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Human NK cells contribute a significant role to host defense as well as xenogeneic cytotoxicity. Previous studies using human 721.221 cell line have shown that peptides derived from the leader sequence of the HLA-G binds and up-regulates the surface expression of HLA-E molecules, which was considered to consequently provide negative signals to human NK cells. However, the direct role of HLA-G in inhibiting human NK cells remains controversial. In this study, we showed that the expression of HLA-G or HLA-E in porcine endothelial cells directly protected sensitive porcine cells from human NK cell-mediated xenogenic cytotoxicity. Blocking assays using 2(2′)-2′-microglobulin; SHP, SHP-1. The direct role of HLA-G in inhibiting human NK cells also provides a unique opportunity to directly study the role of HLA-G and HLA-E in xenotransplantation. Therefore, strategies have to be designed to protect xenograft from polyclonal human NK cells. In addition, the lack of endogenous HLA class I genes also provides a unique opportunity to directly study the role of HLA-G in NK inhibition.
In this study, we tested the hypothesis whether the expression of two nonclassical class I HLA molecules, HLA-G and HLA-E, on porcine aortic endothelial cells (PAEC) would be sufficient to provide negative signals and protect porcine cells from human NK cell-mediated cytotoxicity. Our results indicated that both HLA-E and HLA-G transfection into porcine endothelial cells could directly block human NK-mediated lysis through two distinct pathways. These data strongly support that HLA-G alone can directly provide negative signals to human NK cells in addition to its role in providing leader sequence required for HLA-E expression. These results also will allow strategies to be developed to protect porcine xenograft from human NK cell-mediated immune responses.

Materials and Methods

Cells

HUVEC and K562 were obtained from the American Type Culture Collection (Manassas, VA). PAEC were prepared from Yucatan inbred swine (Sinclair, Columbia, MO) of the z/s haplotype and cultured as previously described (15).

Transfection

Full-length HLA-G and HLA-E genomic DNAs were kindly provided by Dr. Harry T. Orr (University of Minnesota, Minneapolis, MN) (16, 17). The HLA-G and HLA-E were separately transfected into PAEC along with human β2-microglobulin (β2m) genomic DNA using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s instruction. The expression of HLA-G or HLA-E was determined by indirect immunofluorescence.

Immunofluorescence and flow cytometry

Surface expression of HLA-G or HLA-E on PAEC was measured by indirect immunofluorescence. Cells were stained with murine mAb PA2.6, which specifically defines HLA class I framework determinants (American Type Culture Collection), followed by goat anti-mouse FITC-conjugated secondary Ab (Becton Dickinson, Mountain View, CA, USA). Human NK cells were incubated in the presence of PE- or FITC-conjugated mouse anti-human CD3, CD16, and CD56 mAbs (Becton Dickinson). Cells were analyzed by flow cytometry using a FACScan flow cytometer and Lysis II software (Becton Dickinson).

Generation of fresh NK cells and NK cell lines

Human PBLs were isolated from healthy donors by standard Ficoll Hypaque density centrifugation (Pharmacia, Alameda, CA) (15). The fresh PBLs were used immediately in cytotoxicity assays upon isolation. Polyclonal NK cell lines were generated following protocols published previously (18) with modification. Briefly, fresh PBL (10 million) were cultured with irradiated K562 (1 × 10⁶, 10,000 rad) in RPMI 1640-based media supplemented with 25 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM l-glutamine, 10% heat inactivated pooled human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml IL-2 for 4 days. NK cells were further enriched using anti-human CD3 and anti-mouse IgG magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for negative selection. The enriched NK cell populations were maintained in 100U/ml IL-2 and 10% heat inactivated pooled human AB serum during the entire assay period.

NK cell cytotoxicity assay

NK cell cytotoxicity was detected by standard chromium release assay. Briefly, target cells were labeled with 250 μCi ⁵¹Cr (ICN, Costa Mesa, CA) for 60 min. The target cells were washed three times and added to a 96-well round-bottom microtiter plate at 5000 cells/well. Effector cells were added at different E/T ratios in a total volume of 200 μl medium. The plates were incubated for 5 h in a 37°C humidified incubator with 5% CO2. Supernatants were harvested and counted using a Wallac Microbeta counter (Gaithersburg, MD). Spontaneous release was measured in target cells incubated with media alone. Maximal release was measured by treatment of target cells with 1% Triton X-100. Percent specific lysis was determined by the following formula: % specific lysis = (experimental – spontaneous release)/(maximum – spontaneous release) × 100%. All the experiments were repeated at least twice. Spontaneous release was <15% of the maximum release in all assays.

Ab blocking assay

Blocking assays were performed using purified F(ab’)₂ portion of PA2.6 (mouse anti-human HLA class I; American Type Culture Collection), HP-3B1 (mouse anti-human CD94, PharMingen, San Diego, CA), and mouse IgG isotype control. F(ab’)₂ were generated following standard protocol published elsewhere (19) and were used to avoid Ab-dependent cellular cytotoxicity. HP-3B1 was pre-incubated with effector NK cells, whereas PA2.6 were pre-incubated with target PAEC for 10 min at room temperature before NK cell cytotoxicity assay. The Ab was present in the culture media during the entire assay period.

Immunoprecipitations and Western blotting

A total of 2 × 10⁶ human PBL were either untreated or incubated with 4 × 10⁶ HLA-E transfected, HLA-G transfected, or mock-transfected PAECs for 5 min at 37°C. For direct CD94 receptor cross-linking, 20 × 10⁶ human PBL were incubated with HP-3B1 for 15 min on ice, washed twice, then incubated with goat anti-mouse IgG Abs for 5 min at 37°C. After stimulation, cells were collected and then resuspended in lysis buffer (1% Tween 20, 20 mM Tris, pH 8.0, 137 mM NaCl, with protease and phosphatase inhibitors) and incubated for 30 min on ice. Lysates were centrifuged at max speed for 15 min to clear cell debris. Supernatants were preincubated with protein G-agarose beads (Sigma, St. Louis, MO) and then immunoprecipitated with protein G-agarose beads preconjugated with anti-CD94 mAb. Beads were washed three times in TBS plus 0.05% Tween 20. Immunoprecipitated proteins were separated by SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. The filters were blocked with 1% BSA and then probed with different Abs. Proteins reactive to the Abs were visualized with HRP-conjugated goat anti-mouse IgG Ab, followed by an enhanced chemiluminescence Western blot detection system (Amersham, Arlington Heights, IL).

Results

Transfection and expression of nonclassical HLA class I molecules on porcine endothelial cells

The PAEC derived from Yucatan strain of inbred swine were transfected with full-length HLA-G or HLA-E genomic DNA along with full-length human β2m by lipofection method. The surface expression of HLA-G or HLA-E molecules was determined by indirect immunofluorescence using an HLA class I-specific mAb, PA2.6. PA2.6 does not cross-react with any SLA class I molecules. As shown in Fig. 1, both HLA-G- and HLA-E-transfected bulk PAEC showed positively stained for PA2.6 compared with mock-transfected PAEC. The results demonstrated that HLA-G and HLA-E were expressed on transfected PAECs. The transiently transfected bulk lines were used directly in cytotoxicity assays 96 h posttransfection.

Expression of HLA-E and HLA-G protects PAEC from human NK-mediated cytotoxicity

Previous studies have demonstrated that xenogeneic porcine endothelial cells but not the allogeneic human endothelial cells are highly susceptible to human NK cell-mediated lysis despite the fact that both cells express similar level of MHC class I molecules (11). To test if nonclassical HLA class I genes can protect porcine endothelial cells from human NK-mediated cytotoxicity, we examined the human NK cell-mediated lysis against mock-transfected PAEC as well as PAEC cotransfected with human β2m and HLA-G or HLA-E genes. Fresh human PBLs or established human NK cell lines were used as NK effector cells. The population of CD3−CD56− NK cells in PBLs from these donors varied from 5 to 16%. The population of CD3−CD56− NK cells in enriched NK cell lines were >80%. As shown in Table I, mock-transfected PAECs that did not express any HLA class I molecules were lysed by both freshly isolated PBLs as well as established NK cell lines at an E/T of 50:1. The percentage specific lysis of ⁵¹Cr-labeled PAEC ranged from 13.4 to 53.6%. The percentage lysis of
molecules expressed on PAEC. The F(ab\(^9\)) portion of the HLA class I-specific mAb PA2.6. PA2.6 does not cross react with any SLA class I molecules. PA2.6 does not cross react with any SLA class I molecules. The expression of HLA-E or HLA-G on bulk transfectants was detected by FACs analysis using HLA class I-Ig isotype-matched control were preincubated with HLA-E- and HLA-G-transfected PAECs before the addition of human NK effector cells. The F(ab\(^9\)) portion of the PA2.6 or mouse Ig isotype-matched control were preincubated with HLA-E- and HLA-G-transfected PAECs before the addition of human NK effector cells. The F(ab\(^9\)) 2 of the PA2.6 or mouse Ig isotype-matched control were preincubated with HLA-E- and HLA-G-transfected PAECs before the addition of human NK effector cells. The F(ab\(^9\)) 2 were used to avoid Ab-dependent cellular cytotoxicity. As shown in Fig. 2, the F(ab\(^9\)) 2 portion of the PA2.6 significantly increase the NK-mediated lysis of both HLA-E- and HLA-G-transfected PAECs. In contrast, no increase in the NK-mediated lysis of both HLA-E- and HLA-G-transfected PAECs and the mock-transfected PAECs were equally susceptible to lysis by the CTL, which specifically recognized the SLA class I allele expressed on these cells (data not shown).

Anti-HLA class I Ab specifically abrogated HLA-G- or HLA-E-mediated protection

To confirm that the protection of HLA-E- and HLA-G-transfected PAECs was mediated directly by the expression of the HLA-class I molecules, Ab blocking assays were performed using the purified F(ab\(^9\)) 2 portion of the HLA class I-specific mAb, PA2.6. As described earlier, PA2.6 did not cross-react with any SLA class I molecules expressed on PAEC. The F(ab\(^9\)) 2 of the PA2.6 or mouse Ig isotype-matched control were preincubated with HLA-E- and HLA-G-transfected PAECs before the addition of human NK effector cells. The F(ab\(^9\)) 2 were used to avoid Ab-dependent cellular cytotoxicity. As shown in Fig. 2, the F(ab\(^9\)) 2 portion of the PA2.6 significantly increase the NK-mediated lysis of both HLA-E- and HLA-G-transfected PAECs. In contrast, no increase in the NK-mediated lysis were observed with mouse Ig isotype control. These results indicated that the specific protection of PAEC from human NK cells was mediated directly by the expression of HLA-E or HLA-G, but not by the endogeneous porcine SLA class I molecules.

HLA-E and HLA-G protect PAEC from xenoreactive human NK cells through CD94/NKG2-dependent and -independent pathways

Several recent studies have identified the CD94/NKg2 complex as a major inhibitory receptor for HLA-E and possibly for HLA-G (reviewed in Ref. 2). Our next step was to examine if HLA-E and HLA-G protect porcine target cells from human NK cell-mediated cytolysis through the CD94/NKG2 pathway. We showed that although both the HLA-E and HLA-G expression on PAEC protected porcine target cells from human NK cell-mediated cytolysis, Ab blocking assays using human CD94-specific mAb HP-3B1 suggested that the specific protection was mediated through two distinct pathways. As shown in Fig. 3, preincubation of HLA-E-transfected PAEC with anti-CD94 mAb abrogated the HLA-E-mediated protection (a 52.9% increase in NK cytolysis). In contrast, HLA-G-mediated protection of PAECs was not affected in the presence of anti-CD94 mAb.

We next examined the signaling events in the CD94/NKG2 receptor complexes in the NK cells upon incubation with HLA-E- or HLA-G-transfected PAEC. Freshly isolated NK cells were either incubated with HLA-E-transfected PAEC, HLA-G-transfected PAEC, mock-transfected PAEC, or with anti-CD94 mAb cross-linked with anti-mouse IgG. Then, the CD94/NKG2 complexes were immunoprecipitated with anti-CD94 mAb followed by Western blot analysis. The amount of protein loaded in all the wells was comparable as determined by Coomassie blue staining (data not shown). As shown in Fig. 4, incubation of human NK effector cells with HLA-E-transfected PAEC lead to the tyrosine phosphorylation of a 40- to 45-kDa protein that corresponded to the phosphorylated NKG2 subunit of the CD94/NKG2 complex (Fig. 4, first

Table I. Protection of porcine endothelial cells from xenoreactive human NK cells by HLA-E or HLA-G expression

<table>
<thead>
<tr>
<th></th>
<th>Mock Transfection % Lysis</th>
<th>PAEC HLA-G % Lysis (% Inh)</th>
<th>PAEC HLA-E % Lysis (% Inh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cell line-1</td>
<td>53.6</td>
<td>37.0 (31.0)</td>
<td>28.6 (46.6)</td>
</tr>
<tr>
<td>NK cell line-2</td>
<td>44.9</td>
<td>25.7 (42.8)</td>
<td>21.2 (52.8)</td>
</tr>
<tr>
<td>NK cell line-3</td>
<td>26.0</td>
<td>15.0 (42.3)</td>
<td>17.0 (34.6)</td>
</tr>
<tr>
<td>Fresh PBL-1</td>
<td>13.4</td>
<td>2.7 (79.9)</td>
<td>5.0 (62.7)</td>
</tr>
<tr>
<td>Fresh PBL-2</td>
<td>23.7</td>
<td>10.2 (57.0)</td>
<td>6.8 (71.3)</td>
</tr>
<tr>
<td>Fresh PBL-3</td>
<td>16.9</td>
<td>5.5 (67.5)</td>
<td>2.6 (84.6)</td>
</tr>
</tbody>
</table>

* NK cells derived from freshly isolated PBLs or established NK cell lines were used as effector cells at an E:T ratio of 50:1 in standard^{51}Cr release assay with either HLA-E-HLA-G- or mock-transfected porcine endothelial cells.
fect porcine endothelial cells in a standard \( ^{51} \text{Cr} \) release assay (E:T ratio = 100:1) in the presence of 5 \( \mu \text{g/ml} \) of purified F(ab\(^{\prime}\))\(_2\) portion of PA2.6 (anti-HLA class I) or mouse IgG. Results are expressed as mean ± SD and are representative of three different experiments.

The same band was also observed when human NK effector cells were incubated with anti-CD94 mAb, which was used as positive control. Furthermore, the incubation of NK cells with HLA-E-transfected PAEC or with anti-CD94 mAb lead to the recruitment of a Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1), to the CD94/NKG2 complex (Fig. 4, second row). In contrast, no phosphorylation of CD94/NKG2 complex or SHP-1 recruitment was observed in NK cells incubated with HLA-G-transfected PAEC or with mock-transfected and untransfected PAECs. These data indicated that although both HLA-E and HLA-G were able to directly inhibit human NK cell-mediated cytotoxicity, they regulated the NK cells through two distinct pathways. HLA-E protected porcine endothelial cells from xenogeneic human NK cells through the CD94/NKG2-dependent pathway. The engagement of HLA-E lead to the phosphorylation of CD94/NKG2 complex and the recruitment of SHP-1 to the complex. In contrast, HLA-G directly protected porcine endothelial cells from human NK cells through a CD94/NKG2-independent pathway. There was no phosphorylation of the CD94/NKG2 complex or recruitment of SHP-1 upon HLA-G engagement, therefore suggesting that an alternative pathway might be involved.

Although the predicted structures of porcine SLA class I structures resemble human HLA class I molecules, recent studies indicated that the amino acid sequence of SLA class I molecules did not contain the conserved motif at positions required for human KIR binding (13, 14). In humans, amino acid residue at position 77 and 80 in the HLA class I have been implicated to have a dominant role in determining KIR recognition. These motifs were not found in the corresponding SLA class I molecules. Therefore, despite the fact that porcine and human endothelial cells expressed similar level of MHC class I molecules, results shown in this study as well as by others indicated that the porcine endothelial cells were highly susceptible to lysis by both freshly isolated PBL and established human NK cell line. The human anti-porcine xenogeneic cytotoxicity was mediated by NK cells but not by T cells. This was demonstrated by the fact that only the purified CD56\(^{+}\)CD3\(^{-}\)NK cell subpopulation was capable of lysing porcine target cells (data not shown).

A recent study by Seebach et al. demonstrated protection of porcine cells from a subset of human NK cells by the expression of a classical HLA class I molecule on porcine endothelial cells (20). The results showed that the expression of a HLA class I allele, Cw3, on the surface of xenogeneic porcine target cells was able to confer partial protection against part of the human NK clones but not others. Using an HLA-A3 allele, we have observed similar findings that classical HLA class I alleles could not protect porcine cells from polyclonal human NK cells (data not shown). These results, at least in part, might be due to the low percentage of NK cells carrying receptors for a particular HLA class I allele. However, because the xenogeneic cytotoxicity mediated by human NK cells is a polyclonal effect, the blocking of NK-mediated lysis of porcine cells still requires the design of a strategy that will inhibit polyclonal human NK cells. In contrast to the limited percentage of NK cells carrying receptors for a particular classical HLA class I allele, a large percentage of human NK cells carry receptors for nonclassical HLA class I molecules. For example, the tetrameric HLA class I molecule derived from HLA-E was shown to bind a large number of human NK cells (4). Our results indicated that HLA-E expression on porcine cells directly protect these cells against xenoreactive human polyclonal NK cells derived from both fresh PBLs and established NK cell lines. The percentage inhibition observed in some of the experiments was <50%. This might be due to the fact that bulk transfectants were used in these assays. Alternatively, HLA-E might not be the universal inhibitor for all the NK subpopulation; therefore, the

**Discussion**

![FIGURE 4.](http://www.jimmunol.org/)

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variation in the NK subpopulation among different persons would lead to a variation in percentage inhibition observed.

The expression of HLA-E on the cell surface requires the binding of a nonamer peptide derived from the leader sequence from some of the HLA-class I alleles (4–6). So far, all the amino acid sequences of the HLA-E binding peptides were highly conserved with the primary anchor residues at the position 2 and 9 (i.e., VMAPRTLLL; anchor residues are underlined) (21, 22). Alteration of the conserved Met at position 2 or Leu at position 9 was shown to cause a loss of binding and therefore reduced surface expression of HLA-E. Surprisingly, none of the leader sequence of any known SLA class I molecules contains this conserved HLA-E peptide motif. The fact that HLA-E could be transfected and successfully expressed on the surface of the PAEC to inhibit xenoreactive human NK cells suggested that an alternative peptide source or peptide motifs might exist in porcine cells. The source and the sequence of these porcine peptides are currently under investigation.

The role of HLA-G in directly inhibiting NK cells is currently being debated. The initial study using HLA-G-transfected human HLA class I-negative cell line 721.221 concluded that HLA-G expression was able to inhibit human NK cell-mediated lysis (7, 8). However, the HLA-G-transfected 721.221 cell line used in early studies was later found to also express a previously unknown nonclassical HLA class I molecule, HLA-E (4). HLA-E was found to be able to bind leader peptide sequences derived from not only the classical HLA class I molecules but also the HLA-G. Therefore, it was proposed that the up-regulated surface expression of HLA-E upon association with peptides derived from HLA-G leader sequence rather than HLA-G itself provided the main inhibitory signals to NK inhibitory receptors. The direct role of HLA-G in inhibiting NK cell activity has become unclear. This is in part due to the lack of human cell lines that do not express endogenous HLA-E. This issue is further complicated by the fact that even in those human cell lines that express no HLA-E molecules, it is hard to rule out that other nonclassical HLA class I molecules that are equivalent to HLA-E may exist (9, 10). The lack of HLA class I expression in the porcine cells provided us an unique opportunity to directly study the function of HLA-G in regulating human NK cells in the absence of any other HLA class I molecules. The results in this report clearly demonstrate that HLA-G expression on PAEC alone, in the absence of other HLA class I gene expression, was sufficient to directly confer protection against human NK cell-mediated lysis. These results were in agreement with several recent studies that cloned receptors for HLA-G that expressed on all NK cells. (23, 24).

The stable expression of HLA class I generally requires the preassemblage of α-chain, β₂m, and peptide trimeric complexes in the endoplasmic reticulum (25). Class I α-chains alone are unstable on the cell surface and are rapidly degraded (26). Our unpublished data indicated that there were detectable HLA-G or HLA-E expression on PAECs even when they were transfected in the absence of human β₂m. This is possible as a result of the high sequence homology between human and porcine β₂m (27). However, it is not clear at the present time if the human HLA class I α-chain porcine β₂m hybrid molecules were functionally similar in terms of NK inhibition.

In conclusion, we have shown that two nonclassical HLA class I molecules, HLA-E and HLA-G, can be used as an effective strategy to protect porcine cells from human NK cell-mediated cytotoxicity. The expression of HLA-E or HLA-G, in the absence of any other HLA class I molecules, was sufficient to protect these otherwise sensitive porcine endothelial cells from polyclonal human NK cells. However, the mechanism of inhibition by these two nonclassical HLA class I molecules was distinct. Engagement with HLA-E expressed on porcine endothelial cells lead to the phosphorylation of CD94/NKG2 complex and activation of subsequent signaling pathways in human NK cells. In contrast, HLA-G expression directly protected porcine endothelial cells from human NK cells through a CD94/NKG2-independent pathway.

References