Maternal uterine NK cell–activating receptor KIR2DS1 enhances placentation

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Reduced trophoblast invasion and vascular conversion in decidua are thought to be the primary defect of common pregnancy disorders including preeclampsia and fetal growth restriction. Genetic studies suggest these conditions are linked to combinations of polymorphic killer cell Ig-like receptor (KIR) genes expressed by maternal decidual NK cells (dNK) and HLA-C genes expressed by fetal trophoblast. Inhibitory KIR2DL1 and activating KIR2DS1 both bind HLA-C2, but confer increased risk or protection from pregnancy disorders, respectively. The mechanisms underlying these genetic associations with opposing outcomes are unknown. We show that KIR2DS1 is highly expressed in dNK, stimulating strong activation of KIR2DS1+ dNK. We used microarrays to identify additional responses triggered by binding of KIR2DS1 or KIR2DL1 to HLA-C2 and found different responses in dNK coexpressing KIR2DS1 with KIR2DL1 compared with dNK only expressing KIR2DL1. Activation of KIR2DS1+ dNK by HLA-C2 stimulated production of soluble products including GM-CSF, detected by intracellular FACS and ELISA. We demonstrated that GM-CSF enhanced migration of primary trophoblast and JEG-3 trophoblast cells in vitro. These findings provide a molecular mechanism explaining how recognition of HLA class I molecules on fetal trophoblast by an activating KIR on maternal dNK may be beneficial for placentation.

Introduction

During early pregnancy in humans, fetal extravillous trophoblast cells (EVT) invade deeply into the mucosal lining of the uterus, the decidua, to remodel the uterine spiral arteries into high-conductance vessels (1). Reduced trophoblast invasion and vascular conversion results in poor placental perfusion, thought to be the underlying primary defect of common disorders of pregnancy, such as recurrent miscarriage, preeclampsia and fetal growth restriction, although the timing and exact clinical presentation is affected by other genetic and environmental factors (1–3). Conversely, overinvasion can result in life-threatening conditions such as placenta percreta, where trophoblast cells can rupture the uterus (4). Understanding how trophoblast invasion and arterial remodeling are regulated is therefore crucial if we are to predict which pregnancies are at risk and improve management of these disorders.

As they invade, fetal EVT encounter maternal leukocytes, approximately 70% of which are decidual NK cells (dNK) (5, 6). These are functionally and phenotypically distinct from peripheral blood NK cells (pBNK), and a growing body of evidence suggests that they play a role in regulating trophoblast invasion (7–9). EVT express a unique array of HLA class I molecules; HLA-C and nonclassical HLA-E and HLA-G, but not HLA-A or HLA-B (10–12). dNK express cognate receptors for these, such as NKG2A, LILRB1 and members of the killer cell Ig-like receptor (KIR) family (13). Because both KIR and their HLA-C ligands are highly polymorphic genes and trophoblast expresses both paternal and maternal HLA-C allotypes, each pregnancy will be characterized by different combinations of maternal KIR and fetal HLA-C genes (14–16).

Inhibitory KIR that bind to HLA-C are expressed at higher frequencies in dNK than in pBNK during early pregnancy, and dNK show increased binding to HLA-C tetramers (17–19). Conversely, KIR2DL1 and KIR2DS1 Fc-fusion proteins bind directly to primary trophoblast cells, demonstrating the possibility of allogeneic recognition of fetal trophoblast by KIR on maternal dNK (14, 18).

KIR are now recognized as major regulators of NK cell function, and all individuals have HLA-C allotypes that will bind to particular KIR. Inhibitory KIR2DL1 and activating KIR2DS1 bind to allotypes of the HLA-C2 group (C2) with lysine at position 80 while inhibitory KIR2DL2/3 bind to HLA-C1 allotypes with asparagine at 80 (20, 21). There are 2 basic KIR haplotypes, A and B: A have mainly inhibitory receptors, while B have a variable number of additional activating receptors. We have shown that women with 2 KIR A haplotypes (KIR AA genotype) in association with a HLA-C2 group in the fetus are at increased risk of disorders of placentation (14–16, 22). This risk is greatest if the fetal HLA-C2 is inherited from the father and the mother lacks HLA-C2 herself. The telomeric region of KIR B haplotypes (where activating KIR2DS1 is located) provides protection from pregnancy disorders. This suggests that in KIR AA women, binding of inhibitory KIR2DL1 to HLA-C2 on trophoblast results in impaired trophoblast invasion. In women with a KIR B haplotype, activating KIR2DS1 that also binds HLA-C2, may enhance placentation by increasing invasion (14, 15).

The extracellular domain of KIR2DS1 closely resembles that of KIR2DL1 (23) but reagents are now available that allow discrimination of these inhibitory and activating receptors for HLA-C2 (24–26). When KIR2DS1+ pBNK bind HLA-C2+ targets, cytolyis, cytokine production, and cell proliferation are induced (24–28). Since activating KIR2DS1 may be autoreactive in HLA-C2+ individuals, functional responses of KIR2DS1+ pBNK are tuned down or educated in C2/C2 donors compared with those who are C1/C1 (25, 28–30). There have been no studies of expression and function of KIR2DS1 in dNK, and we therefore compared KIR2DS1 and...
KIR2DL1 in dNK with pbNK from the same woman. We show that, like KIR2DL1, KIR2DS1 is expressed more frequently on dNK than their pbNK counterparts. KIR2DS1 is functional and triggers dNK activation. Using expression microarrays, we identified responses generated by dNK when KIR2DS1 binds HLA-C2, including secretion of cytokines such as GM-CSF, which we show enhances migration of primary trophoblast cells in vitro. These results link our genetic findings to functional responses of dNK and suggest how NK allorecognition may determine successful placentation.

Results

Expression of KIR2DL1 and KIR2DS1 by dNK. The HLA-C–specific KIR are expressed at higher frequency in dNK compared with pbNK (17–19). None of these reports distinguished between inhibitory KIR2DL1 or activating KIR2DS1, which both bind HLA-C2. Using 2 mAbs, we analyzed the frequency of KIR2DS1 sp and KIR2DL1 sp cells in matched samples of freshly isolated dNK and pbNK (Figure 1). NK cells were classified into 4 subsets: KIR2DS1 sp, KIR2DL1 sp, dp, KIR2DL1 sp, and dn as shown in A. The frequency of each subset as a percentage of total NK cells was determined in paired samples of fresh NK cells from blood or decidua (data for dn subset not shown, n = 13). Horizontal bars show mean for each group. (C) Blood or dNK were classified into 4 subsets: KIR2DS1 sp, dp, KIR2DL1 sp, and dn as shown in A. The frequency of each subset as a percentage of total NK cells was determined in paired samples of fresh NK cells from blood or decidua (data for dn subset not shown, n = 13). Results show mean for each group. (D) The responsiveness of the KIR2DS1 sp, KIR2DL1 sp, and dp dNK subsets was measured by stimulating either with control IgG (IgG con) or with mAb 11PB6, which crosslinks both KIR2DS1 and KIR2DL1. Degranulation was assessed by staining for surface CD107a or control IgG (n = 5). KIR2DL1 and KIR2DS1 subsets were gated after FACS staining, as shown in A and will thus express a variety of other NK receptors. Basal responses were seen in the dn subset (data not shown). *P < 0.05 and **P < 0.01, Wilcoxon paired samples test. 2DS1 sp, KIR2DS1 sp; 2DL1 sp, KIR2DL1 sp; dp, KIR2DL1+KIR2DS1+.

Figure 1

Frequency of KIR2DS1 sp and KIR2DL1 sp dNK is increased compared with pbNK from the same donors and KIR2DS1 is functional in dNK. (A) Freshly isolated leukocytes from decidua or peripheral blood were gated on live, CD56+CD3– cells and examined for KIR2DS1 and KIR2DL1 expression using mAbs EB6 and 143211. dNK are shown and were also gated on CD9+ cells to avoid contamination with pbNK. (B) Overall frequency of NK cells positive for KIR2DL1 or KIR2DS1 in paired samples from blood and decidua expressed as a percentage of total NK cells (n = 13). (C) Blood or dNK were classified into 4 subsets: KIR2DS1 sp, dp, KIR2DL1 sp, and dn as shown in A. The frequency of each subset as a percentage of total NK cells was determined in paired samples of fresh NK cells from blood or decidua (data for dn subset not shown, n = 13). Horizontal bars show mean for each group. (D) The responsiveness of the KIR2DS1 sp, KIR2DL1 sp, and dp dNK subsets was measured by stimulating either with control IgG (IgG con) or with mAb 11PB6, which crosslinks both KIR2DS1 and KIR2DL1. Degranulation was assessed by staining for surface CD107a or control IgG (n = 5). KIR2DL1 and KIR2DS1 subsets were gated after FACS staining, as shown in A and will thus express a variety of other NK receptors. Basal responses were seen in the dn subset (data not shown). *P < 0.05 and **P < 0.01, Wilcoxon paired samples test. 2DS1 sp, KIR2DS1 sp; 2DL1 sp, KIR2DL1 sp; dp, KIR2DL1+KIR2DS1+.
KIR2DS4) are not expressed equally on the KIR2DS1sp and dp dNK subpopulations (Figure 2, B–D, and Supplemental Figure 4). Therefore, to examine the functional responses of the KIR2DL1/S1 subsets to HLA-C2 in the absence of such confounding effects, dNK expressing these other NKRs were gated out from the analysis, except where specifically stated. Significant degranulation of KIR2DS1sp cells was seen in response to 721.221 cells transfected with HLA-Cw*0602 (221-C2) that was abolished by coincubation with the anti–HLA class I-blocking mAb 6A4 (Figure 3, A and B). As with activation by crosslinking, no significant degranulation was seen in KIR2DL1sp or dp subsets, indicating that inhibition by KIR2DL1 overrides activation of KIR2DS1 by HLA-C2 when they are coexpressed. Coexpression of other NKRs that recognize HLA class I resulted in decreased responses of KIR2DS1sp dNK to 221-C2, but these responses were still significantly higher than in the dp subset (Figure 3C). KIR2DS1sp dNK that are also NKR+ make up approximately 11% of the total dNK population (Supplemental Figure 5A). Individual NKRs differ in their effect on the degranulation response of KIR2DS1sp dNK with 221-C2 target cells, with coexpression of LILRB1 having the least effect and KIR2DL1 the most (Supplemental Figure 5B). Activation of KIR2DS1sp dNK in response to 221-C2 targets is higher in dNK from C1/C1 homozygous individuals compared with those with at least 1 copy of HLA-C2 (C2/X) (Figure 3D).

Because NK cells are sensitive to levels of HLA class I expression, we focused on GM-CSF to demonstrate how activation of KIR2DS1 might enhance placentation. Intracellular staining for GM-CSF confirmed that KIR2DS1sp dNK (with expression of other NKRs but not KIR2DL1) also showed increased intracellular staining for GM-CSF, although this response was lower than for KIR2DS1sp NKR+ cells. This suggests that KIR2DS1 triggering by HLA-C2 upregulates GM-CSF even in cells that coexpress inhibitory NKRs that can bind HLA-C2. These results were confirmed using crosslinking with mAb EB6, which binds both KIR2DS1 and KIR2DL1 in samples of bulk dNK. GM-CSF secretion was detected by ELISA in culture supernatants and was significantly increased in KIR2DS1+ but not KIR2DS1− donors after cross-linking (Figure 5B).
cells that do not coexpress KIR2DL1 represent approximately 13% of total dNK (Supplemental Figure 5A), activation of KIR2DS1 does result in significant GM-CSF secretion.

**GM-CSF produced by dNK after activation of KIR2DS1 stimulates trophoblast migration in vitro**. We then investigated the function of GM-CSF in enabling normal placentation because our genetic data suggests that binding of KIR2DS1 on dNK to trophoblast HLA-C2 is beneficial when there is a HLA-C2 group in the fetus. First, we localized expression of GM-CSF receptor α (GM-CSFRα) in vivo by immunohistochemistry. There was intense expression of GM-CSFRα by interstitial EVT in decidua at sites densely infiltrated by maternal dNK, where dNK can be seen closely associated with EVT (Figure 6, A–E). GM-CSFRα expression was confirmed by flow cytometry using primary trophoblast cells (Figure 6F) and the choriocarcinoma cell line JEG-3 (data not shown). Supernatants of dNK in which KIR2DS1 had been activated by crosslinking contained increased levels of GM-CSF as shown in Figure 5B. These supernatants stimulated JEG-3 migration through fibronectin-coated Transwells and the effect was significantly reduced by addition of neutralizing GM-CSF antibody, compared with supernatant with isotype control mAb added (Figure 6G). Recombinant GM-CSF also increased the migration of primary trophoblast cells through Transwells and enhanced the motility of JEG-3 cells in a scratch assay (Figure 6H and Supplemental Figure 8). Thus, KIR2DS1+ dNK produced GM-CSF following activation, and this enhanced the migration of primary trophoblast and choriocarcinoma cells in vitro.

**Discussion**

The response of maternal uterine NK cells to allogeneic fetal trophoblast at the site of placentation is analogous to the situation during HSC transplantation (HSCT) when donor cells confront allogeneic recipient cells (32–34). Women with KIR AA genotypes who lack HLA-C2 but have a HLA-C2+ fetus are most at risk of pregnancy disorders such as preeclampsia. KIR2DS1 is located in the telomeric region of KIR B haplotypes and is associated with protection from pregnancy disorders when the fetus is HLA-C2+ (14, 15, 22). Similarly, patients with myeloid leukemia who receive grafts from donors with KIR B haplotypes that contain KIR2DS1 show improved survival (35). This effect depends on the HLA-C1 or HLA-C2 status of the donor and is stronger if the donor lacks HLA-C2 but it is present in the recipient. We therefore set out to determine...
how our genetic findings in pregnancy translate into some dNK function beneficial for placentation during early pregnancy.

Initially, we studied the expression of KIR2DS1 in dNK. Overall frequencies of both KIR2DL1 and KIR2DS1 are dramatically increased in dNK compared with pbNK from the same donor. The number of dNK that coexpress both receptors was somewhat larger than predicted by the product rule (36). How the selective increase of KIRs in dNK specific for HLA-C occurs in the decidual microenvironment is unknown, but it is a response to pregnancy, since it is not seen in nonpregnant endometrium (13). The dp cells do proliferate more than the KIR2DS1sp and KIRDL1sp subsets, which may partially explain their prominence in the decidua. IL-15 is transpresented by stromal cells during decidualization and can induce dNK proliferation, maturation, and acquisition of KIR, but not this skewing toward KIR2D specific for HLA-C (13, 37–40). TGF-β can affect the phenotype of dNK and is abundant in decidua (39–41). Changes in MHC class I molecules expressed by stromal cells can also modulate the receptor repertoire of NK cells in vitro or after HSCT (42–44). Although expression of HLA-C on uterine stromal cells increases during decidualization, with the modest numbers studied here, we found no association between maternal HLA-C1 or HLA-C2 and KIR2DL1 or KIR2DS1 frequencies in dNK, as previously shown by others in pbNK (25, 45). Sample numbers did not permit us to determine whether fetal HLA-C1 or HLA-C2 affects KIR2DL1/S1 frequencies independently of maternal HLA-C. Decidual stroma and dNK are in intimate contact but the exact factors responsible for expansion of this dp remain unclear. Nonetheless, the outcome is that whenever a paternal HLA-C2 is expressed by trophoblast, there will be large numbers of dNK potentially able to respond to this nonself HLA-C. The finding that allogeneic mouse matings differing only in paternal MHC produce heavier fetuses than syngeneic matings strongly supports the idea that recognition of paternal MHC by maternal immune cells is a generalized mechanism to regulate placental development (46).

triggering of dNK with mAb to KIR2DL1/S1 or with HLA-C2+ targets results in selective degranulation and cytokine secretion by the KIR2DS1sp cells, but all subsets respond to the same extent when triggered by mAb to NKp46. In addition, a mAb (6A4) known to block binding of KIR2DS1 to HLA-C2+ targets inhib-
its KIR2DS1sp activation (47). The HLA-C1 or HLA-C2 status of the mother is also important as KIR2DS1sp dNK from C1/C1 donors respond better to HLA-C2+ targets than those from C2/C1 heterozygotes. This contrasts with pbNK, where tuning down of KIR2DS1 responses to HLA-C2 was observed only in HLA-C2 homozygotes (25, 26, 29, 30). The rheostat model of NK education proposes that NK cell function is “tuned” by interactions between inhibitory and activating receptors on the cell and their ligands during NK development, and quantity of MHC also affects NK cell responsiveness (48, 49). Indeed, we show that dNK appear to be more sensitive to lower levels of HLA-C, and this may explain why we found evidence of dNK activation via KIR2DS1 even in C2/C1 heterozygotes. Since these mature dNK proliferate in situ, tuning presumably takes place or is maintained within the decidua. These functional findings correlate well with our genetic studies of pregnancy disorders in which the presence/absence of HLA-C2 in the mother as well as the baby influences the outcome (14).

CD107a is a convenient measure of NK cell activation in response to HLA-C2, but cytokine production is more physiologically relevant to pregnancy (7). To avoid nonphysiological responses due to culture with high levels of IL-2 or IL-15, we used primary dNK exposed overnight to a low dose of IL-15, which maintains good viability with minimal activation (50, 51). Under these conditions, IFN-γ is not a useful readout, as the levels produced and number of dNK responding is low (31, 52). Therefore, we used microarrays to identify the mRNA responses of KIR2DL1/S1 subsets following coculture with either 221-C2 or 221-parent cells. In KIR2DS1sp cells, which degranulate in response to HLA-C2, nearly 400 transcripts change, but in KIR2DL1sp cells, which do not degranulate, the transcriptional responses are subdued. In dp cells, KIR2DL1 overrides any degranulation stimulated by KIR2DS1, as it does in pbNK, but other aspects of the response are robust. This suggests divergence of the pathways regulating degranulation and the transcriptional responses mediated by KIR2DS1 in dp cells. Surprisingly, there was very little overlap in the transcriptional responses of the KIR2DS1sp and the dp subsets. Although we excluded confounding factors such as expression of other NKR's that might be responsible for different levels of

Figure 6
Effect of GM-CSF on trophoblast migration. (A) Immunohistochemistry of human implantation site. HLA-G+ EVT (red) and CD56+ dNK (brown). (B) The close association of dNK with EVT is obvious at higher power. (C–E) Immunofluorescence staining of serial section to A. HLA-G+ EVT (green, arrow in C) coexpress GM-CSFRα (red, D) as seen in the overlay in E. Scale bars: 250 μm (A); 25 μm (B–E). (F) Expression of GM-CSFRα on primary EVTs (stained for HLA-G in gate R1 after gating out CD14+ placental macrophages). Histogram shows isotype control (red line) and GM-CSFRα staining (blue line) of the HLA-G+ EVT in R1. (G) JEG-3 cells show increased migration in a Transwell assay in response to supernatant conditioned by dNK from KIR2DS1+ donors after crosslinking with EB6 (super). Addition of neutralizing antibody against GM-CSF to the supernatant (super + GM-CSF mAb) significantly reduced JEG-3 migration compared with supernatant plus isotype control mAb (super + IgG con). Results are presented as the calcein dye fluorescence measured in the lower chamber 48 hours after seeding. Horizontal bars show mean for each group (n = 5). *P < 0.05, Student’s paired t test. (H) Primary trophoblast cells show increased migration in a Transwell assay in response to 10 ng/ml GM-CSF added to the lower chamber. Control wells had no added GM-CSF. Results are presented as the number of EVT counted in the sampled fields (n = 4). *P < 0.01, Student’s t test.
NK cells do not normally kill trophoblast cells unless activated by IL-2, but this cytokine is not found in the decidua. Instead, their function is probably to produce cytokines, enzymes, and other products that may regulate trophoblast invasion (7, 31, 53–55). Given the protective effect of KIR2DS1 on trophoblast invasion in our genetic studies of preeclampsia, we therefore sought proteins induced after stimulation of KIR2DS1 by HLA-C2− targets. Of those we identified, GM-CSF was studied in detail to demonstrate that dNK products induced by ligation of maternal KIR by fetal HLA-C can regulate placental invasion. Secretion of GM-CSF is specifically upregulated in KIR2DS1+ ρet cells, but not dNK after coculture with HLA-C2− target cells. Although the KIR2DS1+ ρet subset accounts for a minority of the total dNK compartment, activation of KIR2DS1 results in increased GM-CSF secretion into supernatants of bulk dNK preparations. The GM-CSF receptor is strongly expressed by the EVT in decidua that are in close contact with dNK. In vitro, GM-CSF enhances invasion by primary trophoblast as well as JEG-3 choriocarcinoma cells. Studies in GM-CSF–deficient mice support the idea that placental development and fetal growth are impaired in the absence of maternal GM-CSF, but the mechanism of this effect is unknown (56). The mechanisms regulating trophoblast migration and transformation of the spiral arteries are complex, and other dNK products induced by activation of KIR2DS1, including chemokines and cytokines, will also be important. Indeed, we only saw partial reduction of trophoblast migration with a GM-CSF–blocking antibody. Our hypothesis was that activation of KIR2DS1 by a HLA-C2− fetus enhances trophoblast invasion and vascular transformation of spiral arteries. This process is frequently defective in pregnancies affected by disorders such as preeclampsia and FGR, as shown by uterine artery Dopplers and anatomical studies of the placental bed (3). We propose that, in a KIR2DS1− woman with a HLA-C2− fetus, the dNK activation that ensues allows trophoblast to invade even more deeply into the decidua. In contrast, women with only KIR2DL1 and a HLA-C2− fetus are at risk of pregnancy disorders where EVT may not effectively transform the more distal myometrial segments of the spiral arteries. Exactly how the process is regulated in vivo in the deeper portions of the decidua and myometrium is not known, but one possibility is that, as well as encouraging migration, KIR2DS1 activation also prevents EVT differentiation to static giant cells. Until more reliable human trophoblast cell lines become widely available, it will be difficult to investigate this further.

In conclusion, our observations suggest how women with KIR2DS1 and a HLA-C2− fetus may have better trophoblast invasion and spiral artery remodeling than women with KIR AA genotypes who are at higher risk of preeclampsia (57). Activation of KIR2DS1 on dNK by HLA-C triggers responses that enhance trophoblast migration and also change the responses of dNK that coexpress the “risky” inhibitory receptor KIR2DL1. Our study thus provides what we believe is the first important link between dNK function and our genetic studies showing that particular combinations of maternal KIR and fetal HLA-C genotypes are associated with different pregnancy outcomes.

Methods

Primary tissue. Decidua were obtained from donors undergoing elective termination between 7 and 12 weeks of pregnancy. Primary trophoblast cells and decidual leukocytes were isolated by enzymatic digestion of placental or decidual tissue as described previously (58). Trophoblast cells were cultured overnight in Ham’s F12, antibiotics, and 20% FCS on fibronectin before analysis by FACS with HLA-G–specific antibody (clone MEM-G/9; AbD Serotec). Decidual samples were analyzed by FACS after staining with anti-CDS5, anti-CD3, and anti-CD9 to evaluate peripheral blood contamination (8, 13). DL preparations were typically composed of more than 50% dNK. For functional assays, DL preparations were cultured overnight in RPMI 1640 medium, antibiotics, 10% FCS, and 2.5 ng/ml IL-15. This low dose of IL-15 maintains dNK viability without significant activation (50). PBMCs were isolated from the same donor at the same week of gestation using Lymphoprep (Axis-Shield) and stained or cultured in same conditions as for DL.

Genotyping of KIR and HLA-C. KIR and HLA-C were typed from genomic DNA by PCR for their presence or absence as described previously (14, 15). KIR genes typed were 3DL1, 3DS1, 2DL1, 2DL2, 2DL3, 2DS5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 2DP1. Typing for HLA-C was performed using a similar approach, which allowed all known HLA-C group HLA-C alleles to be distinguished from HLA-C2 alleles.

Phenotyping NK cells and trophoblast by flow cytometry. 10⁶ fresh cells from blood or decidua were washed with PBS, resuspended in 100 μl FACS buffer (PBS + 1% FCS + 0.1% sodium azide), and incubated with 5 μg/ml human γ-globulins (Sigma-Aldrich) for 15 minutes to block nonspecific binding. To determine the frequency of KIR2DL1 and KIR2DS1, cells were stained with 10 μl KIR2DL1-FITC mAb (clone 143211; R&D Systems) for 15 minutes at room temperature. Then 5 μl KIR2DL1/S1-APC mAb (clone EB6; Beckman Coulter) was added together with antibodies against other surface markers for 20 minutes at 4°C. Other antibodies used were as follows: KIR2DL2/L3/S2-PE (clone GL183), LILRB1-PE (clone HP-F1), NKG2A-PE (clone Z199), and KIR2DS4 (clone FES172) (Beckman Coulter); KIR3DL1-PE (clone DX9; BD Biosciences), CD56−bright violet (clone HCD56; Biolegend), CD3−APC-Cy7 (clone UCHT1; BioLegend), and CD9−PerCP Cy5.5 (M-L13, BD Biosciences — Pharmingen). Further details of antibodies used in multicolor flow cytometry are given in Supplemental Table 2. Dead cells were excluded with near IR fixable live/dead cell dye (Invitrogen). Cells were washed with FACS buffer twice and fixed in 2% paraformaldehyde. Samples were run on LSR Fortessa FACS analyzer (BD Biosciences) and data analyzed using FlowJo 8.0. Freshly isolated primary trophoblast cells and the JEG-3 choriocarcinoma cell line were analyzed by flow cytometry as previously described (12). Briefly, cells were stained with directly conjugated mAb to identify leukocyte or trophoblast cell populations using MEM-G/9-FITC to HLA-G (AbD Serotec) and CD14-PE (clone HCD14; BioLegend).

Functional assays. DL preparations (>50% dNK) with less than 5% pbNK and contamination of CD56+CD69− were used in functional assays. For KIR cross-linking followed by flow cytometry, 96-well plates (Costar EIA) were coated with crosslinking antibody against KIR2DL1/S1 (clone 11Pb6; Miltenyi Biotec) or NKP46 (clone 195314; R&D Systems) in 50 mM HEPES buffer for 4 hours at concentrations between 0 and 10 μg/ml. Optimal antibody con-
centration (2.5 μg/ml) was determined by titration. Matched isotype mAbs were used as controls. 2 x 10^5 dNK per well were incubated for 5 hours. 6 μg/ml Golgi-Stop (BD Biosciences) and Brefeldin (Sigma-Aldrich) were added for the last 4 hours of the incubation. The degranulation response was assessed by staining with anti-CD107a–PerCP Cy5.5 (clone H4A3; BioLegend) after first staining dNK subsets as described above. EB6 was used for KIR2DL1/S1 crosslinking to assess GM-CSF secretion by ELISA with no Golgi-Stop or Brefeldin added. GM-CSF secretion was quantified using the DuoSet ELISA (R&D Systems).

NK cells were also stimulated by incubation with 721.221 HLA-null parent cells (221-parent), or with 721.221 cells transfected either with the group 2 HLA-C allele Cw*0602 (221-C2) or 721.221 cells expressing Cw*0102 (221-C1). Medium alone or phosphor-12-myristate-13-acetate (PMA) (20 ng/ml) and ionomycin (1 μg/ml) were used as negative and positive controls. The effector/target (E/T) ratio was 5:1. To obtain 221-C2 clones with different HLA-C2 surface expression intensity, bulk 221-C2 cells were diluted in 96-well plates at 1 cell per well. Individual clones were stained with mAb W6/32 to identify those with different HLA-C expression. Readouts of NK activation were as follows: CD107a or the γ expression. Readouts of NK activation were as follows: CD107a or the γ expression.

The Wilcoxon signed rank test was used to compare 2 groups of paired data, and the Mann-Whitney test was used for comparison of groups of nonpaired data. Student’s t test (2 tailed) was employed for analyzing normally distributed data obtained from cell lines in vitro. A non-parametric Friedman test with Dunn multiple comparison post test was used to test for differences in between multiple KIR2DS1sp subsets with different NKRs. P < 0.05 was considered statistically significant.

**Study approval.** The Cambridge Research Ethics Committee approved this study (04/Q0108/23 and 08/H0305/40). Informed written consent was obtained from all donors.

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