The HLA-G cycle provides for both NK tolerance and immunity at the maternal–fetal interface

Tamara Tilburgs[a,1,2], J. Henry Evans[a,1], Ângela C. Crespo[a,b], and Jack L. Strominger[a,2]

[a]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138; and [b]Doctoral Programme in Experimental Biology and Biomedicine, Center for Neuroscience and Cell Biology, University of Coimbra, 3004-504 Coimbra, Portugal

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The interaction of noncytotoxic decidual natural killer cells (dNK) and extravillous trophoblasts (EVT) at the maternal–fetal interface was studied. Confocal microscopy revealed that many dNK interact with a single large EVT. Filamentous projections from EVT enriched in HLA-G were shown to contact dNK, and may represent the initial stage of synapse formation. As isolated, 2.5% of dNK contained surface HLA-G. However, surface HLA-G–negative dNK contained internalized HLA-G. Activation of dNK resulted in the disappearance of internalized HLA-G in parallel with restoration of cytotoxicity. Surface HLA-G was reacquired by incubation with EVT. This HLA-G cycle of trogocytosis, endocytosis, degradation, and finally reacquisition provides a transient and localized acquisition of new functional properties by dNK upon interaction with EVT. Interruption of the cycle by activation of dNK by cytokines and/or viral products serves to ensure the NK control of virus infection at the interface, and is illustrated here by the response of dNK to human cytomegalo virus (HCMV)-infected decidual stromal cells. Thus, the HLA-G cycle in dNK can provide both for NK tolerance and antiviral immunity.

pregnancy | decidua | human | HCMV | cytotoxicity

Constitutive HLA-G expression in healthy tissue is restricted to fetal extravillous trophoblasts (EVT) that invade maternal decidual tissue during pregnancy (1–3). HLA-G acts mainly to prevent NK cell cytotoxicity (4–6) and T-cell cytotoxicity (7) and induce tolerance (8, 9). The maternal–fetal interface is a unique tolerized immune compartment. The maternal decidual tissue contains high numbers of maternal immune cells, including decidual natural killer cells (dNK), T cells, and macrophages (10–12). In early pregnancy, when invasion of maternal tissue by HLA-G+ EVT takes place, dNK form the predominant decidual immune cell type and represent ∼70% of total leukocytes.

Besides HLA-G, EVT also express the MHC class I molecule HLA-C and a low level of HLA-E (13). Of these, HLA-C is the only polymorphic antigen capable of eliciting an antigen-specific allo-response, and an HLA-C mismatch has been associated with enhanced T-cell activation and induction of Treg cells at the maternal–fetal interface (14). HLA-C that is represented by allotype groups C1 and C2 and nonpolymorphic HLA-E molecules are also potent ligands for NK inhibitory receptors (6, 15). Therefore, HLA-G may not be necessary to inhibit NK cytotoxicity against EVT at the level of an individual interaction or immune synapse. HLA-G interacts with three receptors, killer cell Ig-like receptor 2DL4 (KIR2DL4 or CD158d) and leukocyte Ig-like receptors B1 (LILRB1, ILT2, or CD85j) and B2 (LILRB2, ILT4, or CD85d), which are expressed by NK cells and macrophages (16). All of these contain an activating as well as an inhibitory motif. Functionally, interaction of HLA-G–expressing cells with NKs has been shown to decrease cytotoxicity (4, 5) and increase production of cytokines such as IL-6 and IL-8 (17–19).

A splice variant of HLA-G exists that leads to shedding of soluble HLA-G (20). Soluble HLA-G has been shown to bind KIR2DL4 on peripheral blood NK cells (pNK) and, upon binding, the KIR2DL4–HLA-G complex internalizes and signaling occurs from an endosomal compartment (18, 21). The fact that signaling from the KIR2DL4–HLA-G complex preferentially occurs from the endosomal compartment indicates that HLA-G may play a greater role than simply protecting HLA-G+ cells from NK cytotoxicity. The importance of HLA-G is further emphasized by the fact that soluble HLA-G can be detected in maternal serum during pregnancy. Although the data are not extensive, reduced levels of soluble HLA-G have been associated with pregnancy complications such as preeclampsia (22).

A study looking at the acquisition of HLA-G by pNK from tumor cells provided insight into a possible mechanism of HLA-G–mediated immune tolerance. This study demonstrated that pNK could acquire HLA-G from a transfected melanoma cell line (M8) via trogocytosis and that after acquisition the pNK were no longer cytotoxic (23, 24), a state reminiscent of dNK that are unable to polarize the microtubule-organizing center (MTOC) and cytolytic granules to the immune synapse (25). Once the HLA-G that had been acquired by pNK was turned over and degraded, the pNK returned to their previous cytotoxic phenotype. Thus, the change in phenotype was transient and reversible. In addition, the expression of the HLA-C2–specific activating receptor KIR2DS1 on pNK enhanced trogocytosis of the chemokine receptor-7 (CCR7) from HLA-C2–expressing targets. KIR2DS1–HLA-C2 interaction also facilitated uptake of plasma membrane fragments and molecules beyond the specific receptor–ligand pair. Furthermore, with the acquisition of CCR7, the pNK acquired migratory properties in response to lymph node chemokines specific for CCR7 (26). Besides its role in trogocytosis, KIR2DS1 is particularly interesting in the context of dNK and EVT interactions, because mothers who have the *KIR2DS1* gene in their genome are protected to some

Significance

Decidual natural killer cells (dNK), the largest population of leukocytes at the maternal–fetal interface, have low cytotoxicity. They are believed to play a role in facilitating invasion of fetal HLA-G+ extravillous trophoblasts (EVT) into maternal tissues, a process essential for establishment of healthy pregnancies. dNK are low-cytotoxic while nonetheless containing cytotoxic granules and functioning in immunity to viral infections, illustrated here with human cytomegalo virus (HCMV). Interaction of dNK with EVT leads to acquisition of HLA-G by dNK (trogocytosis), followed by a cycle of internalization, degradation, and reacquisition of HLA-G. Cytokine activation facilitates HLA-G degradation and coincides with increased cytotoxicity by dNK. Thus, the cycle provides both for NK tolerance and antiviral immune function of dNK.

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[1]T.T. and J.H.E. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: tilburgs@fas.harvard.edu or jlstrom@fas.harvard.edu.

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extent from developing pregnancy complications when its HLA-C2 ligands are expressed by the fetus (27).

In the present study, in vitro coculture of primary fetal HLA-G+ EVT and maternal dNK (obtained from the same pregnancy sample) was used to study their physiological interactions. High-resolution imaging revealed that many immune synapses were formed between dNK and EVT. The dynamics of dNK and HLA-G+ EVT interactions was investigated. Understanding them is essential for elucidating their unique roles in immune tolerance at the maternal–fetal interface and the relation between tolerance and immunity at this site.

Results

Interaction of HLA-G+ EVT with dNK Visualized by Confocal Microscopy. High-resolution confocal microscopy of cocultures of primary EVT and dNK (*Methods*) revealed that the unusually large EVT formed contacts with multiple small dNK (Fig. 1*A*). EVT were distinguished from dNK by their morphology, large nuclei, expression of HLA-G, and absence of perforin. The contacts between EVT and dNK were imaged at higher resolution to examine expression of HLA-G at the contact points. HLA-G was enriched in 25% of these contacts between EVT and dNK (Fig. 1*B* and Fig. S1*A*) and absent in ∼75% of these conjugates (Fig. 1*C* and Fig. S1*B*). Areas of dense F-actin, characteristic of an NK synapse, were present at the contacts (Fig. 1 *B* and *C* and Fig. S1 *A* and *B*). Freshly isolated dNK were not cytolytic, and perforin-containing lytic granules did not polarize to the contact with EVT, whether or not HLA-G was enriched (Fig. S1*C*). EVT projected many filopodia that were characterized by surface HLA-G protein. The filopodia surrounding the contact points frequently reached out and made distinct contacts with dNK (Fig. 1*D*). Moreover, examples of dNK were found that had separated from EVT and on which HLA-G could be detected (white boxes in Fig. 1*E*).

Both Cell-Surface and Intracellular HLA-G Protein, but Not HLA-G mRNA, Is Detected on dNK. Direct ex vivo FACS analysis with the mAb MEM-G/09 that recognizes mature HLA-G revealed that HLA-G was present on the cell surface of ∼2.5% of CD45+ CD56+CD14− dNK but not on pNK (Fig. 2 *A* and *B*). HLA-G+ dNK were viable, as demonstrated by lack of 7AAD (7-amino-actinomycin D) staining, and did not include NK cells conjugated to other HLA-G+ decidual cells (Fig. S2). Next, the surface HLA-G− and HLA-G+ dNK populations separated by FACS were analyzed for total HLA-G protein by Western blot of cell lysates. When probed with the antibody for denatured HLA-G (MEM-G/01), a band of the expected size (∼39 kDa) was observed for dNK with surface HLA-G. Surprisingly, dNK lacking surface HLA-G also showed the 39-kDa HLA-G band in the Western blot, indicating the presence of intracellular HLA-G (Fig. 2*C*). Quantification of HLA-G protein relative to HSP70 protein expression demonstrated no significant difference in the amount of HLA-G between surface HLA-G− and HLA-G+ dNK populations (Fig. 2*D*), indicating that surface HLA-G− dNK also contained HLA-G but that it had been internalized. Next, HLA-G mRNA in FACS-sorted surface HLA-G–positive and surface HLA-G–negative dNK was quantified by quantitative (q)RT-PCR. HLA-G mRNA was detected in HLA-G+ EVT but was not detected in any of the dNK samples (Fig. S3). The data are consistent with the acquisition of HLA-G from EVT by dNK via trogocytosis, followed by internalization by endocytosis (18, 24).

Cytokine Activation of dNK Results in Loss of Both Surface and Internalized HLA-G. Freshly isolated dNK have little or no cytolytic activity and fail to polarize the MTOC and cytotoxic granules to the synapse with target cells (25). Culture of dNK with IL-15 results in reacquisition of cytolytic activity accompanied by restoration of polarization (25). Moreover, culture with IL-15 also resulted in the disappearance of both surface and intracellular HLA-G, as demonstrated by FACS and Western blot analysis of fresh and IL-15–stimulated (36 h) dNK (Figs. 3 and 4*A*). The activation of dNK by IL-2 and IL-12 also resulted in the significant loss of HLA-G after 36 h (Fig. 3). Some evidence of HLA-G ubiquitination has been reported, and could lead to HLA-G degradation (28).

Coculture of Activated dNK or pNK with EVT Results in Reacquisition of Surface HLA-G. To examine whether EVT can be the source of HLA-G found on dNK, primary FACS-sorted dNK were cultured

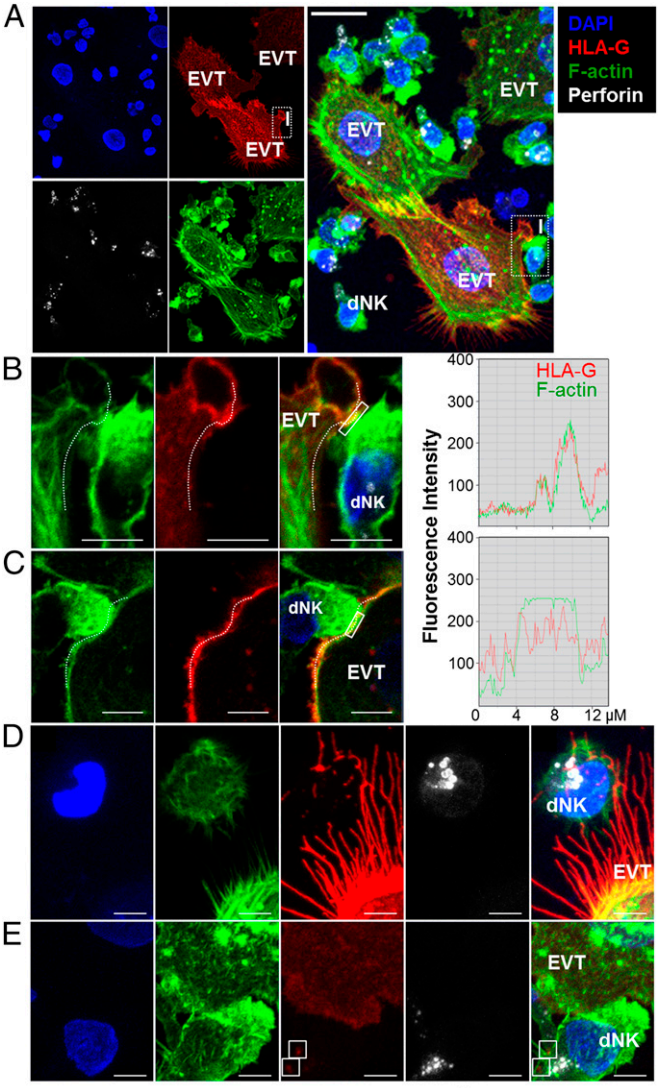

Fig. 1. Immune synapse formation between dNK and EVT. EVT and sample-matched dNK were coincubated for 2 h. The cocultures were stained for HLA-G (red) and then fixed, permeabilized, and stained with filamentous-actin (F-actin) (green), DAPI (blue), and perforin (white). (*A*) Images of three HLA-G–expressing EVT and many perforin-containing dNK. (*Left*) Single colors. (*Right*) Merged image of all four colors. (Scale bar, 20 μm.) The dotted box indicates a dNK–EVT interaction imaged at higher magnification in *B*. (*B* and *C*) dNK and EVT contacts in which HLA-G is (*B*) enriched and (*C*) not enriched at the contacts between EVT and dNK. (Scale bars, 5 μm.) Graphs depict HLA-G (red) and F-actin (green) intensity on the dotted profile lines following the EVT membrane. The fluorescence intensity within the white boxes are depicted in Fig. S1. (*D*) Images showing HLA-G–coated filopodia extending from EVT to dNK. Panels show single colors as well as a merged image. (Scale bars, 5 μm.) (*E*) Images showing HLA-G staining on dNK. The HLA-G staining on dNK is not continuous with the EVT. Panels show single colors as well as a merged image. White boxes indicate trogocytosed HLA-G (red) on dNK. (Scale bars, 5 μm.)

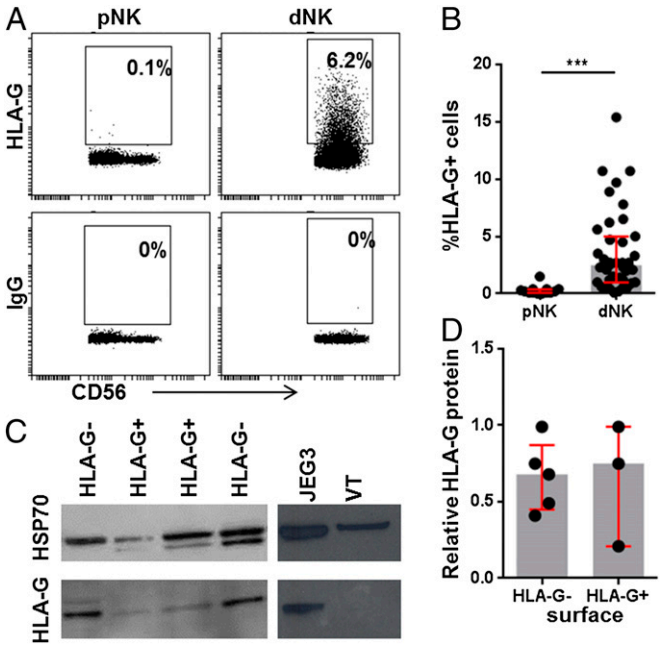

Fig. 2. HLA-G is present on dNK both as surface HLA-G and as internalized HLA-G. (*A* and *B*) FACS plots (*A*) and percentage (*B*) of surface HLA-G+ CD45+ CD56+CD14−CD3− pNK and dNK analyzed directly ex vivo. (*C*) Western blot images of fresh dNK sorted into surface HLA-G+ and HLA-G− populations. Bands for HLA-G (39-kDa) and HSP70 (70-kDa) proteins are depicted. Negative and positive controls include HLA-G− VT and HLA-G+ EVT. (*D*) Quantification of HLA-G relative to HSP70 expression within surface HLA-G− and surface HLA-G+ dNK. Bars depict median percentages and lines depict interquartile range. ***$P < 0.005$.

alone or in the presence of sample-matched EVT. During culture with IL-15 but in the absence of EVT, dNK lost or internalized surface HLA-G in 18 h and HLA-G was completely gone from the cell surface after 36 h (Fig. 4 *A* and *B* compared with Fig. 2 *A* and *B*; 2.5% ex vivo vs. 1.3% at 18 h vs. 0.1% at 36 h). A significantly higher proportion of dNK cultured with IL-15 and EVT for 18 h had surface HLA-G than those that were cultured with IL-15 alone (Fig. 4 *A* and *B*). Furthermore, IL-15 preactivation significantly increased the uptake of HLA-G by dNK (Fig. 4*B*). Coculture of pNK with EVT also resulted in the acquisition of HLA-G (Fig. 4*C*).

Direct transfer following cell contact (trogocytosis) is the likely mechanism for HLA-G reacquisition from EVT by dNK. Alternative mechanisms would include (*i*) soluble HLA-G could be shed into the medium by EVT for subsequent acquisition by dNK, or (*ii*) HLA-G–containing exosomes could be released by EVT for uptake by dNK. To discriminate between these alternatives, dNK or pNK were physically separated from EVT using Transwell separation membranes. Transwell separation of the dNK and pNK from the EVT abrogated acquisition of HLA-G (Fig. S4). Thus, acquisition of HLA-G from EVT is contact-dependent, consistent with trogocytosis being the primary mechanism of HLA-G acquisition by NK cells.

HLA-G Acquisition and NK Cytolytic Capacity. The loss of HLA-G from the dNK surface (Fig. 4) and the degradation of intracellular HLA-G (Fig. 3) during incubation with IL-15 coincide with an increase in dNK cytotoxicity (25). To examine the influence of HLA-G on the cytolytic capacity, FACS-sorted surface HLA-G− and HLA-G+ dNK were incubated with the MHC-negative 721.221 targets (221) and analyzed for cytolytic activity using a FACS-based CD107a degranulation assay. Little degranulation (~10% CD107a+ dNK) toward 221 targets was observed, and no difference between the surface HLA-G− and HLA-G+ dNK was found (Fig. S5 *A* and *B*). Next, surface HLA-G− and HLA-G+ dNK were incubated with IL-15 for 18 h. Degranulation of both dNK subsets in the presence of 221 targets significantly increased. However, again, no difference between surface HLA-G− and HLA-G+ dNK was observed (Fig. S5*B*). Furthermore, if EVT were substituted for 221 as targets, no signs of cytotoxicity to EVT were found, based on both evaluation by light microscopy and the CD107a degranulation assay (Fig. S5 *C*–*E*).

In addition, pNK and dNK were harvested after coculture with EVT and assessed for degranulation capacity toward 221. As a negative control, dNK and pNK were also cocultured with MHC-negative villous trophoblasts (VT). Interestingly, no difference in degranulation was observed between dNK that lost HLA-G during culture with IL-15 alone, or with IL-15 and VT, and dNK that retained HLA-G during culture with IL-15 and EVT (Fig. S6 *A* and *B*). Similarly, pNK that acquired HLA-G during coculture with IL-15 and EVT did not show a difference in degranulation against 221 compared with pNK cultured in IL-15 alone or with IL-15 and VT (Fig. S6*C*). The same results were obtained using a ^{51}Cr cytolytic assay (Fig. S6 *D* and *E*). Thus, under the conditions used here, HLA-G (re)acquisition by pNK and dNK did not change their degranulation levels or cytotoxicity to 221.

IL-15 Is Required for Lysis of Human Cytomegalo Virus-Infected Decidual Stromal Cells by dNK. A recent study demonstrated that dNK can kill human cytomegalo virus (HCMV)-infected decidual stromal cells (DSC) upon coincubation, but only after a lag of 6 h or longer (29). Here, fresh dNK were cocultured with DSC and HCMV-infected DSC in the presence or absence of IL-15. In the absence of IL-15, after 10 h of coincubation, dNK failed to respond to HCMV-infected DSC but degranulated at a low level in the presence of IL-15 (Fig. 5*A*), similar to that observed previously (29). However, after preincubation of dNK with IL-15 for 18 h no lag in degranulation was observed, and after 10 h of coincubation 18% of dNK degranulated, half of which was

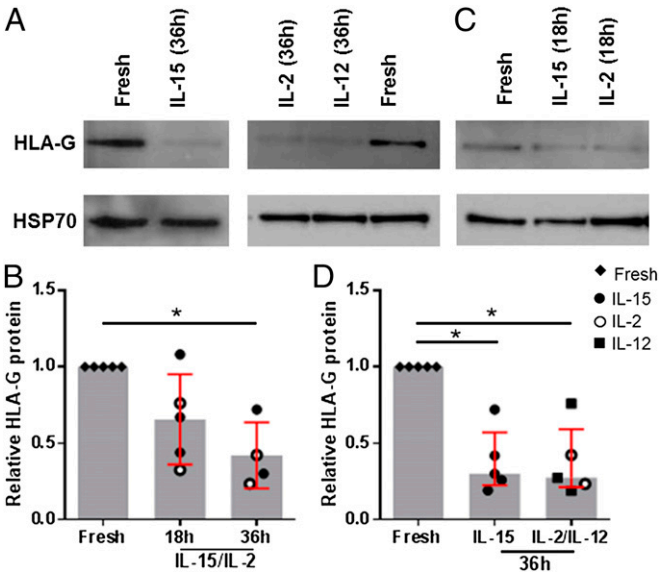

Fig. 3. Degradation of HLA-G on dNK by cytokine stimulation. (*A* and *B*) Western blot images (*A*) and quantification (*B*) of HLA-G relative to HSP70 protein of fresh dNK (◆) and dNK cultured with IL-15 (●) or IL-2 (○) for 36 h. (*C* and *D*) Western blot images (*C*) and quantification (*D*) of HLA-G relative to HSP70 protein of fresh dNK (◆) and dNK cultured with IL-15 (●), IL-2 (○), or IL-12 (■) for 36 h. Bars depict median percentages and lines depict interquartile range. *$P < 0.05$.

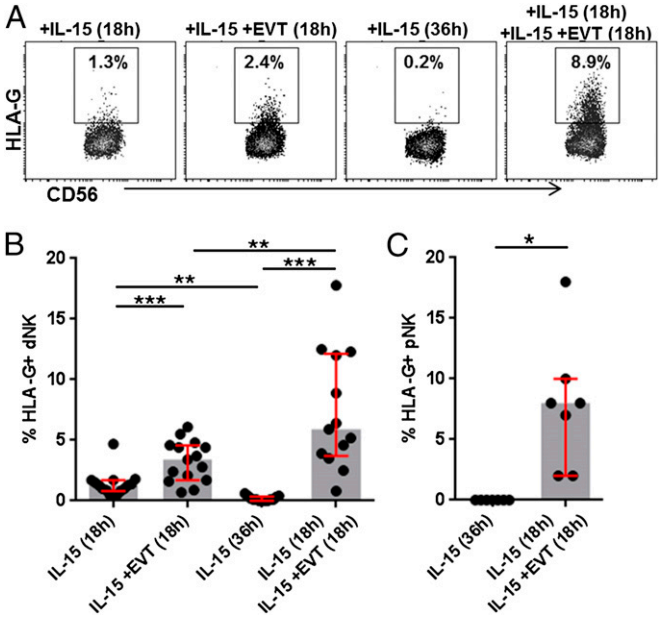

Fig. 4. dNK and pNK reacquire HLA-G from EVT. (*A*) Representative FACS plots of CD56 and HLA-G expression on dNK cultured with IL-15 or with IL-15 and EVT for 18 or 36 h. (*B* and *C*) Graphs depict percentages of (*B*) HLA-G+ dNK and (*C*) HLA-G+ pNK after culture with or without EVT. Bars depict median percentages and lines depict interquartile range. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

specific to HCMV infection (Fig. 5*B*). These experiments illustrate the specific requirement of IL-15 to activate dNK cytotoxicity both in response to uninfected DSC and specifically in response to HCMV-infected DSC.

KIR2DL4- and KIR2DS1-Expressing dNK Have Increased Levels of Surface HLA-G. To investigate the role of HLA-G receptors in controlling HLA-G acquisition, fresh dNK were stained for HLA-G and analyzed for surface ILT2, surface KIR2DL4, and intracellular KIR2DL4 expression. Interestingly, HLA-G+ dNK had significantly higher levels of intracellular KIR2DL4, but neither surface ILT2 nor surface KIR2DL4 was significantly different between HLA-G+ and HLA-G− dNK (Fig. S7 *A–D*). Furthermore, fresh and IL-15–preactivated dNK were cocultured with EVT in the presence of blocking antibodies for ILT2 and KIR2DL4 or isotype controls. However, neither KIR2DL4 nor ILT2 antibodies were able to block the acquisition of HLA-G (Fig. S7 *E* and *F*) (16, 19). This may suggest that KIR2DL4 and ILT2 are not required for HLA-G uptake.

The KIR2DS1 receptor is particularly interesting to study in the context of dNK and EVT interactions. One study demonstrated that KIR2DS1 facilitated trogocytosis of HLA-C2 molecules and associated membrane fragments from antigen presenting cells (APC) by pNK (26). A protective effect of the *KIR2DS1* gene in the development of pregnancy complications was demonstrated (27). Therefore, dNK were typed for the expression of KIR2DS1 using flow cytometry (30) (Fig. S8*A*). Women who have the *KIR2DS1* gene (~1/3 of the US population) did not have a higher level of surface HLA-G on dNK than individuals who lack the *KIR2DS1* gene in the genome (Fig. S8*B*). Moreover, HLA-C2 expression by fetal EVT did not influence HLA-G expression on dNK (Fig. S8*C*). However, when dNK were subdivided into the four KIR2DL1/S1 subsets (L1−S1−, L1+S1−, L1−S1+, L1+S1+), a significant increase in the percentage of HLA-G+ dNK was found within the KIR2DS1+ dNK subsets (L1−S1+, L1+S1+) directly ex vivo as well as after coculture with IL-15 and EVT (Fig. S8 *D* and *E*). Specific blocking antibodies for KIR2DS1 are currently unavailable, and whether KIR2DS1 can directly contribute to the uptake of HLA-G cannot be addressed. However, the activating properties of KIR2DS1 may enhance dNK–EVT interaction and increase the uptake of membrane fragments including HLA-G.

Discussion

Interactions of dNK and HLA-G+ EVT are difficult to study due to the low EVT numbers that can be obtained from tissue and the lack of proliferative capacity of both dNK and EVT. For this reason, many previous studies have relied on a tumor cell line (JEG3), HLA-G–transfected cells (the lymphoblastic cell line 4C4 or the melanoma cell line M8), or recombinant soluble HLA-G to mimic dNK interactions with HLA-G or EVT (18, 19). In the present study, coculture of primary HLA-G+ EVT and dNK obtained from the same pregnancy sample was used to address direct interactions ex vivo and functional effects on dNK by EVT. High-resolution imaging demonstrated an abundance of contacts formed between a single EVT with many dNK. Perforin did not localize to the synapse in the vast majority of dNK–EVT interactions, indicating that a lytic synapse is not required for acquisition of HLA-G by dNK. Furthermore, filopodia-like structures formed by EVT were seen frequently to reach out and make distinct contacts with the dNK, and may either represent an intermediate stage in synapse formation or provide an alternative manner for the transfer of HLA-G.

Coincubation of IL-15–activated sample-matched dNK or allogeneic pNK with EVT resulted in enhanced transfer of HLA-G from EVT to NKs. No evidence of NK cytotoxicity on EVT in this coculture was evident. Furthermore, the proportion of dNK with surface HLA-G following coculture with EVT was consistent with the level of HLA-G observed on fresh dNK ex vivo, and thus may reflect a homeostatic balance between acquisition and HLA-G turnover. Acquisition of HLA-G led to internalization, as demonstrated by the presence of intracellular HLA-G in surface HLA-G− dNK. Both surface and intracellular HLA-G was lost or degraded in dNK by 36 h in the presence of IL-15.

The cycle of HLA-G uptake by trogocytosis, internalization, degradation, and reacquisition (Fig. 6) may provide novel functional properties to dNK. Incubation of dNK or pNK with EVT that either prevented HLA-G loss from dNK or resulted in de novo acquisition of HLA-G by pNK did not change degranulation levels or cytotoxicity to 221. Thus, under the conditions used here, the relatively low levels of HLA-G expressed on the surface through reacquisition did not result in the more general state of dNK in which cytotoxicity is suppressed. An important constraint in the

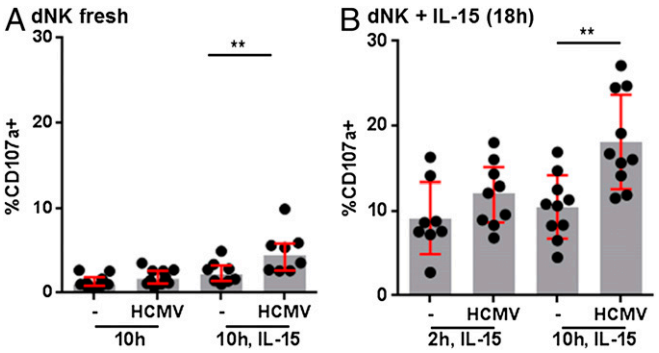

Fig. 5. IL-15–activated dNK specifically degranulate in response to HCMV-infected DSC. (*A*) The graph depicts the percentage of CD107a+ dNK in response to DSC and HCMV-infected DSC in the presence or absence of IL-15. Cells were coincubated for 10 h. (*B*) Similar graph of IL-15–preactivated dNK (18 h) cocultured for 2 or 10 h with DSC and HCMV-infected DSC.

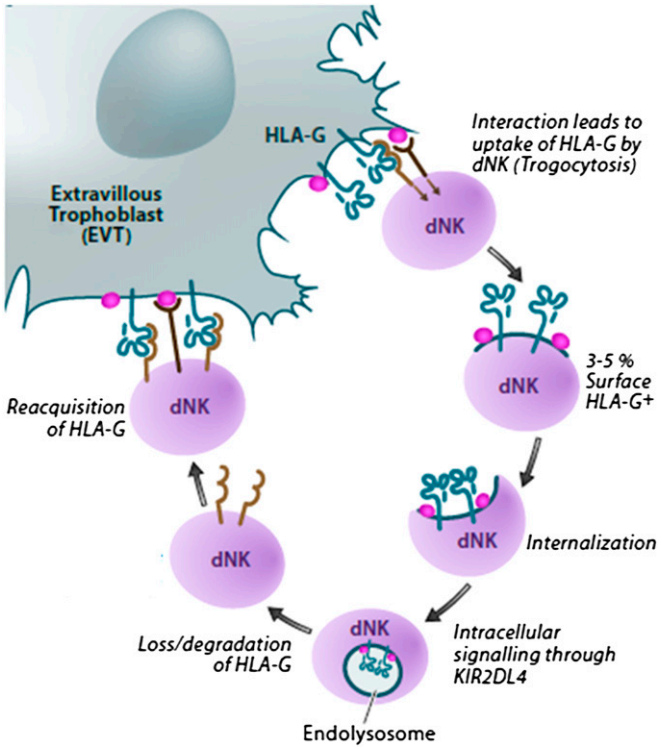

Fig. 6. HLA-G cycle. Trogocytosis, endocytosis, degradation, and finally reacquisition provide a transient and localized acquisition of new functional properties by dNK upon interaction with EVT.

experimental setup is the necessity to use IL-15 in the cultures. IL-15 is required for the survival of dNK in culture but is also a potent activator of NK cells. Its presence does not necessarily reflect the decidual environment in which dNK acquire HLA-G. Activation of NK cells with IL-15 is likely to override the possible inhibitory effects of HLA-G uptake under more antiinflammatory conditions, as found at the fetal–maternal interface. How dNK are suppressed in their cytolytic responses and the role of HLA-G both in that phenomenon and in possible additional endosomal signaling events are key questions for future studies.

Overexpression of HLA-G on 221 cells inhibits cytotoxicity of pNK and dNK. dNK contain an abundance of cytolytic granules, and the low cytotoxicity of freshly isolated dNK is the result of the failure to polarize the MTOC and the associated cytolytic granules to the synaptic region (25). The inhibition of dNK cytotoxicity that results is clearly an important facet of maternal–fetal tolerance. However, it raises the problem of how the pregnant female is able to respond to events that require participation of cytotoxic dNK, for example during a placental HCMV infection, a common viral infection at this site (29, 31). One possibility could be that the HLA-G cycle between EVT and dNK provides direct inhibition of cytotoxicity at an individual EVT–NK synapse as well as a prolonged but temporary inhibition of the dNK cytolytic machinery during HLA-G endocytosis and endolysosomal signaling events. The restoration of dNK cytolytic capacity by activation with proinflammatory cytokines such as IL-15 may be crucial in providing dNK with the ability to clear viral infections. The control of virus infection at the interface by dNK may require interruption of the HLA-G cycle, which could include inhibition either of reacquisition or endocytosis of HLA-G and is illustrated in the case of HCMV infection. No cytotoxicity after coculture of dNK with HCMV-infected DSC for 4 h was observed but, after 6–18 h of contact, dNK were fully able to kill the HCMV-infected DSC (29). In addition, in the present experiment, dNK were fully able to degranulate in response to HCMV-infected DSC without a lag and within 10 h of culture with IL-15, whereas in the absence of IL-15, dNK did not degranulate in response to HCMV-infected DSC. Furthermore, dNK preactivated by IL-15 for 18 h were able to respond to HCMV-infected DSC within 2 h of contact. Both failure to polarize the MTOC and up-regulation of NK cytotoxicity receptors on NK cells and/or NK ligands on the infected cells are plausible mechanisms. However, neither of the latter phenomena has been observed for HCMV infection. Incubation of dNK with IL-15 has been shown to restore both polarization of the cytolytic machinery as well as cytotoxicity of dNK (31). Here, preactivation of dNK by IL-15 was shown to eliminate the lag time in degranulation of dNK in response to HCMV-infected DSC. These results pose the interesting question of whether HCMV-infected cells activate dNK through the same mechanism as IL-15. DSC are known to secrete IL-15 (32). HCMV may up-regulate IL-15 secretion. Alternatively, HCMV-infected DSC may activate dNK through an entirely different mechanism.

A "danger" or proinflammatory cytokine signal resulting from the infection could be required to restore cytolytic capacity of dNK and interrupt the HLA-G cycle. The difficulty of clearing HCMV infection at the interface may be related to the conflict between tolerance and immunity that occurs at this site (29, 33). The detailed mechanisms that lead to inhibition of polarization as well as the restoration of cytolytic capacity by activation of dNK by IL-15 or the loss of acquired HLA-G by dNK are key questions for the future.

Trogocytosis and the intercellular transfer of membrane fragments and associated cell-surface proteins between immune cells are common and affect many stages of an immune response (34, 35). Trogocytosis of target cell molecules can lead to enhanced or sustained intracellular signaling pathways and induce de novo functional properties in receiving cells. Through acquisition and incorporation of target cell proteins in the cell membrane, the receiving cells can obtain new opportunities for interaction with other immune cell types for which ligands or receptors were previously not expressed (26, 36). Through the acquisition of HLA-G and possible other EVT membrane-associated molecules, the fraction of dNK with surface HLA-G may gain the ability to interact with other decidua-resident immune cells such as decidual T cells and macrophages. This mechanism could therefore constitute an important function for immune regulation of dNK activity and facilitate the spread of immune-regulatory functions at the fetal maternal interface. Although fresh HLA-G+ dNK expressed higher levels of intracellular KIR2DL4, surprisingly, KIR2DL4 (and ILT2) was not required for the acquisition of HLA-G. Intracellular HLA-G–KIR2DL4 complexes may result in sustained intracellular signaling after abrogation of the immune synapse and uptake of HLA-G (8, 18, 37).

Similar to a previous study that demonstrated that KIR2DS1 expression represented a major advantage for acquiring CCR7 from HLA-C2+ allogeneic dendritic cells and T-cell blasts (26), a small but consistent increase of HLA-G acquisition on KIR2DS1+ dNK was demonstrated. This small increase may suggest an increase in the interaction of KIR2DS1+ dNK with EVT. Prolonged intracellular signaling and possibly distinct functional properties may result. The role of KIR2DS1 in pregnancy is highlighted by the observation that women who express KIR2DS1 on their NK cells are protected from pregnancy complications whereas women who lack *KIR2DS1* but do carry a fetus that expresses an HLA-C2 group ligand for KIR2DS1 are at increased risk (27).

The fetal–maternal interface illustrates how evolution has met the challenge of generating a localized and specific tolerized immune compartment that does not reject an allogeneic graft (the fetus) while maintaining a systemic immune system capable of fighting infections (33). Our data provide novel insight into

this unique immune compartment and suggest that trogocytosis of HLA-G from fetal EVT by dNK and the subsequent HLA-G cycle may contribute to the multiple redundant mechanisms that help to maintain this essential balance.

Methods

Tissue Samples and Lymphocyte and Trophoblast Isolation. Discarded human placental and decidual material (gestational age 6–12 wk) was 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 Massachusetts General Hospital. 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 (38). Briefly, decidual and villous tissues were macroscopically identified and separated. Decidual tissue was washed, minced, and thereafter digested with 0.1% collagenase type IV and 0.01% DNase I (Sigma-Aldrich) gently shaking in a water bath for 1 h at 37 °C. Released cells were washed with RPMI, 10% (vol/vol) FBS (8 min, 650 × *g*) and filtered through 100-, 70-, and 40-μm sieves (BD; Labware). Filtered cells were dissolved in 20 mL 1.023 g/mL Percoll (GE Healthcare) and layered on a Percoll gradient (10 mL 1.080 g/mL; 12.5 mL 1.053 g/mL) for density gradient centrifugation (30 min, 800 × *g*). Lymphocytes were isolated from the 1.080–1.053 g/mL interface. Collected cells were directly stained for flow cytometric analysis or FACS sorting. pNK were isolated using a RosetteSep human NK cell enrichment mixture (StemCell Technologies) followed by Ficoll (GE Healthcare) density gradient centrifugation (20 min, 800 × *g*). For all NK cell isolates, >95% purity was obtained.

Trophoblasts were isolated as described previously (38). In short, villous tissue was gently scraped from the basal membrane. Thereafter, the tissue was digested for 8 min at 37 °C with a trypsin (0.2%) EDTA (0.02%) solution. Trypsin was quenched with F12 medium containing 10% (vol/vol) newborn calf serum (NCS) and penicillin, streptomycin (all from Gibco). Cells were filtered over a gauze mesh and washed once with complete F12 medium and layered on Ficoll (GE Healthcare) for density gradient centrifugation (20 min, 800 × *g*). Cells were collected, washed once, and incubated 20 min at 37 °C in a tissue-culture dish for removal of macrophages. Trophoblasts were collected and directly stained for flow cytometric analysis or FACS sorting.

Cocultures of NKs and EVT, flow cytometry, confocal imaging, Western blots, quantitative real-time PCR, degranulation assay, chromium release assay, HCMV infection of DSC, and statistical analyses used are described in *SI Methods*.

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1. Ellis SA, Sargent IL, Redman CW, McMichael AJ (1986) Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line. *Immunology* 59(4):595–601.
2. Geraghty DE, Koller BH, Orr HT (1987) A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment. *Proc Natl Acad Sci USA* 84(24):9145–9149.
3. Kovats S, et al. (1990) A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 248(4952):220–223.
4. Pazmany L, et al. (1996) Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science* 274(5288):792–795.
5. Poehlmann TG, et al. (2006) Inhibition of term decidual NK cell cytotoxicity by soluble HLA-G1. *Am J Reprod Immunol* 56(5-6):275–285.
6. Long EO, Kim HS, Liu D, Peterson MES, Rajagopalan S (2013) Controlling natural killer cell responses: Integration of signals for activation and inhibition. *Annu Rev Immunol* 31:227–258.
7. Le Gal FA, et al. (1999) HLA-G-mediated inhibition of antigen-specific cytotoxic T lymphocytes. *Int Immunol* 11(8):1351–1356.
8. LeMaoult J, et al. (2007) Immune regulation by pretenders: Cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells. *Blood* 109(5):2040–2048.
9. Brown R, et al. (2012) CD86+ or HLA-G+ can be transferred via trogocytosis from myeloma cells to T cells and are associated with poor prognosis. *Blood* 120(10):2055–2063.
10. Koopman LA, et al. (2003) Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med* 198(8):1201–1212.
11. Tilburgs T, et al. (2008) Evidence for a selective migration of fetus-specific CD4+ CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. *J Immunol* 180(8):5737–5745.
12. Houser BL, Tilburgs T, Hill J, Nicotra ML, Strominger JL (2011) Two unique human decidual macrophage populations. *J Immunol* 186(4):2633–2642.
13. Le Boutellier P, Sargent IL (2000) HLA class I molecules in the placenta: Which ones, where and what for? A workshop report. *Placenta* 21(Suppl A):S93–S96.
14. Tilburgs T, et al. (2009) Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J Reprod Immunol* 82(2):148–157.
15. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL (1993) HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci USA* 90(24):12000–12004.
16. Rajagopalan S, Long EO (1999) A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med* 189(7):1093–1100.
17. Hanna J, et al. (2006) Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* 12(9):1065–1074.
18. Rajagopalan S, et al. (2006) Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol* 4(1):e9.
19. Li C, Houser BL, Nicotra ML, Strominger JL (2009) HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells. *Proc Natl Acad Sci USA* 106(14):5767–5772.
20. Fujii T, Ishitani A, Geraghty DE (1994) A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. *J Immunol* 153(12):5516–5524.
21. Rajagopalan S, Moyle MW, Joosten I, Long EO (2010) DNA-PKcs controls an endosomal signaling pathway for a proinflammatory response by natural killer cells. *Sci Signal* 3(110):ra14.
22. Hackmon R, et al. (2007) Reduced third-trimester levels of soluble human leukocyte antigen G protein in severe preeclampsia. *Am J Obstet Gynecol* 197(3):255.e1–255.e5.
23. LeMaoult J, Caumartin J, Carosella ED (2007) Exchanges of membrane patches (trogocytosis) split theoretical and actual functions of immune cells. *Hum Immunol* 68(4):240–243.
24. Caumartin J, et al. (2007) Trogocytosis-based generation of suppressive NK cells. *EMBO J* 26(5):1423–1433.
25. Kopcow HD, et al. (2005) Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc Natl Acad Sci USA* 102(43):15563–15568.
26. Marcenaro E, et al. (2013) KIR2DS1-dependent acquisition of CCR7 and migratory properties by human NK cells interacting with allogeneic HLA-C2+ DCs or T-cell blasts. *Blood* 121(17):3396–3401.
27. Hiby SE, et al. (2004) Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 200(8):957–965.
28. Alegre E, et al. (2013) In vivo identification of an HLA-G complex as ubiquitinated protein circulating in exosomes. *Eur J Immunol* 43(7):1933–1939.
29. Siewiera J, et al. (2013) Human cytomegalovirus infection elicits new decidual natural killer cell effector functions. *PLoS Pathog* 9(4):e1003257.
30. Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, Michaëlsson J (2010) Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* 115(6):1166–1174.
31. Pereira L, Maidji E (2008) Cytomegalovirus infection in the human placenta: Maternal immunity and developmentally regulated receptors on trophoblasts converge. *Curr Top Microbiol Immunol* 325:383–395.
32. Okada S, et al. (2000) Expression of interleukin-15 in human endometrium and decidua. *Mol Hum Reprod* 6(1):75–80.
33. Tilburgs T, Strominger JL (2013) CD8+ effector T cells at the fetal-maternal interface, balancing fetal tolerance and antiviral immunity. *Am J Reprod Immunol* 69(4):395–407.
34. Carlin LM, Eleme K, McCann FE, Davis DM (2001) Intercellular transfer and supramolecular organization of human leukocyte antigen C at inhibitory natural killer cell immune synapses. *J Exp Med* 194(10):1507–1517.
35. Davis DM (2007) Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. *Nat Rev Immunol* 7(3):238–243.
36. McCann FE, Eissmann P, Onfelt B, Leung R, Davis DM (2007) The activating NKG2D ligand MHC class I-related chain A transfers from target cells to NK cells in a manner that allows functional consequences. *J Immunol* 178(6):3418–3426.
37. Favier B, Lemaoult J, Lesport E, Carosella ED (2010) ILT2/HLA-G interaction impairs NK-cell functions through the inhibition of the late but not the early events of the NK-cell activating synapse. *FASEB J* 24(3):689–699.
38. Tilburgs T, et al. (2015) Human HLA-G+ extravillous trophoblasts: Immune-activating cells that interact with decidual leukocytes. *Proc Natl Acad Sci USA* 112(23):7219–7224.
39. Alter G, Malenfant JM, Altfeld M (2004) CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods* 294(1-2):15–22.
40. Kamil JP, Coen DM (2007) Human cytomegalovirus protein kinase UL97 forms a complex with the tegument phosphoprotein pp65. *J Virol* 81(19):10659–10668.
41. Bradley AJ, et al. (2009) High-throughput sequence analysis of variants of human cytomegalovirus strains Towne and AD169. *J Gen Virol* 90(Pt 10):2375–2380.