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# HLA-G Inhibits Rolling Adhesion of Activated Human NK Cells on Porcine Endothelial Cells[1]

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**Human NK cells adhere to and lyse porcine endothelial cells (pEC) and therefore may contribute to the cell-mediated rejection of vascularized pig-to-human xenografts. Since MHC class I molecules inhibit the cytotoxic activity of NK cells, the expression of *HLA* genes in pEC has been proposed as a potential solution to overcome NK cell-mediated xenogeneic cytotoxicity. HLA-G, a minimally polymorphic HLA class I molecule that can inhibit a wide range of NK cells, is an especially attractive candidate for this purpose. In this study we tested whether the expression of HLA-G on pEC inhibits the molecular mechanisms that lead to adhesion of human NK cells to pEC and subsequent xenogeneic NK cytotoxicity. To this end two immortalized pEC lines (2A2 and PED) were stably transfected with HLA-G1. Rolling adhesion of activated human NK cells to pEC monolayers and xenogeneic cytotoxicity against pEC mediated by polyclonal human NK lines as well as NK clones were inhibited by the expression of HLA-G. The adhesion was partially reversed by masking HLA-G on pEC with anti-HLA mAbs or by masking the HLA-G-specific inhibitory receptor ILT-2 on NK cells with the mAb HP-F1. The inhibition of NK cytotoxicity by HLA-G was only partially mediated by ILT-2, indicating a role for other unknown NK receptors. In conclusion, transgenic expression of HLA-G may be useful to prevent human NK cell responses to porcine xenografts, but is probably not sufficient on its own. Moreover, the blocking of rolling adhesion by HLA-G provides evidence for a novel biological function of HLA molecules.** ***The Journal of Immunology*****, 2001, 167: 6002–6008.**

Under physiological circumstances NK cells play an important role in the cellular immune response against viral infections and tumors (1). The observation that human NK cells are able to lyse porcine endothelial cells (pEC)[3] in vitro suggested that NK cells also participate in the rejection of pig-to-human xenografts (2). This idea was supported by the finding of NK cell infiltrates in pig organs perfused with human blood ex vivo (3) as well as in pig-to-nonhuman primate xenografts (4) and in small animal models of both concordant and discordant xenotransplantation (5). In agreement with these observations, several adhesion receptor pairs seem to be intact across the human-porcine species barrier in vitro, providing the basic requirements for human leukocytes, including NK cells, to interact with pEC (6, 7).

The function of NK cells is regulated by a concert of activating and inhibitory signals (8–10). After binding to potential target cells several receptor-ligand interactions take place between proteins on the surface of the NK cell and the target cell, determining the fate of the latter. A number of recently identified NK cell-specific activating receptors, NKp44, NKp46, and NKp30, respectively (9, 11), and the human homologue of mouse 2B4 (11) scan potential target cells for their ligands. The presence of such corresponding ligands results in activation of NK cells and initiates their killing mechanisms, while the absence of these putative ligands confers relative or complete resistance (12). At the same time, inhibitory NK receptors recognize potential MHC class I molecules on the target cells (13). HLA-C locus alleles interact with the two-domain Ig superfamily member KIR2DL1–3 (CD158a/b) receptors, whereas *HLA-A* and *-B* loci products interact with the three-domain KIR3DL receptors. The ligands for the KIR2DL4–5 and the other Ig-like Ig-like transcript (ILT) receptors are less well defined, but include both classical and nonclassical *HLA* class I alleles and homologous proteins, some of which are virally derived (14). Finally, the nonclassical HLA-E was found to be the ligand for a family of lectin-like CD94/NKG2 heterodimers (14). It is generally believed that inhibitory signals elicited by any of the above ligand-receptor interactions are dominant and will protect target cells from NK cell-mediated damage. On the other hand, both the two- and three-domain killer Ig-like receptor (KIR) molecules as well as the lectin-like CD94-NKG2 complex exist in activating forms. These receptors differ in their transmembrane and intracellular domains from the inhibitory equivalents and, using a different signaling pathway, enhance cytotoxicity (15). Apart from the direct cytotoxicity described above, NK lysis is also triggered by Ab coating of target cells (16), resulting in Ab-dependent cellular cytotoxicity.

The susceptibility of pEC to human NK cytotoxicity may be explained by the failure of human NK inhibitory receptors to recognize swine MHC class I molecules (SLA). This hypothesis was supported by our previous demonstration that the expression of HLA-Cw3 on pEC provides protection from human NK lysis (17).

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[3] Abbreviations used in this paper: pEC, porcine endothelial cells; Co, control; SLA, swine leukocyte Ag; KIR, killer Ig-like receptor; ILT, Ig-like transcript; VLA4, very late Ag 4.



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However, since *HLA-C* is a polymorphic allele, its transgenic expression on a vascularized xenograft might result in T cell allorecognition. In contrast, HLA-G is a minimally polymorphic molecule present on cytotrophoblasts in every individual during fetal development (18, 19) and does not induce T cell alloresponses (20, 21). Consequently, HLA-G is an attractive candidate to prevent NK cell-mediated damage of porcine grafts. To date, the experimental results regarding this issue are still conflicting, with some groups reporting partial and others no detectable HLA-G-mediated target cell protection (22–26). Also, it has recently been proposed that the expression of certain MHC molecules on target cells could negatively influence the adhesion of NK cells (27, 28). This view is not in line with earlier studies showing that MHC recognition does not interfere with adhesion to target cells (29). However, there has been no study to date examining the effect of HLA-G on the adhesion of activated NK cells.

The immunological function of HLA-G is still poorly understood. The observation that maternal NK cells accumulate in the uterus during pregnancy suggested that the HLA-G-positive extravillous cytotrophoblast cells form a protective shell around the implanting fetus, shielding it from lysis by maternal NK cells (30). Other investigators, however, proposed that HLA-G may induce the release of growth factors from NK cells that are necessary for successful implantation of the fetus (30, 31). Although in vitro studies with human cells confirmed the potential of HLA-G to inhibit NK cytotoxicity (31, 32), the nature of the NK cell receptors responsible for this phenomenon was a matter of debate. It is now evident that HLA-G inhibits NK cells via two discrete pathways. First, the leader peptide of HLA-G is used to assemble HLA-E. Upon reaching the cell surface, HLA-E interacts with the CD94-NKG2 complex, providing an indirect pathway of inhibition for HLA-G (33, 34). More recently, new findings provided evidence for direct recognition of HLA-G by NK cells. It was reported that the Ig-like NK receptors, ILT-2 (35) and p49/KIR2DL4 (36, 37), respectively, bind HLA-G. ILT-2 transduces an inhibitory signal to NK cells, while KIR2DL4 is an activating receptor that induces NK cytotoxicity and IFN-$\gamma$ secretion (38).

The primary aim of the present study was to examine whether HLA-G protects xenogeneic pEC from the lysis mediated by human NK cells and to determine which receptors are involved in this recognition. The use of porcine cells permitted analysis of the direct recognition of HLA-G by NK receptors in the absence of HLA-E. We demonstrate that HLA-G is able to partially protect pEC from direct lysis mediated by human NK cells. Moreover, using NK cell clones we observed that ILT-2 is only partially responsible for the HLA-G-mediated inhibition of NK cytotoxicity. Finally, the expression of HLA-G on pEC had implications for the rolling adhesion of human NK cells, indicating a new biological function for HLA molecules.

## Materials and Methods

### *Cells*

Two SV40-immortalized pEC lines were established and characterized in our laboratory: the bone marrow-derived microvascular 2A2 and the aortic PEDSV.15 (PED) lines (39). The generation of porcine 2A2 and human 721.221 cell lines stably transfected with HLA-A2, HLA-B27, or HLA-Cw3, as well as the isolation of human PBMC, the purification of NK cells, and the generation of polyclonal NK lines and NK clones have been described previously (17). The human erythroleukemic cell line K562 and the clonal NK cell line NK92 (40) were obtained from American Type Culture Collection (Manassas, VA). NK92 cells were maintained in MyeloCult H5100 medium (StemCell Technologies, Vancouver, BC, Canada) containing 500 U/ml human IL-2 (Chiron, Palo Alto, CA).

### *Transfection*

The plasmid construct containing the neomycin resistance gene and the SR$\alpha$ promoter controlling the expression of *HLA-G1* cDNA and the use of the calcium phosphate technique for stable transfection of 2A2 cells were previously described (17, 41). Transfected 2A2 cells were selected in G418 (Life Technologies, Gaithersburg, MD) at a concentration of 250 $\mu$g/ml, and growing colonies were analyzed for the expression of HLA-G by flow cytometry. The establishment of the HLA-G-transfected PED cell line has been described in detail previously (25).

### *Immunostaining and flow cytometry*

Surface expression of MHC class I molecules on transfected 2A2 cells was analyzed by indirect immunofluorescence using primary mouse anti-HLA Abs and secondary FITC-conjugated goat anti-mouse Abs (Roche, Indianapolis, IN) on a FACScan (BD Biosciences, Basel, Switzerland). After trypsinization (0.25% trypsin; Life Technologies), 2A2 cells were resuspended at $5 \times 10^5$ cells/tube in staining buffer (PBS, 0.1% BSA, and 0.05% $NaN_3$) and incubated for 30 min at 4°C with saturating concentrations of the following mAbs: 2.27.3A (anti-SLA class I, IgG2a; J. S. Arn, Harvard Medical School, Boston, MA), DX17 and W6/32 (anti-HLA class I, IgG1; L. Lanier, University of California, San Francisco, CA; and IgG2a, Sigma, Buchs, Switzerland, respectively), and the anti-HLA-G mAb 87G (IgG2a; D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), MEM-G8, -G9, and -G10 (IgG1; V. Horejsi, Institute of Molecular Genetics, Prague, Czech Republic). For the expression of adhesion molecules on pEC the following mAb (all IgG1) were used: 3F4 (anti-VCAM-1; Alexion, New Haven, CT), 1.2B6 (anti-E-selectin; Serotec, Kidlington-Oxford, U.K.), and 12.C5 (anti-P-selectin; D. Haskard, Hammersmith Hospital, London, U.K.).

Phenotypic analysis of NK cell lines was conducted by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (BD PharMingen, San Diego, CA). The expression of inhibitory/activating receptors on NK cells was analyzed with the anti-ILT-2 mAb HP-F1 (IgG1; M. Colonna, Basel Institute of Immunology, Basel, Switzerland) and the following mAb was purchased from Beckman-Coulter (Fullerton, CA): EB6 (anti-KIR2DL1, IgG1), GL183 (anti-KIR2DL2, IgG1), DX9 (anti-KIR3DL1, IgG1), and HP-3B1 (anti-CD94, IgG2a). The expression of adhesion molecules on human NK cells was examined by indirect immunofluorescence using the mAb 25.3.1 (anti-CD11a), 7E4 (anti-CD18), HP2/1 (anti-CD49d/very late Ag 4 (VLA4)), and DREG56 (anti-CD62 ligand; all IgG1 from Beckman-Coulter) and KPL-1 (anti-CD162; Chemicon, Temecula, CA). Irrelevant isotype-matched mAb were used as controls, and propidium iodide gating was used to exclude dead cells in all experiments. To compare the levels of surface expression, the geometric mean fluorescence intensity ratios were calculated by dividing the mean fluorescence intensity of staining with the mAb of interest by the mean fluorescence intensity of the control mAb.

### *Isoelectric focusing*

Isoelectric focusing was conducted using a previously described protocol (42). Briefly, $10^7$ cells transfected cells were cultured in methionine-free RPMI 1640 medium (Life Technologies) for 1 h before radiolabeling with 100 $\mu$Ci of [$^{35}$S]methionine (Pharmacia Biotech, Dubendorf, Switzerland) for 4 h. Cells were than lysed in 20 mM Tris (pH 7.9), 150 mM NaCl, 10 mM EDTA, and 0.5% Nonidet P-40; the nuclei were pelleted; and the supernatant was collected. The lysate was precleared by Pansorbin (Calbiochem-Novabiochem, San Diego, CA). HLA class I molecules were immunoprecipitated with mAb W6/32 and protein A-Sepharose (Pharmacia Biotech). The beads containing the mAb-MHC class I complexes were washed four times and separated from MHC class I molecules by adding 9.5 M urea. Denaturing isoelectric focusing gels covering the pH 3.5–10 range were run in a Bio-Rad Protein II Gelbox at 880 V, 12 mA, and 8 W for 13–16 h. The gels were fixed in 10% acetic acid, treated with Amplify (Pharmacia Biotech), and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY).

### *Adhesion assay*

Rolling adhesion of human NK cells on PED cells was analyzed using a modified Stamper-Woodruff assay (43, 44). Briefly, porcine endothelial cells were grown to confluence in a 30-mm culture dish within the limits of a circle of 20 mm in diameter administered with a nontoxic silicon oil coat (dimethylpolysiloxane; Sigma-Aldrich). The resulting monolayers were washed and overlaid with 100 $\mu$l of Weissmann buffer containing purified activated NK cells ($10^7$ cells/ml), glucose (5.55 mM), and human albumin (5 mg/ml; Fluka, Deisenhofen, Germany). The dishes were then

rotated at 64 rpm in a prewarmed (37°C) horizontal shaker-incubator (Infors, Bottmingen, Switzerland). This rotation speed induces a continuous flow of NK cells on pEC monolayers, thus simulating the shear stress and contact times present in the postcapillary vascular system. After 10 min the assay was stopped by rapidly placing the dishes on ice and prefixing the cells for 2 min with 1% paraformaldehyde in PBS. The monolayers were then gently washed, fixed for an additional 15 min, and finally protected with a glass coverslip. For quantification, four fields of 0.16 $mm^2$ were defined at a distance of 0.6 cm from the center of rotation, and the number of adhering cells was counted by light microscopy. In blocking experiments untransfected or HLA-G-transfected PED (PED-G) monolayers were preincubated with DX17 or the isotype control Ab (MOPC21, IgG1; Sigma-Aldrich) at a concentration of 20 $\mu$g/ml for 30 min at 4°C. Alternatively, NK cells were pretreated with 20 $\mu$g/ml HP-F1, GL183, HP-3B1, or MOPC21 for 30 min at 4°C. After incubation with mAb the cells were washed twice with Weissmann buffer, then used in adhesion assays. Statistical significance was evaluated by paired *t* testing using StatView software (Abacus Concepts, Berkeley, CA).

### *Cytotoxicity assays*

The cytotoxic activity of polyclonal human NK cell lines and clones was tested in standard $^{51}Cr$ release assays in serum-free AIM-V medium as described previously (17). Briefly, target cells were added to triplicate samples of serial 2-fold dilutions of NK cells in round-bottom 96-well plates at E:T cell ratios ranging from 25:1 to 3:1. After incubation for 4 h the release of radioactive $^{51}Cr$ was analyzed on a gamma counter, and the percentage of specific lysis was calculated. All NK cells used as effectors efficiently lysed K562 cells, which were used as positive control targets.

## Results

### *Analysis of HLA-G expression by porcine endothelial cells*

After transfection, 2A2 cells were analyzed for surface expression of HLA-G1 by flow cytometry. As shown in Fig. 1*a* both the pan HLA class I mAb W6/32 and the HLA-G-specific mAb 87G stained HLA-G-transfected 2A2 cells (2A2-G), while the paternal 2A2 line as well as mock-transfected 2A2 control cells (2A2-Co) were W6/32 negative. Neither W6/32 nor 87G cross-reacts with porcine molecules, including SLA-class I, on the surface of pEC. HLA-G expression on 2A2-G was further confirmed by staining with other HLA class I (DX17) and HLA-G-specific (MEM-G8, -G9, and -G10) mAb, none of which cross-reacted with porcine Ags (data not shown). Similar levels of endogenous porcine MHC class I expression were found on 2A2-G and 2A2-Co cells by immunostaining with 2.27.3A (Fig. 1*a*). The pattern of HLA-G and SLA class I surface expression on microvascular 2A2-G was similar to that found on aortic PED cells transfected with HLA-G (PED-G), which has been described previously (25).

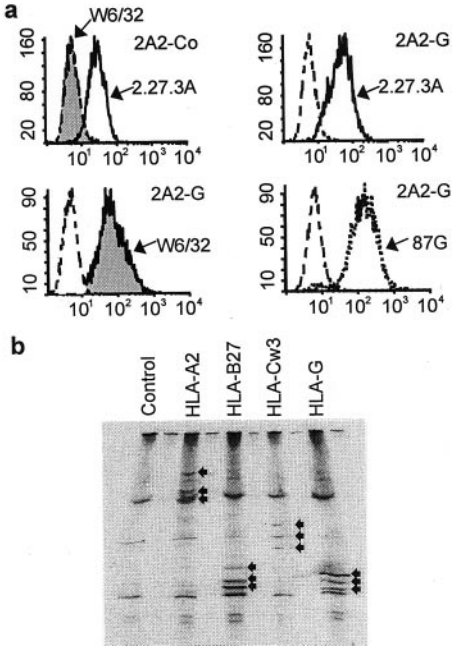


**FIGURE 1.** *a*, Cell surface expression of HLA-G and porcine MHC class I molecules on 2A2 cells transfected with HLA-G. 2A2-Co, and 2A2-G cells were analyzed by indirect immunofluorescence using mAb specific for porcine (2.27.3A; solid line) or human (W6/32; filled histogram) MHC class I molecules and for HLA-G (87G; dotted line). The histogram for an isotype-matched control Ab is also depicted (dashed line). *b*, One-dimensional isoelectric focusing analysis of 2A2 cells transfected with various HLA class I molecules. Lysates of metabolically labeled transfected and untransfected 2A2 cells were immunoprecipitated with W6/32 Ab and resolved by isoelectric focusing. The position of HLA class I heavy chain is indicated by arrows.

To compare the glycosylation patterns of HLA molecules, porcine 2A2 and human 721.221 cells, transfected with the same HLA genes, were immunoprecipitated with W6/32 and run on a denaturing isoelectric focusing slab gel. The proportions of non-, mono-, and bisialylated forms of HLA class I molecules found in porcine 2A2 cells (Fig. 1*b*) were very similar to those present in human 721.221 cells (42). Each MHC class I molecule was represented by three distinct bands due to the maturation process from the nonsialylated form to the mono- and bisialylated proteins. Taken together, these data demonstrate a similar surface expression and glycosylation pattern of HLA molecules in human and transfected porcine cells.

### *HLA-G partially protects porcine endothelial cells from xenogeneic human NK cytotoxicity*

As demonstrated previously, HLA-Cw3 expressed on pEC partially inhibited xenogeneic NK cytotoxicity mediated by polyclonal human NK cell lines. In contrast, reports on the inhibitory potential of HLA-G are controversial. Similar to our previous results with aortic pEC (PED) transfected with HLA-G (25), the lysis of 2A2-G cells by human NK cells was partially inhibited compared with the lysis of 2A2-Co cells. Table I shows a summary of the direct cytotoxic activity of seven polyclonal NK lines isolated from various healthy donors against a panel of different HLA class I-transfected 2A2 cells. Similar to 2A2-Cw3 and 2A2-B27, the percentage of specific killing of 2A2-G cells was reduced between 54 and 84%.

### *Clonal analysis of human NK lysis of HLA-G-transfected porcine endothelial cells*

One possible explanation for the failure of HLA-G to protect 2A2-G cells completely from the lysis mediated by polyclonal human NK cell populations could be the simultaneous presence of NK cells that express different patterns of inhibitory receptors. To verify this hypothesis we generated a panel of NK cell clones from two different donors and analyzed their NK receptor repertoire as

Table I. *Relative percentages of lysis of 2A2-Co and HLA-transfected 2A2 cells*[a]

| NK Line | 2A2-Co | HLA-B27 | HLA-Cw3 | HLA-G |
|---|---|---|---|---|
| FI | 100 | 58 | 47 | 54 |
| KH | 100 | 62 | 66 | 81 |
| JS-1 | 100 | 65 | 67 | 61 |
| BA | 100 | NT[b] | 53 | 70 |
| JS-2 | 100 | NT | 46 | 61 |
| GS | 100 | NT | 54 | 84 |
| JS-3 | 100 | NT | 34 | 65 |

[a] Values are given as the mean percent specific killing compared to lysis of 2A2-Co targets, calculated at four different E:T ratios (20:1, 10:1, 5:1, 2.5:1).
[b] NT, not tested.

well as their cytotoxic activity against 2A2-G and 2A2-Cw3 cells (Table II and Fig. 2). Xenogeneic killing of approximately half the NK clones (54.5%) was inhibited by the expression of HLA-G on 2A2 target cells, while the remaining clones lysed 2A2-G cells to the same extent as untransfected 2A2-Co cells (Fig. 2). The expression of HLA-Cw3 consistently provided either complete (93 ± 7%) or no protection against NK clones, whereas HLA-G provided only incomplete protection (47 ± 18%). The xenogeneic cytotoxicity of NK92, a $CD16^-$ NK cell line, was also partially inhibited (40 ± 9%) by the expression of HLA-G on PED cells (Table II).

With the exception of a single clone (BA.10.14), the potential of HLA-Cw3 to inhibit a given NK clone correlated well with GL183 positivity, indicating the expression of the HLA-Cw3-specific KIR2DL2 inhibitory receptor. However, no correlation was found between the expression of ILT-2, an HLA-G-specific NK receptor, and the protection mediated by HLA-G (Table II). In fact, the inhibition of clonal NK cytotoxicity against HLA-G-transfected pEC occurred either in the absence (BA.10.6 and BA.10.12) or in the presence (GS.60 and NK92) of ILT-2.

To investigate species-specific differences in the susceptibility of HLA-G-transfected cell lines to NK cytotoxicity, selected human NK clones were also tested against human 721.221 cells transfected with either HLA-Cw3 or HLA-G. Some NK clones (BA.10.13) were inhibited by HLA-G regardless of whether it was expressed on pig or on human cells (Fig. 3, *a* and *b*). However, other NK clones were only inhibited by HLA-G when expressed on 721.221 cells and not on pig cells (Fig. 3, *c–f*). Again HLA-Cw3 provided either no or complete inhibition of NK cytotoxicity, whereas HLA-G inhibition was less efficient. NK clone BA.10.14, which was positive for GL183 (KIR2DL2/KIR2DS2) and HP-F1 (ILT-2), but not for HP-3B1 (CD94) staining, was inhibited by HLA-Cw3 and HLA-G expression on the surface of human 721.221, but not on porcine 2A2 cells (Fig. 3, *e* and *f*). This apparent lack of inhibition of BA.10.14 by HLA-Cw3 and HLA-G expressed on pig cells might be explained by the recognition of strong activating porcine molecules, since the absence of CD94 on this NK clone excludes the possibility that 721.221 cells were protected indirectly by the expression of HLA-E.

### *HLA-G inhibits rolling adhesion of activated human NK cells to pEC*

Before target cell lysis in vivo NK cells have to adhere to pEC, a process that includes rolling and, subsequently, firm adhesion.

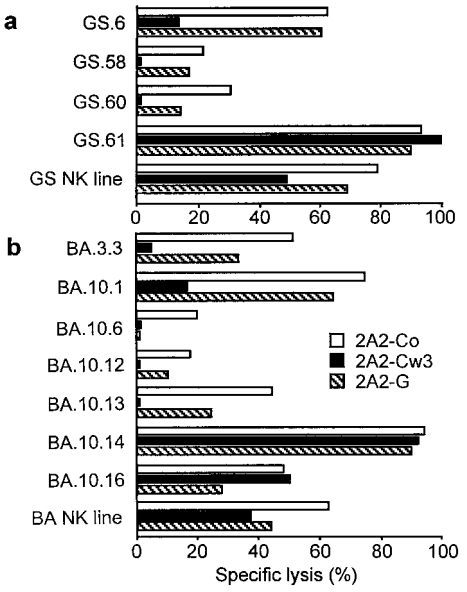


**FIGURE 2.** HLA-G mediates inhibition of the cytotoxic activity of several human NK clones. The cytotoxicity of NK cell clones purified from donors GS (*a*) and BA (*b*) was studied against 2A2-Co cells and 2A2 cells expressing either HLA-G (2A2-G) or HLA-Cw3 (2A2-Cw3) in a standard 4-h $^{51}Cr$ release assay. All results are expressed as the percent specific lysis and were obtained at an E:T cell ratio of 10:1.

Therefore, the question was addressed of whether HLA-G surface expression interferes with rolling adhesion of human NK cells on PED monolayers, the first step of the adhesion process. All IL-2-activated polyclonal human NK cell lines as well as the NK92 cells used in these experiments expressed bright levels of the $\beta_2$ integrin LFA-1 (CD11a/CD18), the VLA-4 integrin $\alpha$-chain (CD49d), and P-selectin glycoprotein ligand-1 (CD162; data not shown). L-selectin was expressed on subportions (2–30%) of NK lines, but not on NK92. PED-Co and PED-G expressed VCAM-1 (CD106), the ligand for VLA-4, at similar levels, whereas the expression of porcine ICAM could not be tested because mAb were not available. P- and E-selectin (CD62P and CD62E), the ligands for P-selectin glycoprotein ligand-1 (CD162), were expressed at lower levels on PED-G than on PED-Co (data not shown). However, this difference was not relevant, as VLA-4-VCAM-1 interactions play

Table II. *Analysis of the expression of NK receptors on NK clones and the NK92 cell line*

| NK Clones | GL183 | EB6 | NKB1 | ILT-2 | CD94 | Inhibition[a] HLA-G | Inhibition[a] HLA-Cw3 |
|---|---|---|---|---|---|---|---|
| BA.3.3 | 42[b](99%)[c] | 2 (82%) | Neg. | NA[d] | 10 (92%) | 40 | 94 |
| BA.10.1 | 64 (99%) | Neg. | Neg. | NA | 6 (86%) | 13 | 85 |
| BA.10.6 | 80 (14%) | Neg. | Neg. | Neg. | 12 (100%) | 79 | 87 |
| BA.10.12 | 21 (98%) | Neg. | Neg. | Neg. | Neg. | 59 | 97 |
| BA.10.13 | 84 (100%) | Neg. | Neg. | Neg. | 4 (94%) | 74 | 94 |
| BA.10.14 | 84 (100%) | 8 (98%) | 8 (100%) | 7 (85%) | Neg. | 0 | 0 |
| BA.10.16 | 15 (14%) | Neg. | 8 (96%) | 10 (85%) | 13 (36%) | 42 | 0 |
| GS.6 | 152 (99%) | 147 (100%) | NA | Neg. | 22 (99%) | 1 | 79 |
| GS.58 | 237 (99%) | 18 (99%) | NA | Neg. | 2 (63%) | 37 | 95 |
| GS.60 | 342 (99%) | 29 (99%) | NA | 7 (98%) | 3 (92%) | 45 | 97 |
| GS.61 | Neg. | Neg. | NA | Neg. | 12 (98%) | 0 | 0 |
| NK92 | Neg. | Neg. | Neg. | 5 (100%) | 6 (100%) | 44 | NA |

[a] Expression (MFIR) of NK receptor on clones from donor BA and GS and NK92 cells examined by FACS analysis.
[b] Percentage of positive cells.
[c] Percentage of inhibition of lysis mediated by NK clones against 2A2-Cw3 and 2A2-G target cells.
[d] NA, Not available.

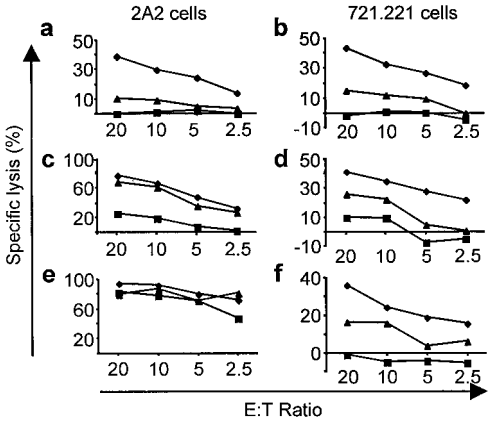


**FIGURE 3.** Comparison of HLA-G-mediated protection in human and pig target cells. The cytotoxic activities of three representative NK clones, BA.10.13 (*a* and *b*), BA.10.1 (*c* and *d*), and BA.10.14 (*e* and *f*), against porcine 2A2 cells (*a*, *c*, and *e*) and human 721.221 cells (*b*, *d*, and *f*) expressing either HLA-Cw3 (■) or HLA-G (▲) were compared with that of untransfected control cells (♦). Cytotoxicity was determined at four different E:T cell ratios (20:1–2.5:1) in a standard 4-h $^{51}$Cr release assay and was expressed as the percent specific lysis.

the pivotal role in rolling adhesion of human NK cells to PED, while blocking of P- and E-selectin has no or only minor effects (6). Nevertheless, the expression of HLA-G on PED-G monolayers significantly inhibited the number of adherent NK cells. On the average, a reduction of 50 ± 19% was observed ($p < 0.0001$) when PED-G monolayers were compared with untransfected PED-Co cells (Fig. 4*b*).

To test whether HLA-G was directly responsible for this reduction, Ab-blocking experiments were performed using CD16-negative NK92 cells to avoid FcγRIII-mediated effects on adhesion. HLA-G-mediated inhibition of rolling adhesion was more pronounced for NK92 cells than that observed with activated polyclonal NK lines and was completely reversed by blocking with the anti-MHC class-I mAb DX17 (Fig. 5*a*). Next, we addressed the

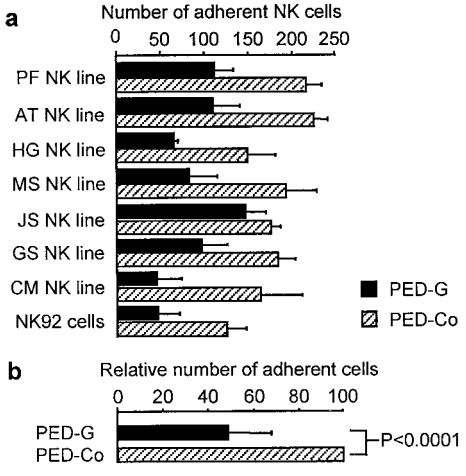


**FIGURE 4.** Expression of HLA-G inhibits rolling adhesion of human NK cells on pEC monolayers. *a*, Rolling adhesion of seven different NK cell lines and NK92 cells on PED-G (■) and PED-Co (▨) monolayers is shown. The absolute number (±SD) of adhering NK cells was calculated as the mean of four separate counting fields per sample. *b*, The percentage of adherent-activated NK cells on PED-G (■) monolayers compared with the respective intra-assay control PED-Co (▨; index = 100) was calculated from seven independent experiments. Statistical analysis of differences was performed by paired *t* testing.

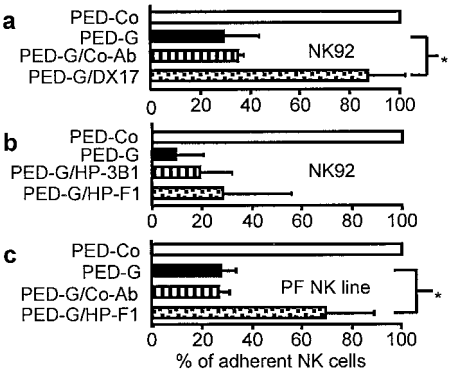


**FIGURE 5.** The inhibition of rolling NK cell adhesion to PED-G monolayers is reversed by anti-MHC class I and anti-ILT-2 mAb. *a*, PED-Co or PED-G monolayers were pretreated with medium alone (PED-Co and PED-G), the anti-MHC class I mAb DX17 (PED-G/DX17), or an isotype co-Ab (PED-G/Co-Ab) before analysis of rolling adhesion of NK92 cells. Results are calculated from three independent experiments. Alternatively, NK 92 cells (*b*; ILT-2$^+$, CD94$^+$) or activated NK cells (*c*; PF NK line, 64% positive for ILT-2 by FACS) were pretreated with medium alone (PED-G and PED-Co), the mAb HP-F1 (PED-G/HP-F1), the mAb HP-3B1 (PED-G/CD94), or an isotype co-Ab (PED-G/Co-Ab) before examination of the rolling adhesion to PED-G and PED-Co monolayers. All results are expressed as the mean percentage ± SD of adhering NK cells compared with the respective intraassay control (PED-Co, index = 100). ∗, Statistical significance ($p < 0.01$), as calculated by paired *t* testing.

question of whether interactions between HLA-G and ILT-2, which are involved in the control of NK cytotoxicity, are also important in the regulation of rolling adhesion. Despite the expression of ILT-2 on the surface of NK92 cells (Table II) the inhibition of rolling adhesion by HLA-G was only slightly reversed (18%; $p = 0.18$) by masking ILT-2 with HP-F1 (Fig. 5*b*). In contrast, HP-F1 significantly reversed the inhibition of rolling adhesion of three different NK cell lines on PED-G monolayers (mean inhibition, 48%; range, 24–61%), as shown in a representative experiment with the PF NK line (Fig. 5*c*). The percentage of NK cells expressing ILT-2 ranged from 64 to 84%. In these experiments no significant reversion (9%; $p = 0.49$) was observed by blocking CD94 with HP-3B1 (Fig. 5*b*) or KIR2DL2, the NK receptor specific for HLA-Cw1, -3, -7, and -8, with GL183 (data not shown).

## Discussion

Based on previous reports indicating that HLA-G is a potent inhibitor of NK cell cytotoxicity, we tested the potential of HLA-G to protect pEC from xenogeneic human NK cytotoxicity. Our results demonstrate that the expression of HLA-G in either microvascular (2A2) or aortic (PED) pEC provides only partial protection against NK cytotoxicity mediated by polyclonal NK lines, the NK92 cell line, and a panel of NK clones. Since it has been shown that human NK cells may be inhibited, although weakly, by SLA class I molecules (45), differences between HLA-G-transfected pEC and control pEC in the expression of SLA class I may theoretically also explain our results. However, comparable amounts of SLA class I molecules were found on the HLA-G-transfected pEC, and the reversion of the HLA-G-mediated protection observed in blocking experiments using an anti-HLA-class I Ab (data not shown) confirms the ability of HLA-G to inhibit NK cytotoxicity against pEC (25). These findings are also consistent with the results of Sasaki et al. (23), who reported partial inhibition (31–43%) of xenoreactive human anti-pig NK cytotoxicity against primary aortic pEC transiently transfected with genomic HLA-G along with the human *β$_2$-microglobulin* gene. In contrast, Dorling

et al. (24) did not find a protective effect of HLA-G in a stably transfected aortic pEC line against the lysis mediated by human PBMC, NK, and LAK cells. Since a different pig cell line and cytotoxicity assay (lactate dehydrogenase release) was used in the latter study, and the NK cells were frozen and stored overnight at −80°C after isolation and before functional studies, the discrepancy may relate to technical details.

The importance of the relative strength of activating and inhibitory signals in the regulation of NK cytotoxicity was stressed by our finding that the inhibitory signal transduced by receptors specific for HLA-G appears to be weaker than the signal transduced by HLA-Cw3-specific receptors. In theory, insufficient expression levels on transfected pEC could explain the lower efficiency of HLA-G. Alternatively, the frequency, expression levels, and/or relative potency of HLA-G-specific inhibitory receptors on NK cells may not be sufficient to inhibit NK cytotoxicity to the same degree as the HLA-Cw3-specific KIR. Indeed, a reduction of HLA-G surface expression was associated with a loss of protection from NK cytotoxicity, confirming previous reports on the role of HLA-G expression levels in the regulation of NK cytotoxicity (25, 41). On the other hand, HLA-G, even at high levels of expression, rarely provided complete protection against NK clones; hence, it is possible that HLA-G-induced signals are weaker than signals elicited by HLA-Cw3.

For many years, the nature of HLA-G-specific NK receptors was a matter of debate, until recently when new receptors, ILT-2 and p49/KIR2DL4, which recognize HLA-G directly, were identified. The frequency of HLA-G-specific inhibitory receptors and KIR on peripheral blood-derived NK cells seems to be comparable, since the number of NK clones that were inhibited by HLA-G and HLA-Cw3, respectively, did not differ significantly in our analysis. We found that the ILT-2 receptor was only expressed on a fraction of the cells present in polyclonal NK lines (23–84%) and that the expression levels of ILT-2 on both NK lines and clones were clearly lower than those of KIR2DL2. Moreover, the clonal analysis revealed no correlation between the expression of ILT-2 or any other receptor and the protective effect of HLA-G, whereas the expression of KIR2DL2 correlated well with HLA-Cw3-mediated protection. Unfortunately, blocking experiments could not be performed due to the short survival of these NK clones. The inhibition of ILT-2-negative NK clones could not be ascribed to KIR2DL4, which is an activating receptor (38). Therefore, the present data are compatible with the existence of as yet unknown HLA-G-specific receptors that together with ILT-2 are responsible for the observed effect of HLA-G.

In pig to human xenotransplantation rolling adhesion is the main mechanism for the capture of human NK cells from the circulation (46) and is followed by firm adhesion, transendothelial migration, and infiltration into the porcine graft. Molecules that control the adhesion of NK cells to target cells and/or the adhesion mechanisms leading to NK cell extravasation and tissue infiltration are important in the regulation of NK cell function (47). When we analyzed the effect of HLA-G expression on pEC in adhesion assays, we could clearly demonstrate that HLA-G inhibited rolling adhesion of activated human NK cells. This finding raised the question of whether the observed protective effect of HLA-G against NK cytotoxicity was caused by an impairment of adhesion. Our data do not provide direct evidence for this idea, since the in vitro assay used to assess NK cytotoxicity against PED-G cells does not require rolling adhesion. In addition, the respective levels of inhibition of rolling adhesion and cytotoxicity did not correlate well among the NK lines. However, a link between adhesion and cytotoxicity seems possible; blocking of the interactions between ILT-2 and HLA-G had an effect on both adhesion and cytotoxicity, suggesting that HLA-G might decrease NK-mediated killing at least partially by reducing the capacity of NK cells to adhere to pEC. Our finding that masking ILT-2 with HP-F1 reversed the inhibition of rolling adhesion only partially when polyclonal NK lines were studied and failed to reverse the inhibition of adhesion using NK92 cells indicated that HLA-G may inhibit rolling adhesion also via NK receptors different from ILT-2. Two recent studies support our view of a link between MHC class I molecule expression and adhesion mechanisms. Eriksson et al. (27) showed that mouse NK cells interact longer with MHC class I-deficient target cells compared with NK-resistant cells that express MHC class I. Furthermore, using a flow cytometry assay for conjugate formation, Burshtyn et al. (28) demonstrated that the LFA-1-dependent adhesion of YTS NK cells transfected with KIR2DL1 was strongly reduced by the expression of HLA-Cw4 on 721.221 target cell. Taken together, our data obtained from functional adhesion assays for the first time demonstrate that interactions between HLA class I molecules and inhibitory NK receptors regulate adhesion, possibly by interfering with the expression of adhesion molecules on NK cells.

In conclusion, transgenic expression of HLA-G on porcine endothelium reduces rolling adhesion of activated human NK cells and provides direct protection against NK cell-mediated xenogeneic cytotoxicity in the absence of HLA-E. These results increase our understanding of the fundamental biological functions of HLA-G and indicate a potential direct role for HLA-G in the regulation of NK cell functions. Although this potential needs to be further explored in animal models, HLA-G expression alone will not be successful as a strategy to overcome NK cell-mediated xenograft rejection unless used in combination with other approaches.

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