

Composition and dynamics of the uterine NK cell KIR repertoire in menstrual blood

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Uterine natural killer (NK) cells are abundantly present in endometrium and decidua. Their function is governed by interactions between killer cell immunoglobulin-like receptors (KIRs) and cognate human leukocyte antigen (HLA) class I ligands. These interactions have implications for reproductive success. Whereas most uterine NK cells are known to express KIRs, little information is available about KIR repertoire formation and stability over time. This is primarily due to inherent difficulties in gaining access to human uterine tissue. As endometrial immune cells are shed during menstruation, menstrual blood may serve as a source for studies of KIRs on uterine NK cells. Here, we performed a combined assessment of six inhibitory and activating KIRs on uterine NK cells from paired menstrual and peripheral blood. Menstrual blood contained a high frequency of uterine NK cells expressing KIRs. The uterine NK cell KIR repertoires were markedly different from those in peripheral blood NK cells, biased toward KIR2D-receptor expression, and formed independently of selection conferred by cognate HLA class I molecules. Moreover, uterine NKG2C⁺ self-KIR⁺ NK cell expansions were detected. Finally, the distinct KIR repertoires of uterine NK cells were stable over multiple menstrual cycles. Our results provide novel insight into KIR repertoire formation on human uterine NK cells.

INTRODUCTION

Uterine natural killer (NK) cells are phenotypically and functionally specialized NK cells present in the endometrium of the non-pregnant uterus and the maternal part of the placenta (decidua) during pregnancy.^{1–3} The endometrium undergoes marked changes with each menstrual cycle. The sex hormone progesterone acts on endometrial stromal cells leading to decidualization of the endometrium. Progesterone also promotes stromal cell interleukin (IL)-15 production.^{4,5} IL-15 regulates NK cell homeostasis and its increase in the endometrium promotes uterine NK cell proliferation.⁵ If a fertilized embryo implants into the decidualized endometrium and pregnancy is initiated, the expansion of uterine NK cells continues.³ As a result of this, uterine NK cells constitute up to 70% of all leukocytes present in first trimester decidua.³ During early stages of pregnancy, decidual NK cells interact with and regulate invasion of fetal-derived trophoblasts.³ This interaction is thought to occur via killer cell immunoglobulin-like receptors (KIRs) expressed on uterine NK cells.^{3,6} Uterine NK

cells can also directly contribute to regulation of spiral artery remodeling.^{7–11} Both interaction with trophoblasts and spiral artery remodeling are important for successful implantation and likely influence pregnancy outcome. Indeed, population genetic studies have revealed associations between KIRs and KIR ligands, and pregnancy disorders such as recurrent miscarriage and preeclampsia.^{12–16} However, difficulties in accessing primary uterine tissue have hampered efforts to understand uterine NK cell KIR repertoire composition.

KIRs represent a polymorphic and polygenic family of activating and inhibitory receptors that recognize distinct human leukocyte antigen (HLA) class I alleles.¹⁷ NK cells are the main KIR-expressing immune cells,^{18,19} and KIR expression on NK cells is variegated, seemingly to ensure broad specificity and capacity to sense the presence or absence of single HLA class I alleles.²⁰ At steady state the acquisition of KIR expression on peripheral blood NK cells is a random process not subjected to any positive or negative selection.²¹ However, during viral infections, most notably during

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Received 29 July 2015; accepted 4 May 2016; published online 8 June 2016. doi:10.1038/mi.2016.50

cytomegalovirus (CMV) infection, studies suggest the NK cell compartment to be influenced by cognate HLA class I ligands. In peripheral blood, this is evident by the presence of expanded NK cell populations expressing CD57 and the activating receptor NKG2C together with self-inhibitory KIRs.^{22–25} Many uterine NK cells express KIRs,^{26–30} and the receptor profile of uterine NK cells from decidua is biased toward expression of KIR2D-receptors.^{6,31,32} However, little information is available about the composition of KIR repertoires on uterine NK cells from non-pregnant uteri. Specifically, it is not known whether HLA class I molecules influence uterine NK cell KIR expression, if NKG2C⁺ expansions with narrow self-KIR⁺ repertoires also exist within the uterine NK cell compartment, and whether the same KIR repertoire is regenerated on uterine NK cells with each menstrual cycle.

In this study we utilized the fact that, in non-pregnant women, endometrial immune cells are shed with the menses.^{33,34} We obtained matched menstrual and peripheral blood from a cohort of 25 women, of which 10 were sampled longitudinally over at least two menstrual cycles. We characterized the composition of the KIR repertoire on uterine NK cells by simultaneously assessing six inhibitory and activating KIRs. Our data provide novel insight into uterine NK cell KIR repertoire formation and specifically into the composition and stability of inhibitory and activating KIRs on uterine NK cells.

RESULTS

Uterine NK cells are present at high levels in menstrual blood

To study uterine NK cells, we collected matched menstrual and peripheral blood from 25 women of child-bearing age (Table 1). Using flow cytometry, NK cells were identified as alive CD45⁺CD3⁻CD4⁻CD14⁻CD15⁻CD19⁻ lymphocytes expressing CD56 and/or CD16, (Figure 1a). On

average, 17% of menstrual blood CD45⁺ lymphocytes were NK cells by these criteria (range: 10–40%) as compared with 10% in peripheral blood of the same donors (range: 4–16%).

Next, we studied the differentiation status of NK cells from menstrual blood by assessing the combinatorial expression of CD16, CD57, NKG2A, and CD9 (Figure 1b and Supplementary Figure 1 online). Expression of CD16, CD57, and NKG2A can be used to characterize conventional peripheral blood NK cell differentiation.¹⁹ Previous work has shown that uterine NK cells exhibit a CD56^{bright}NKG2A⁺CD9⁺ phenotype while lacking expression of CD16 and CD57.^{29–31} Cells with a CD16⁻CD57⁻NKG2A⁺ phenotype (hereafter referred to as uterine NK cells) were indeed the most prevalent NK cells in menstrual blood (Figure 1c). Furthermore, in menstrual blood, this phenotype was associated with a high expression of CD9 and CD56 (Figures 1d and e). Of note, on average 25% (range: 3–60%) of the menstrual blood NK cells had a phenotype more reminiscent of conventional peripheral blood CD56^{dim} NK cells with expression of CD16, CD57, and/or NKG2A (Figure 1c). Finally, uterine NK cells in menstrual blood exhibited signs of ongoing proliferation, unlike CD16⁺ NK cells (Supplementary Figure 1). This corroborates early work showing expression of Ki67 in ‘endometrial stromal granulocytes’ toward the end of the menstrual cycle.³⁵

Taken together, we find that menstrual blood is a viable source of uterine NK cells.

Uterine NK cells from menstrual blood have a distinct KIR specificity compared with conventional NK cells

It is well established that most uterine NK cells isolated from either endometrium or decidua express KIRs.^{6,26–30} Extending these reports, we here characterized total expression of six inhibitory and activating KIRs on uterine NK cells from menstrual blood (Figure 2a). On average, 58% of the uterine NK cells (CD16⁻CD57⁻NKG2A⁺) expressed KIRs (Figure 2b). Conventional (CD16⁺) NK cell subsets in menstrual blood had a KIR expression frequency similar to that of the corresponding peripheral blood subsets (Figure 2b). As an alternative approach to visualize KIR expression, we first gated on pan-KIR⁺ NK cells, and subsequently analyzed the distribution of these cells within subsets co-expressing NKG2A, CD16, and CD57 (Figure 2c). This analysis demonstrated that uterine NK cells (CD16⁻CD57⁻NKG2A⁺) constituted the major KIR-expressing population in menstrual blood (Figure 2c).

The presence of conventional NK cells in menstrual blood indicated a potential concern for contamination in our downstream analysis of uterine NK cell KIR expression. To address this, we determined total KIR expression on CD16⁻ menstrual blood NK cells using different definitions for the uterine NK cells. Similar to NKG2A, CD9 and CD69 are both reportedly expressed by most uterine NK cells.^{36,37} Our analysis revealed that the inclusion of KIR-expressing cells was largest when NKG2A was used to define uterine NK cells (Supplementary Figure 2). With this approach, inevitably, a minute fraction of conventional CD56^{bright}CD16⁻ NK cells

Table 1 Donor characteristics

	Donors (n = 25)
Age, median (range)	30 years (21–50)
Sampled longitudinally	10/25 (40%)
Contraceptives	10/25 (40%)
Copper IUD	7/25 (28%)
Vaginal ring	1/25 (4%)
COC	2/25 (8%)
Previous pregnancy	15/25 (60%)
Previous miscarriage	6/25 (24%)
Endometriosis	0/25 (0%)
Previous unprotected heterosexual coitus	25/25 (100%)
Menstruation duration, median (range)	3.5 days (2–8)
Length of menstrual cycle, median (range)	27 days (23–32)
HCMV positive	18/20 (90%) ^a

Abbreviations: COC, combined oral contraceptive pill; HCMV, human cytomegalovirus; IUD, intrauterine device.

^aInformation not available from five donors.

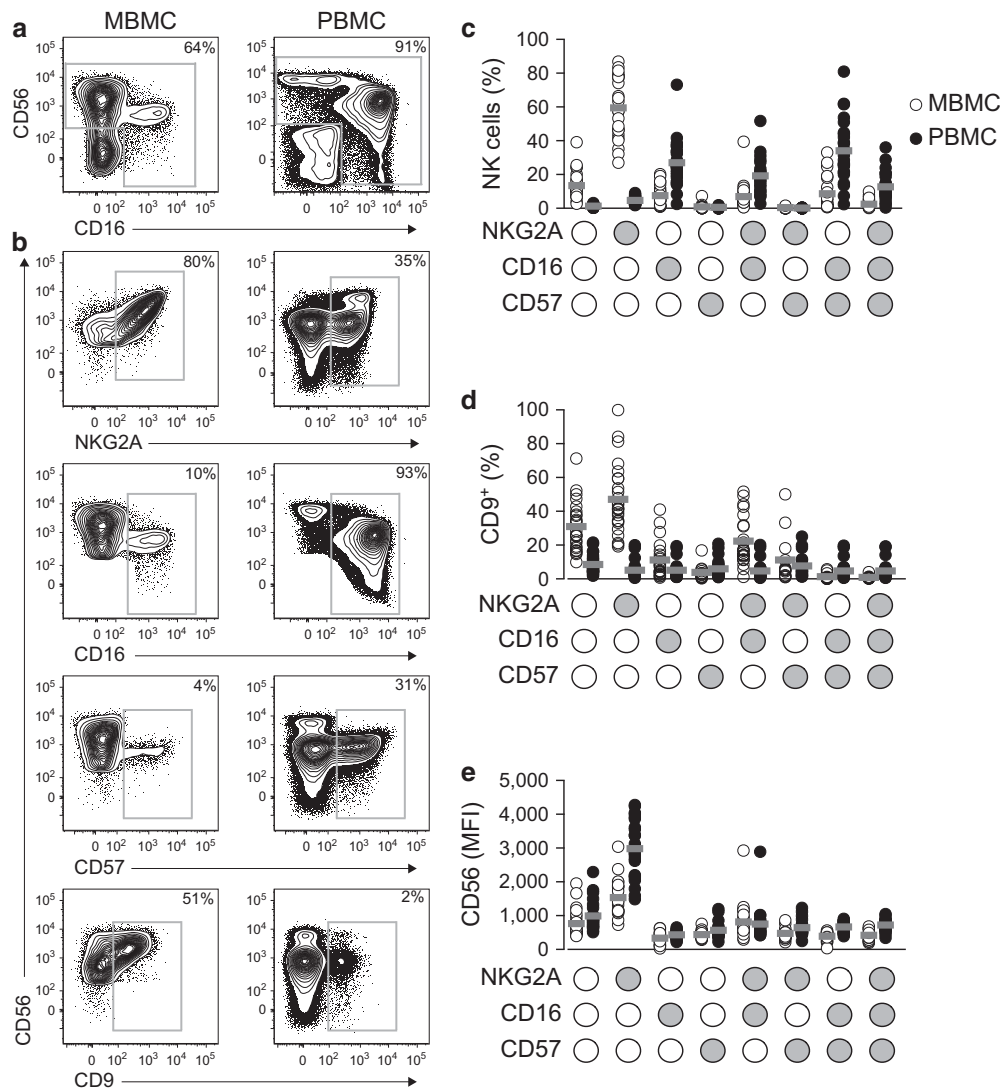


Figure 1 Menstrual blood contains a high frequency of uterine NK cells. (a) Staining for CD56 and CD16 on alive CD45⁺CD3⁻CD4⁻CD14⁻CD15⁻CD19⁻ lymphocytes from menstrual and peripheral blood to identify NK cells. (b) Representative staining for NKG2A, CD16, CD57, and CD9 on NK cells from menstrual (MBMC) and peripheral (PBMC) blood. (c) Summary of data for frequency of cells out of total NK cells expressing different combinations of CD16, CD57, and NKG2A in menstrual and peripheral blood ($n=25$). (d) Frequency of CD9⁺ cells out of NK cell subsets defined by different combinations of CD16, CD57, and NKG2A in menstrual and peripheral blood ($n=25$). (e) Mean fluorescence intensity (MFI) of CD56 expression within NK cell subsets defined by different combinations of CD16, CD57, and NKG2A in menstrual and peripheral blood ($n=25$).

would end up in our final uterine NK cell gate. However, as conventional CD56^{bright} NK cells are KIR negative this would not affect the downstream analysis.

Finally, to assess KIR specificity, expression of individual KIRs was studied. Previous reports have indicated the KIR specificity of uterine NK cells from decidua, but not from endometrium, to be biased toward KIR2D receptors.^{6,31,32} Notably, fewer uterine NK cells obtained from menstrual blood expressed KIR3DL1, and instead a bias toward KIR2D-receptor expression was noted as compared with peripheral blood (Figure 2d). KIR2DS4 did not follow this pattern of expression, as it was generally lower on all menstrual blood NK cells as compared with peripheral blood (Figure 2d).

In summary, menstrual blood contains high levels of KIR⁺ uterine NK cells and the KIRs expressed by uterine NK cells differ in composition compared with matched peripheral blood NK cells.

Uterine NK cells frequently co-express multiple KIRs

After concluding that expression of inhibitory and activating KIRs differ between uterine and conventional NK cells, we next assessed KIR co-expression patterns. The frequency of NK cells expressing one to six KIRs was compared between uterine NK cells and conventional NK cells from menstrual and peripheral blood. The KIR co-expression patterns of conventional NK cells from menstrual and peripheral blood were similar (Figure 3a).

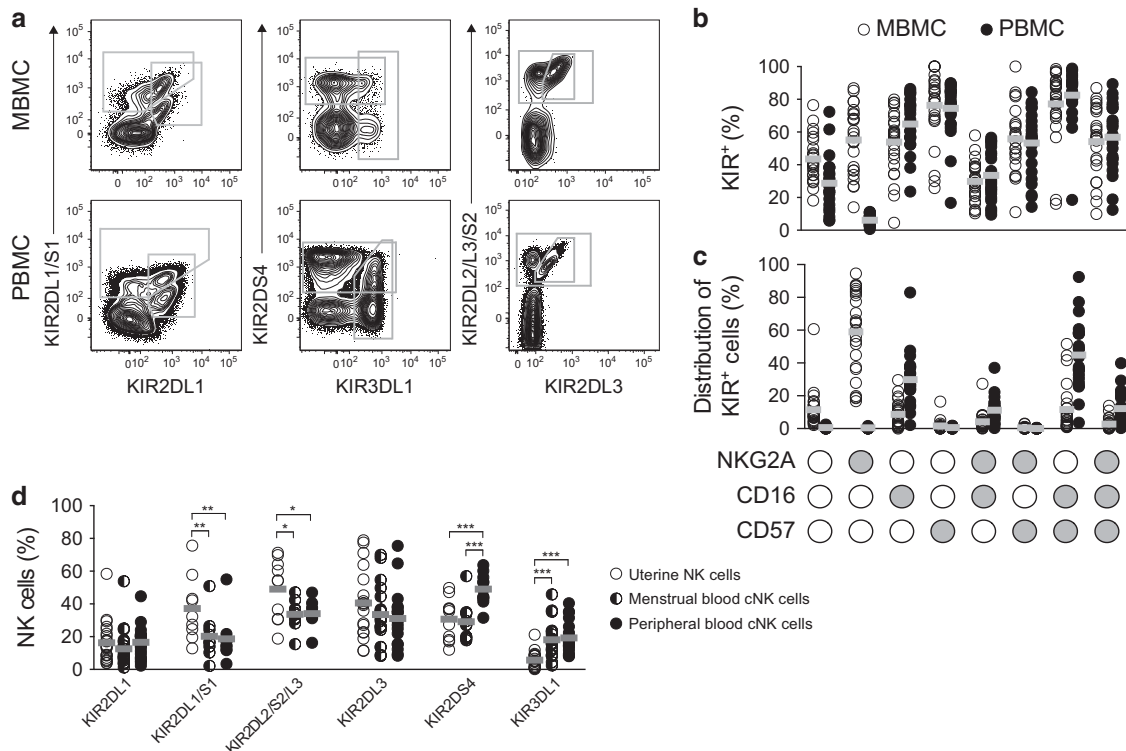


Figure 2 Menstrual blood uterine NK cells express high levels of KIRs. **(a)** Representative staining for KIR2DL1, KIR2DL1/S1, KIR2DL2/L3/S2, KIR2DL3, KIR2DS4, and KIR3DL1 on NK cells from menstrual (MBMC) and peripheral (PBMC) blood. **(b)** Frequency of pan-KIR⁺ cells (defined by Boolean-combinations of KIR gates) within subsets defined by different combinations of CD16, CD57, and NKG2A in menstrual and peripheral blood NK cells (defined as in **Figure 1**, $n = 25$). **(c)** Frequency of cells expressing different combinations of CD16, CD57, and NKG2A in menstrual and peripheral blood ($n = 25$) after gating on pan-KIR⁺ cells NK cells. **(d)** Frequency of menstrual blood uterine (CD16⁻CD57⁻NKG2A⁺) and conventional (CD16⁺) NK cells, and peripheral blood CD16⁺CD56^{dim} NK cells expressing the indicated KIR ($n = 23, 9, 10, 18, 11$, and 20 , respectively, $*P < 0.05$, $***P < 0.001$, ANOVA non-parametric test with Dunn's test).

In contrast, significantly fewer uterine NK cells expressed one KIR, similar co-expression was noted for two KIRs, and significantly more uterine NK cells expressed three or four KIRs (**Figure 3a**).

Considering that expression of a given KIR occurs largely independent of other KIRs, another method to assess KIR co-expression patterns is to apply the 'product rule', a special case of Baye's rule.²¹ It assesses the probability of two random events (here co-expression of two or more KIRs) to co-occur. We applied the product rule to all co-expression possibilities of two and three KIRs, and performed a linear regression of the observed (from the flow cytometry data) and expected (given by the product rule) frequencies of KIR expression (**Figures 3b and c**). On uterine NK cells, co-expression was more frequent for two and three KIRs (deviating by 31% and 134%, respectively) as compared with peripheral blood NK cells (**Figures 3b and c**). The KIR co-expression patterns for conventional NK cells (**Figures 3b and c**), both from menstrual and peripheral blood, was in line with what has previously been reported studying peripheral blood NK cells.²¹

Together, we find that not only are KIR specificities distinct between uterine and peripheral blood NK cells (**Figure 2d**), but also the overall structures of the KIR-repertoires differ significantly.

Cognate HLA class I ligands have no impact on the uterine NK cell KIR repertoire

To explore whether the formation of distinct uterine NK cell KIR-repertoires was a result of HLA class I-mediated selection, we examined the influence of KIR ligands on KIR expression. Donors were genotyped for the presence or absence of KIR ligands HLA-C1 (ligand for KIR2DL3), HLA-C2 (KIR2DL1), and HLA-Bw4 (KIR3DL1) (**Figure 4a**). We focused on individuals with two out of three KIR ligands present, and analyzed the observed frequencies of self- and non-self KIRs with respect to the presence or absence of cognate HLA class I ligands. Combinatorial rules predict a greater chance of expressing a self over a non-self KIR in the presence of two out of three possible ligands.²¹ On uterine NK cells, 30% of the expressed KIRs had a self ligand present compared with 23% with a non-self ligand (**Figure 4b**). The observed distribution was not significantly different from the theoretical values of KIR expression expected from a random distribution, without any selective influence of cognate ligands (**Figure 4b**). A similar random distribution was seen for menstrual blood conventional NK cells (**Figure 4c**), and in agreement with previous findings,^{18,21} also for peripheral blood conventional NK cells (**Figure 4d**). Our data indicate that although the KIR-repertoires of uterine and conventional NK cells differ, both

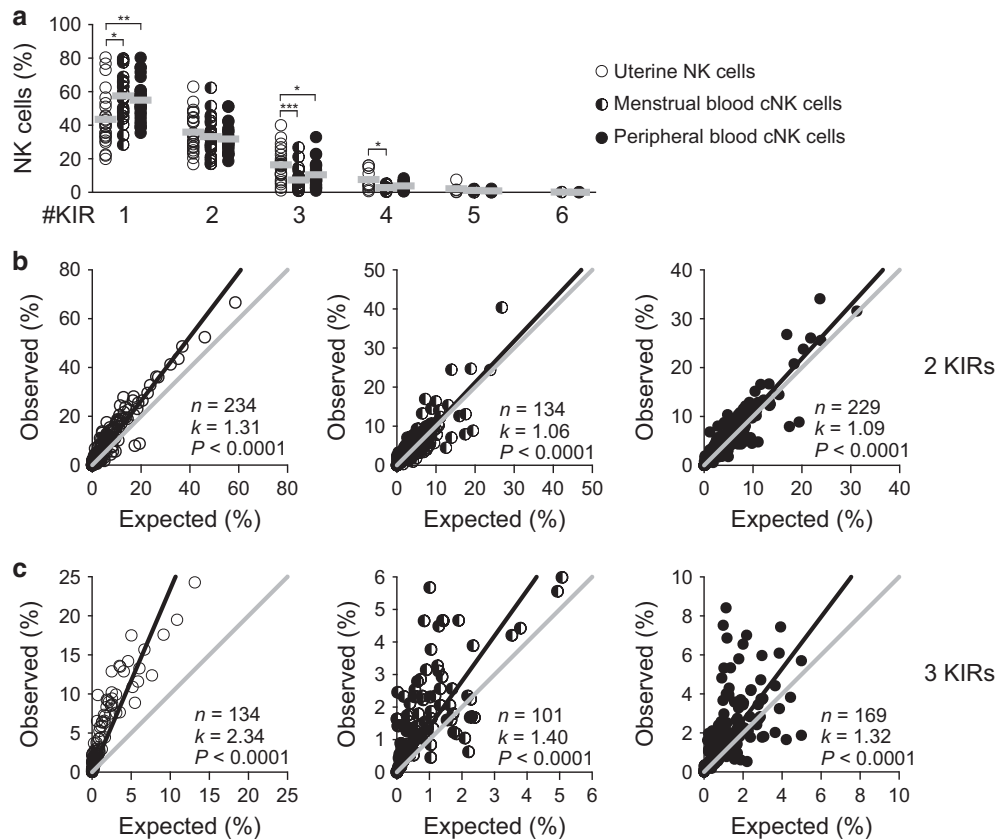


Figure 3 KIR co-expression patterns on uterine NK cells. **(a)** Frequency of menstrual blood uterine (CD16⁻CD57⁻NKG2A⁺) and conventional (CD16⁺) NK cells and peripheral blood CD16⁺CD56^{dim} NK cells expressing 1–6 KIRs ($n=2-25$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ANOVA parametric test, with Dunnett test). **(b)** Observed frequencies of NK cells co-expressing two KIRs (14 pairs analyzed) plotted against those expected from the product rule. K -values were derived from the slope of the linear regression of observed data, relative to a perfect fit with the product rule. P -values were derived from a linear regression analysis. Deviation from the product rule in percent is obtained by the formula $(k - 1) \times 100$. **(c)** Product rule assessment for NK cells co-expressing three KIRs (11 combinations analyzed). For **(b)** and **(c)**, if expression of two KIRs is an independent event, their co-expression frequency is predicted by the product rule, i.e., by the product of the expression frequencies for the respective KIRs ($\%KIR-A^+ \times \%KIR-B^+ = \%KIR-AB^+$). The gray line, illustrating a 1:1 relation between observed and expected frequencies, depicts this.

with respect to specificity and co-expression patterns, the two populations of NK cells have a random distribution of self and non-self KIRs.

The KIR repertoire of uterine NK cells is stable over several menstrual cycles

The exploratory analysis of KIR expression and the identification of the KIR repertoire characteristics on uterine NK cells represent snapshots of repertoires present at the end of any given menstrual cycle. Uterine NK cells uniquely increase in frequency during each menstrual cycle up to 400 times during a woman's life. Notably, little is known about dynamic changes in the uterine NK cell phenotype and KIR repertoire over time. To address this question, we studied menstrual blood uterine NK cells longitudinally in 10 donors. Comparing bulk expression of CD16, CD57, NKG2A, and pan-KIR on total NK cells (uterine and conventional) over several menstrual cycles revealed a marked variation in CD16 and CD57, but not in NKG2A and pan-KIR expression (Figure 5a and Supplementary Figure 3). This likely represents variations in the amount of conventional

NK cells that were retrieved from menstrual blood in different menstrual cycles.

Next, we analyzed the full KIR repertoires of uterine NK cells over sequential cycles. It has previously been shown that the KIR repertoire of peripheral blood NK cells in a healthy adult individual is stable over several years.²⁴ However, in contrast to peripheral blood, the endometrium undergoes marked physiological changes with each menstrual cycle, and changes in uterine NK cell KIR repertoires would thus not be surprising. Here, by simultaneously analyzing expression of six inhibitory and activating KIRs we could visualize 64 possible combinations of KIR expression in menstrual blood uterine NK cells (Figure 5b). Strikingly, the KIR repertoire of uterine NK cells was recapitulated in samples collected sequentially from the same donors (Figure 5b and Supplementary Figure 3). Moreover, the uterine NK cell KIR repertoire was clearly distinct from that of matched peripheral blood NK cells (Figure 5b).

In summary, in a given individual, each menstrual cycle sees the recapitulation of a highly similar uterine NK cell KIR repertoire.

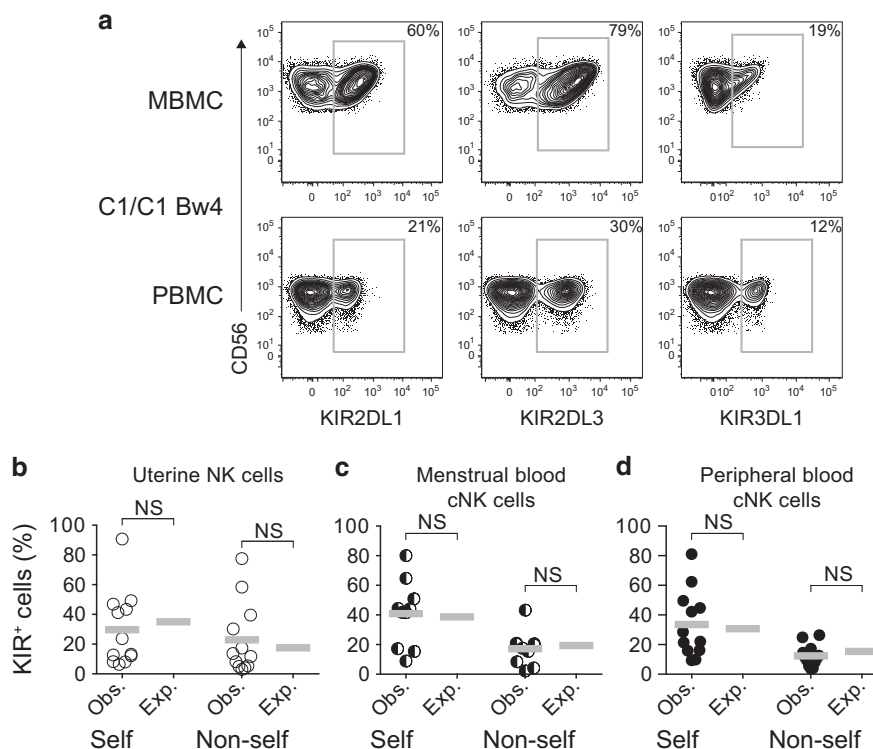


Figure 4 KIR expression on uterine NK cells is independent of cognate HLA class I ligands. **(a)** Stainings for KIR2DL1, KIR2DL3, and KIR3DL1 on matched uterine and peripheral blood NK cells from one representative C1/C1 Bw4 donor. **(b–d)** Observed (Obs.) frequencies of menstrual blood uterine NK cells **(b)**, menstrual blood conventional (CD16⁺) NK cells **(c)**, and peripheral blood CD16⁺CD56^{dim} NK cells **(d)** expressing self- and non-self KIRs compared with theoretical values of random KIR expression (expected; Exp.) analyzed for 12 individuals with 2 KIR ligands present (NS, not significant, Wilcoxon test).

The uterine NK cell compartment contains NKG2C⁺ self-KIR⁺ expansions

Peripheral blood NK cells can adapt to viral infections, most often to CMV, by generating clonal-like expansions of NKG2C⁺CD57⁺ populations displaying narrow KIR repertoires often characterized by expression of a single self-KIR.^{22–25} It is not known whether such expansions exist also in the uterus. In line with previous reports analyzing NK cells from decidua,^{38,39} we found that also uterine NK cells from menstrual blood uniformly expressed NKG2C **(Figure 6a)**. A more detailed analysis revealed that primarily the KIR⁺ uterine NK cells in menstrual blood expressed NKG2C **(Figures 6b and c)**.

The relatively low and homogeneous staining pattern of NKG2C on KIR⁺ uterine NK cells, in addition to the absence of CD57 expression **(Figure 1)**, did not permit the use of these receptors to identify NK cell expansions. Instead, we used our high-resolution KIR repertoire analysis to assess whether the uterine NK cell compartment contained KIR⁺ NK cell expansions. Among the 1,600 (64 × 25 donors) KIR co-expression frequencies assessed **(Figure 6d)**, seven KIR⁺ expansions were identified within the uterine NK cell compartment **(Figure 6e, f, and Supplementary Figure 4)**. As only two of the donors were CMV negative **(Table 1)**, it was not possible to associate presence or absence of KIR⁺ expansions with CMV status. Four of the uterine NK cell expansions were unique to that

compartment and not found in matched peripheral blood, whereas three donors had expansions shared between the uterine and peripheral blood NK cell compartments. Similar to NK cell expansions identified in peripheral blood,²⁴ all KIR⁺ expanded subsets identified within uterine NK cells expressed at least one inhibitory self-KIR **(Supplementary Figure 4)**.

Together, this indicates that the human uterine NK cell compartment can contain NKG2C⁺ self-KIR⁺ expansions.

DISCUSSION

In this study we have examined KIR expression on uterine NK cells obtained from menstrual blood. In menstrual blood, most uterine NK cells expressed KIRs, and the KIR repertoires were characterized by specificities distinct from matched peripheral blood NK cells, and with a composition biased toward KIR2D-receptor expression. Moreover, the uterine NK cell repertoires displayed a high degree of receptor co-expression and formation of the repertoires occurred independently of selection conferred by cognate HLA class I ligands. Longitudinal analysis revealed the KIR repertoires to be stable over time, despite that the endometrium regenerates with each menstrual cycle. Finally, NKG2C⁺ self-KIR⁺ uterine NK cell expansions were identified, which in some individuals were present independently of expansions in peripheral blood.

Previous work has shown that both endometrial and decidual tissues contain a high frequency of KIR⁺ uterine

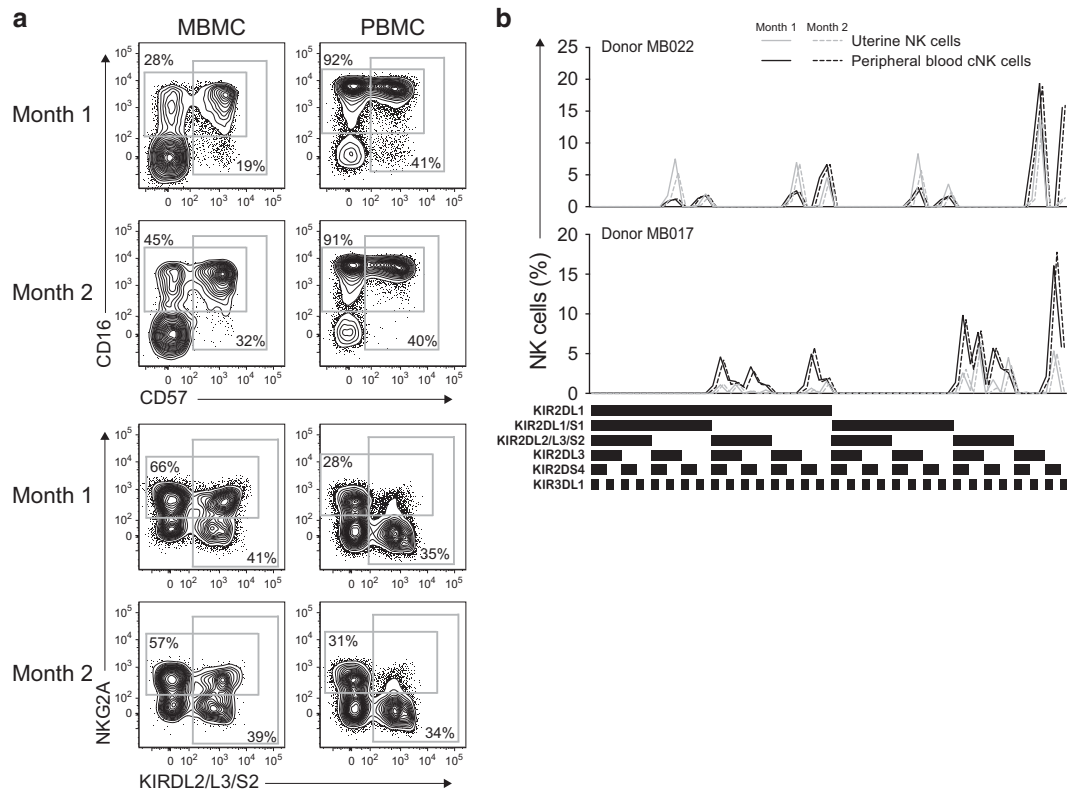


Figure 5 Longitudinal analyses of the uterine NK cell KIR repertoire over multiple menstrual cycles. **(a)** Representative staining of CD57, CD16, KIR2DL2/L3/S2, and NKG2A on matched menstrual (MBMC) and peripheral (PBMC) blood NK cells isolated from consecutive menstrual cycles. **(b)** KIR-repertoire plots of uterine NK cells (CD16⁺CD57⁺NKG2A⁺, gray filled or dashed lines) and CD16⁺CD56^{dim} peripheral blood NK cells (black filled or dashed lines). Boolean-combinations of the six gates for KIR (**Figure 2a**) were used to generate up to 64 distinct phenotypes. Data are representative of 10 donors.

NK cells.^{26–30} However, this earlier work often relied on one- or two-dimensional KIR expression analysis, without taking the exact specificities of anti-KIR antibodies into account. The high-resolution KIR analysis performed here, combining KIR genotyping with expression analysis of six distinct inhibitory and activating receptors, adds additional depth to our knowledge about KIR expression patterns on uterine NK cells.

A common notion is that the bias of KIR expression toward KIR2D receptor expression arises uniquely in pregnancy.^{6,31,32} Contrary to this, our data indicated that uterine NK cell KIR expression is distinct from matched peripheral NK cells and skewed toward KIR2D-receptors also in the endometrium of non-pregnant women. The potential role for maternal or fetal HLA class I molecules in selecting for certain KIRs to be expressed has previously been investigated on uterine NK cells from decidua.^{6,31} In these studies, neither maternal nor fetal HLA-C seemed to affect KIR2D expression. Our results are in line with previous reports, as we observed a random expression of self and non-self KIRs also on uterine NK cells isolated from menstrual blood.

Dynamics of uterine NK cell KIR expression has previously been assessed to some extent where KIR expression on uterine NK cells isolated from decidua has been shown to decrease with gestational age.^{28,37} We provide additional insight into uterine NK cell KIR repertoire dynamics by showing that the uterine

NK cell KIR repertoires are stable over menstrual cycles. This corroborates data on peripheral blood NK cells, where the KIR repertoire in healthy adults also changes very little over time.^{20,24} However, the peripheral blood NK cell KIR repertoire can adopt throughout the life span of an individual as exemplified by the KIR expression in newborns and young children being much lower compared with in adults.^{40,41} Moreover, during certain viral infections, clonal-like expansions of NKG2C⁺self-KIR⁺ peripheral blood NK cells can arise also leading to perturbations in the overall KIR repertoires.^{22–25} With respect to uterine NK cells, it is conceivable that menarche, pregnancy, and menopause might represent events that could cause alterations in the KIR repertoire. Furthermore, although the menstrual blood uterine NK cell KIR repertoire is similar at the end of each cycle, it is still unknown if dynamic changes occur throughout the menstrual cycle. For instance, the relationship between uterine NK cell KIR repertoires at the implantation window, i.e., the period when the uterus is receptive for implantation, to that found in menstrual blood (resembling decidualized endometrium) should be determined.

Uterine NK cells from women with unexplained infertility have been reported to display a bias toward KIR2D-receptor expression.²⁶ In contrast to this, poor spiral artery remodeling as well as insufficient trophoblast invasion has been associated

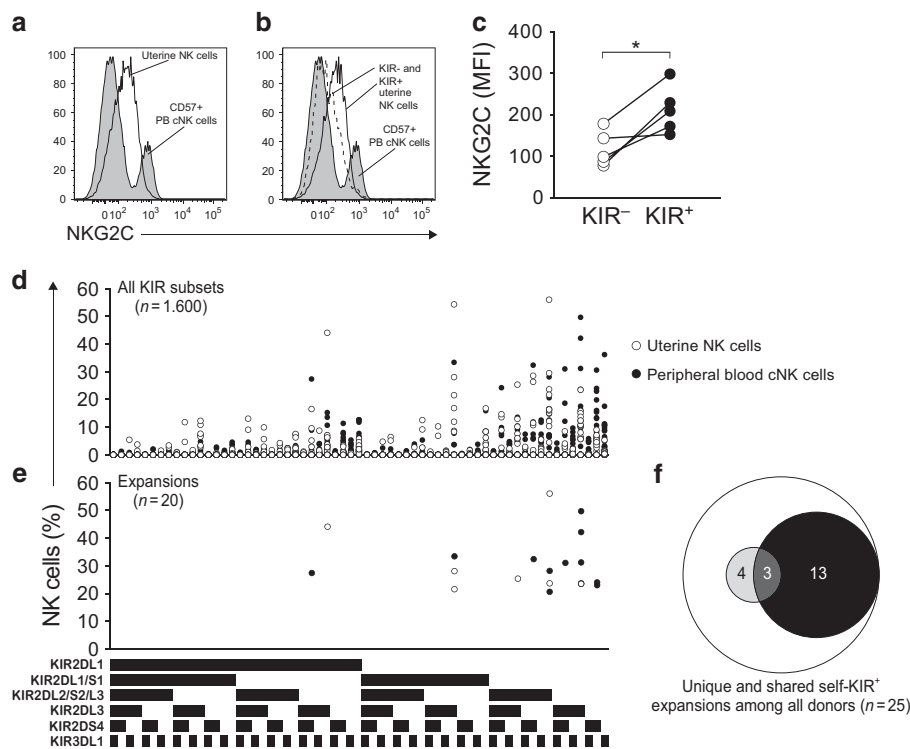


Figure 6 Menstrual blood contains NKG2C⁺ self-KIR⁺ uterine NK cell expansions. (a) Representative staining for NKG2C on CD9⁺ uterine NK cells (solid line) and CD57⁺ NKG2A⁻ CD16⁺ CD56^{dim} peripheral blood NK cells (gray histogram) from the same donor. (b) Representative staining for NKG2C on KIR⁺ CD9⁺ uterine NK cells (solid line), KIR⁻ CD9⁺ uterine NK cells (dotted line), and CD57⁺ NKG2A⁻ CD16⁺ CD56^{dim} peripheral blood NK cells (gray histogram). (c) Mean fluorescence intensity (MFI) of NKG2C expression on KIR⁺ and KIR⁻ uterine NK cells ($n = 5$, $*P < 0.05$, Wilcoxon test). (d) KIR repertoire plots of uterine NK cells (CD16⁻ CD57⁻ NKG2A⁺, open white circles) and NKG2A⁻ CD16⁺ CD56^{dim} peripheral blood NK cells (black circles) ($n = 25$ and 1,600 data points). (e) KIR repertoire plots of donors with expanded KIR⁺ uterine NK cell populations (open white circles) and/or NKG2A⁻ CD16⁺ CD56^{dim} peripheral blood NK cell populations (black circles). Expansions were defined with Tukey's range test in combination with frequency of parent population and of total NK cells. (f) Venn diagram illustrating unique or shared expansions between uterine (gray) and peripheral blood (black) NK cells in proportion to the total number of investigated donors (open circle).

with lower KIR2DL1 and/or KIR2DS1 expression.^{6,42} These studies investigate mechanisms underlying population genetic associations between KIRs and pregnancy disorders. In our analysis, we found no differences in uterine NK cell KIR expression when stratifying for use of contraceptives, previous pregnancies, or age. As KIR repertoires are highly variable between individuals, future studies should include significantly larger cohorts. For this, menstrual blood represents an attractive cellular source given its non-invasive and scalable nature for acquisition of clinical material.

We further report that uterine NK cells can harbor NKG2C⁺ self-KIR⁺ expansions. In peripheral blood NK cells, such imprinting of KIR repertoires has previously been linked to viral infections such as CMV.^{22–25} In the uterus it is conceivable that the semi-allogeneic setting present during pregnancy could promote the emergence of self-KIR⁺ expansions within the decidual NK cell compartment. However, in the current study we could detect self-KIR⁺ uterine NK cell expansions also in nulliparous women. To identify the underlying mechanisms generating such expansions in the uterus, future studies should aim at characterizing self-KIR⁺ expansions longitudinally in larger cohorts of women of child-bearing age, before and after a pregnancy, as well as in uterine NK cells obtained from decidua.

In conclusion, we have performed a high-resolution analysis of KIRs on uterine NK cells obtained from menstrual blood and find these cells to have unique KIR repertoires, with a high degree of KIR co-expression. The repertoires are cyclically regenerated and NKG2C⁺ self-KIR⁺ expansions can be found within the uterine NK cell population. These findings provide new perspectives for understanding how variegated KIR repertoires are formed and challenges the notion that biased expression of KIR repertoires arises uniquely during pregnancy. Finally, our results open up for future studies using menstrual blood to mechanistically assess how KIRs and their HLA class I ligands regulate uterine NK cells, and how this may influence pregnancy outcomes.

METHODS

Collection of menstrual and peripheral blood NK cells. Blood samples were collected after oral and written informed consent had been retrieved and with the approval from the Regional Ethics Review Board, Stockholm, Sweden. Menstrual blood was collected as previously described.^{33,34} In brief, women of child-bearing age with regular menstrual cycles and no known gynecological diseases (Table 1) were provided with a menstrual cup (Lunette, Lune Group, Finland), collection tubes, and plastic pipettes. Collection tubes contained 10 ml collection medium Rosewell Park Memorial Institute

(10% Fetal Calf Serum, Penicillin, Streptomycin, 40 U ml⁻¹ heparin (LEO Pharma, Zaventem, Belgium), 50 µg ml⁻¹ Gentamicin (Gibco, Carlsbad, CA), 2.5 µg ml⁻¹ Fungizone (Gibco)) and were kept refrigerated until use. Menstrual blood was collected during menstruation days 1 and 2. Mononuclear cells from menstrual blood and peripheral blood were isolated using lymphoprep (GE Healthcare, Uppsala, Sweden). Isolated cells were cryopreserved for subsequent batched analysis. Net menstrual blood volumes varied between 5 and 40 ml. Out of the 25 women, seven donated blood twice, two women three times, and one woman four times. The median age of the cohort was 30 years, 40% of the women reported using contraceptives, 60% had been previously pregnant, 6 out of 25 had experienced a previous miscarriage, the median menstruation duration was 3.5 days, and the median menstrual cycle length was 27 days (Table 1).

Antibodies and flow cytometry. CD3 (UCHT1, PE-Cy5, Beckman Coulter, Atlanta, GA, or OKT3, Brilliant Violet 785, BioLegend, or SK7, APC, BD Biosciences, San Jose, CA), CD4 (OKT4, PE-Cy5, BioLegend, San Diego, CA, or RPA-T4, BB515, BD Biosciences), CD8 (SK1, APC-H7, BD Biosciences), CD9 (eBioSN4, eFluor 450, eBioscience, San Diego, CA, or M-L13, Horizon V450, BD Biosciences), CD14 (M5E2, Horizon V500, BD Biosciences, or 61D3, PE-Cy5, eBioscience), CD15 (MMA, Horizon V500, BD Biosciences), CD16 (3G8, FITC, APC-Cy7, or Brilliant Violet 711, BioLegend), CD19 (HIB19, Horizon V500, BD Biosciences, or PE-Cy5, BioLegend), CD45 (HI30, Alexa Fluor 700, BioLegend), CD45RA (HI100, Brilliant Violet 785, BioLegend), CD56 (N901, ECD, Beckman Coulter), CD57 (TB01, purified, eBioscience, or NK-1, Brilliant Violet 605, BD Biosciences), CD69 (TP1.55.3, ECD, Beckman Coulter), CD161 (191B8, biotin, Miltenyi Biotech, Bergisch Gladbach, Germany), CD197 (G043H7, Brilliant Violet 421, BioLegend), IFN-γ (4S.B3, Brilliant Violet 711, BioLegend), Ki67 (Ki67, BV421, BioLegend), KIR2DL1 (REA284, APC-Vio770, Miltenyi), KIR2DL1/S1 (EB6, PE-Cy7, Beckman Coulter), KIR2DL2/3/S2 (GL183, PE-Cy5.5, Beckman Coulter), KIR2DL3 (180701, FITC, R&D Systems), KIR3DL1 (DX9, Alexa Fluor 700 or Brilliant Violet 711, BioLegend), NKG2A (Z199, APC, Beckman Coulter), NKG2C (134591, PE, R&D Systems, Minneapolis, MN), and TNF (MAb11, PE-CF594, BD Biosciences). Purified KIR2DS4 (179315, R&D Systems) was biotinylated using a Fluoreporter Mini-biotin-XX protein labeling kit (Life Technologies). Biotinylated antibodies were detected using streptavidin-Qdot 585 (Invitrogen, Carlsbad, CA). Purified CD57 antibody (IgM) was detected using a secondary anti-IgM (eFluor650, eBioscience). All samples were stained with Live/Dead Aqua or Green (Invitrogen) to discriminate live and dead cells. Detection of KIR2DS1 was achieved as previously described.⁴³ Foxp3/Transcription Factor staining kit (eBioscience) was used to fix and permeabilize cells. After cryopreservation, viability was assessed by double staining with a viability dye (Live/Dead Near infrared, Invitrogen) and for Annexin-V (FITC, BD) expression, which consistently gave a viability of 80–90%. Samples were analyzed using a BD LSR Fortessa equipped with four lasers and 20 detectors. Data were analyzed using FlowJo (Tree Star, Ashland, OR). As all flow cytometry stainings were performed on cryopreserved cells, validation experiments were performed comparing matched freshly isolated and previously cryopreserved menstrual blood NK cells. From these, a slight reduction in MFI of markers studied was noticed on previously cryopreserved cells. However, cryopreservation did not impact the possibility to gate on positive and negative events.

HLA and KIR genotyping. Genomic DNA was isolated from blood using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). KIR genotyping was performed using PCR-SSP technology with a KIR typing kit (Olerup-SSP, Stockholm, Sweden). KIR ligands were determined with the KIR HLA ligand kit (Olerup-SSP), which detects the HLA-C1, -C2, and -Bw4 motifs.

CMV^{pp65}-stimulation assay. PBMC or menstrual blood mononuclear cells were thawed and distributed in a 96-well U-bottom plate at a final

concentration of 10 × 10⁶ cells ml⁻¹. Subsequently, cells were incubated in the presence or absence of CMV^{pp65}-overlapping peptides (JPT Peptide Technologies, Berlin, Germany, 1 mg ml⁻¹ for each peptide). Brefeldin A was added 4 h after adding the peptides. After 18 h of total incubation time, the cells were surface stained, permeabilized, and stained for intracellular interferon (IFN)-γ and Tumor necrosis factor (TNF). Donors were considered CMV positive when >0.1% of T cells responded with IFN-γ and TNF or when a distinct IFN-γ⁺TNF⁺ population could be identified in response to CMV^{pp65}-overlapping peptides.

Statistical methods and outlier identification. The 64 KIR-expressing subsets were not normally distributed and, therefore, we could not perform outlier analysis using the Chauvenet criterion as previously described.²⁴ Instead, Tukey's range test was used to identify outliers. This method is not dependent on distributional assumptions and ignores the mean and s.d., making it resistant to influence from the extreme values in the range. First and third quartiles were calculated for each KIR co-expressing subset (63 in total, excluding KIR-negative cells). Next, each data point was determined to be either inside or outside the interquartile range, using 1.5 multiplication of the range. Similar to previous reports on peripheral blood NK cells,²⁴ we set as inclusion criteria that a KIR⁺ subset had to represent at least 20% of the parent population (uterine NK cells or CD16⁺ conventional NK cells), as well as at least 5% of total NK cells (defined as in Figure 1), to be considered as an expansion. Combinatorial analysis and product rule analysis was performed as previously described.^{18,21}

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

ACKNOWLEDGMENTS

This work was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, the Swedish Cancer Society, the Swedish Society for Medical Research, the Cancer Research Foundations of Radiumhemmet, the Swedish Society of Medicine, the Novo Nordisk Foundation, Åke Olsson's Foundation, Bengt Ihre's Foundation, Magnus Bergwall's Foundation, Julin's Foundation, Åke Wiberg's Foundation, and the Stockholm County Council. MAI is funded by the Wenner-Gren Foundations. We thank D. Hans-Gustaf Ljunggren for critically reviewing the manuscript, medical student Daniel Samsami, Dr Aino Fianu Jonasson, and research midwives Margaretha Ström and Maria Karlsson for assistance with samples, and all donors that participated in the study.

DISCLOSURE

The authors declared no conflict of interest.

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