Killer Ig-Like Receptor Expression in Uterine NK Cells Is Biased toward Recognition of HLA-C and Alters with Gestational Age[1]

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Immunogenetic studies suggest that interactions between maternal killer Ig-like receptor (KIR) expressed by uterine NK (uNK) cells, and fetal HLA-C molecules on trophoblast, influence the success of human placentation. However, the exact functional response of fresh uNK cells to trophoblast HLA-C molecules is unknown. In this study, we show by quantitative RT-PCR and FACS that both activating and inhibitory KIR specific for HLA-C are expressed at higher levels and on an increased proportion of NK cells in the human decidua compared with blood. In contrast, expression of KIR3DL1/S1, which is specific for HLA-B, is similar in both NK cell populations. Remarkably, there is also a temporal change in the expression pattern of HLA-C-specific KIR, with a decline in both intensity of expression and frequency on uNK cells throughout the first trimester of pregnancy. This selective up-regulation of KIR has functional consequences because uNK cells show increased binding of HLA-C tetramers compared with blood NK cells. Ab cross-linking shows that these KIR are functional and results in increased cytokine secretion. uNK cells, therefore, exhibit a unique KIR profile that enhances their ability to recognize trophoblast cells expressing HLA-C at the materno-fetal interface. This is the first report to demonstrate selective regulation of KIR expression over time in vivo in a normal physiological situation and suggests that KIR expression by uNK cells is regulated by the tissue microenvironment in the decidua. ***The Journal of Immunology,*** **2008, 181: 39–46.**

The uterine decidua contains large numbers of $CD56^{bright}$ NK cells in the first half of pregnancy. At this stage of gestation when the placenta is formed, extravillous trophoblast cells derived from the fetus migrate deeply into the decidua and myometrium. These trophoblast cells home to the maternal spiral arteries and destroy the muscular wall resulting in vascular dilatation that increases the maternal blood flow to the placenta, a process essential for normal fetal growth and development (1, 2). Failure to adequately transform these spiral arteries results in poor placental perfusion and underlies many common diseases of pregnancy including fetal growth restriction, pre-eclampsia, and recurrent miscarriage (3).

How trophoblast invasion is regulated is unknown, but several lines of evidence indicate that the uterine immune system is involved, in particular the uterine NK (uNK)[4] cells that amass at the implantation site. In an immunogenetic study, we showed that specific combinations of two polymorphic gene systems, killer Ig-like receptors (KIR) in the mother and HLA-C in the fetus were associated with pre-eclampsia (4). In the decidua, KIR are expressed only by uNK cells and their cognate ligands include HLA-C molecules that are expressed by trophoblast (5–8).

KIR can function either as inhibitory or activating receptors distinguished by the length of the cytoplasmic tail; long-tailed (L) are inhibitory and short-tailed (S) are activating (9, 10). KIR haplotypes differ in two ways: there is variation in the number of *KIR* genes present and also allelic polymorphism at each individual *KIR* locus. "A" haplotypes have seven *KIR* genes that are mostly inhibitory while "B" haplotypes have a variable number of additional genes that mainly have activating functions. For those KIR that recognize HLA-C, all HLA-C allotypes can be divided into two groups that differ at position 80: HLA-C1 (Asn^{80}) are bound by KIR2DL2/3 whereas HLA-C2 (Lys^{80}) are bound by KIR2DL1. There are short-tailed activating KIR with similar extra-cellular domains that also have the potential to bind to C1 and C2 groups; these are KIR2DS2 and 2DS1, respectively. Almost all individuals have inhibitory KIR for both HLA-C groups but the equivalent activating KIR that are found on the B haplotype are only present in some individuals (9, 10).

We found that mothers with the *KIRAA* genotype, in which inhibitory KIR predominate, were at increased risk of pre-eclampsia, but only when confronted by a fetus expressing a group 2 HLA-C

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[4] Abbreviations used in this paper: uNK, uterine NK cell; KIR, killer Ig-like receptor; pbNK, peripheral blood NK cell; VEGF, vascular endothelial growth factor.

allele (HLA-C2) (4). Insight into how these immunogenetic findings may translate into a functional mechanism has come from a recent study that shows that decidual NK cells may control trophoblast migration and also directly affect the maternal vasculature (11). uNK cells differ phenotypically from both the $CD56^{bright}$ and $CD56^{dim}$ subsets in blood (12, 13). They produce chemokines (IP-10 and IL-8) that directly enhance trophoblast invasion as well as the angiogenic factors vascular endothelial growth factor (VEGF), placenta growth factor, and Ang2 (11, 14, 15). The secretion of these growth factors by uNK cell clones is reduced following KIR2DL1 binding to HLA-C2 (11). Thus, KIR/HLA-C interactions can regulate factors known to influence trophoblast invasion and local vascular remodeling.

The functional responses of uNK cells to trophoblast in each pregnancy will vary depending on which KIR are displayed by the mother's uNK cells and the HLA-C status of the trophoblast. In a previous study, we found that the KIR known to recognize HLA-C were expressed by a higher proportion of decidual NK cells compared with peripheral blood NK (pbNK) cells (5). The expression levels of these HLA-C-specific KIR by uNK cells were also higher than in blood. These experiments were performed with mAbs that cannot distinguish between the inhibitory and activating forms of these KIR due to the similarity of their extra-cellular domains. In addition, the KIR genotype of patients in the study was unknown. The relative expression in uNK cells of activating and inhibitory KIR that recognize HLA-C is, therefore, unclear. This will greatly influence the functional outcome that results from triggering of KIR by trophoblast HLA-C.

We have now investigated the expression of individual KIR in fresh uNK cells from women of known *KIR* genotypes during the first trimester. We show that in uNK cells at the start of pregnancy, there is a selective increase in mRNA and protein expression of both activating and inhibitory KIR that recognize HLA-C compared with pbNK cells, and expression of these KIR in uNK cells declines as gestation proceeds. We show for the first time that uNK cells exhibit enhanced binding of HLA-C tetramers compared with blood NK cells, due to up-regulation of KIR2DL3 and that ligation of KIR alters cytokine secretion by fresh uNK cells. Given that most studies in humans have used blood NK clones or cytokine-stimulated NK cells, these studies are important in understanding how fresh uNK cells will respond to trophoblast in different pregnancies.

Materials and Methods

Primary cells and tissue

PBMC were obtained from fresh venous blood of normal adult volunteers and depleted of T cells using the RosetteSep system (Stem Cell Technologies). Decidual leukocytes were isolated by enzymatic digestion of maternal decidual tissue from elective first trimester terminations of pregnancy (between 6 and 12 wk) as described previously (16). Primary trophoblast cells were isolated from first trimester placental villi and cultured overnight on fibronectin before analysis by FACS (16). Ethical approval was obtained from the Cambridge Local Research Ethics Committee and the University of Western Australia Human Ethics Committee. Genomic DNA for KIR typing and total RNA were isolated from purified cells using the QIAamp and RNeasy Mini kits, respectively (Qiagen).

Genotyping of KIR and HLA-C

KIR and HLA-C were genotyped in patient's genomic DNA using previously published methods (4, 17). In brief, the *KIR* genes were genotyped for presence or absence by PCR with sequence-specific primers using two pairs of primers per gene or allele. In some cases, primers with mismatched bases next to the specific 3′ terminal base were used to improve discrimination. *KIR* genes typed were *2DL1*, *2DL2*, *2DL3*, *2DS1*, *2DS2*, *3DL1*, and *2DL5*. Typing for HLA-C was performed using a similar approach, which allowed all known HLA-C group C1 alleles to be distinguished from C2 alleles (16).

Table I. *Primers and probes for genotyping and also for real-time RT-PCR for individual KIR cDNA transcripts*[a]

Gene	Primer	Size (bp)
2DL1		
For1	GTTGGTCAGATGTCATGTTTGAA	127
Rev1mm	GGTCCCTGCCAGGTCTTG**G**G	
Probe	ACTTCCTTCTGCACAGAGAGGG	
2DL2		
For2mm	GAGGGGGAGGCCCATGA**G**T	150
Rev2mm	TCGAGTTTGACCACTCGT**G**T	
Probe	TTCTCTGCAGGGCCCAAGGTCAA	
2DL3		
For2	TCCTTCATCGCTGGTGCTG	255
Rev2	GGCAGGAGACAACTTTGGATCA	
Probe	AAATGCTGTTGTAATGGACCAAGAGCCTGCA	
2DS1		
For1mm	CTTCTCCATCAGTCGCATG**T**A	100
Rev1	AGGGTCACTGGGAGCTGACAA	
Probe	AAGACCTGGCAGGGACCTACAGATGCTAC	
2DS2		
For2	CGGGCCCCACGGTTT	240
Rev2	GGTCACTCGAGTTTGACCACTCA	
Probe	AGAGCGTGACCTTGTCCTGCAGCT	
2DL5		
For1	GGAGGACATGTGACTCTTCT	197
Rev1	GACCACTCAATGGGGGAGC	
Probe	TGTCGCTCTCGTCTTGGGTTTAC	
3DL1		
For1	CGCTGTGGTGCCTCGA	197
Rev1	GGTGTGAACCCCGACATG	
Probe	TTCCCTGCATGTGCTGTGGTCA	

[a] Mismatched bases (mm, used to improve specificity) are shown in bold and underlined. Probes were labeled with FAM dye at the 5′ end and TAMRA at the 3′ end.

Quantitation of KIR mRNA levels by real-time RT-PCR

Levels of individual *KIR* transcripts were measured by real-time RT-PCR using a fluorescently labeled TaqMan probe designed to bind to the PCR product produced by the same primers as used for genotyping. Total RNA was prepared from PBMC from female ($n = 10$) and male ($n = 9$) volunteers after T cell depletion and also from samples of decidual mononuclear cells ($n = 26$). First strand cDNA was prepared from 1 μg of each RNA sample using random hexamers and Superscript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR was performed for specific KIR cDNAs using the ABI PRISM 7500 detection system (Applied Biosystems). Primers were used at a final concentration of 900 nM and probes at 200 nM, diluted in ABsolute QPCR polymerase mix (ABgene). Primer and probe sequences are shown in Table I. After denaturation at 95°C for 15 min, 7 cycles consisting of 15s at 95°C and 60s at 65°C were followed by 36 cycles of 15s at 95°C and 60s at 60°C. The specificity of the assay for each *KIR* was tested by amplification of a panel of 10 plasmids each containing a different KIR cDNA. Primers were only accepted when the only cDNA amplified was from the correct target *KIR* (data not shown). This method was used to measure the relative levels of each *KIR* transcript in RNA from total decidual leukocytes taken at the same gestational age (9–10 wk). This was compared with *KIR* expression in PBMC isolated from normal men and non-pregnant women following T cell depletion of blood. The cell fractions compared are, therefore, $CD56^+CD3^-$ blood NK cells and $CD56^+$ uNK cells. FACS analysis showed that all samples contained between 60 and 75% NK cells and were, therefore, comparable.

Expression levels of each *KIR* mRNA (except *2DL3*) were measured in arbitrary units relative to a standard curve generated by serial dilution of a genomic DNA sample containing all the KIR genes. Because the 2DL3 primers cross an intron, *2DL3* mRNA expression was measured using a standard curve of a plasmid containing *2DL3* cDNA, diluted to the same number of copies as the genomic DNA. The endogenous control, *18S ribosomal RNA* was measured in the same samples using probes supplied by Applied Biosystems. Expression values obtained for each KIR transcript were normalized against ribosomal *18S* to correct for loading differences. Expression levels of KIR between different groups were compared using the non-parametric Mann-Whitney *U* test. Differences were considered statistically significant when $p < 0.05$.

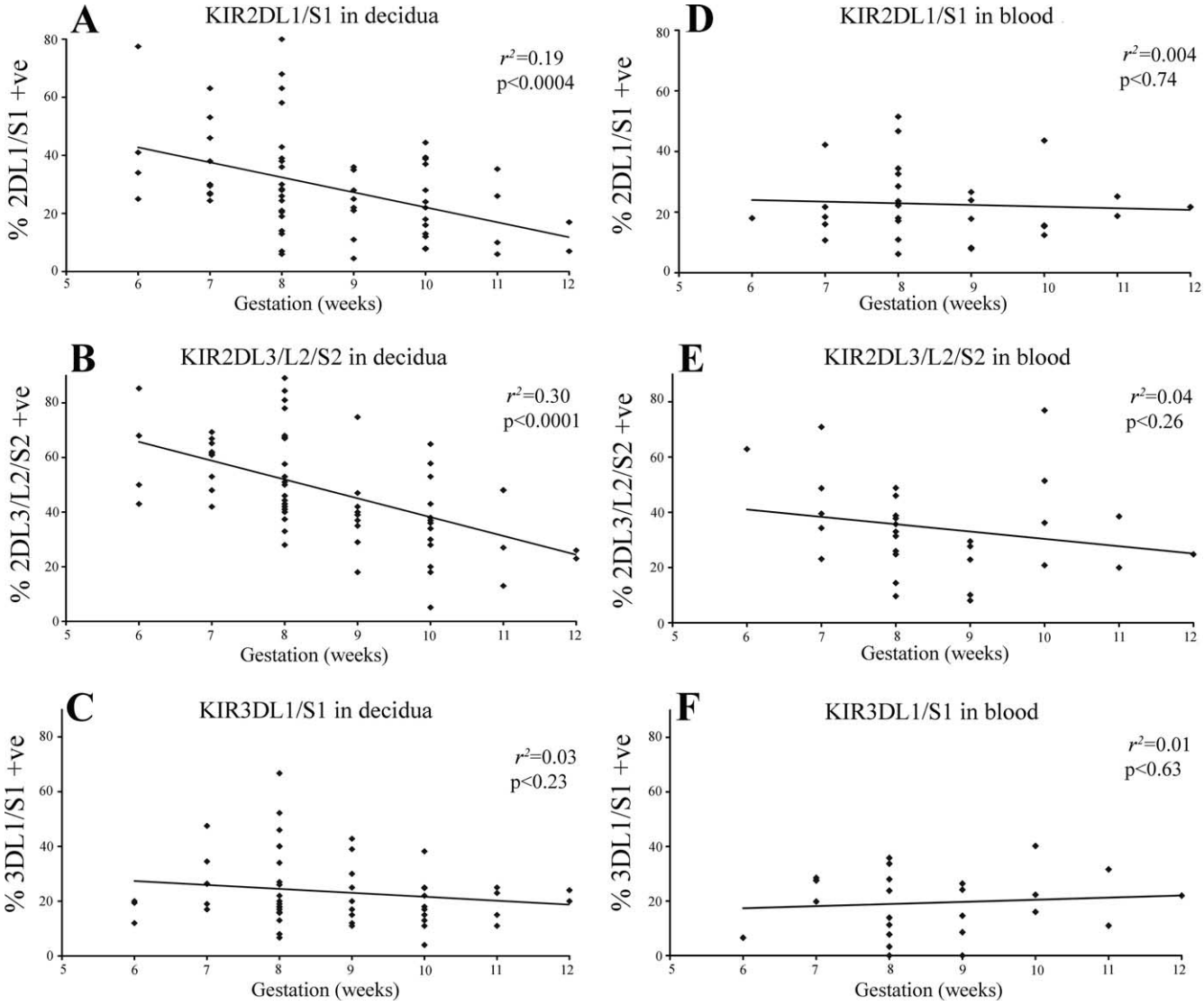

FIGURE 1. Analysis of KIR expression in uterine and pbNK cells during the first trimester of pregnancy. $CD56^+$ NK cells from blood and decidua (6- to 12-wk gestation) were analyzed by two-color immunofluoresence analysis using anti-CD56 and anti-KIR mAbs recognizing either KIR2DL1/S1 (*A* and *D*), KIR2DL3/L2/S2 (*B* and *E*), or KIR3DL1/S1 (*C* and *F*). Each symbol represents a different patient sample gated on $CD56^+$ cells. For each Ab, a best-fit line was drawn and the *p*-value indicates whether the correlation coefficient (r^2) differs significantly from zero. The percentage of uNK cells ($n = 62$ patients) staining with Ab to KIR2DL1/S1 (*A*) or KIR2DL3/L2/S2 (*B*) declined significantly during the first trimester, whereas the proportion expressing KIR3DL1/S1 (*C*) did not. KIR expression was also analyzed in peripheral NK cells in paired blood samples obtained from 29 of these patients and showed no significant change in KIR expression during this period (*D–F*).

Cell surface staining for flow cytometry

Staining for flow cytometric analysis was performed on 10^6 cells using a FACScan cytometer (BD Biosciences). Expression of KIR2D molecules was monitored using the Abs EB6 and GL183 (Immunotech), followed by a goat anti-mouse-FITC secondary Ab (Sigma-Aldrich). GL183 is specific for KIR2DL3/2DL2/2DS2, which recognize group 1 HLA-C alleles (HLA-C1), whereas EB6 recognizes KIR2DL1/2DS1, which bind to HLA-C2 alleles (18). Free secondary Ab binding sites were blocked with 50 μg/ml polyclonal mouse IgG (Sigma-Aldrich) and cells were then stained using directly conjugated Abs against CD56 (PE) (BD Biosciences). Expression of KIR3DL1 and 3DS1 was detected using Z27 or DX9 (BD Biosciences). All Abs for cell surface FACS were of the IgG1 isotype and relevant IgG1 mAb controls were used in each case. For FACS analysis of primary cells using a fluorescent tetramer of HLA-C1, 1×10^6 cells were incubated with 1 μg of tetramer conjugated to R-PE in the presence of CD56-FITC or the relevant FITC-labeled isotype control. The recombinant tetramer was HLA-Cw*0102 refolded using the peptide VAPWNSLSL; this is an HLA-C1 allotype (ProImmune). The negative control for tetramer staining was IgG2a-PE. Following staining, cells were fixed for 10 min in 2% paraformaldehyde before analysis. Primary trophoblast cells were stained with PE-conjugated tetramers of KIR2DL1*00301 (1 μg/ml) and KIR2DS1*001 (25 μg/ml) produced as described previously (19). The mAb 6A4 (IgG1), which recognizes HLA class 1 molecules, was used at 10 μg/ml to block KIR-tetramer binding (19, 20). Trophoblast cells were identified by staining with the mAb G233-FITC from our laboratory (21). Differences in the percentage of cells staining for HLA-C tetramer in blood and decidua were compared using the non-parametric Mann-Whitney *U* test. The relationship between KIR expression and gestational age was analyzed by calculating the Pearson correlation coefficient (r^2).

Cytokine production by decidual leukocytes

Individual wells of a 24-well plate were coated with the anti-KIR Ab HP-3E4 (22), or its isotype control (IgM), and freshly isolated total decidual leukocytes were cultured for 16 h in DMEM/F12, 10% FCS, 2 mM glutamine and antibiotics. The concentration of IFN-γ in the culture supernatants was measured using the DuoSet ELISA (R&D Systems). To identify the source of IFN-γ, intracellular staining for IFN-γ was performed on parallel cultures using mAb clone 25723.11 (IgG2b) labeled with FITC or the negative control clone 27–35-FITC (BD Pharmingen). Brefeldin (Sigma-Aldrich) was added to the cultures at 10 μg/ml, 12 h

after the culture was started, to increase intracellular cytokine staining. Cells were surface labeled 4 h later with anti-CD56 PE and anti-CD9 PC5 (BD Biosciences), fixed for 15 min in 3% paraformaldehyde in PBS, and then intracellular staining was performed using the manufacturer's protocol (BD Pharmingen).

Results

The frequency of uNK cells expressing KIR specific for HLA-C decreases during the first trimester

Our previous study showed that the pattern of KIR expression in uNK cells during pregnancy is clearly different to that in pbNK cells from pregnant or non-pregnant individuals (5). To establish when these differences arise, we examined KIR expression by flow cytometry in uNK cells isolated from 62 samples throughout the first trimester (6–12 wk). This corresponds to the main period of trophoblast invasion into the decidua. $CD56^+$ uNK cells were stained with mAbs that recognize KIR2DL3/L2/S2 and KIR2DL1/S1. It was striking that a very high percentage of $CD56^+$ cells in decidua expressed KIR specific for HLA-C1 and C2 groups at 6- to 7-wk gestation (40–90% are $2DL3/L2/S2^+$ and 25–80% are $2DL1/S1^+$; Fig. 1, *A* and *B*). There was a significant decline in the frequency of $CD56^+$ cells staining for these KIR throughout the first trimester ($p < 0.0004$). KIR3DL1 and 3DS1 are specific for HLA-Bw4 allotypes that, unlike HLA-C, are never expressed by trophoblast.

Peripheral blood samples were obtained at the same time from 29 of these women. In contrast to uNK cells, KIR expression in blood NK cells showed no significant change throughout the first trimester (Fig. 1, *D–F*). During early pregnancy, the percentage of uNK cells expressing KIR2DL3/L2/S2 or KIR2DL1/S1 was significantly higher than in the corresponding pbNK cells from the same patient. However, as expression of these KIR declined in uNK cells with gestation, the difference in KIR expression between decidua and blood disappeared (Fig. 1). In contrast, expression of KIR3DL1/S1 was similar in blood and uNK cells and showed no significant change throughout early pregnancy (Fig. 1, *C* and *F*).

These results show that during the very early stages of placentation, expression of those KIR specific for HLA-C is significantly increased in $CD56^+$ uNK cells compared with other KIR specific for HLA-B alleles. There is a gradual decline in frequency of uNK cells expressing these HLA-C-specific KIR during the first three months of pregnancy. The KIR repertoire is, therefore, regulated differently in blood and uNK cells during the first trimester of pregnancy and individual KIR show different expression profiles in the decidua over this period.

Quantitation of individual KIR transcripts in blood and decidua

Because the mAbs that recognize KIR2DL3/L2/S2 and KIR2DL1/S1 cannot distinguish between activating and inhibitory forms of the relevant KIR, flow cytometry cannot reveal whether the up-regulation of HLA-C-reactive KIR is due to enhanced expression of inhibitory and/or activating KIR. We developed a quantitative RT-PCR method based on the primers used for genotyping to measure the relative expression levels of those KIR RNA transcripts known to bind HLA-C, the activating KIR, 2DS1, and 2DS2 and the inhibitory KIR, 2DL1, 2DL2, and 2DL3. For comparison, we also measured the expression of KIR2DL5 and KIR3DL1.

To minimize differences in expression solely due to gestational age, the relative levels of each KIR transcript were measured in RNA from total decidual leukocytes taken between 9 and 10 wk of gestation. This was compared with KIR expression in PBMC following T cell depletion of blood from normal men and non-pregnant women. FACS analysis showed that all samples used for RNA isolation contained between 60 and 75% $CD56^+$ NK cells

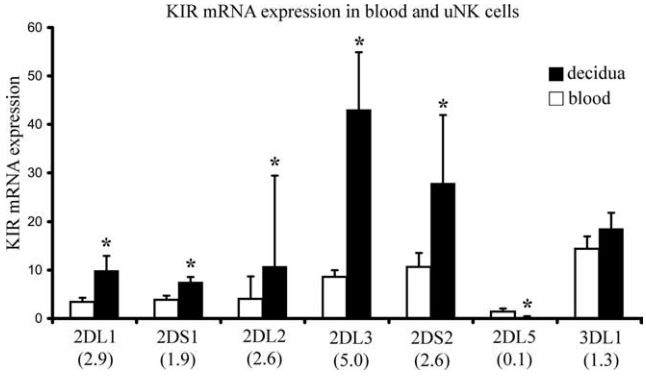

FIGURE 2. Relative expression of transcripts for KIR in blood and uNK was measured by real-time RT-PCR. The transcript values plotted are the median and SE measured in arbitrary units and corrected for 18S expression between samples. Figures in brackets indicate the fold difference in expression for each KIR transcript in uNK compared with blood NK cells. * indicates the up- or down-regulation in uNK cells compared with blood was statistically significant ($p < 0.02$).

and were, therefore, comparable. Median expression levels for each KIR in blood and decidual NK cells were determined using only those patients who typed positive for that particular KIR (Fig. 2). We found no significant differences in expression for any of the KIR measured in pbNK cells from men and women. KIR expression data from pbNK cells from both sexes was, therefore, used in subsequent comparisons with uNK cells. Expression levels of KIR3DL1 were not significantly different between NK cells from blood and decidua. In contrast, all KIR specific for HLA-C were expressed in uNK cells at levels between 1.9- and 5-fold higher than in pbNK cells. Surprisingly, KIR2DL5 expression in uNK cells was nearly 10-fold lower than in blood. In blood NK cells, the KIR transcripts showing the highest median expression levels encode 3DL1 followed by 2DS2 and 2DL3. In uNK cells, the expression pattern is different, with 2DL3 and 2DS2 more abundant than 3DL1 whereas 2DL5 was almost absent. uNK cells, therefore, show up-regulation of both activating and inhibitory KIR that recognize HLA-C. The fact that KIR3DL1 expression is similar in both compartments suggests the independent regulation of certain KIR mRNA transcripts in NK cells between blood and decidua.

uNK cells show increased binding to HLA-C tetramers

As well as the increased frequency of uNK cells expressing HLA-C-specific KIR, the levels of expression detected on each cell as assessed by the intensity of staining by the relevant mAbs was also increased compared with pbNK cells (data not shown). To determine whether this altered the functional interactions between the uNK cells and fetal trophoblast, we examined binding of tetrameric complexes of HLA-C to freshly isolated uNK cells. HLA class I tetramers have been used to identify HLA-specific primary T cells in peripheral blood but the affinity of the KIR/HLA interaction is low and binding of HLA-C tetramers to primary NK cells has not been described (23). We found that a tetramer of HLA-Cw*0102, an allotype previously shown to bind to KIR2DL3/L2 (24), bound to uterine $CD56^+$ cells (Fig. 3*C*). This tetramer binding was completely abolished by prior incubation with mAb specific for KIR2DL3/L2/S2, indicating that binding of this HLA-C1 tetramer is mediated by the KIR recognized by this Ab (Fig. 3*D*). As expected, preincubation with a control mAb to KIR2DL1/S1 had no significant effect on HLA-C1 tetramer binding (Fig. 3*E*). In contrast, the same tetramer showed significantly lower binding to pbNK cells ($p < 0.01$, Mann-Whitney *U* test; Fig. 4). A total of

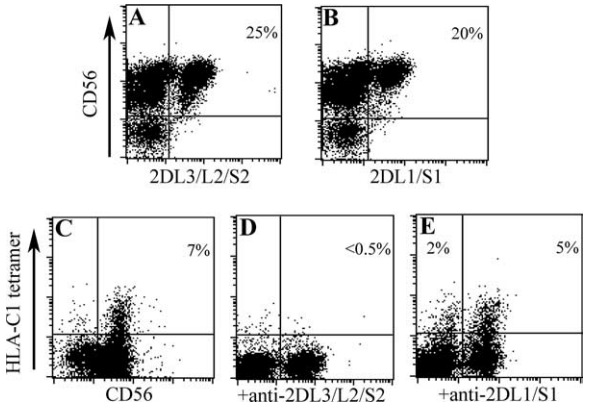

FIGURE 3. HLA-C1 tetramers bind to fresh uNK cells. Decidual leukocytes were analyzed by FACS following staining with mAbs to CD56 and KIR. *A* and *B*, KIR expression is confined to $CD56^+$ uNK cells, and, in this patient, 25 and 20% of decidual leukocytes stain positive for KIR2DL3/L2/S2 or KIR2DL1/S1, respectively. *C*, Staining of the same cells with HLA-C1 tetramer shows binding to 7.5% of decidual leukocytes; almost all of this binding is to $CD56^+$ cells. *D*, Tetramer binding is blocked after preincubation with mAb specific for KIR2DL3/L2/S2, before HLA-C1 staining. *E*, Preincubation with Ab to KIR2DL1/S1 had no effect on total tetramer binding.

8.3% (±2.2) of $CD56^+$ NK cells in decidua stained positive for the HLA-C1 tetramer, compared with 2.1% (±0.4) of blood $CD56^+$ NK cells. This formally demonstrates that up-regulation of KIR surface expression on primary uNK cells results in significantly increased binding to HLA-C molecules.

The increased binding of the HLA-C1 tetramer in uNK cells could be due to up-regulation of the inhibitory KIR that bind this molecule (2DL3/L2) or of the activating receptor 2DS2. The mAb GL183 cannot distinguish between these molecules. By combining flow cytometry using anti-CD56 and GL183, with KIR genotyping of donors, we could examine expression of 2DL3/L2/S2 in blood and uNK cells (Fig. 5*A*). Through genotyping, we were able to identify those patients who were *KIR2DL3*$^+$*/L2*$^-$*/S2*$^-$. In these patients, the increased staining in uNK cells with this mAb is due to increased 2DL3 protein expression alone, because 2DL2 and 2DS2 are absent. Secondly, in these same patients, binding of Cw1 tetramer to uNK cells is due to 2DL3 alone, because it was blocked by GL183, which recognizes 2DL3/L2/S2 (Fig. 4). In support of this observation, the staining intensity of uNK cells with this mAb correlated well with binding of the HLA-C1 tetramer, in patients genotyped as *KIR2DL3*$^+$*/L2*$^-$*/S2*$^-$. (Pearson correlation coefficient $r = 0.89$, $p = 0.003$; Fig. 5*B*). Taken together, these data show that in patients where KIR2DL3 is the only KIR present recognized by GL183, the increased binding of HLA-C1 tetramers to uNK cells can be attributed to increased expression of 2DL3 protein on the cell surface.

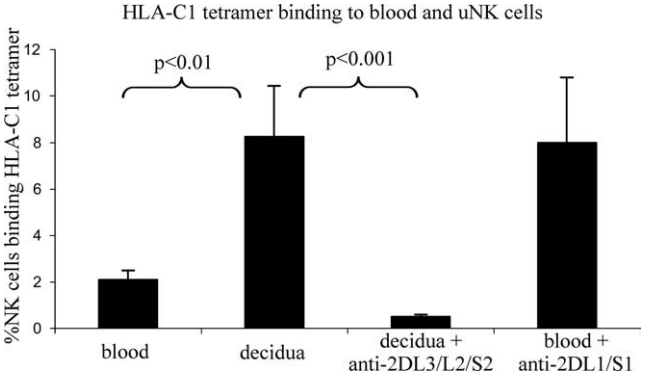

FIGURE 4. HLA-C tetramers show increased binding to NK cells from decidua compared with blood NKs. Blood ($n = 9$) and decidual ($n = 16$) leukocytes were analyzed by FACS following staining with HLA-C tetramer and anti-CD56. Preincubation of NK cells with anti-KIR2DL3/L2/S2 mAb abolished HLA-C tetramer binding to decidual NK cells (decidua plus anti-2DL3/L2/S2, $p < 0.001$) but preincubation with mAb specific for KIR2DL1/S1 had no effect.

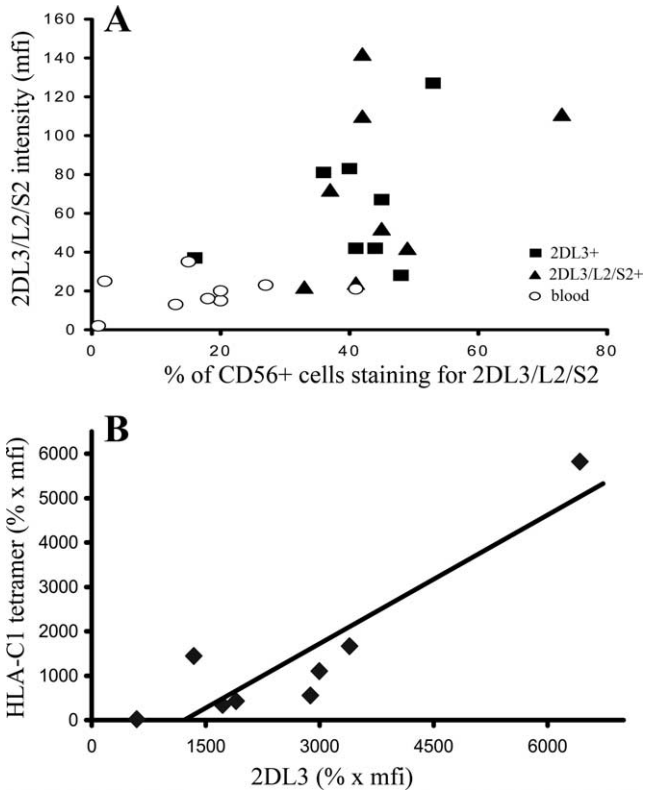

FIGURE 5. Decidual leukocytes cells show increased expression of KIR specific for HLA-C compared with blood NK cells. *A*, Expression of KIR2DL3/L2/S2 was analyzed by FACS in uNK ($n = 15$) and blood NK cells ($n = 9$), in patients genotyped for KIR. Blood $CD56^+$ NK cells are shown as (○). $CD56^+$ uNK cells show increased staining intensity and a greater proportion of the cells are KIR^+ compared with blood NK cells. There was no significant difference in frequency or mean fluorescent intensity (mfi) for uNK samples genotyped as *KIR2DL3*$^+$*/L2*$^+$*/S2*$^+$ (▲) or as *KIR2DL3*$^+$*/L2*$^-$*/S2*$^-$ (■). *B*, The expression of KIR2DL3 in uNK cells and binding of HLA-C1 tetramer as measured by FACS is strongly correlated. In patients genotyped as *KIR2DL3*$^+$*/L2*$^-$*/S2*$^-$, staining of uNK cells with anti-KIR2DL3/L2/S2 mAb is due to KIR2DL3 expression. The staining intensity for KIR2DL3 is correlated with the intensity of HLA-C1 tetramer binding (Pearson correlation coefficient, $r = 0.89$, $p < 0.003$). Overall staining intensity for KIR2DL3 or HLA-C1 tetramer binding was measured as the percentage of uNK cells staining positive, multiplied by the mean fluorescent intensity.

KIR tetramers bind to primary trophoblast

HLA-C is the only classical HLA class I molecule expressed on primary trophoblast cells (7, 8). To see whether trophoblast HLA-C molecules can bind KIR, we used flow cytometry to detect binding of tetramers of KIR2DL1 or 2DS1 to primary trophoblast cells (16, 19). Trophoblast cells were identified by staining with mAb G233, which recognizes the non-classical HLA-G that is expressed only on trophoblast cells at the materno-fetal interface (21). No significant binding of the KIR2DS1 tetramer to HLA-G$^+$

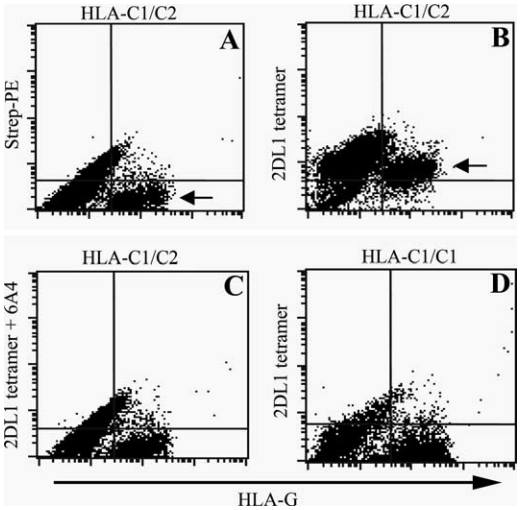

FIGURE 6. Tetramers of KIR2DL1 bind to trophoblast cells. Primary trophoblast cells of HLA-C genotype *C1/C2* (*A–C*) or *HLA-C1/C1* (*D*) were double stained with PE-conjugated tetramer of KIR2DL1 and the HLA-G specific mAb G233. *A*, streptavidin-PE negative control. Some autofluorescence is unavoidable using these primary cells but the HLA-G$^+$ trophoblast cells are clearly distinguishable (arrowed). *B*, KIR2DL1 tetramer binds to HLA-G$^+$ trophoblast cells genotyped as *HLA-C1/C2* as well as to HLA-G negative fetal fibroblasts that contaminate the trophoblast preparation. *C*, KIR2DL1 binding was abolished by prior incubation with the anti-HLA class 1 mAb 6A4. *D*, The KIR2DL1 tetramer did not bind significantly to trophoblast cells genotyped as *HLA-C1/C1*.

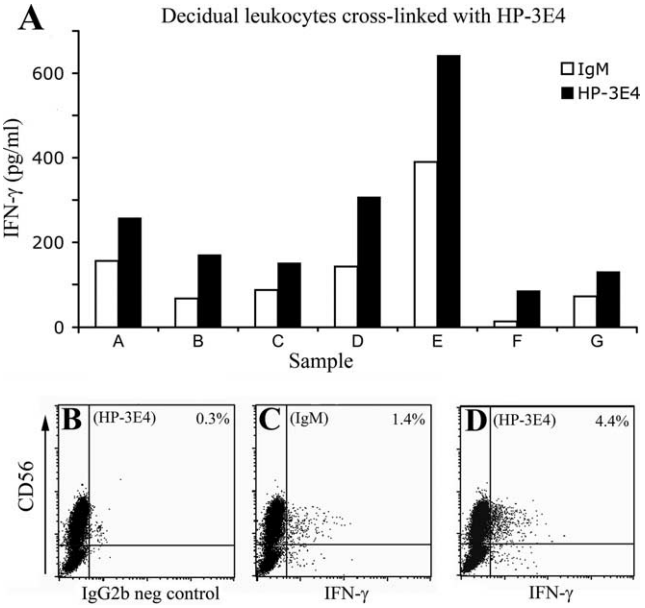

FIGURE 7. Cross-linking of KIR using Ab results in increased secretion of IFN-γ by decidual leukocytes. *A*, freshly isolated decidual leukocytes were cultured for 16 h in wells coated with anti-KIR mAb HP-3E4 (■) or with control IgM (□). IFN-γ secretion was significantly increased in the supernatant by culture with HP-3E4 as measured by ELISA ($n = 7$, $p < 0.02$, Wilcoxon paired test). Because KIR are only expressed on CD56$^+$ decidual NK cells, we used intracellular FACS to confirm the phenotype of the IFN-γ producing cells. Fresh decidual leukocytes were cultured overnight in wells coated with mAb HP-3E4 (*D*) or with control IgM (*C*) and then stained for IFN-γ (FITC) and CD56. The level of nonspecific intracellular staining was determined by staining cells with IgG2b-negative control (FITC) and CD56 after culture in wells with HP-3E4 (*B*). The percentage of CD56$^+$ cells showing specific intracellular staining with anti-IFN-γ Ab was increased from 1.1% to 4.1% after culture on IgM control, or HP-3E4, respectively (*C* and *D*).

trophoblast cells was detected (data not shown). However, tetramers of KIR2DL1 did bind to HLA-G$^+$ trophoblast cells as well as to HLA-G$^-$ fetal fibroblasts from placental villi that do contaminate the trophoblast preparation (16) (Fig. 6*B*). The trophoblast cells in this sample were genotyped as heterozygous for *HLA-C1* and *C2* and so should express HLA-C of both allotypes (7). The tetramer binding was blocked by prior incubation with the 6A4 Ab, which has previously been shown to block KIR tetramer binding to HLA-C (19) (Fig. 6*C*). However, KIR2DL1 tetramer did not bind to trophoblast cells that were shown by genotyping to be homozygous for *HLA-C1*, indicating that KIR2DL1 binding required expression of HLA-C2 by trophoblast cells (Fig. 6*D*). KIR can, therefore, bind to the form of HLA-C molecules displayed at the surface of primary trophoblast cells.

IFN-γ secretion following KIR ligation on primary uNK cells

To establish whether KIR on uNK cells are functional, we cultured freshly isolated decidual leukocytes for 16 h in wells coated with the anti-KIR Ab HP-3E4 or IgM control. Cross-linking with HP-3E4, which reacts with several KIR including 2DL1, 2DS1, and 2DS4, resulted in increased secretion of IFN-γ as detected by ELISA using culture supernatants (Fig. 7*A*). Genotyping of these patients showed they all had at least one activating KIR recognized by HP-3E4. FACS analysis confirmed that intracellular staining for IFN-γ in CD56$^+$ uNK cells increased following cross-linking with HP-3E4 (Fig. 7, *B–D*). Triple staining with CD9 confirmed that IFN-γ was produced by CD56$^+$CD9$^+$ uNK cells, rather than contaminating CD56$^+$CD9$^-$ blood NK cells (data not shown). Culture of uNK cells in the presence of IL-15 (2 ng/ml) resulted in a 2-fold increase in basal IFN-γ secretion, but the response to KIR ligation was the same (data not shown). We conclude that cross-linking of KIR, which are only expressed on CD56$^+$ cells in the decidua, results in increased IFN-γ production. These KIR are, therefore, functional and can stimulate cytokine production by freshly isolated uNK cells.

Discussion

Our analysis of the KIR repertoire of fresh uNK cells shows that it is quite different from blood NK cells. A key finding is that the KIR repertoire in uNK cells is dynamic and changes dramatically during the early weeks of pregnancy. Both the proportion of CD56$^+$ cells expressing KIR specific for HLA-C and the levels of expression of these KIR are up-regulated. The up-regulation of KIR is particular for those that bind to HLA-C, because the HLA-B-specific KIR3DL1 did not increase significantly. This is relevant because trophoblast cells, which invade into the decidua at this time, express HLA-C and not HLA-A or HLA-B (12). The increased expression of HLA-C-specific KIR in uNK cells is a transient phenomenon, and, by 12 wk of pregnancy, KIR expression in uNK cells resembles that seen in pbNK cells. This suggests that decidua is able to regulate the KIR repertoire in a coordinated manner, with individual KIR responding independently to this unique microenvironment.

Although the HLA-C-reactive KIR are expressed on the majority of uNK cells between 6- and 8-wk gestation, there is a gradual decline in the frequency of uNK cells expressing these KIR throughout the first trimester. This means that maximal expression of these KIR coincides with the period when trophoblast migrates through the decidua to transform the spiral arteries. By the end of the first trimester, decidual colonization by trophoblast is complete, although invasion continues into the myometrium for a further few weeks (1). We have not been able to answer the question of when the up-regulation of KIR takes place, because decidual

tissue from the first 6 wk of pregnancy is rarely available. We did, however, previously report that uNK cells do express KIR in the secretory (luteal) phase of the non-pregnant endometrium (25). Another study using flow cytometry suggested KIR expression levels in uNK cells from endometrium were similar to blood (26). KIR expression is not, therefore, a pregnancy-related phenomenon and does not depend on the presence of trophoblast. The transient increase in KIR specific for HLA-C appears to be established by the sixth week of gestation; this coincides with the decidualization of stromal cells that begins at implantation and takes place during the early weeks of pregnancy.

Our findings contrast with observations on the KIR repertoire in pbNK cells. In healthy individuals, both the frequency and expression levels of KIR are genetically determined and remain fixed for years (27–30). The frequency and level of surface expression of a particular KIR depends on which allele is present and is also related to the expression of its cognate ligand in that individual (27, 28, 31). Changes in KIR repertoire have been described after bone marrow transplantation following donor NK cell engraftment. In this situation, $CD56^{bright}KIR^{-}$ cells are the first to appear but are then replaced by $CD56^{dim}KIR^{+}$ cells (32, 33). Our description of the change in KIR repertoire in uNK cells during pregnancy is the first report of the regulation of individual KIR in a normal physiological setting.

The origin of uNK cells is relevant in considering how they acquire their distinctive KIR phenotype. Selective recruitment of NK cells with the appropriate phenotype from the blood (or loss of specific cells due to apoptosis) could give rise to the unusual KIR repertoire observed in the uNK population (34, 35). Endometrial extracts promote the selective migration of $CD16^{-}$ blood NK cells. This effect is probably mediated by IL-15, a progesterone-regulated product of uterine stromal cells (36). Alternatively, the KIR repertoire of uNK cells could arise in response to local signals acting on NK cells within the decidua. Conditioned medium from uterine stromal cells up-regulates KIR in $CD56^{bright}$ blood NK cells and this effect was blocked by mAbs to TGF-α (37). Decidual stromal cells could, therefore, modulate KIR expression in NK cells derived from blood. However, there is also evidence for local NK progenitors in the uterus. $CD34^{+}CD45^{+}$ cells have been described in endometrium and can be induced to proliferate and differentiate into NK cells (37, 38). Moreover, when human endometrium was transplanted into non-obese diabetic/severe combined immunodeficient/γ-C null (NOG) mice, which are deficient in NK activity, $CD56^{+}$ NK cells proliferated in the human explants in response to estrogen and progesterone just as in a normal menstrual cycle (39). Whatever their origin, the interaction with decidualized stromal cells is likely to provide a unique microenvironment enabling uNK cells to establish their distinctive KIR repertoire.

Previous studies of KIR expression in uNK cells used patients whose genotype was not known (5, 6, 26). In addition the Abs used were not able to discriminate between activating and inhibitory KIR. By using flow cytometry and quantitative RT-PCR on genotyped patients, we have demonstrated that both inhibitory and activating KIR specific for HLA-C are selectively up-regulated. These differences in relative levels of activating and inhibitory KIR will cause blood and uNK cell populations to respond differently to the same HLA-C ligand. Expression of KIR3DL1 did not alter, and KIR2DL5 expression in uNK cells actually decreased compared with blood. The function and ligand of KIR2DL5 are unknown but it seems unlikely that it is involved in placentation. Of interest is the fact that the most highly up-regulated KIR was KIR2DL3, compared with KIR2DL2 or KIR2DL1. KIR2DL3/L2 are allelic and show functional differences in binding HLA-C allotypes (40). That these are important is shown by the correlation of HLA-C1 and KIR2DL3 (but not KIR2DL2) with resolution of Hepatitis-C infection (41). We have not yet determined whether the HLA-C status of the mother in the decidual microenvironment affects the KIR repertoire of uNK cells. The presence of KIR ligand is known to affect the frequency and levels of KIR expression in pbNK cells (27). It is also possible that interactions between uNK cells and the HLA-C status of invading fetal trophoblast cells may influence the KIR repertoire, and whether it differs in first and subsequent pregnancies in a woman is an interesting question. Overall, our results show that the expression of each KIR locus studied is independently regulated and that this regulation differs between blood and decidual NK cells.

We have also demonstrated that the increased levels of KIR expression result in higher binding of HLA-C tetramers by uNK compared with blood NK cells. This is likely to be functionally important because it reflects an increased ability of uNK cells to bind HLA-C in vivo, including that on trophoblast. The conformation of HLA-C molecules on trophoblast is unusual in that it exists predominantly in the stable β2m-associated form, with negligible levels of the unfolded HLA-C conformers that are seen on somatic cells (8). We also show that tetramers of KIR2DL1 specifically bind to HLA-C molecules on primary trophoblast cells. All these findings suggest that both KIR and HLA-C in the placental bed have unusual features, which result in an uNK cell population that is specifically adapted for enhanced recognition of HLA-C on trophoblast.

Evidence that the interaction between KIR and HLA-C is critical to the proper regulation of placentation comes from immunogenetic studies where particular combinations of maternal KIR and fetal HLA-C genotypes were found to be associated with pre-eclampsia and recurrent miscarriage (4, 17). Functional studies have shown the uNK cells show poor killing activity but make a variety of cytokines and angiogenic factors including IFN-γ, IP-10, IL-8, VEGF-A, VEGF-C, and Ang1 (11, 13–15). These are all known to influence either vascular function or trophoblast migration, both of which are abnormal in disorders of pregnancy such as pre-eclampsia. Secretion of several of these factors by uNK clones is altered following engagement of inhibitory KIR by HLA-C ligands, but the response of fresh uNK cells has not been reported (11). We have now shown that Ab ligation of KIR on fresh uNK cells does regulate IFN-γ secretion. This cytokine inhibits migration of human trophoblasts in vitro, and localized production of IFN-γ by uNK cells is required for normal vascular modification and decidualization during early pregnancy in mice (42–44). Because both KIR and HLA-C are polymorphic, the interaction between uNK cells and trophoblast will vary in each pregnancy. Experiments are underway to compare how fresh uNK cells with different KIR phenotypes respond to HLA-C1 and HLA-C2 groups.

In summary, we have shown that uNK cells show a transient up-regulation of both activating and inhibitory KIR responsible for recognition of HLA-C. The high frequency and expression levels of these KIR differ significantly from pbNK cells and enhances the ability of uNK cells to bind HLA-C. This supports the results of genetic studies that implicate KIR/HLA-C interactions in regulating a normal physiological situation, namely placentation (4, 17). It also suggests a novel potential mechanism that may contribute to conditions such as pre-eclampsia, because women who fail to adequately up-regulate these KIR in uNK cells may be at increased risk of pregnancy complications. The unique decidual microenvironment clearly provides a niche for differentiation of a distinctive KIR repertoire that is primed for recognition of HLA-C. Identification of the local factors responsible for this transient up-regulation will be important in understanding how the KIR repertoire in uNK cells is controlled.

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Disclosures

The authors have no financial conflict of interest.

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