ORIGINAL ARTICLE

Natural killer cell profiles in recurrent pregnancy loss: Increased expression and positive associations with TACTILE and LILRB1

Denise H. J. Habets[1,2,3] | Anna Schlütter[1] | Sander M. J. van Kuijk[4] | Marc E. A. Spaanderman[1,3,5] | Salwan Al-Nasiry[1,3] | Lotte Wieten[2,3]

[1]Department of Obstetrics and Gynecology, Maastricht University Medical Centre, Maastricht, the Netherlands

[2]Department of Transplantation Immunology, Maastricht University Medical Centre, Maastricht, the Netherlands

[3]GROW school for Oncology and Developmental Biology, Maastricht University, Maastricht, the Netherlands

[4]Department of Clinical Epidemiology and Medical Technology Assessment, Maastricht University Medical Centre, Maastricht, the Netherlands

[5]Department of Obstetrics and Gynecology, Radboud University Medical Centre, Nijmegen, the Netherlands

Correspondence
Denise Habets, Department of Obstetrics and Gynecology, Maastricht University Medical Centre, Maastricht, the Netherlands.
Email: denise.habets@maastrichtuniversity.nl

Funding information
Maastricht University Medical Centre

Abstract

Problem: NK cells are important for healthy pregnancy and aberrant phenotypes or effector functions have been associated with RPL. We compared expression of a broad panel of NK cell receptors, including immune checkpoint receptors, and investigated their clinical association with RPL as this might improve patient stratification and prediction of RPL.

Method of study: Peripheral blood mononuclear cells were isolated from 52 women with RPL and from 2 women with an uncomplicated pregnancy for flowcytometric analysis and plasma was used to determine anti-CMV IgG antibodies.

Results: Between RPL and controls, we observed no difference in frequencies of T-, NKT or NK cells, in CD56dimCD16+ or CD56brightCD16- NK cell subsets or in the expression of KIRs, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46 or DNAM1. NK cells from women with RPL had a higher expression of LILRB1 and TACTILE and this was associated with the number of losses. The immune checkpoint receptors PD1, TIM3 and LAG3 were not expressed on peripheral blood NK cells. In RPL patients, there was a large variation in NKG2C expression and higher levels could be explained by CMV seropositivity.

Conclusion: Our study identified LILRB1 and TACTILE as NK cell receptors associated with RPL. Moreover, we provide first support for the potential role of CMV in RPL via its impact on the NK cell compartment. Thereby our study could guide future studies to confirm the clinical association of LILRB1, TACTILE and NKG2C with RPL in a larger cohort and to explore their functional relevance in reproductive success.

KEYWORDS
cytomegalovirus, immune checkpoint, natural killer cell, recurrent pregnancy loss

1 | INTRODUCTION

Recurrent pregnancy loss (RPL), defined by the Dutch national guidelines as two or more pregnancy losses below twenty weeks of gestation, affects 1–2% of women trying to conceive.[1,2] RPL imposes a heavy burden on the affected couple and often results in a variety of emotional feelings, such as grief, guilt and anxiety.[3] Until now, the cause of RPL, such as chromosomal abnormalities, thrombophilia, endocrine or uterine anomalies, is known in about half of the cases, whereas the other half of RPL cases cannot be explained yet.[4] It has been hypothesized that a significant proportion of these unexplained pregnancy losses may have an immune-related etiology leading to defective placentation.[5]

During placentation, extravillous trophoblast cells (EVTs) from the fetus invade the decidua and spiral arteries.[6,7] Inadequate invasion and remodeling have been described to be related to obstetric complications, such as RPL and pre-eclampsia.[8,9] From an immunological point of view, invading EVTs are a unique challenge to the maternal immune system, as the fetus is genetically half different from the mother. Hence, a tight balance needs to be established between proper invasion of the EVTs without rejection and impeding over-invasion of the trophoblast cells into the decidua of the mother, on the one hand, as well as being able to adequately respond to pathogens, on the other hand.[10] Natural killer (NK) cells have been proposed to play a critical role in maintaining this balance.[10] NK cells are present in high numbers in the decidua during early pregnancy, in which they surround the invading trophoblast cells and the spiral arteries.[6] During early pregnancy, they secrete a variety of cytokines, chemokines and growth factors, among vascular endothelial growth factor A/C and placental growth factor, which modulate invasion and homing of trophoblasts to the spiral arteries.[7,10] The production of these factors peaks during gestational weeks 8 to 14, the time during which spiral artery remodeling is maximal.[11]

Unlike other lymphocytes, NK cells do not require antigen presentation by human leukocyte antigen (HLA) molecules in order to differentiate between diseased- or foreign cells and healthy cells of self-origin. NK cells have a broad receptor repertoire that regulates their function by providing activating or inhibiting signals upon binding with a ligand and the balance of these signals determines whether NK cells become activated or not.[12] Two of the receptor families most frequently studied in relation to reproductive success are the family of killer-cell immunoglobin-like receptors (KIR), including, for example, the inhibitory KIR2DL1, KIR2DL2/3 and KIR3DL1 that interact with HLA class I, and the family of c-type lectin-like receptors including NKG2A, NKG2C, both interacting with HLA-E, and NKG2D binding to MHC class I chain-related (MIC) proteins and UL16 binding proteins (ULBP).[13] Besides, there is a family of natural cytotoxicity receptors (NCR), a group of activating receptors that are primarily expressed on NK cells, including NKp30, Nkp44 and NKp46 interacting with diverse ligands on tumor cells (e.g., B7-H6, NKp44L, vimentin) but also more general with heparan sulfate proteoglycans.[13] and a leukocyte immunoglobulin-like receptor family including the NK cell associated LILRB1 receptor with specificity for HLA-G.[14] Additionally, there is a group of immune checkpoint receptors, which regulate the degree of immune activation. The inhibitory receptors in this group are important for immunological tolerance and PD1, TIM3, LAG3 and TACTILE are examples of this group of receptors.[15,16,17] Although the role of this receptor family in pregnancy is not yet known, a recent study has shown that TIM3 positive NK cells are associated with an immunosuppressive phenotype with reduced cytotoxicity and production of anti-inflammatory cytokines.[18]

Given their suggested important role in pregnancy, several studies explored the association between NK cell numbers and reproductive success, though the results between these studies are not fully conclusive yet.[19] Moreover, in women with RPL, differences in peripheral blood NK cell receptor expression have been described for example by Fukui et al.[20] Comins et al.[21] and Strobel et al.[22] who primarily focused on activating receptors, and by Zhu et al.,[23] Ntrivalas et al.[24] and Emmer et al.[25] focusing on CD16 in combination with inhibitory KIRs and NKG2A. The immune checkpoint receptors have not been thoroughly studied in relation to RPL, except for TIM-3.[18] and TIGIT.[21] However, they can dampen immune reactivity against tumors and have been shown to be distinctive for patients versus controls in cancer immunology.[26] The many overlapping mechanisms of immunological tolerance between cancer and pregnancy warrant further evaluation of the impact of these receptors on pregnancy outcome.

In our study we compared NK cell phenotype profiles, including novel immune checkpoint inhibitors, in women with a history of recurrent pregnancy loss and women with a previous uncomplicated pregnancy and investigated their clinical association with RPL.

2 | MATERIAL AND METHODS

2.1 | Study population

In this retrospective study, fifty-two women with recurrent pregnancy loss and twenty-two women with a previously vascular uncomplicated pregnancy were included for characterization of peripheral blood (PB) leukocytes. Women with two or more reported pregnancy losses before 20 weeks of gestation, were included at least 3 months after pregnancy loss. Women were excluded if outcomes from the clinical RPL evaluation indicated abnormal parental karyotype, thrombophilia (presence of anti-phospholipid antibodies/Factor V Leiden mutation/Prothrombin mutation/Lupus anticoagulant or deficiency of Protein C, Protein S, antithrombin), endocrine abnormality (e.g., thyroid dysfunction) or uterine anomalies. Upon written informed consent, according to the Medical Ethical Committee of the Maastricht University Medical Centre (Maastricht UMC+) NL67368.068.18, information on baseline characteristics and cells were obtained. All PB samples were processed immediately after collection. Plasma was available of 30 women with RPL and of 16 controls.

2.2 | Isolation of PB leukocytes

Leukocytes derived from ethylene diamine tetra acetic acid (EDTA) blood samples were isolated by incubation of whole blood with red blood cell lysis buffer (.155 mol/L NH_4Cl, 10 mmol/L $KHCO_3$ and .1 mol/L EDTA (Na_2) with pH adjusted to 7.6) in a 1:5 ratio followed by centrifugation. After lysis the pellet with leukocytes was washed twice with PBS. Viability of cells and cell numbers were determined by trypan blue staining in a counting chamber.

2.3 | Antibodies and flowcytometric analysis

PB leukocytes were stained with conjugated antibodies for 30 minutes at 4°C in order to determine NK cell phenotype (CD3(REA613), CD56(REA196), CD158b (DX27), CD158e (DX9), CD159a (REA110), CD158a (REA284), CD4(REA623), CD8(BW135/80), CD85j (GHI/75), CD159c (REA205), CD337(REA823), CD336(2.29), CD335(REA808), CD314(REA797), CD279(PD1.3.1.3), CD226(DX11), CD96(REA195), CD366(REA635), CD223(REA351). After washing twice, samples were measured on a FACS Canto II (BD Biosciences San Jose, CA, USA) and analyzed with the BD FACSDiva Software v.8.0.2(BD Biosciences, San Jose, CA). Acquisition of sample was stopped at 5000 CD3-CD56 NK cells and this criterium was met for all samples. All measured events were included in the analysis. SI Figure 1 shows the gating strategy for determining cell populations and NK receptors. NK cell receptors were analyzed on total population of CD3-CD56+ NK cells and additionally on CD3-CD56^{dim} and CD3-CD56^{bright} NK cells. In order to reduce inter-experimental variations, data acquisition was standardized with application setting and daily calibration with CST beads. In a pilot study, we observed that there was no consistent impact of timing of sampling with respect to the menstrual cycle on peripheral cell populations or NK cell receptors (manuscript submitted).

2.4 | CMV status

Anti-CMV IgG antibodies in plasma were measured by chemiluminescence immunoassay according to manufactures procedures (CMV IgG elecsys, Roche, Mannheim, Germany).

2.5 | Statistical analysis

Data was assessed for normality by visual inspection of histograms. Data of baseline characteristics was either presented as average and standard deviation and analyzed with the independent-samples t-test or presented as percentage and analyzed with the Pearson's chi-square test. Data of immune markers from PB samples and CMV status of women with RPL and controls was presented as median with interquartile range and analyzed with the non-parametric Mann-Whitney-U test. Data of right-skewed immune markers was normalized by logarithmic transformation before linear regression analysis. Linear regression

TABLE 1 Baseline characteristics of study population

Baseline Characteristics	RPL (n = 52)	Controls (n = 22)	P
Age	31.3 ± 3.5	31.8 ± 3.1	.579
Height	168 ± 8	170 ± 5	.305
Weight	74 ± 18	67 ± 10	.052
BMI	26.0 ± 6.3	22.8 ± 4.2	.021
Smoking	18	6	.252
Alcohol use	39	44	.721
Drugs use	4.8	0	.374
Medication use	23	19	.741
Allergies	40	44	.770
Menarche	13.1 ± 1.7	13.3 ± 1.3	.703
Regular cycle	90	77	.214
Gravida	5 ± 2	2 ± 1	<.001
Para	0 ± 1	1 ± 1	.001
Pregnancy losses	4 ± 2	0 ± 0	<.001

Baseline characteristics of study population, data are presented as average ± standard deviation or as percentage.

analysis was performed to estimate associations between immune markers and having multiple pregnancy losses. The estimated associations were presented as regression coefficient (B) and 95% confidence interval, and were additionally adjusted for BMI. Levene's test was used to test whether standard deviations between groups differed. Moreover, linear regression analysis was performed to estimate correlations, presented as correlation coefficient (r), between specific immune markers and the number of pregnancy losses. A P-value below .05 was considered statistically significant. All statistical analyses were conducted with IBM SPSS statistics version 25(IBM Corp, Los Angeles, USA).

3 | RESULTS

A total of 52 women with RPL was included in the analysis in addition to 22 controls. Women with RPL had on average more previously confirmed pregnancies versus controls (5 versus 2, P < .001), more pregnancy losses (4 versus 0, P < .001) and fewer births (0 versus 1, P = .001). Furthermore, women with RPL had higher body mass index versus controls (26.0 kg/m^2 versus 22.8 kg/m^2, P = .021). For all other variables, no significant differences were found (Table 1).

3.1 | NK cells from women with RPL have a higher expression in LILRB1 and TACTILE

First, we observed no differences in percentages of the main lymphocyte subsets of T cells
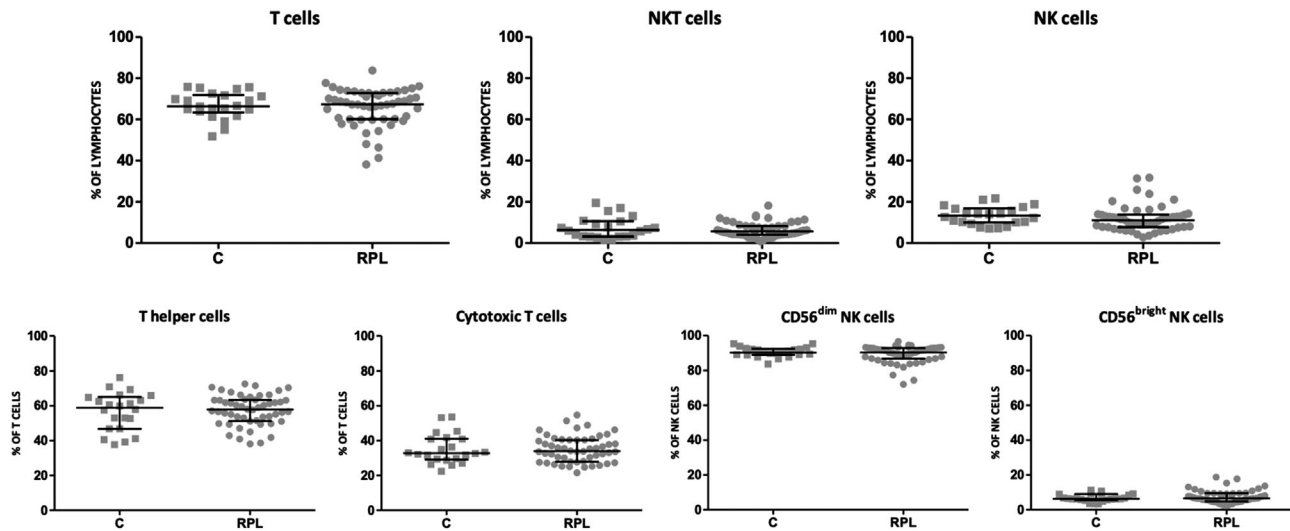
FIGURE 1 Frequencies of cell populations in women with a previous uncomplicated pregnancy (C n = 22) and women with recurrent pregnancy loss (RPL n = 52). Cell populations are presented as percentage of lymphocytes (NKT cells (CD3$^+$CD56$^+$), T cells (CD3$^+$CD56$^-$) and NK cells (CD3$^-$CD56$^+$)), as percentage of T cells (T helper cells (CD3$^+$CD56$^-$CD4$^+$) and cytotoxic T cells (CD3$^+$CD56$^-$CD8$^+$)) or as percentage of NK cells (CD56^{dim} NK cells (CD3$^-$CD56$^+$CD16$^+$) and CD56^{bright} NK cells (CD3$^-$CD56^{++}CD16$^-$)). All populations were measured by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range

(CD3$^+$CD56$^-$), NKT cells (CD3$^+$CD56$^+$) and NK cells (CD3$^-$CD56$^+$) between women with RPL and women with a previous uncomplicated pregnancy (Figure 1, SI Table 1). In addition, we did not observe a difference in T helper cells (CD3$^+$CD56$^-$CD4$^+$), cytotoxic T cells (CD3$^+$CD56$^-$CD8$^+$), cytotoxic CD56^{dim} NK cells (CD3$^-$CD56$^+$CD16$^+$) or cytokine producing CD56^{bright} NK cells (CD3$^-$CD56^{++}CD16$^-$) (Figure 1, SI Table 1). Second, we analyzed activating receptors to investigate potential differences in the percentage of positive NK cells or in expression levels depicted as mean fluorescent intensity (MFI) of total NK cells (CD3$^-$CD56$^+$). There were no differences in expression of the percentage of NK cells positive for NKG2C (CD159c) or in the expression levels of DNAM1(CD226), NKG2D (CD159c), NKp46(CD335) and NKp30(CD337) when comparing women with RPL and women with a previous uncomplicated pregnancy. NKp44(CD336) was not detected on NK cells (Figure 2, SI Table 1). Third, expression of inhibitory receptors was compared between both groups of women. No difference in the percentage of positive NK cells was observed for KIR2DL1(CD158a), KIR2DL2/3(CD158b), KIR3DL1(CD158e) and NKG2A (CD159a). We did observe a higher MFI for LILRB1(CD85j) in women with RPL (RPL 771 versus control 298, P = .007) (Figure 3, SI Table 1). Fourth, analysis of inhibitory immune checkpoint receptors showed that TACTILE (CD96) was higher expressed on NK cells in women with RPL (RPL 747 versus control 370, P = .046) (Figure 3, SI Table 1). PD1(CD279), LAG3(CD223) or TIM3(CD366) expression was not detected on the cell surface of NK cells (Figure 3, SI Table 1).

In addition to the analysis of total NK cells, we compared expression of activating and inhibitory receptors between RPL woman vs controls for the CD56^{dim} NK cell subset (SI Figure 2, SI Table 2) and CD56^{bright} NK cell subsets (SI Figure 3, SI Table 2). On CD56^{dim} cells, we observed a higher MFI for TACTILE (RPL 859 versus control 308, P = .005) and

LILRB1(RPL 673 versus control 363, P = .016) in RPL as compared to controls (SI Figure 2), while this difference was not observed on the CD56^{bright} subset (SI Figure 3). On the CD56^{bright} subset, we observed a lower percentage of NKG2A positive cells in women with RPL compared to controls (RPL 83.4 versus control 91.9, P = .042) while this difference was not observed in total CD56 NK cell subset or in the CD56^{dim} subset. On both dim and bright subsets of NK cells, all the other receptors were equivalently expressed in women with RPL and controls (SI Figure 2, SI Figure 3, SI Table 2).

3.2 | Expression of LILRB1 and TACTILE is associated with RPL and with the number of pregnancy losses

Regression analysis showed that expression levels of TACTILE (B = .39; P = .050) and LILRB1(B = .64; P = .020) were associated with RPL (Table 2). After adjustment for BMI, the associations not only persisted but even increased (B = .60; P = .006 and B = .97; P = .002, respectively, Table 2). Furthermore, regression analysis showed positive associations for both TACTILE (R = .330; P = .004) and LILRB1(R = .279; P = .017) with the number of pregnancy losses (Figure 4, SI Table 3). For the other receptors, there was no association with the number of pregnancy losses (SI Table 3).

3.3 | Percentage of NKG2C positive NK cells is related to CMV seropositivity in RPL

When comparing standard deviations (SD) in cell populations and receptor expression between women with RPL and controls (SI Table 4), SD of NKG2C expression was increased in women with
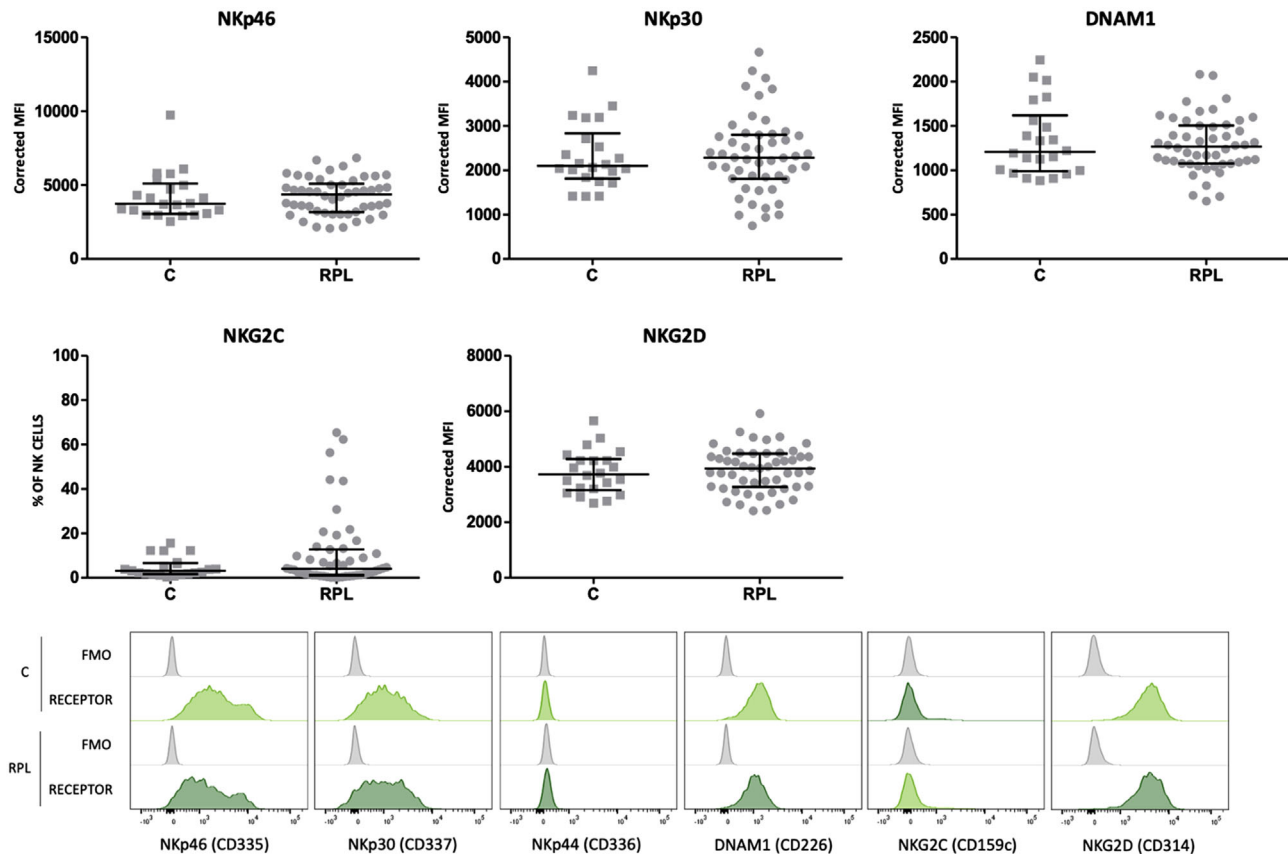
FIGURE 2 Expression of activating receptors in women with a previous uncomplicated pregnancy (C n = 22) and women with recurrent pregnancy loss (RPL n = 52). Data show percentage NKG2C positive cells of total NK cells (CD3⁻CD56⁺) or corrected mean fluorescent intensity (MFI) of total NK cells (CD3⁻CD56⁺) for NKG2D, DNAM1, NKp46 and NKp30. Correction was done by subtracting the MFI of the fluorescence minus one (FMO). All data were acquired by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range. Bottom: representative histograms of activating receptors on peripheral NK cells, grey histograms represent the FMO (no receptor staining) and green histograms represent the receptor staining in RPL and C

RPL (SD 1.65 versus 1.02; P = .028). Human cytomegalovirus (CMV) has been shown to impact the NK cell repertoire, both functionally and phenotypically and an increased expression of NKG2C has been correlated to CMV status.[27,28,29] Hence, we examined anti-CMV IgG antibodies in available sera of 30 women with RPL and of 16 controls. There was no difference in percentages of NKG2C-positive cells between CMV⁺ RPL women and CMV⁺ controls (P = .407) and no difference between CMV⁻ RPL women and CMV⁻ controls (P = .368, Figure 5). In the control group, percentages of NKG2C positive cells were comparable between CMV⁺ and CMV⁻ controls (P = .759, Figure 5), however the CMV+ group was rather small (n = 5). Interestingly, in the group of RPL women, we observed an increased expression of NKG2C in CMV⁺ women when compared to CMV⁻ women (P = .043).

4 | DISCUSSION

The primary goal of this study was to phenotypically profile NK cells in women with unexplained RPL and women with a previously uncomplicated pregnancy and to study the clinical association with RPL. Our

results show that two receptors, LILRB1 and the immune checkpoint receptor TACTILE are differently expressed in RPL women. Furthermore, we show a positive association of LILRB1 and TACTILE with RPL and with the number of pregnancy losses.

As 50% of RPL cases are yet without an identifiable cause, there is a growing need for the development and clinical use of predictive biomarkers. Given the potential importance of NK cells for successful pregnancy, several studies used flow cytometry to compare human NK cell numbers and phenotypes between RPL and pregnancies without complications.[18,20–25] The partly conflicting data from these studies illustrate that comparing pNK cell numbers or pNK cell subsets based on CD56^dim and CD56^bright will not be sufficiently robust as biomarker. To better capture the breath of the receptor repertoire we designed antibody panels covering several of the NK receptors previously associated with RPL as well as a selection of immune checkpoint receptors, i.e., PD1, LAG3, LIRLB1 and TACTILE, that have not been studied before for this purpose. Although, our analysis was still based on a selection of NK receptors, and a fully comprehensive analysis of the repertoire would require a Cytof-, spectral cytometry- or single cell RNAseq approach, the identification of LILRB1 and TACTILE as interesting candidates for further analysis.
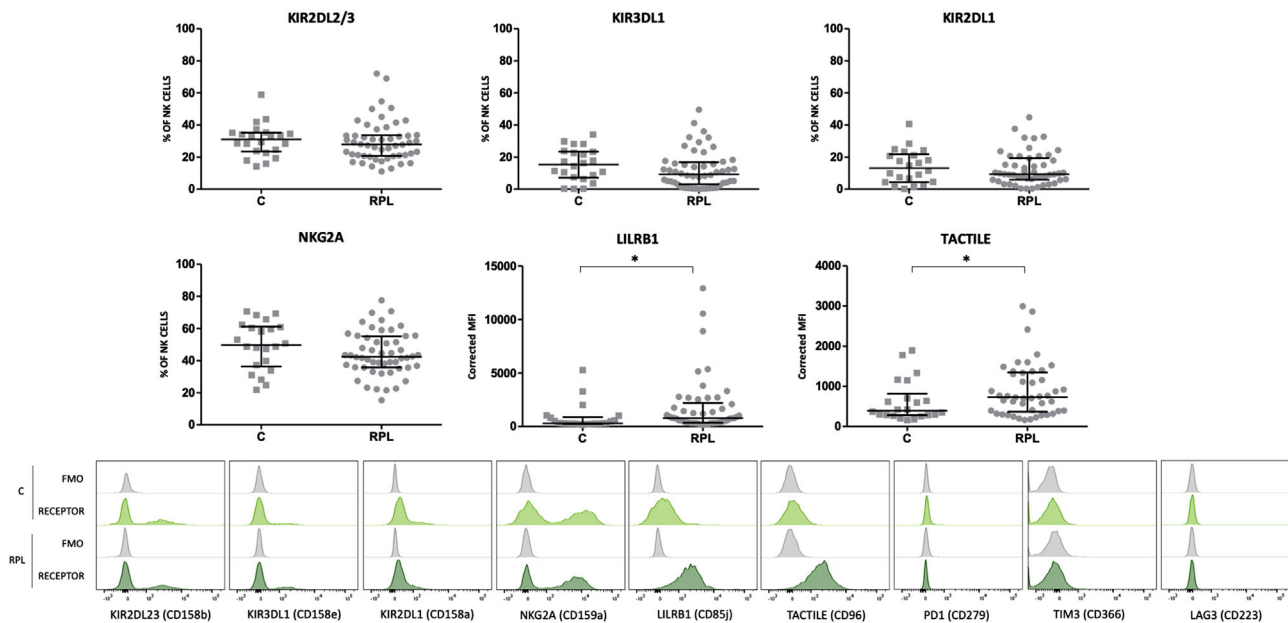
FIGURE 3 Expression of inhibitory receptors in women with a previous uncomplicated pregnancy (C n = 22) and women with recurrent pregnancy loss (RPL n = 52). Data show percentage of KIR2DL2/3, KIR3DL1, KIR2DL1, NKG2A positive cells of total NK cells (CD3⁻CD56⁺) or corrected mean fluorescent intensity (MFI) of total NK cells (CD3⁻CD56⁺) for LILRB1 and TACTILE. Correction was done by subtracting the MFI of the fluorescent minus one (FMO). All data were acquired by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range. Bottom: representative histograms of inhibitory receptors on peripheral NK cells, grey histograms represent the FMO (no receptor staining) and green histograms represent the receptor staining in RPL and C

TABLE 2 Regression coefficient table

Immune Markers		Crude		Adjusted for BMI	
		B [95% CI]	P	B [95% CI]	P
CELL POPULATIONS	T cells	−.02 [−.10, .05]	.526	−.04 [−.13, .05]	.341
	NKT cells	−.07 [−.39, .26]	.686	−.06 [−.44, .32]	.751
	NK cells	−.18 [−.42, .06]	.135	−.12 [−.41, .16]	.396
	T helper cells	.02 [−.07, .11]	.648	−.05 [−.15, .05]	.306
	Cytotoxic T cells	−.00 [−.11, .11]	.988	.08 [−.04, .20]	.192
	CD56dim NK cells	−.02 [−.04, .01]	.216	−.00 [−.04, .03]	.801
	CD56bright NK cells	.04 [−.18, .27]	.712	−.05 [−.31, .21]	.688
ACTIVATING RECEPTORS	NKp46	.00 [−.15, .16]	.947	−.06 [−.25, .12]	.497
	NKp30	−.03 [−.22, .17]	.801	−.10 [−.34, .13]	.377
	DNAM1	−.03 [−.16, .11]	.665	.11 [−.03, .25]	.131
	NKG2C	.18 [−.59, .96]	.641	.36 [−.55, 1.27]	.428
	NKG2D	.02 [−.09, .12]	.747	.09 [−.03, .21]	.153
INHIBITORY RECEPTORS	KIR2DL23	−.05 [−.25, .15]	.601	.03 [−.21, .27]	.826
	KIR3DL1	−.41 [−1.19, .38]	.304	−.44 [−1.36, .48]	.340
	KIR2DL1	−.07 [−.66, .52]	.808	−.60 [−1.23, .03]	.062
	NKG2A	−0.11 [−0.28, 0.06]	0.197	−0.09 [−0.30, 0.11]	0.356
	LILRB1	0.64 [− 0.10, 1.18]	0.020	0.97 [0.37, 1.57]	0.002
	TACTILE	0.39 [−0.00, 0.78]	0.050	0.60 [0.18, 1.03]	0.006

All data on immune markers in women with RPL (n = 52) and controls (n = 22) were acquired by flow cytometry and subsequently normalized by logarithmic transformation for linear regression analysis; data of estimated associations between immune markers and having recurrent pregnancy losses are presented as regression coefficient (unstandardized B) and [95% confidence interval].
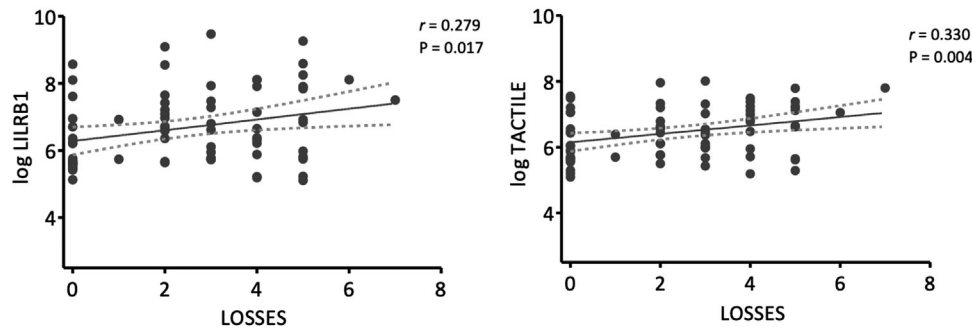
FIGURE 4 Linear regression between logarithmically transformed LIRLB1 and TACTILE expression and number of pregnancy losses (n = 74), presented as regression line with correlation coefficient (r) and 95% confidence interval (dotted lines)
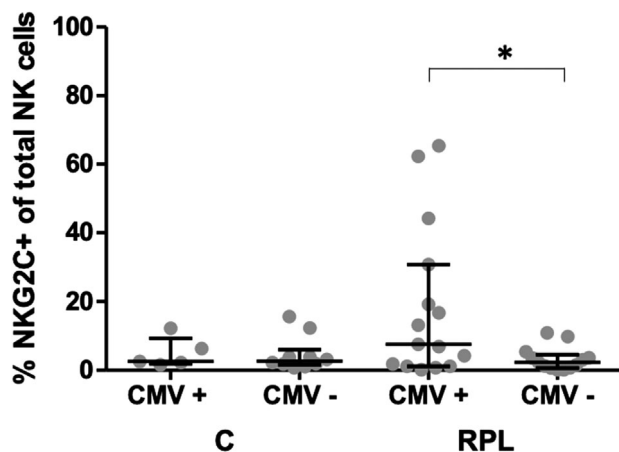
FIGURE 5 Frequencies of natural killer (NK) cells expressing NKG2C are shown for women with a previous healthy pregnancy (C n = 16) and women with recurrent pregnancy loss (RPL n = 30) being either positive or negative for the cytomegalovirus (CMV). Dots represent individuals, lines indicate median and interquartile range

LILRB1 is an immunoglobulin-like receptor that interacts with MHC class I proteins and preferentially binds to HLA-G, a non-classical MHC class I protein expressed on EVTs in the decidua during pregnancy.[30] Interestingly, LILRB1 is an inhibitory receptor for peripheral blood NK (pNK) cells, but it has been shown to act as an activating receptor for NK cells in the decidua hence promoting the production of proangiogenic cytokines, EVT invasion and spiral artery remodeling.[31,32,33] LILRB1 has been shown to be expressed by decidual NK cells present in both the decidua basalis and decidua parietalis.[34] Moreover, in CMV positive woman, LILRB1 expression was expressed at higher levels on NKG2C positive endometrial NK cells from multigravid women as compared to woman without previous pregnancies.[35] In line with this, a second study, showed increased expression of LILRB1 on NKG2C positive endometrial and decidual NK cells from woman with multiple as compared to first time pregnant women.[36] These NK cells, termed 'pregnancy trained decidual NK cells' had unique transcriptomic and epigenetic signatures, and secreted IFN-γ and VEGFα upon activation which promoted capillary sprouting. The subsequent observation that secretion could be blocked by monoclonal antibody blockade of

LILRB1 clearly illustrated the potential functional relevance of LILRB1 in controlling NK cell function in the uterus.[37]

The functional relevance of TACTILE remained obscure for many years, however, in the context of anti-cancer immune responses it has been shown to negatively control cytokine production by NK cells by antagonizing NK cell activation mediated by the DNAM1 receptor.[38] Although the role of TACTILE in the decidua is not known, expression of its ligand, the poliovirus receptor (PVR or CD155), on EVT and maternal endothelial cells, has been demonstrated by single cell RNAseq analysis of first trimester placentas.[39] Moreover, we do know that TIGIT, an immune checkpoint receptor that shares the same ligand as TACTILE, has been found on decidual NK cells and has been assumed to have a putative inhibitory interaction between the decidual NK cell and the EVT and has been suggested to promote the tolerogenic functions of dendritic cells and regulatory T cells at the fetal-maternal interface.[39]

Based on our current data, we cannot provide a direct link on the potential relation between LILRB1 or TACTILE expression on peripheral blood NK cells and expression on NK cells in the uterus. Hence, it would be interesting to follow up on our findings with a paired analysis of LILRB1 or TACTILE expression profiles from peripheral blood NK cells and uterine NK cells in RPL versus control women, for example, to assess whether recruitment of LILRB1 or TACTILE positive subsets to the uterus is disturbed in RPL women.

Inhibitory KIRs and NKG2A are the best characterized inhibitory receptors for NK cells, they are highly expressed on dNK cell and have been proposed to regulate dNK cell effector function by interaction with HLA-C and HLA-E.[40] In line with two previous studies.,[24,25] but in contrast to two other studies.,[23,41] we did not observe an association between RPL and percentages of pNK cells positive for KIR2DL1, KIR2DL2/3 or NKG2A. Additionally, we could not confirm the reduced TIM-3 and higher NKG2D expression observed on pNK cells in peripheral blood of women with RPL reported by two other studies.[18,23] The lack of consistency of these results may be explained by variation between the studies in the definition of RPL, in patient inclusion criteria, or technical differences in e.g., the used antibody clones and set up of the flow cytometer. Moreover, a weakness of most of these studies, including ours, is the relatively low study power. This emphasizes the importance of confirmatory studies, preferably with large patient

cohorts, as well as harmonization of study protocols for adequate comparison of studies, to ultimately obtain truly reliable biomarkers.

Another important point to consider is the location of sampling since it is becoming increasingly clear that NK cells in peripheral blood and in tissues are very different.[42] In peripheral blood, we could not detect the immune checkpoint receptors PD1, LAG3 and TIM3. Our data are in line with the concept that these receptors are primarily expressed on highly activated NK cells or on subsets of tissue-resident NK cells.[43] This underlines the importance of including NK cells obtained from the (pregnant) uterus in future studies exploring the causal relation between NK cells and pregnancy outcome or NK cell-based targets for intervention. However, for a biomarker to be predictive such a causal relation with the disease is not *per se* required. Moreover, sampling of uterine NK cells, e.g., trough biopsies, is a relatively invasive procedure. Although obtaining NK cells from menstrual blood is an attractive alternative,[44] caution should be taken as some of the predictive surface markers may be lost during the isolation procedure. Hence, an advantage of phenotyping pNK cells is that it can be more easily implemented in routine diagnostics for in- or outpatient clinics. Evidently, this is provided that a robust and predictive NK cell phenotype can be identified and confirmed in a standardized setting.

To further assess whether the peripheral blood NK compartment can predict for the phenotype and function of uterine NK cells, a comparison between phenotypes and function of both peripheral blood and uterine NK cells from control versus RPL women will be needed as this cannot be concluded from the current study. Exploring the potential relation between peripheral blood and uterine NK cells is also interesting to better understand the origin of uterine NK cells for which several possibilities have been proposed. The first possibility is that NK cells are recruited from the peripheral blood to the uterus, where they undergo further tissue specific differentiation under the influence of the uterine environment.[10,45] The second possibility is that uterine NK cells already reside in the tissue.[46] The third possibility is that uterine NK cells are likely to be a heterogeneous population arising from both.[47]

Because of the complexity of the immune response, the integration of multiple parameters might be necessary for accurate prediction. First of all, this could be done by the analysis of co-expression of receptors, which may reveal additional information on the relation between specific NK cell subsets and pregnancy outcome. Co-expression can be assessed by conventional two-dimensional analysis using a predefined and sequential gating strategy. However, this approach may lead to losses of important information, including for example the interplay between specific cell types or receptors.[48] Hence, more in-depth information can be obtained by multivariate-models (e.g., T-SNE, ViSNE) or a recently developed method based on Principal Component Analysis (PCA), called Discriminant Analysis of Multi Aspect Cytometry (DAMACY), that can quantitatively compare the immune cell composition between groups after the fusion of the data from the different flow cytometry panels.[48,49] For future analysis it would be exciting to analyze if several receptors possibly cluster in women with RPL or women with a previous healthy pregnancy using those more advanced analysis models.

Viral status is another feature to include in future comprehensive NK cell profiles. CMV seropositivity or reactivation is the most prominently studied example of the impact of viruses on the NK cell repertoire, and it has been linked to increased expression of NKG2C, KIR and LILRB1 and the induction of 'memory NK cells', characterized by a longer lifespan and enhanced effector function.[27,28,29] Interestingly, a study with 25 healthy controls showed that CMV status was not related to NKG2C expression on menstrual blood NK cells while it was positively associated with higher NKG2C expression on NK cells in peripheral blood.[50] We did not observe a clear association between NKG2C and CMV status in controls but we did observe an increased percentage of NKG2C positive NK cells in CMV$^+$ women with RPL when compared to CMV$^-$ women with RPL. Although CMV has been suggested to negatively impact reproductive success, its role in pathogenesis of RPL is still quite controversial.[51,52,53,54] Unfortunately, we determined CMV status only in a limited number of women (30 RPL and 16 controls) since plasma samples were not available for all individuals in our cohort. Especially the CMV positive control group was underpowered (n = 5) to strongly conclude that there was no difference in NKG2C expression between CMV positive versus CMV negative control women. Since the epidemiology of CMV infection can be diverse, as various populations might be affected differently, larger cohorts with a broader socioeconomic distribution of RPL cases are needed to get more clear results on the role of CMV in RPL, and it would be highly interesting to integrate evaluation of the NK cell repertoire in those studies as well.

In summary, our study showed that LILRB1 and TACTILE were higher expressed in women with RPL and positively associated with the number of pregnancy losses. Moreover, we showed CMV seropositivity was associated with higher expression of NKG2C in RPL woman, providing a first indication that CMV may influence reproductive success through its impact on the NK cell compartment. Thereby, it provides an interesting starting point for future studies in large- and standardized cohorts to confirm their predictive value for RPL as well as functional studies to investigate a potential causal relation with pregnancy outcome.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

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

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Additional supporting information can be found online in the Supporting Information section at the end of this article.