Expression of KIR2DS1 by decidual natural killer cells increases their ability to control placental HCMV infection

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The combination of the activating killer cell Ig-like receptor 2DS1 (KIR2DS1) expressed by maternal decidual natural killer cells (dNK) and the presence of its ligand, the HLA-C allotype HLA-C2, expressed by fetal trophoblasts, reduces the risk of developing pregnancy complications. However, no molecular or cellular mechanism explains this genetic correlation. Here we demonstrate that KIR2DS1+ dNK acquired higher cytotoxic function than KIR2DS1− dNK when exposed to human cytomegalovirus (HCMV)-infected decidual stromal cells (DSC), particularly when DSCs express HLA-C2. Furthermore, dNK were unable to degranulate or secrete cytokines in response to HCMV-infected primary fetal extravillous trophoblasts. This emphasizes the immunological challenge to clear placental viral infections within the immune-privileged placenta. Activation of dNK through KIR2DS1/HLA-C2 interaction increases their ability to respond to placental HCMV infection and may limit subsequent virus-induced placental pathology. This mechanism is directly related to how KIR2DS1 expressed by dNK reduces development of severe pregnancy complications such as miscarriages and preterm delivery.

human | HLA-C | trophoblast | pregnancy | immune tolerance

One of the most interesting observations in the past decade in reproductive immunology research is that women who have the activating killer cell Ig-like receptor 2DS1 (*KIR2DS1*) in their genome have lower risks of developing pregnancy complications. In contrast, women who lack *KIR2DS1* are at increased risk (1, 2). The protective effect of maternal KIR2DS1 expression is most obvious when the fetus expresses the ligand for KIR2DS1, the HLA-C2 group allotype of HLA-C (2). Recently, the presence of *KIR2DS5* was also associated with lower risk of developing pregnancy complications in African women, whereas the effect of *KIR2DS1* seems to be characteristic of European populations (3). The presence or absence of the *KIR2DS1* gene is due to the various rearrangements in the *KIR* locus during evolution (4, 5). This resulted in two main KIR haplotypes, KIR-A and KIR-B. The KIR-A haplotype lacks most activating KIR including *KIR2DS1*, whereas the KIR-B haplotypes include one or more activating KIR genes. Both the KIR-A and KIR-B haplotypes contain multiple inhibitory KIR including KIR2DL1 that, similarly to KIR2DS1, binds HLA-C2 molecules. Although engagement of KIR2DL1 with HLA-C2 leads to inhibition of natural killer (NK) function (e.g., cytotoxicity), engagement of KIR2DS1 with HLA-C2 leads to NK activation (6). Protection from pregnancy complications by the KIR-B haplotype includes severe maternal and fetal syndromes that occur in high frequencies in the population such as miscarriages (20–30%), fetal growth restriction (3–10%), and preeclampsia (3–5%) (1, 2).

KIR2DS1 is expressed with increased frequency on decidual NK cells (dNK) at the maternal–fetal interface compared with peripheral blood NK cells (pNK) (7). The current hypothesis suggests that the protective effect of *KIR2DS1* lies in the activation of dNK through the binding of HLA-C2 molecules expressed by fetal extravillous trophoblasts (EVT). The activation through HLA-C2/KIR2DS1 is postulated to provide dNK with the ability to secrete beneficial cytokines and growth factors, especially granulocyte–monocyte colony stimulation factor (GM-CSF), to facilitate trophoblast invasion and placental growth (2). This increase in cytokine production was observed when dNK were stimulated with anti-KIR2DS1 antibodies and classical NK target cells that expressed HLA-C2. However, primary EVT do not elicit cytokine responses by dNK even when KIR2DS1 and HLA-C2 are present (8). Therefore, these genetic associations demand further investigation into the molecular and cellular mechanisms underlying the reduced pregnancy risk linked to activating KIR, and in particular KIR2DS1. Our laboratory and others have shown that dNK have many differences in gene expression, cytokine secretion, and cytolytic capacity in comparison with pNK (2, 9–12). Furthermore, dNK form immune synapses with EVT in which perforin is not localized to the synaptic region, a feature typical of a nonlytic synapse (13). During these immune synapses, dNK acquired HLA-G from the EVT through trogocytosis. Interestingly, KIR2DS1+ dNK incorporated increased levels of HLA-G into their membrane, suggesting that prolonged intracellular signaling and possibly distinct functional properties may result.

In addition to the beneficial effect of KIR2DS1 in pregnancy, individuals who carry the KIR-B haplotype have a significantly improved outcome after viral infections (14, 15). The combined presence of KIR3DS1 and its ligand HLA-Bw4 was associated with slower progression to AIDS, lower viral load, and slower decline of CD4+ T cells (14, 16). KIR3DS1 and KIR2DS1 were

Significance

The etiology of pregnancy complications such as miscarriage, preterm birth, and preeclampsia are largely unknown. The combination of the maternal Killer Immunoglobulin-like Receptor-2DS1 (KIR2DS1) with fetal HLA-C was identified as a factor determining risk of pregnancy complications. Thus decidual natural killer cells (dNK) that express high levels of KIR may have a predominant role in pregnancy outcome. Although the dNK effect has been associated with cytokine and growth factor production to promote placentation, here we show that the combination of maternal KIR and HLA-C is also a factor that determines protection against placental human cytomegalovirus (HCMV) infections. Expression of KIR2DS1 by dNK increases the cytotoxic response of dNK to HLA-C2+ HCMV-infected maternal decidual stromal cells, preventing viral spread and placental pathology.

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shown to be protective against respiratory papillomatosis caused by human papillomavirus (17). Moreover, KIR3DS1+ NK cells inhibited HIV-1 replication in vitro (18). Finally, activating KIR play a role in NK-mediated clearance of human cytomegalovirus (HCMV) infection following stem-cell or solid-organ transplantation (19–21). The importance of activating receptors for self-MHC in the clearance of HCMV infection has also been demonstrated in mice, where NK cells expressing activating receptors displayed increased responses to infected cells and were involved in the differentiation of murine cytomegalovirus (MCMV)-specific memory NK cells (22). The NK receptors, their HLA ligands, and clinical benefits are listed in *SI Appendix*, Table S1. Viral infections such as HCMV, HIV, herpes simplex virus, and influenza virus can cause severe maternal and fetal morbidity when they occur during pregnancy (23–25). Infections have been reported to cause spontaneous abortions at a rate of about 4%. However, difficulties in demonstrating the pathogenic role of a wide variety of pathogens may result in under-diagnosis (26). HCMV is the most common congenital infection and occurs in 0.5–2% of all live births. HCMV infects the placenta before the fetus and can inhibit trophoblast differentiation and invasion, which can lead to placental insufficiency and fetal growth restriction (27–30). In addition to NK cells, CD8+ T cells are the most important cells to respond and clear viral infections (31). However, in early pregnancy decidual tissue, CD8+ T cells form a minor population representing only 2–7% of total lymphocytes. Furthermore, decidual CD8+ effector-memory T cells contain reduced levels of the cytolytic molecules perforin and granzyme B and may have a limited cytolytic potential (32). Moreover, dNK are capable of killing HCMV-infected placental fibroblasts (33). This study investigated the functional differences of primary KIR2DS1+ and KIR2DS1− dNK as well as the influence of KIR2DS1 expression by dNK on the response to HCMV-infected maternal decidual stromal cells (DSC) and primary fetal EVT. Moreover, primary dNK and EVT were obtained from the same pregnancy sample to accurately represent maternal–fetal responses (8).

Results

Frequency of KIR2DS1+ NK Cells Was Significantly Increased in Decidua. To identify *KIR2DL1* and *KIR2DS1* gene carriers, a previously described flow cytometry based strategy was used (34). This allowed the identification of four NK populations in *KIR2DL1+ KIR2DS1+* gene carriers: KIR2DL1 single-positive (L1+S1−), KIR2DS1 single-positive (L1−S1+), KIR2DL1+ and KIR2DS1+ double-positive (L1+S1+), and double-negative (L1−S1−). In *KIR2DL1+* but *KIR2DS1−* gene carriers, two NK populations were identified: KIR2DL1 positive (L1+) and KIR2DL1 negative (L1−) (Fig. 1*A*). Among the *KIR2DS1* gene carriers, the frequency of both L1−S1+ and L1+S1+ was significantly higher in dNK than in pNK (Fig. 1*B*). In *KIR2DS1−* individuals, L1+ NK were more frequent in decidua than in blood (Fig. 1*B*). These results confirm previous reports demonstrating a skewing of dNK receptor expression toward recognition of HLA-C (for which KIR2DL1 and KIR2DS1 are receptors) and, particularly, the potential of dNK to develop an activating response to HLA-C2 through KIR2DS1 (2, 35).

KIR2DS1 Expression on dNK Correlated with a Relatively High Content of Cytolytic Proteins. To determine whether the presence of KIR2DS1 influenced the cytolytic potential of NK cells, freshly isolated dNK and pNK were examined for the expression of the cytolytic molecules granzyme B, perforin, and the 9-kDa active form of granulysin (36, 37). A higher percentage of dNK expressed granulysin compared with pNK, and granulysin was also expressed at a higher level in dNK (*SI Appendix*, Fig. S1 *A–C*). pNK expressed higher levels of perforin compared with dNK, whereas no difference was found between expression of granzyme B by dNK and pNK. Furthermore, dNK and pNK of *KIR2DS1+* individuals were divided into the four NK subtypes, and S1+ (both L1−S1+ and L1+S1+) dNK expressed higher levels of granzyme B, perforin, and granulysin compared with the L1−S1− dNK subset (Fig. 1 *C* and *D*). The increased expression of cytolytic molecules was specific for S1+ dNK and was not observed in S1+ pNK (Fig. 1*C*; *SI Appendix*, Fig. S1*D*).

dNK Express High Levels of Phosphorylated Signaling Molecules. The binding of KIR2DS1 to its ligand HLA-C2 triggers DAP12 phosphorylation and recruitment and phosphorylation of the adaptor proteins SYK and ZAP-70 (38). To determine the potential for activation of these signaling pathways, dNK and pNK from *KIR2DS1+* and *KIR2DS1−* donors were cultured with and without the phosphatase inhibitor pervanadate. A significantly higher percentage of dNK had p-SYK/p-ZAP70 expression than pNK after pervanadate treatment (*SI Appendix*, Fig. S2 *A* and *B*). In addition, the expression levels of p-SYK/p-ZAP70 were significantly increased in dNK compared with pNK (*SI Appendix*, Fig. S2*C*). No difference was found between total expression of SYK or ZAP70 between pNK and dNK. This suggests an activation of dNK in vivo due to the ability of pervanadate to inhibit dephosphorylation of proteins that were already phosphorylated previously. Instead, it can also suggest a higher capacity for dNK to signal through p-SYK/p-ZAP70 due to the ability of pervanadate to promote phosphorylation of proteins toward a maximum/saturated level. However, no differences in p-SYK/p-ZAP70 were found in

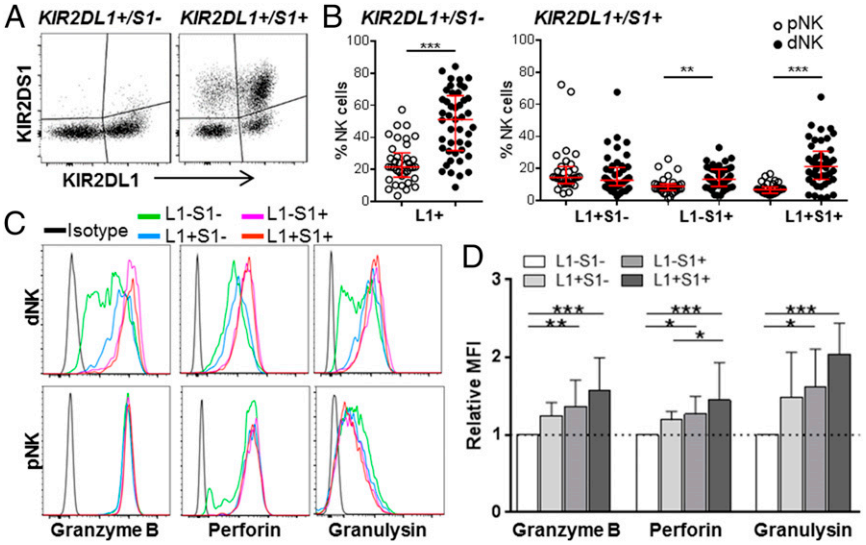

Fig. 1. KIR2DS1 expression increases granule expression in dNK. (*A*) FACS plots of KIR2DL1 and KIR2DS1 expression in freshly isolated dNK from *KIR2DL1+/S1−* (*Left*) and *KIR2DL1+S1+* (*Right*) donors. (*B*) Percentages of dNK and pNK subsets defined by KIR2DL1 (L1) and KIR2DS1 (S1) expression from *KIR2DL1+/S1−* (*Left*) and *KIR2DL1+S1+* (*Right*) donors. (*C*) FACS plots of granzyme B, perforin, and granulysin expression in each of the four L1/S1 dNK (*Top*) and pNK (*Bottom*) subsets. (*D*) Relative expression of the cytolytic molecules in the four L1/S1 subsets of dNK. The expression level is depicted as the relative mean fluorescence intensity (MFI) compared with the L1−S1− subset (n = 10). dNK and pNK are identified by CD45+CD14−CD8−CD56+ (*SI Appendix*, Fig. S11). Bars indicate median and interquartile range; $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

dNK from *KIR2DS1*+ and *KIR2DS1*− donors, whereas pNK from *KIR2DS1*+ donors had a small but not significant increase in p-SYK/p-ZAP70 compared with pNK from *KIR2DS1*− donors (*SI Appendix*, Fig. S2 *A* and *B*).

dNK from KIR2DS1+ Mothers Are Not Efficiently Inhibited by HLA-C2. Next, cytotoxicity of dNK and pNK from KIR2DS1− and KIR2DS1+ individuals in response to MHC class I negative targets 721.221 (221) and 221 that express either HLA-C1 (221.C1) or HLA-C2 (221.C2) were compared. As reported previously (10, 39), degranulation of pNK in response to 221 was significantly higher than that of dNK (*SI Appendix*, Fig. S3*A*; Fig. 2 *A*–*D*) (2). HLA-C1 binds the inhibitory KIR2DL2/3 receptors, and 221.C1 significantly inhibits cytotoxicity of NK cells compared with 221. In contrast, HLA-C2 can inhibit NK cytotoxicity by engaging with KIR2DL1 or activate NK through KIR2DS1. Expression of HLA-C2 on 221.C2 significantly inhibited cytotoxicity of all pNK and dNK obtained from KIR2DS1− donors and pNK from KIR2DS1+ donors (*SI Appendix*, Fig. S3*A*; Fig. 2 *A*, *C*, and *D*). No difference was observed between $CD56^{dim}$ and $CD25^{bright}$ pNK (*SI Appendix*, Fig. S3 *B* and *C*). Interestingly, dNK from KIR2DS1+ women were not as effectively inhibited by 221.C2 (Fig. 2*B*). In addition, the percentage of S1+ single-positive dNK, but not pNK, positively correlated with the degranulation level of total dNK in response to 221.C2 (R^2 0.418; $P = 0.047$; Fig. 2 *E* and *F*). HLA typing of the women and blood donors was performed and did not demonstrate significant differences in dNK or pNK responses from HLA-C1/C1 or HLA-C2/Cx individuals in response to 221 and 221.C2 (*SI Appendix*, Fig. S3 *D* and *E*). Thus, NK education by presence or absence of HLA-C2 did not influence the increase in degranulation that correlated with the higher frequencies of KIR2DS1+ cells. These results suggest that the activation of KIR2DS1 single-positive dNK through KIR2DS1/HLA-C2 interaction outweighs the inhibition of KIR2DL1 by HLA-C2 in KIR2DL1+ dNK but not pNK. The presence of a subset of single-positive KIR2DS1+ dNK (which is absent in mothers without the *KIR2DS1* gene) shifts the response of the total dNK population toward higher degranulation to HLA-C2 targets. This effect was independent of the presence of HLA-E, as blocking of NKG2A had no effect. Moreover, the four L1/S1 dNK subsets were analyzed separately for degranulation in response to 221, 221.C1, and 221.C2. The degranulation of S1 single-positive dNK in response to 221.C2 was significantly higher than all other dNK subsets (Fig. 3 *A* and *D*). The response of S1 single-positive dNK to 221.C1 and 221 was comparable to the double-negative subset and lower than the L1+ subsets, as expected due to NK licensing (40) (Fig. 3 *B* and *C*). Thus, HLA-C2–induced activation of S1+ dNK resulted in downstream signaling and degranulation.

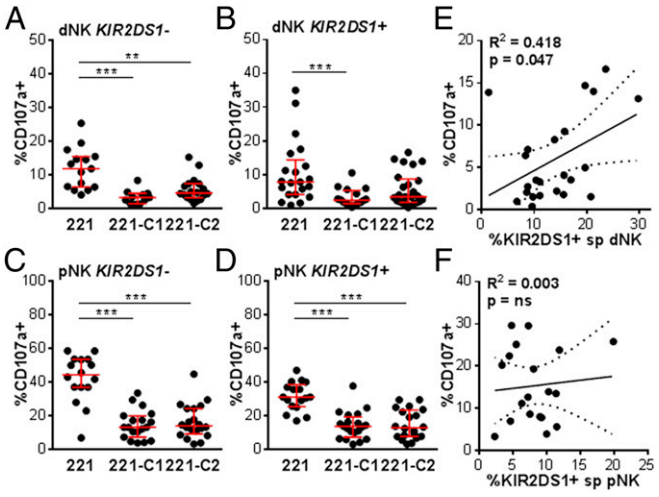

Fig. 2. dNK from *KIR2DS1*+ women are not efficiently inhibited by HLA-C2+ targets. (*A*) dNK from *KIR2DS1*−, (*B*) dNK from *KIR2DS1*+, (*C*) pNK from *KIR2DS1*−, and (*D*) pNK from *KIR2DS1*+ donors in response to 221, 221.C1, and 221.C2. Correlation between the percentage of S1+ single-positive cells and percentage of CD107a+ (*E*) dNK and (*F*) pNK in response to 221.C2. dNK were identified by CD45+CD14−CD8−CD56+ (*SI Appendix*, Fig. S11). $^{**}P < 0.01$, $^{***}P < 0.005$.

dNK Degranulate and Produce Cytokines in Response to HCMV-Infected DSC but Not to Infected JEG3 or EVT. In addition to the reduced incidence of pregnancy complications in *KIR2DS1*+ mothers, *KIR2DS1* and other activating KIR genes (e.g., *KIR2DS2*, *KIR2DS5*, or *KIR3DS1*) have been associated with a more efficient clearance of viral infections (15, 17, 19–21). We therefore postulated that activation of KIR2DS1+ dNK may lead to a more efficient clearing of virus-infected placental cells. To test this hypothesis, DSC, the HLA-G+ choriocarcinoma cell line JEG3, and primary fetal HLA-G+ EVT were infected with HCMV-AD169-GFP or HCMV-Toledo-GFP. HCMV-AD169 infection resulted in ∼100% HCMV+ DSC (∼70% immediate early (IE)-GFP+ and ∼30% late antigen (pp65+) GFP− cells), ∼40% HCMV+ JEG3 (10% IE-GFP+, and ∼30% pp65+GFP− cells), and ∼100% HCMV+ EVT (100% IE-GFP+ and 50% pp65+ GFP+ cells) after 48 h (*SI Appendix*, Fig. S4 *A* and *B*). Active HCMV infection was confirmed by detection of HCMV-UL55, -UL123, and -UL125 mRNA transcripts in DSC, JEG3, and EVT (*SI Appendix*, Fig. S5). Both dNK and pNK showed significantly higher levels of degranulation in response to HCMV-infected DSC compared with healthy DSC (Fig. 4*A*; *SI Appendix*, Fig. S4*C*). Interestingly, when JEG3 and EVT were infected with HCMV, pNK demonstrated significantly increased degranulation in response to these cells compared with uninfected cells. However, dNK did not degranulate in response to HCMV-infected JEG3 or EVT (Fig. 4 *B* and *C*; *SI Appendix*, Fig. S4 *D* and *E*). Similar results were obtained using the HCMV strains AD169 and Toledo (*SI Appendix*, Fig. S6). The JEG3 and all EVT used expressed HLA-C1 and -C2. These data illustrate the difficulty dNK have in responding to placental viral infections. In addition, pNK secreted significantly more IFNγ, TNFα, and GM-CSF in response to HCMV-AD169–infected DSC, JEG3, and EVT compared with uninfected healthy cells. Similarly, dNK secreted more of these cytokines in response to HCMV-infected DSC compared with healthy DSC (Fig. 4 *D*–*F*; *SI Appendix*, Fig. S7). However, whereas dNK secreted low levels of TNFα and GM-CSF in response to healthy EVT, no increase of these cytokines was observed following coculture with HCMV-infected EVT. Furthermore, no significant difference was observed in cytokine secretion between pNK or dNK from *KIR2DS1*− or *KIR2DS1*+ donors (*SI Appendix*, Fig. S7). A limitation of this study is the lack of information on HCMV status of all tissues used for experiments. HCMV infection was previously shown to specifically expand cytolytic NK populations that express the HLA-E receptor NKG2C+ as well as the NK maturation marker CD57 in HCMV-seropositive individuals (41, 42). The capacity of NKG2C+ and CD57+ pNK and dNK to degranulate in response to HCMV-infected DSC was analyzed. No correlation between degranulation levels and the percentage of NKG2C+ pNK or dNK was observed, suggesting that expansion of these populations, possibly due to previous infection, does not influence the NK response to HCMV-infected DSC (*SI Appendix*, Fig. S8). Although virtually none of the dNK expressed CD57, a high percentage of pNK expressed CD57. Moreover, CD57+ pNK had significantly higher levels of degranulation in response to HCMV-infected DSC (*SI Appendix*, Fig. S8*H*). Coculture of dNK or pNK with HCMV-infected DSC or HCMV-infected EVT led to a decrease of HCMV-IE-GFP+ cells, compared with HCMV-infected cells that were cultured alone (*SI Appendix*, Fig. S9). The decrease of HCMV-IE-GFP+ DSC and EVT in the presence of pNK and the decrease of HCMV-IE-GFP+ DSC in the presence of dNK reflect the degranulation data (Fig. 4). However, the loss of HCMV-IE-GFP+ EVT upon coculture with dNK is unexpected and may indicate that dNK use other mechanisms independently of degranulation and cytokine secretion to clear HCMV when EVT are infected.

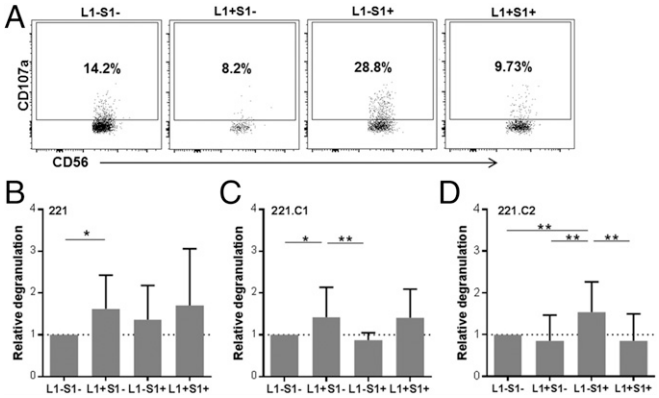

Fig. 3. KIR2DS1+ single-positive dNK have higher levels of degranulation in response to HLA-C2+ targets. (*A*) FACS plots of CD107a expression of four dNK subsets (L1−S1−, L1+S1− L1−S1+ and L1+S1+) in response to 221.C2 targets. Graphs depict degranulation of L1+S1−, L1−S1+, and L1+S1+ subsets relative to the L1−S1− subset in response to (*B*) 221 (n = 21), (*C*) 221-C1 (n = 16), and (*D*) 221.C2 (n = 24). dNK were identified by CD45+CD14−CD8−CD56+ (*SI Appendix*, Fig. S11). Bars indicate median and interquartile range; $*P < 0.05$, $**P < 0.01$.

Degranulation of KIR2DS1+ dNK in Response to HCMV-Infected HLA-C2+ DSC Is Increased. Next, the influence of KIR2DS1 and HLA-C2 expression on the capacity to respond to HCMV infection was investigated. A DSC line homozygous for HLA-C1 (DSC.C1) and a DSC line expressing both HLA-C1 and -C2 (DSC.C2) were infected with HCMV. dNK that express KIR2DS1 (L1−S1+ and L1+S1+) demonstrated a significantly increased response to HCMV-infected DSC.C2 compared with L1−S1− dNK (Fig. 5*B*). A similar increase was demonstrated in response to infected DSC.C1, but this was not significant (Fig. 5*A*). Moreover, the percentage of total S1+ dNK (both L1−S1+ and L1+S1+) positively correlated with the relative degranulation in response to infected DSC.C2 (Fig. 5*C*). In pNK, the L1+S1+ double-positive subset also showed an increased response to HCMV-infected DSC in C1 homozygous or C1/C2 (Fig. 5 *D* and *E*). Furthermore, S1+ pNK did not correlate with the degranulation level in response to infected DSC.C2 (Fig. 5*F*). These data demonstrate that the combination of KIR2DS1 expression on dNK and the presence of HLA-C2 on DSC increased the ability of dNK to respond to decidual HCMV infection.

HCMV Infection Reduced HLA-C on DSC, JEG3, and EVT. HCMV has many molecular strategies to circumvent immune recognition and avoid lysis of infected cells. Immune evasion strategies by HCMV include the blockade of peptide presentation and down-regulation of cell-surface expression of MHC class I (43, 44). This prevents cytolysis by CD8+ T cells but also increases the susceptibility to lysis by NK cells through missing self-recognition. Down-regulation of HLA-C could also prevent activation of NK cells through KIR2DS1 (6, 45). To shed further light on this situation, HCMV-infected and uninfected DSC, JEG3, and EVT were analyzed for expression of HLA-C, HLA-E, and HLA-G 48 h post infection. At this time, cells that express the IE HCMV gene (IE-GFP), the late HCMV antigen pp65, and both HCMV antigens can be found (*SI Appendix*, Fig. S4*B*). HCMV-infected DSC that expressed HCMV-IE-GFP lost essentially all HLA-C expression (*SI Appendix*, Fig. S10 *A* and *B*). In contrast, HLA-C expression was reduced on ∼50% of infected JEG3 and EVT that expressed HCMV-IE-GFP although residual HLA-C expression was evident (*SI Appendix*, Fig. S10 *A* and *B*). Importantly, HLA-C expression was increased on HCMV-infected DSC and JEG3 that did not express HCMV-IE-GFP, but did express the late HCMV antigen pp65 in comparison with uninfected DSC and JEG3 (*SI Appendix*, Fig. S10 *A* and *C*). Thus, the HLA-C ligand for KIR2DS1 is present on late HCMV-infected cells. HCMV infection resulted in a modest reduction of HLA-E on infected DSC but did not change HLA-E and HLA-G expression on JEG3 and EVT (*SI Appendix*, Fig. S10*A*). The antibody used for detection of HLA-C (DT9) has been reported to cross-react with HLA-E (46). However, HLA-E expression was not significantly affected by HCMV infection, showing that the change in DT9 staining is due to changes in HLA-C expression. Furthermore, HCMV infection did not induce MICA or MICB, two potent activating ligands that bind the activating NK receptor NKG2D, on any of the HCMV-infected cells.

Discussion

HLA-C expressed by fetal EVT has a unique role in pregnancy as the only polymorphic MHC molecule that can present viral peptides to maternal T cells and as the main molecule to which immune tolerance needs to be established (31). The importance of HLA-C was also demonstrated by the observation that the combination of the HLA-C allotype HLA-C2 (expressed by fetal trophoblasts) and the presence of its receptor KIR2DS1 (expressed by maternal NK cells) reduced the risk for pregnancy complications and is related to fetal birth weight (2, 47). Here we present a mechanism to explain how expression of KIR2DS1 by dNK increases the ability of dNK to control placental HCMV infection and thus may limit subsequent virus-induced placental inflammation and pathology.

The previous observation that dNK express higher levels of KIR2DL1 and S1 than pNK was confirmed (2, 7). In addition, KIR2DS1+ dNK (both L1−S1+ and L1+S1+ subsets) increased expression of key cytolytic molecules (perforin, granzyme B, and granulysin). Furthermore, dNK from *KIR2DS1*+ mothers were less efficiently inhibited by HLA-C2 expression on target cells, and the percentage of S1+ dNK (but not pNK) significantly correlated with the ability to degranulate in response to HLA-C2+ targets. Overall, these data indicate a skewing of dNK

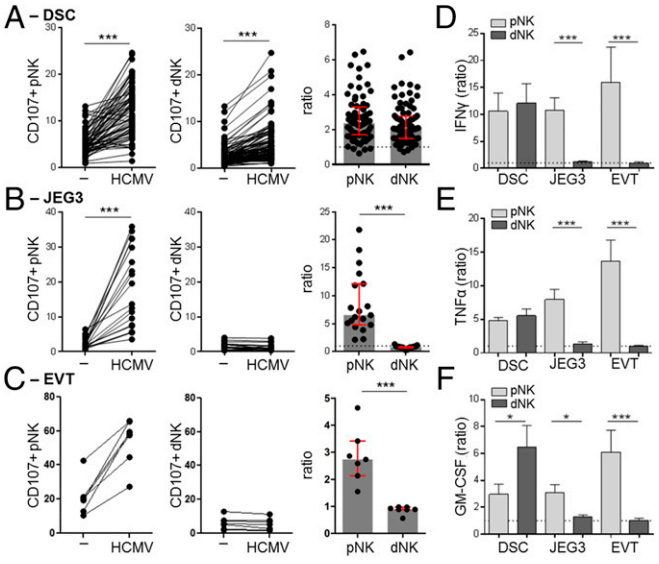

Fig. 4. dNK degranulate and produce cytokines in response to HCMV-infected DSC but not to HCMV-infected JEG3 and EVT. Percentage of CD107a+ pNK (*Left* panels in *A*–*C*) and dNK (*Middle* panels in *A*–*C*) after coculture with healthy and HCMV-infected (*A*) DSC, (*B*) JEG3, and (*C*) EVT for 10 h in the presence of 2.5 ng/mL IL-15. Relative degranulation of pNK and dNK in response to HCMV-infected cells relative to healthy cells are also depicted (*Right* panels in *A*–*C*). Cell culture supernatants of dNK and pNK incubated with healthy and HCMV-infected DSC (pNK n = 27, dNK n = 35), JEG3 (pNK n = 14, dNK n = 14), and EVT (pNK n = 7, dNK n = 9) were analyzed for (*D*) IFNγ, (*E*) TNFα, and (*F*) GM-CSF. Cytokine production is depicted as the ratio of concentrations in HCMV-infected cultures relative to uninfected cultures. All ratios >1 indicate an increased response to HCMV-infected versus uninfected cells. $*P < 0.05$, $***P < 0.005$.

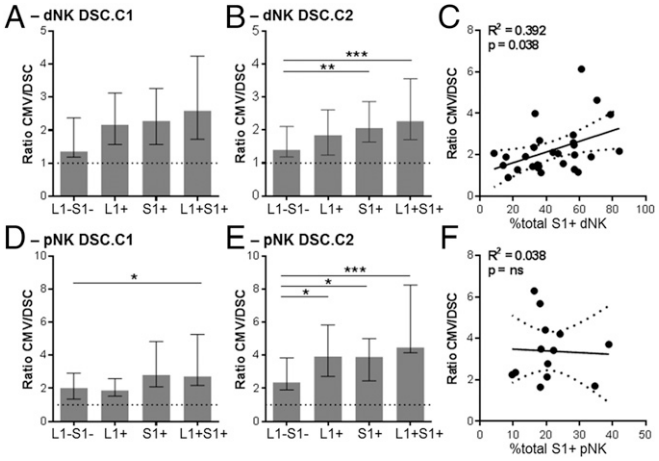

Fig. 5. Increased response of KIR2DS1+ dNK to HCMV-infected HLA-C2+ DSC. Specific degranulation of the four L1/S1 NK subsets in response to HCMV-infected DSC. Data are depicted as the ratio of the percentage of CD107a+ NK in response to HCMV-infected relative to uninfected DSC of (*A*) dNK/DSC.C1 ($n = 24$), (*B*) dNK/DSC.C2 ($n = 13$), (*C*) pNK/DSC.C1 ($n = 13$), and (*D*) pNK/DSC.C2 ($n = 10$). Correlation between specific degranulation in response to HCMV-infected DSC.C2 and the percentage of S1+ (S1+ and L1+ S1+) in (*E*) dNK and (*F*) pNK. Bars indicate median and interquartile range; $*P < 0.05$, $***P < 0.005$.

receptor expression toward recognition of HLA-C and, particularly, the potential of dNK to develop an activating response through KIR2DS1 and its interaction with HLA-C2. Although activation of KIR2DS1 by HLA-C2 does not override the inhibition through KIR2DL1-HLA-C2 interaction on the same cell (L1+S1+ double-positive dNK are efficiently inhibited by HLA-C2), the presence of a KIR2DS1 single-positive subset shifts the response of the total dNK population toward higher degranulation to HLA-C2+ targets. More importantly, dNK and, in particular, KIR2DS1+ dNK degranulated in response to HCMV-infected DSC, and L1+S1+ double-positive dNK were not inhibited by HCMV-infected HLA-C2+ DSC. The initial decrease of HLA-C expression on HCMV-infected DSC may lead to a lack of NK inhibition and response by KIR2DL1+ cells (including L1+ single-positive and L1+S1+ double-positive cells) through missing-self recognition. Another possibility is that HCMV infection leads to up-regulation of an unknown activating ligand for KIR2DS1 and to higher levels of degranulation of KIR2DS1+ cells. Similarly, a recent study identified HLA-F as a ligand for KIR3DS1. Interaction of HLA-F and KIR3DS1 increased degranulation of KIR3DS1+ NK cells and inhibited HIV replication (18).

In contrast to infected DSC, dNK failed to increase degranulation and cytokine production in response to HCMV-infected JEG3 and EVT. Thus, EVT and JEG3 seem protected from a dNK response even when infected. Although previous studies have demonstrated that JEG3 and EVT have many genomic differences, JEG3 and EVT share the same MHC expression pattern on their cell surface (8). In this study, responses to HCMV-infected JEG3 and EVT were similar. Interestingly, even in the absence of dNK degranulation and cytokine secretion in response to HCMV-infected EVT (HCMV-IE-GFP+ EVT), a significant loss of HCMV-IE-GFP+ EVT was observed upon coculture with dNK. These data may indicate that dNK use other mechanisms that are independent of degranulation and cytokine secretion to clear HCMV when EVT are infected. Future studies should investigate these mechanisms that include the expression of cell-surface death receptors (e.g., FASL and PD-1) or miRNAs that were shown to be effective in HCMV clearance and may lead to discovery of unexpected mechanisms as to how dNK control viral infections in the placenta. Importantly, the failure of dNK to induce either cytolysis or proinflammatory cytokine secretion in response to HCMV-infected trophoblasts must be related to the high prevalence of HCMV+ individuals and development of congenital infections.

Many molecules, in particular HLA-G, but also others (e.g., surface expression of PD-L1, CTRAM, and secretion of anti-inflammatory cytokines such as TGFβ and EBI3) have been shown to be expressed on EVT and may explain how EVT are protected from degranulation and cytotoxicity (8). Interestingly, pNK degranulated and produced proinflammatory cytokines (IFNγ, TNFα, and GM-CSF) in response to HCMV-infected JEG3 and EVT. pNK are not in direct contact with EVT in vivo, and whether influx of pNK to HCMV-infected decidua contributes to clearance of HCMV-infected EVT requires further investigation. Furthermore, degranulation and cytokine production of pNK in response to HCMV-infected EVT demonstrates that EVT are not completely resistant to killing by immune cells. Additional activation of dNK during virus-induced inflammation may be required for dNK to clear infected EVT. Furthermore, the presence of high proportions of other decidual immune cells, such as macrophages and regulatory T cells, may further influence the capacity of dNK to respond to healthy or virus-infected EVT (31). Decidual macrophages in particular have been shown to restrain killing of the semiallogeneic cytotrophoblasts by dNK by a TGF-β–dependent mechanism (48). Further investigation is needed to determine if there are conditions under which dNK are able to kill infected EVT. In the present study, dNK were activated with a low dose of IL-15 (2.5 ng/mL). However, during a placental viral infection, other decidual immune cells, most importantly macrophages, can secrete high levels of proinflammatory cytokines (TNFα, IL-1β, IL-6, IL-12) that can enhance dNK activation (49).

DSC required a multiplicity of infection (MOI) of 0.5 to be infected whereas EVT required an MOI of 3–4 to become infected to the same level. This may suggest that DSC become infected before EVT in vivo and that dNK, in particular KIR2DS1+ dNK, may limit the spread of HCMV through the placenta by lysis of infected DSC. Previously, miRNAs produced and secreted by EVT exosomes were shown to limit HCMV infection of trophoblast cells (50). Regardless, HCMV-infected EVT were found in placental tissue (51), and other pathogens such as *Listeria monocytogenes* were shown to preferentially infect EVT (52). This demonstrates the difficulty the maternal immune system faces to provide immunity to infection in the tolerogenic environment of the placenta. Moreover, HCMV infection can inhibit trophoblast differentiation and invasion and result in underdeveloped placentas. Many pregnancy complications are thought to be the result of insufficient trophoblast invasion, inadequate spiral artery remodeling, and placental insufficiency. The interaction of maternal immune cells with fetal EVT at the maternal–fetal interface regulates these processes. However, no clear mechanisms are presented that explain the failure of these processes. A large variety of infections during pregnancy, which include HCMV reactivation during pregnancy, contribute to the occurrence of miscarriages and preterm birth (53). HCMV replicates in cytotrophoblasts isolated from early and late gestation placentas in vitro (29), and HCMV DNA was detected in decidual and placental biopsies in 69% of 282 healthy pregnancies using a PCR-based method (51, 54). Approximately 15% of pregnant women with primary HCMV infections spontaneously abort in early gestation, and HCMV seropositive women have a 1.5-fold increased risk of developing preeclampsia (55). However, due to the high variety of pathogens involved and the subclinical nature of many infections, occurrence of infections during pregnancy is underdiagnosed (26).

Here we demonstrate that expression of KIR2DS1 by dNK increases their ability to respond to placental HCMV infection. This may reduce or prevent virus-induced pathology of the placenta, improve placental function, and limit development of pregnancy complications. In contrast, women who lack *KIR2DS1* may have a lower ability to control placental HCMV infection and are prone to develop complications. Similarly, *KIR2DS5* may have a similar role in African populations in which the burden of infections with many pathogens and pregnancy

complications are prevalent. Improving the diagnostics of infections during pregnancy, monitoring the development of pregnancy complications, and investigating the relationship to HLA and KIR genotypes are key to improving maternal–fetal health. This approach will provide an understanding of how the maternal immune system balances immunity to infections and fetal tolerance and whether the immune system is capable of maintaining both simultaneously.

Methods

Trophoblasts and dNK were freshly isolated from discarded human placental, and decidual material (gestational age 6–12 wk) obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Peripheral blood leukocytes were isolated from discarded leukopacks from healthy volunteer blood donors at the Massachusetts General Hospital, Boston. All of the human tissue used for this research was deidentified, discarded clinical material. The Committee on the Use of Human Subjects [the Harvard institutional review board (IRB)] determined that this use of placental and decidual material is exempt from the requirements of IRB review (exemption determination no. F15835). The procedure to isolate EVT, dNK, and pNK has recently been described (8). Further details on the isolation of EVT, DSC, dNK, and pNK as well as methods for flow cytometry; degranulation assay; cytokine secretion assays; HCMV infection of DSC, JEG3, and EVT; fluorescence microscopy; quantitative real-time PCR; and statistical analyses used are described in *SI Appendix, SI Methods*.

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