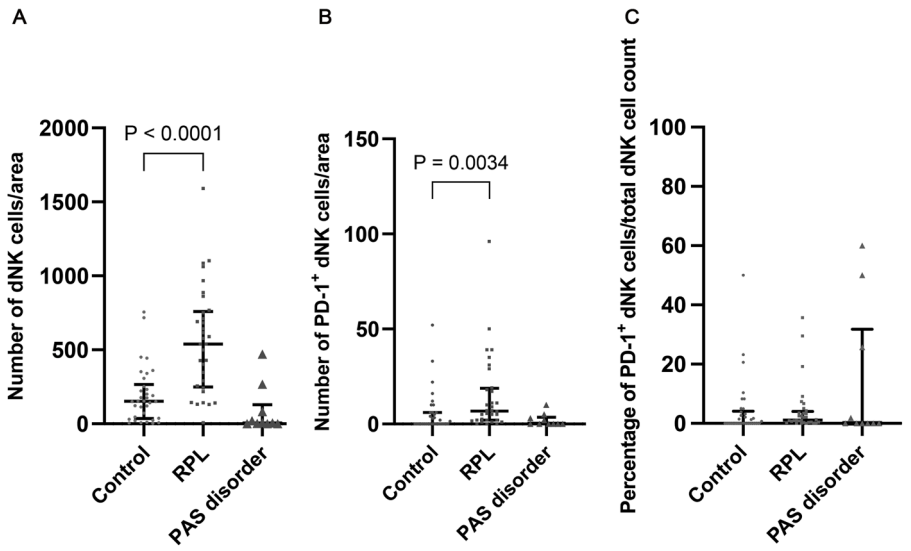


Fig. 3. The number of dNK cells per area, PD-1$^+$dNK cells per area and the percentage of dPD-1$^+$NK cells per total dNK cell count in the three groups. The number of dNK cells (A) and of PD-1$^+$ dNK cells (B) per area is significantly higher in the RPL groups compared to both other groups. There is no difference between the percentage of PD-1+ dNK cells of total dNK cell count between the three groups (C). Data are presented as median ± IQR. Each dot represents one patient.

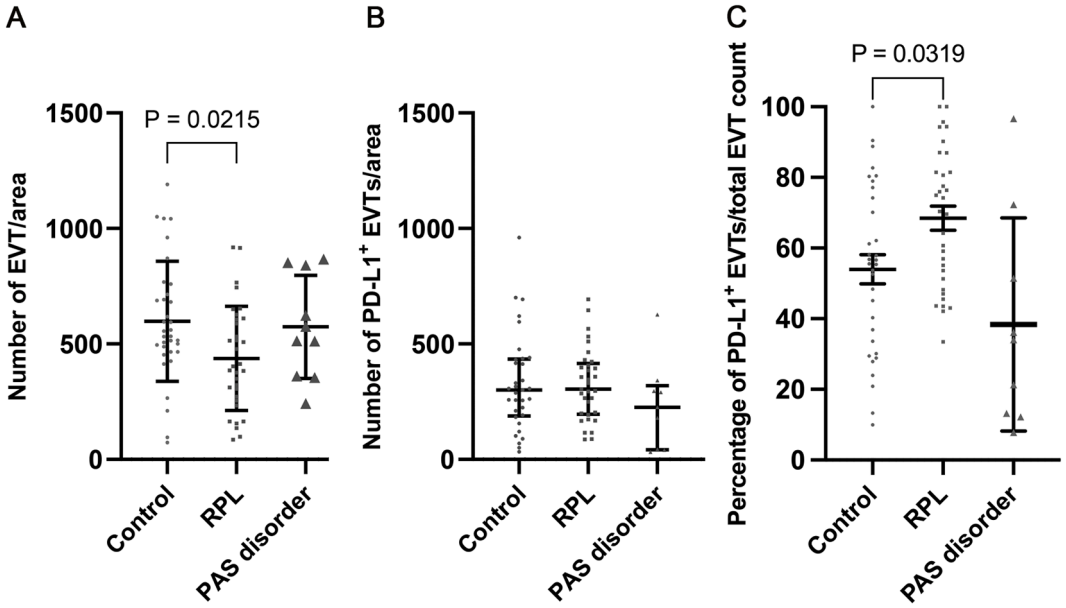


Fig. 4. The number of EVTs per area (A), the number of PD-L1$^+$EVTs per area (B) and the percentage of PD-L1$^+$EVTs per total EVT cell count (C) in the three groups. The invasion of EVTs in the RPL group is significantly lower than in the control group. The percentage of PD-L1$^+$ EVT per total EVT count is significantly higher in the RPL groups compared to both other groups. Data are presented as mean ± SD (A, C) or as median ± IQR (B). Each dot represents one patient.

2. The number of PD-1$^+$dNK cells is higher in the RPL group compared to the control group, however the ratio of PD-1$^+$dNK cells to total dNK cell count is the same in all three groups.
3. The number of EVTs is reduced in the RPL group compared to the control group.
4. The proportion of PD-L1$^+$ cells in all EVT cells is higher in the RPL group compared to the control group, whereas there is no difference in the total number of PD-L1$^+$ EVT cells between the groups.
5. There were no differences found between the control and the PAS group.

4. Discussion/Conclusion

The focus of this study was to compare numbers of dNK cells, PD-1$^+$ dNK cells, EVT cells and PD-L1$^+$ EVT cells in the human decidua basalis between women with a history of RPL, women with a PAS disorder and women with previous uncomplicated pregnancies (control group). To our knowledge no other study compared both receptor and corresponding ligand with immunohistochemical stainings from human samples of these three groups of women.

The reduction of EVTs in the RPL group was expected as RPL is characterized by inadequate invasion of trophoblasts into the decidua [7]. We did not find an increased number of EVTs per area in the PAS group, however. This could be due to the small sample size of the PAS group or the fact that the depth of invasion does not necessarily mean that there are more EVTs in specific areas. The goal of this study was to find an underlying explanation for the differences in invasion patterns in the three groups.

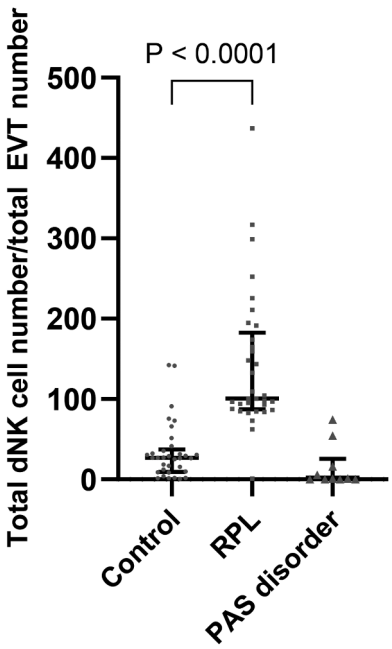


Fig. 5. Total dNK cell number per EVT cell count in the three groups. There are significantly more dNK cells per EVT cell in the RPL group compared to both other groups. Data are presented as median ± IQR. Each dot represents one patient.

First, we investigated the total number of dNK cells in the decidua basalis. dNK cell number in the decidua was significantly higher in women with RPL than in both other groups and there was no difference between dNK cell number between the PAS group and the control group. A higher amount of dNK cells in women with RPL compared to controls was found in other studies as well [7,24,25]. It is still unknown whether the observed increase in dNK cell numbers in RPL samples is the result or the cause of RPL, but it may sustain the idea of dNK cell mediated regulation of EVT invasion. Regarding the PAS group, the study by Schwede et al. [11] supports our findings and our data aligns with their conclusion that the sole amount of dNK cells does not seem to influence the higher invasiveness seen in PAS disorder. In another study, a significantly reduced number of dNK cells in patients with placenta accreta was found [26]. In this study, decidual tissue was obtained from term placentas during caesarean sections, which could explain the discrepancies to our findings as Moffett and Shreeve [2] found a lower percentage of dNK cells in term placenta compared to the first trimester.

Second, we focused on the amount of PD-1 and PD-L1 in all samples. We found that the number of PD-1$^+$dNK cells and the ratio of PD-L1$^+$ cells to EVT cells is higher in the RPL group compared to both other groups. Drawing on previous findings that PD-1$^+$ dNK cells exhibit reduced expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) [20,27], and considering that these cytokines can inhibit trophoblast invasion by, among other mechanisms, inducing EVT apoptosis [7,28], we initially hypothesized that the RPL group would display lower levels of PD-1 and/or PD-L1, whereas elevated levels would be observed in the PAS group. However, we show that the total amount of (PD-1$^+$) dNK cells is higher in the RPL group. It can be reasoned that PD-1$^-$ dNK cells might influence the pathogenesis of RPL in a more differentiated manner. One can assume that both upregulation and downregulation of PD-1 can result in dysregulation of dNK cells. While the suppression of pro-inflammatory cytokine production appears beneficial for a healthy pregnancy, since high levels of cytokines like TNF-α can induce abortion in mice [29], these cytokines are also crucial during the first trimester. Excessive reduction of TNF-α and IFN-γ may lead to decreased levels of angiogenic factors and metalloproteinases, which are essential for proper trophoblast invasion and spiral artery remodeling [7,29]. Hence, further studies are needed to elucidate the exact mechanism by which PD-1 regulates dNK cell function during healthy pregnancy and pregnancy complications.

To date, only limited studies have investigated PD-1 and PD-L1 in RPL and PAS disorder during the first trimester. In contrast to our data, the IHC data from Vacca et al. [20] showed that PD-1 expression is lower in spontaneous miscarriage samples compared to healthy control pregnancies. However, in their study PD-1 expression was investigated in a limited number of patients (n = 5) and on decidual Group 3 innate lymphoid cells (ILC) rather than dNK cells. Both are part of ILC family, however ILC3 is believed to be more involved in mucosal and barrier immunity. Some murine studies show that inhibiting PD-L1 leads to a fivefold increased risk of a pregnancy loss [19] and results in reduced litter sizes [30]. However, these studies focus on PD-1$^+$*T* cell/PD-L1$^+$ interaction instead of PD-1$^+$ NK cells. In addition, one in-vitro study showed that in a trophoblast cell line and in early villi, PD-L1 knockdown resulted in reduced migration and invasion of the trophoblasts [31]. In this study, only the sole presence or absence of PD-L1 on trophoblast cells resulted in the described effect. An in-vivo interaction of PD-L1 binding to its receptor on dNK cell in patients could result in different outcomes as additional factors might exert different effects.

Although these studies provide evidence suggesting that PD-1 and PD-L1 may play a significant role in pregnancy disorders, the small number of studies and the varying methodological approaches make it challenging to directly compare their findings with our results. As suggested by Zhang et al. [32], the PD-1/PD-L1 interaction seems to influence a wide range of immune responses, and it is possible that a sole number of PD-1 receptors/PD-L1 ligands falls short in describing the complex interplay of the different cells in the decidual microenvironment. Therefore, novel studies are needed to focus on additional receptor/ligand pairs and more extensively on the interaction of different immune cells expressing these receptors and ligands.

In addition to the small sample size of the PAS group, another limitation of our study is the difference in sampling methods between the three groups of women. Whereas tissue from the control and PAS group was obtained during surgical terminations of pregnancy, tissue from the RPL group was obtained after intrauterine fetal death. Therefore, differences in dNK cell numbers could be due to the influx of these immune cells as a response to fetal demise. Last, this study was limited by the availability of clinical information, which prevented the identification of a well-defined cohort of patients with unexplained RPL who had undergone a complete clinical work-up in accordance with established guidelines [33]. Given the heterogeneous nature of the RPL population, it is anticipated that not all patients exhibit features of inadequate placentation. Nevertheless, the observed findings revealed promising and robust significant differences. Future studies involving larger cohorts with more detailed clinical characterization are essential to determine which specific RPL subgroups are predisposed to markedly elevated levels of PD-1$^+$ dNK cells or PD-L1$^+$ EVTs, thereby refining our understanding of the immunological landscape in RPL.

In the current study, dNK cells were identified based solely on CD56 expression; however, additional markers could be useful to accurately discriminate specific dNK subsets (e.g., dNK1, dNK2, dNK3), which possess distinct functional capacities such as differing degranulation potential and IFN-γ secretion in response to nonspecific stimuli [34]. These subset-specific profiles may be masked when analyzing the general CD56$^+$ dNK cell population. Moreover, expanded phenotyping could enhance spatial mapping of dNK cell subsets within the decidua, providing insight into their location and possible interactions with surrounding immune cells. Beyond enumeration, assessing dNK cell functionality is essential to fully elucidate their role in biological processes such as pregnancy and pregnancy complications. Lastly, while CD56 predominantly marks NK cells, it is also expressed by NKT cells, which co-express T cell receptors. Given that T cells can express PD-1, distinguishing between PD-1$^+$ dNK and PD-1$^+$ dNKT cells [35] presents a challenge. Nevertheless, as the proportion of dNKT cells in the human

decidua is negligible [13], their potential impact on the current findings is expected to be minimal.

In conclusion, this is the first study comparing decidual PD-1 positive dNK cells and PD-L1 positive EVT expression in patients with RPL and PAS disorder to healthy controls. Our findings show that both PD-1 and PD-L1 are differentially expressed on dNK cells and EVTs in the RPL group compared to the control group. This may affect the cytokine secretion of the dNK cells and therefore the cytokine microenvironment in the decidua, possibly leading to a disruption of the proper immune regulation at the maternal-fetal interface. No significant differences were found between the PAS group and the control group.

Although we cannot draw functional conclusions, our data suggest that PD-1/PD-L1 interaction might influence dNK cells resulting in inadequate placentation. Future studies with larger cohorts and functional testing are needed to determine which threshold of PD-1/PD-L1 signaling is optimal for the maintenance of a healthy pregnancy or disturbance in pregnancy disorders like RPL. Moreover, a more extensive view on diverse receptors present on different subsets of dNK cells or even additional decidual immune cell subsets and ligands present on the EVT are required to see which other factors determine this complex regulation of [in]correct EVT invasion.

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CRediT authorship contribution statement

Anna Moscheik: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Denise Habets:** Conceptualization, Methodology, Writing – review & editing. **Irmgard Classen-Linke:** Conceptualization, Supervision, Writing – review & editing. **Ulrike von Rango:** Conceptualization, Investigation, Methodology, Resources, Supervision, Writing – review & editing. **Carmen A H Severens-Rijvers:** Investigation, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

None.

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Erklärung § 5 Abs. 1 zur Datenaufbewahrung

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten

bei mir, Anna Moscheik, Rosenweg 23, 46414 Rhede

hinterlegt sind.

Erklärung gemäß § 5 Abs. (1) und (2), und § 11 Abs. (3) 12. der Promotionsordnung

Hiermit erkläre ich, **Anna Moscheik,** an Eides statt, dass ich den wesentlichen Anteil an der Publikation:

Moscheik A., Habets D., Classen-Linke I., von Rango U., P., Severens-Rijvers CAH.: Decidual NK cells, extravillous trophoblast and their PD-1/PD-L1 interaction: New insights into recurrent pregnancy loss and placenta accreta spectrum disorders; Immunology Letters.; 2026, 279 geleistet habe.

Die Anteile an der Arbeit waren wie folgt:

	Doktor-andin A. Moscheik	Prakt. Betreuer PD. Dr. U. von Rango	Prakt. Betreuer Dr. C. Severens-Rijvers	Assistentin Dr. D. Habets	Doktormutter Prof. Dr. rer. nat. I. Claßen-Linke	Summe (%)
Studienüberwachung		40	30		30	100
Studiendesign/Konzeption	55	20	20	5		100
Datenerhebung	65	20	15			100
Datenauswertung	95	5				100
Durchführung der Experimente • Auswahl der Proben • Durchführung der Immunohistochemie • Mikroskopieren und Quantifikation	 85 20 100	 5 5	 10 75			 100 100 100
Statistische Auswert.	95			5		100
Bereitstellung von Materialien		25	75			100
Interpretation der Datenauswertung	75	10	5	5	5	100
Verfassung des Manuskripts	**100**					100
Korrektur des Manuskripts		35	20	25	20	100

Aus diesem wesentlichen Anteil ergibt sich selbstverständlich die Stellung als Erstautorin.

Unterschrift der Doktorandin

Als Doktormutter bestätige ich die Angaben von Anna Moscheik und in Vertretung für die nicht mehr erreichbaren und **nicht deutschsprachigen** Koautoren Denise Habets und Carmen A.H. Severens-Rijvers

Unterschrift der Doktormutter

Unterschrift des korresp. Autors

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