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Killer Ig-like Receptor 2DL4 Does Not Mediate NK Cell IFN-γ Responses to Soluble HLA-G Preparations

Michael E. L. Le Page,*† Jodie P. Goodridge,*† Elisabeth John,* Frank T. Christiansen,* and Campbell S. Witt*§

The MHC class I b molecule HLA-G has previously been reported to be the ligand for the NK cell receptor killer Ig-like receptor (KIR)2DL4, but this remains controversial. In this study, we investigated IFN-γ production by freshly isolated NK cells in response to both soluble and solid-phase ligands, including anti-KIR2DL4 mAbs and rHLA-G. Although freshly isolated CD56bright NK cells produced IFN-γ in response to soluble HLA-G preparations, the response was found to be absolutely dependent on the presence of small numbers of contaminating CD56+, CD14+, CD11c+ myeloid dendritic cells (mDCs). HLA-G tetramers bound only to the contaminating mDCs in the NK preparations, and Abs to KIR2DL4 and HLA-G did not block NK cell IFN-γ production. NK cells did not respond to plate-bound HLA-G. Freshly isolated NK cells also produced IFN-γ in response to unpurified soluble anti-KIR2DL4 mAb but not to low endotoxin affinity–purified Ab. The data suggest that previous reports of functional interactions between KIR2DL4 and HLA-G may have resulted from the use of purified NK cells that were contaminated with mDCs and HLA-G preparations that were contaminated with material capable of stimulating mDCs to produce cytokines that stimulate NK cells to produce IFN-γ. The Journal of Immunology, 2014, 192: 732–740.

Killer Ig-like receptor (KIR)2DL4 is a framework gene of the KIR complex, a genetically polymorphic family of receptors expressed by NK cells. Different members of the KIR family activate or inhibit NK cell cytotoxicity and cytokine secretion in response to their ligands, the class I HLA molecules (1–3). KIR2DL4 is unusual among KIRs in both its structure and its expression. It has an extracellular region with a D0-D2 domain structure, whereas other KIR have D0-D1-D2 or D1-D2 structures. Inhibitory KIRs have two ITIMs in their cytoplasmic tail, whereas activating KIR have a positively charged amino acid in their transmembrane (TM) domain that enables them to partner with the activating adapter molecule DAP12. In contrast, KIR2DL4 has structural features of both inhibitory and activating KIRs including a single ITIM motif and a positively charged amino acid in its TM domain (4), which enables it to partner with the adaptor molecule FcRγ (5). All other KIRs are expressed constitutively on a subset of CD56dim NK cells (6, 7), whereas KIR2DL4 is primarily expressed on CD56bright NK cells, although its expression on all NK cells can be induced by culture with IL-2 (8, 9). However, membrane expression of KIR2DL4 occurs only at low density and only in individuals with at least one copy of the 10A-A TM (-TM) allele, and not in individuals homozygous for the common 9A-ΔTM and 10A-B alleles (9, 10). The 10A-A, 10A-B, and 9A-ΔTM alleles all occur at frequencies >20% (11, 12), suggesting balancing selection between the alleles, the cause of which is yet to be determined.

Functional studies suggest that ligation of KIR2DL4 activates NK cells as demonstrated by NK cell IFN-γ production and, to a lesser extent, cytotoxicity (8, 9, 13). Some evidence that KIR2DL4 is potentially capable of mediating inhibition has also been presented (14, 15). KIR2DL4 is also unique among KIR in that freshly isolated NK cells have been reported to be activated through KIR2DL4 by soluble ligands (13), whereas other KIRs require cross-linking. Soluble molecules reported to activate NK cells through KIR2DL4 include the mAb 33 (13) and a soluble form of the reported ligand, HLA-G (16). However, the evidence that HLA-G is the ligand for KIR2DL4 has been contradictory. Binding of recombinant chimeric KIR2DL4 to cells expressing HLA-G was first reported in 1998 (15, 17), although equivalent binding to alleles at other HLA loci was also shown. Similar evidence was reported by other groups (17–19). Recombinant GST-KIR2DL4 was also observed to precipitate with HLA-G expressed in K562 cells in a pull-down experiment (20). In contrast, HLA-G tetramers were reported not to bind to cells transfected with KIR2DL4 (21), and surface plasmon resonance studies failed to show any interaction between monomeric or dimeric HLA-G and recombinant KIR2DL4 (22). Evidence for functional consequences of HLA-G binding to KIR2DL4 on NK cells has been limited to secretion of IFN-γ and other cytokines from purified NK cells after incubation with soluble mAb 33 or soluble HLA-G (sHLA-G) (16).

In view of the somewhat contradictory evidence for HLA-G being the ligand for KIR2DL4 and the limited evidence of functional consequences of HLA-G binding to KIR2DL4, we investigated the production of IFN-γ by NK cells incubated with HLA-G using flow cytometry and ELISA.

Materials and Methods

Acquisition and culture of cells

All subjects were consenting adults previously typed for KIR2DL4 genotype. PBMCs were isolated from fresh blood by Ficoll-Paque gradient...
centrifugation. Where required, PBMCs were cryopreserved for later use. Purification of NK cells by RosetteSep NK cell isolation kit (15065; Stemcell Technologies, Vancouver, BC, Canada) or EasySep NK cell isolation kit (19055; Stemcell Technologies) were performed according to manufacturer’s instructions. At the time of this study, Abs in the RosetteSep kit were CD3, CD4, CD19, CD36, CD66b, and GlyA. Additional Abs in the EasySep kit were CD20, CD14, HLA-DR, and CD123. Purified NK cells were incubated with freshly irradiated Daudi feeder cells and 200 IU/ml rIL-2 (T3267; Sigma, St. Louis, MO) as described previously (10).

Cytokine release assays

NK cell IFN-γ response to stimuli was measured in both PBMCs and purified NK cells exposed to soluble and plate-bound ligands. Error bars in all figures indicate SEM. Abs for KIR2DL4 were mAb 2238 (MAB2238; R&D, Minneapolis, MN), mAb 33 (gift from S. Rajagopalan), low endotoxin affinity (LEAF)–purified mAb 33 (347003; BioLegend, San Diego, CA), and mAb531 Ab (M. Coloma courtesy of K. Campbell). Abs to HLA-G were 87G (10-437-C100; Exbio Praha, Czech Republic) and W6/32 (13-9983-82; ebioScience), as well as 4H84 (557577; BD Biosciences, Franklin Lakes, NJ), MEMG1 (ab7759; Abcam, Cambridge, U.K.), and MEMG9 (ab7758; Abcam). The Ab to Ig-like transcript (ILT)–4 was 27D6 (16-5148-81; ebioScience), and the Ab to IL-12 was QS-12p70 (ab25036; Abcam). rHLA-F and rHLA-G were supplied by J. Goodridge and D. Geraghty.

In assays using soluble ligands, 50,000 NK cells or ~500,000 PBMCs (this number was adjusted such that 50,000 NK cells were present) were incubated with ligand (and blocking Ab/isotype control where appropriate) in a volume of 150 μl at 37°C for 18 h. sHLA-F or sHLA-G was used at a final concentration of 3.6 μg/ml, whereas Abs (used for blocking or stimulation) were at concentrations optimized for staining by FACS. After this, the plate was incubated for a further 4 h with 50 μl brefeldin A (B7651-5MG; Sigma) at a final concentration of 12 mM. Supernatant was acquired for IFN-γ ELISA, and cell pellets were stained for intracellular IFN-γ by flow cytometry.

For assays using plate-bound ligands, flat-bottom, nontissue culture-treated, 96-well plates were preincubated overnight at 4°C with Ab, HLA-G, or HLA-F, diluted in ELISA bicarbonate coating buffer (15 mM Na2CO3, 30 mM NaHCO3, pH 9.5). Plates were blocked with 90% RPMI 1640/10% FCS at 37°C for a minimum of 30 min and washed before addition of cells. Fifty thousand NK cells (or equivalent PBMCs) were incubated in a volume of 150 μl at 37°C, after which brefeldin A was added for 4 h and IFN-γ production assayed as before.

Flow cytometry

Primary Abs for KIR2DL4 were mouse anti-human mAbs 2238, 33 or 53.1, and secondary Abs were goat anti-mouse IgG, IgG1, or IgG2a conjugated to fluorochrome as appropriate. For intracellular IFN-γ staining, the IntraPrep Permeabilization kit (IM2389; Beckman Coulter, Brea, CA) was used with directly conjugated Abs. Cells were washed and fixed with 1% paraformaldehyde and then evaluated on a FACS Canto Flow Cytometer. NK cell-surface marker levels were determined using directly conjugated Abs CD56-PE (555392), and IFN-γ, CD3-PE (IM1282U), and CD14-FITC (IM0645U) from Beckman Coulter, and HLA-DR–allophycocyanin–H7 (641393), CD11c-PE (555392), and IFN-γ–FITC (555718) from BD Biosciences. The gating strategy consisted of an initial lymphocyte gate based on forward and side scatter, followed by child gates based on surface marker staining levels.

Flow sorting experiments required that PBMCs or RosetteSep NK cells be stained with a single-step stain (CD56-PE, CD11c-PE, CD14-FITC), before sorting on a FACSAria Flow Sorter. In this case, the initial gating step consisted of all live cells based on forward and side scatter. Child gates were chosen based on surface marker staining levels as described earlier. After this, purified CD56+CD14+CD11c+ cells (or RosetteSep NK cells depleted of this population) were collected in sterile 5-ml tubes that had been washed with pure FCS. All cells were counted at this point: myeloid dendritic cells (mDCs) were incubated for 30 min with sHLA-G or control media, before being washed once, diluted to the appropriate concentration, and then added to RosetteSep NK cells as described earlier to achieve a concentration of mDCs equivalent to that present in the purified RosetteSep preparation.

IFN-γ ELISA

IFN-γ content of supernatant obtained from cellular assays was determined with the Opti-ELISA kit (555142; BD) “half-volume” assay in 96-well ELISA plates (351172; BD) according to the manufacturer’s instructions.

Characterization of HLA-G

HLA-G plasmid and recombinant sHLA-G protein was produced as described previously (23). HLA-G fractions were eluted from an HPLC size separation column and assessed for IFN-γ stimulatory ability as shown in Fig. 10. An example of the HLA-G used in each experiment can be seen in Fig. 10C. This fraction contained β2m (~13 kDa), β2m-associated HLA-G H chain (~26 kDa), β2m-associated HLA-G (~39 kDa), HLA-G dimers (~80 kDa), and high m.w. forms of HLA-G.

Quantitation of β2m-free HLA-G by ELISA used mAb 4H84 (557577; BD) as the capture Ab and biotinylated MEMG1 (120-26034; Abcam) as the detection Ab. Quantitation of β2m-associated HLA-G used MEMG9 (11-292-M01; Exbio, Vestec, Czech Republic) as the capture Ab and biotinylated W6/32 (13-9983; ebioScience, San Diego, CA) as the detection Ab. In each case, ExtrAvidin Peroxidase (E2886; Sigma) was used to develop the signal.

Fractions eluted from the HLA-G column were loaded onto a 10–20% Bio-Rad Criterion gel (Bio-Rad, Hercules, CA) with Precision Plus protein molecular mass standards and run for 160 min at 140 V. The gel was stained using Cooomassie brilliant blue G-250. The identity of HLA-F and HLA-G preparations was verified by mass spectrometry of gel bands (Proteomics International, Perth, WA, Australia).

Results

Freshly isolated NK cells, but not cultured NK cells, produce IFN-γ in response to sHLA-G

We have previously shown that plate-bound anti-KIR2DL4 mAb stimulates IFN-γ production from 14-d IL-2–cultured NK cells from donors with at least one KIR2DL4 10A-A allele, which was confirmed in this study using the EasySep kit. In contrast, cultured NK cells or freshly isolated PBMCs, which were not depleted of mDCs, did not produce IFN-γ in response to sHLA-G. We also found that purified, freshly isolated NK cells failed to produce IFN-γ in response to sHLA-G (Fig. 1A). In contrast, purified, 14-d cultured NK cells produced ~500 pg/ml of IFN-γ in response to sHLA-G. As shown in Fig. 1B, this response was dependent upon the presence of β2m on the HLA-G protein. HLA-G fractions depleted of β2m and β2m-associated HLA-G produced ~0 pg/ml of IFN-γ in response to sHLA-G, whereas HLA-G fractions enriched for β2m (~26 kDa) produced ~500 pg/ml of IFN-γ.

FIGURE 1. IFN-γ production in 14-d cultured NK cells induced by anti-KIR2DL4 Abs or sHLA-G (histograms show mean ± SEM of duplicate wells). (A) Solid-phase, not soluble mAb to KIR2DL4 (gray bars), stimulates NK cell IFN-γ. Appropriate isotype controls (black bars) did not stimulate IFN-γ. (B) Plate-bound anti-KIR2DL4 stimulates IFN-γ, but both plate-bound and sHLA-G (and HLA-F control) failed to stimulate IFN-γ.

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encodes a membrane-expressed receptor (10). In view of reports that soluble anti-KIR2DL4 stimulates IFN-γ from freshly isolated NK cells, we tested the ability of three different soluble anti-KIR2DL4 mAbs to stimulate IFN-γ in 14-d cultured NK cells. Fig. 1A shows that only plate-bound and not soluble KIR2DL4 mAbs induced IFN-γ production in cultured NK cells. Because sHLA-G has also been reported to stimulate IFN-γ production in freshly isolated NK cells, we tested the ability of plate-bound HLA-G and sHLA-G to stimulate IFN-γ in 14-d cultured NK cells. Fig. 1B shows that neither plate-bound nor sHLA-G induced NK cell IFN-γ production in cultured NK cells. An anti–HLA-G ELISA confirmed coating of the plates with sHLA-G.

Because soluble ligands were ineffective on cultured NK cells, we turned to freshly isolated NK cells. Fig. 2A shows that sHLA-G stimulated IFN-γ in freshly isolated NK cells, whereas media alone or recombinant sHLA-F did not stimulate. IFN-γ was produced by CD56bright NK cells [previously shown to express membrane KIR2DL4 in Goodridge et al. (8)], but not CD56dim NK cells [previously shown not to express membrane KIR2DL4], in response to sHLA-G. Fig. 2B shows that the response was largely, although not completely, restricted to cells expressing KIR2DL4. Fig. 2C shows that the response to sHLA-G was dose dependent. Fig. 2D shows that in a panel of PBMCs from 30 donors, the IFN-γ responses measured by ELISA correlated with stimulation by sHLA-G or soluble anti-KIR2DL4 mAb 33 (shown to induce IFN-γ by Rajagopalan et al. [13]), suggesting that sHLA-G and mAb 33 were acting through the same receptor. Fig. 2E shows that, in contrast with sHLA-G, plate-bound HLA-G did not stimulate IFN-γ from fresh NK cells.

**NK cell IFN-γ production in response to sHLA-G requires myeloid DCs.**

Fresh NK cells, purified by two different methods, exhibited a marked difference in the IFN-γ response to sHLA-G. Fig. 3 shows that NK cells purified using a Rosette-Sep kit produced IFN-γ in response to sHLA-G, whereas NK cells purified by an EasySep kit were completely unresponsive. RosetteSep and EasySep NK purification kits (Stemcell Technologies) both use negative selection principles, but comparison of the Ab cocktails used to remove non-NK cells revealed that the EasySep kit con-
tained additional Abs (anti–HLA-DR and anti-CD123) for removal of monocytes, mDCs, and plasmacytoid dendritic cells.

Exploratory FACS analysis (Fig. 4A, right) determined that RosetteSep NK preparations, but not EasySep NK preparations, contained a contaminating population of CD56<sup>dim</sup>, HLA-DR+, CD11c+ cells, ruling out both plasmacytoid dendritic cells (CD11c<sup>+</sup>) and monocytes (CD14+). The phenotype of the contaminating cells was consistent with mDCs. The RosetteSep kit did remove some mDCs compared with the PBMCs from which they were derived, but the EasySep NK cell preparation was completely devoid of mDCs (Fig. 4B).

To investigate the contribution of the contaminating mDCs to the IFN-γ response to sHLA-G, we performed cell depletion and add-back experiments by cell sorting the contaminating mDCs. Donor PBMCs were sorted to obtain CD56<sup>+</sup>, CD14<sup>-</sup>, CD11c<sup>-</sup> cells are necessary for IFN-γ production by freshly isolated NK cells stimulated with sHLA-G as measured by flow cytometry (upper panels) and ELISA (lower panels, with error bars). RosetteSep-purified NK cells from two 10A-A donors were stimulated with HLA-G before and after depletion of mDCs and after adding back an equivalent number (×1) or twice as many (×2) mDCs. In these donors this was equivalent to between 1 and 5% of all cells. EasySep-purified NK cells did not respond to stimulation by sHLA-G in the absence of mDCs, but addition of an equivalent number of mDCs to that present in PBMCs restored a partial response. ELISA histograms show mean ± SEM of duplicate wells.

FIGURE 3. NK cells from a KIR2DL4<sup>10A</sup> individual were purified by RosetteSep or EasySep purification kits and stimulated with sHLA-G. HLA-G stimulates IFN-γ from RosetteSep-purified NK cells, but not from EasySep-purified NK cells. Percentages indicate the proportion of CD56<sup>high</sup> and CD56<sup>dim</sup> NK cells positive for IFN-γ, respectively. Representative data from five experiments are shown.

FIGURE 4. Characterization of contaminating cells in RosetteSep NK cell purifications. (A, left panel) Contaminating cells were CD56<sup>+</sup>, CD11c<sup>-</sup>, and the cells purified by cell sorting (right panel) were CD14<sup>-</sup> consistent with their being mDCs. Representative data from five experiments are shown. (B) Staining with CD56 and HLA-DR in NK cells purified from five subjects showed a contaminating population of HLA-DR<sup>+</sup>, CD56<sup>-</sup> cells in RosetteSep-purified NK cells that were completely absent from EasySep-purified NK cells.

FIGURE 5. CD56<sup>+</sup>, CD14<sup>-</sup>, CD11c<sup>-</sup> cells are necessary for IFN-γ production by freshly isolated NK cells stimulated with sHLA-G as measured by flow cytometry (upper panels) and ELISA (lower panels, with error bars). RosetteSep-purified NK cells from two 10A-A donors were stimulated with HLA-G before and after depletion of mDCs and after adding back an equivalent number (×1) or twice as many (×2) mDCs. In these donors this was equivalent to between 1 and 5% of all cells. EasySep-purified NK cells did not respond to stimulation by sHLA-G in the absence of mDCs, but addition of an equivalent number of mDCs to that present in PBMCs restored a partial response. ELISA histograms show mean ± SEM of duplicate wells.
Discussion

KIR2DL4 is a difficult receptor to study due to its low-level membrane expression relative to other KIR receptors and the

NK cells that had not been exposed to HLA-G (Fig. 7). Fig. 7A shows that HLA-G-pulsed mDCs were sufficient to stimulate IFN-γ from fresh NK cells. Fig. 7B shows that 14-d cultured NK cells that were completely unresponsive to sHLA-G and mDCs were present. Fig. 7C shows that mDCs alone do not produce IFN-γ when pulsed with sHLA-G, but they assist 14-d cultured NK cells to produce IFN-γ.

IFN-γ-stimulating activity of sHLA-G is not blocked by Abs to KIR2DL4, HLA-G, or ILT-4

All of the preceding results were consistent with mDCs binding sHLA-G via a receptor like ILT-4 and presenting HLA-G as a plate-bound ligand to NK cells. However, an alternative explanation was that the sHLA-G preparation contained a contaminant that induced mDCs to secrete a cytokine that stimulated IFN-γ production from NK cells. In the first scenario, it should be possible to block NK cell IFN-γ production using blocking Abs to KIR2DL4, HLA-G, or ILT-4. Fig. 8 demonstrates that addition of Abs to KIR2DL4, (LEAF 33, 2238) HLA-G (W6/32, 87G), or ILT-4 (27D6) to freshly isolated RosetteSep NK cells stimulated with sHLA-G did not block IFN-γ production. All blocking Abs were used at concentrations demonstrated to give maximum fluorescence in flow cytometric assays when incubated with cells expressing KIR2DL4 or HLA-G as appropriate.

sHLA-G and mAb 33 stimulate IL-12 production from myeloid cells

Because Abs to KIR2DL4 and HLA-G did not block IFN-γ production, we examined the possibility that the sHLA-G preparation induced accessory cells to produce IL-12, which, in turn, stimulated NK cell IFN-γ production. Because contaminating mDCs in RosetteSep NK preparations were too few to analyze by FACS after an 18-h incubation period, PBMCs were stimulated and IL-12 production was assessed in monocytes. We stimulated PBMCs from individuals previously shown to be high or low IFN-γ producers in response to sHLA-G, with sHLA-G or mAb 33, and assessed monocyte IL-12 production by FACS (Fig. 9A, 9B). We found that both sHLA-G and mAb 33 stimulated monocyte IL-12 production, and that a higher proportion of monocytes from the IFN-γ high responder produced IL-12 compared with the IFN-γ low responder. Fig. 9C shows that the addition of anti–IL-12 Ab to RosetteSep-purified NK cells stimulated with sHLA-G was sufficient to abrogate the IFN-γ response. Fig. 9D shows that (in contrast with non-LEAF mAb 33 used in Figs. 1A, 9A, 9B) soluble LEAF mAb 33 did not stimulate NK cell IFN-γ production.

Because it is difficult to obtain HLA-G verified to be endotoxin free, we examined the relationship between IFN-γ-stimulating activity and HLA-G in column fractions obtained during purification of HLA-G. Fig. 10A shows that IFN-γ-stimulating activity was uniformly high in column fractions 3–15, after which it fell away. In contrast, an ELISA using Ab MEMG/9 that reacts only with denatured HLA-G (W6/32, 87G), or ILT-4 (27D6) to freshly isolated RosetteSep NK cells stimulated with sHLA-G did not block IFN-γ production. All of the preceding results were consistent with mDCs binding sHLA-G via a receptor like ILT-4 and presenting HLA-G as a plate-bound ligand to NK cells. However, an alternative explanation was that the sHLA-G preparation contained a contaminant that induced mDCs to secrete a cytokine that stimulated IFN-γ production from NK cells. In the first scenario, it should be possible to block NK cell IFN-γ production using blocking Abs to KIR2DL4, HLA-G, or ILT-4. Fig. 8 demonstrates that addition of Abs to KIR2DL4, (LEAF 33, 2238) HLA-G (W6/32, 87G), or ILT-4 (27D6) to freshly isolated RosetteSep NK cells stimulated with sHLA-G did not block IFN-γ production. All blocking Abs were used at concentrations demonstrated to give maximum fluorescence in flow cytometric assays when incubated with cells expressing KIR2DL4 or HLA-G as appropriate.

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Because mDCs express the ILT-4 receptor (LIRB2), which binds to the α-3 domain of HLA-G (24), leaving the α-1 and α-2 domains available for presentation to other cells, we hypothesized that mDCs might present HLA-G to KIR2DL4 on NK cells. To investigate this possibility and exclude the binding of sHLA-G to the α-3 domain of HLA-G (24), leaving the α-1 and α-2 domains available for presentation to other cells, we hypothesized that mDCs might present HLA-G to KIR2DL4 on NK cells. To investigate this possibility and exclude the binding of sHLA-G directly to NK cells, we performed a number of “pulse” experiments where PBMC-sorted mDCs were exposed to sHLA-G, washed, and then incubated with fresh or autologous cultured
fact that membrane expression is confined to the CD56<sup>bright</sup> subset of freshly isolated NK cells from individuals with at least one 10A-A allele (8–10). Low-level membrane expression of KIR2DL4 can be induced on proliferating CD56<sup>dim</sup> NK cells during in vitro culture (8, 9), and for cellular studies this may be the best source of NK cells that are both KIR2DL4<sup>+</sup> and mDC free.

Fig. 1 and previous data (9, 10) demonstrate that all Abs to KIR2DL4, including mAb 33, when presented as plate-bound arrays, stimulate IFN-γ from cultured NK cells. Other groups have reproduced this finding (9). This is consistent with KIR2DL4 being expressed on the membrane of cultured NK cells (8) and the fact that we have observed only robust IFN-γ production when the NK cells are derived from an individual with at least one KIR2DL4<sup>+</sup> and mDC free. KIR2DL4<sup>+</sup> NK cells were incubated with sHLA-G, mDCs pulsed with sHLA-G (DC–HLA-G), or nonpulsed mDCs (DC–Ctl). (B) IFN-γ production by 14-d cultured NK cells cultured with sHLA-G, nonpulsed mDCs, or sHLA-G-pulsed mDCs. (C) mDCs exposed to sHLA-G in the absence of NK cells do not produce IFN-γ. However, mDCs pulsed with HLA-G-stimulated IFN-γ from 14-d cultured NK cells. In all cases, NK cell IFN-γ production was measured by flow cytometry (upper panels) and ELISA (lower panels). ELISA histograms show mean ± SEM of duplicate wells.

FIGURE 7. HLA-G–pulsed mDCs are sufficient to induce IFN-γ production by freshly isolated NK cells and by 14-d cultured NK cells. (A) RosetteSep-purified NK cells were incubated with sHLA-G, mDCs pulsed with sHLA-G (DC–HLA-G), or nonpulsed mDCs (DC–Ctl). (B) IFN-γ production by 14-d cultured NK cells cultured with sHLA-G, nonpulsed mDCs, or sHLA-G-pulsed mDCs. (C) mDCs exposed to sHLA-G in the absence of NK cells do not produce IFN-γ. However, mDCs pulsed with HLA-G-stimulated IFN-γ from 14-d cultured NK cells. In all cases, NK cell IFN-γ production was measured by flow cytometry (upper panels) and ELISA (lower panels). ELISA histograms show mean ± SEM of duplicate wells.

The demonstration that HLA-G tetramers bind to mDCs and not to NK cells led us to speculate that HLA-G may be presented by mDCs to KIR2DL4 on NK cells. However, the lack of blocking by Abs to KIR2DL4 (2238 and LEAF 33), HLA-G (87G, W6/32), and ILT-4 (27D6), together with the fact that both non-LEAF mAb 33 and sHLA-G stimulated IL-12 production in monocytes suggested that the IFN-γ production by NK cells may have been driven by accessory cell–derived cytokines. Further evidence that HLA-G was not driving IFN-γ production was provided by the fact that IFN-γ-stimulating activity did not correlate with the column fractions containing HLA-G. We did not have adequate quantities of non-LEAF mAb 33 to perform a similar column fractionation experiment, but the failure of LEAF mAb 33 to stimulate IFN-γ...
strongly suggests that the Ab fraction of this preparation was not the active component.

The evidence for HLA-G being a ligand for KIR2DL4 has been controversial. Although several groups have provided evidence from binding studies (15, 17–20), other binding studies of HLA-G tetramer binding to KIR2DL4-transfected cells (21) and surface plasmon resonance (22) failed to detect any interaction. In some studies that showed evidence of binding to HLA-G, alleles from other HLA loci bound to a similar degree (15), and in others the difference between binding of HLA-G and control alleles was small (3-fold difference in mean channel fluorescence) (18). In unpublished experiments using an ELISA-like assay, equivalent low-avidity binding of KIR2DL4 has been observed with recombinant forms of HLA-G, HLA-A2, and H-2Kβ (K. Campbell, personal communication [26]), suggesting that these are all background binding interactions. It is possible that a unique form of HLA-G not represented in our preparations is a ligand for KIR2DL4, but our preparation included proteins of apparent molecular mass characteristic of simple HLA-G/b2m heterodimers, dimers of the heterodimers, and b2m-free H chain and larger aggregates. Our preparation did not include any of the HLA-G isotypes in which one or another exon is spliced out, but none of the publications providing evidence of HLA-G binding to KIR2DL4 included these isotypes either (15–20).

Earlier reports of IFN-γ production (measured by ELISA) by freshly isolated NK cells in response to soluble mAbs (13) may have been because of NK cell preparations containing small numbers of mDCs and mAb preparations contaminated by mDC-stimulating components. Of course, the fact that our particular HLA-G preparation included IL-12-stimulating contaminants does not mean that all HLA-G preparations used in previous publications are similarly contaminated, but this possibility must be considered. Indeed, some batches of sHLA-G tested by us completely failed to stimulate IFN-γ production despite containing the same forms of HLA-G (detected by SDS-PAGE) as IFN-γ-stimulating batches. We assume that the batch of HLA-F used as a negative control in these experiments failed to stimulate IFN-γ because of its lack of contaminants. Rajagopalan et al. (16) have shown colocalization of endocytosed HLA-G with KIR2DL4 in cytoplasmic endosomes. Because there is evidence that KIR2DL4 is capable of endocytosing CpG oligonucleotides (ODNs) (27), it is possible that ODNs are the relevant contaminant in some HLA-G solutions resulting in coendocytosis of HLA-G along with ODNs, thereby explaining the colocalization of HLA-G and KIR2DL4 in endosomes.

The present studies do not conclusively rule out the possibility that some form of HLA-G is a ligand for KIR2DL4, but they raise serious doubts about many published results suggesting that IFN-γ production by NK cells can be directly stimulated through KIR2DL4 by sHLA-G or soluble Abs. In view of our results, we suggest a series of criteria that should be used to assess future claims in regard to ligands for KIR2DL4. Given the powerful effect of small numbers of contaminating mDCs, future claims should include: 1) enumeration of mDC numbers in purified NK cell preparations, 2) the use of LEAF-purified ligands, and 3) convincing blocking studies with both anti-ligand and anti-KIR2DL4 Abs. The possibility that soluble Abs to KIR2DL4 might stimulate an IFN-γ response rather than block the receptor has influenced experimental design in the past. The data presented in this article demonstrate that soluble Abs to KIR2DL4, including mAb 33, do not directly stimulate IFN-γ production and, therefore, may be useful as blocking Abs. A biological response by NK cells from a donor with at least one 10A-A allele, but not by NK cells from a donor lacking this surface-expressed allele, would
also convincingly demonstrate a requirement for a functional KIR2DL4 receptor. It is noteworthy in this respect that our panel of 30 individuals showed no relationship between IFN-\(\gamma\) response to soluble ligands and KIR2DL4 genotype (data not shown), whereas the response to plate-bound anti-KIR2DL4 was genotype dependent (10, 25). Defining a physiologically relevant ligand for KIR2DL4 in this way will be necessary to establish the function of this receptor in human immunity.

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References