

# Human Histocompatibility Leukocyte Antigen (HLA)-G Molecules Inhibit NKAT3 Expressing Natural Killer Cells

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## Summary

The crucial immunological function of the classical human major histocompatibility complex (MHC) class I molecules, human histocompatibility leukocyte antigen (HLA)-A, -B, and -C, is the presentation of peptides to T cells. A secondary function is the inhibition of natural killer (NK) cells, mediated by binding of class I molecules to NK receptors. In contrast, the function of the nonclassical human MHC class I molecules, HLA-E, -F, and -G, is still a mystery. The specific expression of HLA-G in placental trophoblast suggests an important role for this molecule in the immunological interaction between mother and child. The fetus, semiallograft by its genotype, escapes maternal allorecognition by downregulation of HLA-A and HLA-B molecules at this interface. It has been suggested that the maternal NK recognition of this downregulation is balanced by the expression of HLA-G, thus preventing damage to the placenta. Here, we describe the partial inhibition of NK lysis of the MHC class I negative cell line LCL721.221 upon HLA-G transfection. We present three NK lines that are inhibited via the interaction of their NKAT3 receptor with HLA-G and with HLA-Bw4 molecules. Inhibition can be blocked by the anti-NKAT3 antibody 5.133. In conclusion, NK inhibition by HLA-G via NKAT3 may contribute to the survival of the fetal semiallograft in the mother during pregnancy.

In the last five years three major functions of the classical human MHC class I molecules, HLA-A, -B, and -C have been established: (a) presentation of peptides to T cells (1, 2); (b) inhibition of NK cells via inhibitory NK receptors, KIR<sup>1</sup> (3–6); and (c) activation of NK cells via activatory NK receptors, KAR (7–9).

In contrast, the function of the nonclassical human MHC class I molecules is only poorly understood (10). Of the 19 nonclassical MHC I genes, 3 genes, HLA-E, -F, and -G, were found to be expressed as proteins (11). The HLA-G gene has the same general structure as the classical MHC class I genes with five exons and three introns. It gives rise to five differently spliced mRNAs lacking zero, one, or even two exons. All these mRNAs are translated; one leads to a soluble HLA-G molecule without the transmembrane and intracellular domains (12, 13). Both the soluble and the largest membrane-associated HLA-G molecules assemble trimeric complexes with  $\beta$ 2-microglobulin ( $\beta$ 2m) and en-

dogenously derived peptides that show a distinct peptide motif similar to that of peptides bound to classical MHC class I molecules (14, 15).

HLA-G is selectively expressed at the fetomaternal interface. Extravillous cytotrophoblasts that invade maternal tissue as well as amniotic epithelium express no HLA-A or HLA-B molecules (16–18) but do express HLA-C molecules (19). In addition, high levels of HLA-G are present on the cell surface and at least some components, LMP7 and TAP, of the endogenous antigen presentation pathway are overexpressed (20, 21).

This restricted expression, as well as the potential of HLA-G to assemble trimeric classical MHC class I-like complexes, led to the hypothesis that HLA-G might play an important role in the immunological interaction between mother and child (22). Therefore, two hypotheses have been put forward. One suggests that HLA-G-restricted CTL survey the trophoblast for viral infections or malignancy through the presented peptide pool (10). The second hypothesis, which is supported by this report, proposes an inhibitory effect of HLA-G on those NK cells that would normally recognize the absence of the classical MHC class I

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<sup>1</sup>Abbreviations used in this paper: KAR, killer activatory receptor; KIR, killer inhibitory receptor; LGL, large granular lymphocyte.

molecules and therefore would destroy the fetal cytotrophoblast (14, 23).

NK cells were found to detect the absence of MHC I molecules on the cell surface and this finding led to the missing-self hypothesis (24). Subsequently two groups of receptors were reported on NK cells: activatory and inhibitory receptors (25). Engagement of the activatory receptors leads to target cell killing, while stimulation of the inhibitory receptors prevents killing. In humans, the members of both groups belong to the immunoglobulin superfamily and recognize public epitopes in MHC class I molecules. They differ mainly in the transmembrane and cytoplasmic domains, which suggests that the recognition pattern is similar, but the signaling different (7, 9). Until now, four human inhibitory NK receptors and their specificities have been identified, NKAT1–4 (3; Table 1). The expression of the inhibitory NK receptors is tightly regulated: receptors for self-MHC class I molecules are upregulated, while the general expression pattern for KIRs seems to be inherited (26).

To prove that NK inhibition by HLA-G is an effective protection for the fetus, inhibition of the majority of NK cells in the mother has to be demonstrated and the mediating receptors have to be identified. Partial inhibition of NK cells in a CD56<sup>+</sup> bulk culture has been found (27, 28), but to date no interacting receptor could be identified. In this study, we address the question of NK bulk culture inhibition and show for the first time NKAT3 inhibition by HLA-G.

## Materials and Methods

**NK Lines and Populations.** PBL from healthy donors were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation using FicoLite-H (Linaris, Bettingen, Germany). The buffy coats were obtained from the blood banks in Heidelberg and Tübingen. To isolate NK cells, 10<sup>7</sup> PBL in 80 µl MACS buffer (PBS + 0.5% BSA + 2 mM EDTA) were incubated with 20 µl CD56 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 15 min at 6–12°C. CD56<sup>+</sup> PBL were separated on MiniMACS separation columns (Miltenyi Biotec

using a VarioMACS separation magnet. Retained cells were cultured in RPMI + 5% human serum (HS) + 1000U/ml rhIL-2 (Proleukin, Chiron GmbH, Germany) + 2 mM glutamin + 1 mM sodium-pyruvate + 1× nonessential amino acids (Sigma Chem. Co., St. Louis, MO) in the presence of irradiated human PBL of any donor. NK lines were created by dilution in 96-well plates. CD56<sup>+</sup> PBL were distributed at 10, 3.3, and 1.1 cells/well. After 7 d, each well was split in three and after 7 d more, two of the three sets of plates were used for assays against LCL721.221 and LCL721.221.G, transfected with the full-length HLA-G locus 5.4 kb genomic DNA cells. Wells that showed high killing of LCL721.221 and low killing of LCL721.221.G were picked from the third set of plates and expanded.

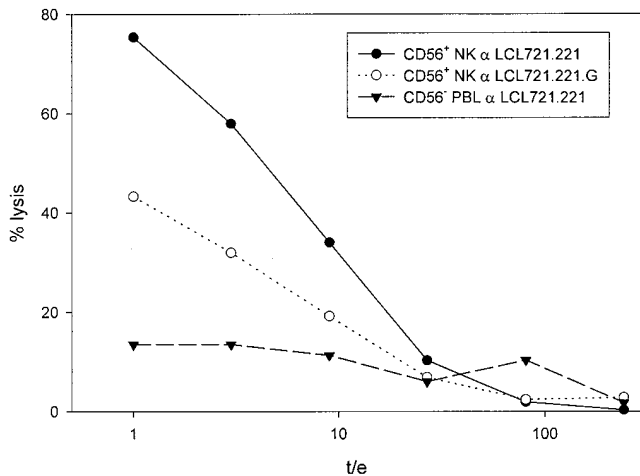
**Flow Cytometry.** 10<sup>6</sup> cells were labeled either with directly FITC-labeled antibodies against CD4 (Immunotech Luminy, France), CD8 (PharMingen, San Diego, CA), CD56 (Becton-Dickinson, Mountain View, CA) or with primary antibodies B73.1, αCD16 (a gift from Dr. B. Perussia, Philadelphia, PA) and 5.133, αNKAT3/4, combined with a FITC-labeled goat anti-mouse antibody (Dianova, Hamburg, Germany). For each staining, 5–10 × 10<sup>3</sup> cells were analyzed using a FACSCalibur<sup>®</sup> flow cytometer (Becton-Dickinson, San Jose, CA) and CellQuest software.

**Cytolytic Assays.** For measurement of cytolytic activity, <sup>51</sup>Cr release assays were performed. The following cell lines were used as targets: human HLA-null lymphoblastoid cell line LCL721.221 (29) and its HLA-G transfectant, LCL721.221.G, (27); human MHC class I-reduced cell line C1R (30) and its HLA-B8 (provided by Dr. M. Takiguchi, Tokyo, Japan), -B\*2705 (provided by Dr. P. Cresswell, New Haven, CT), and -B\*5101 (31) transfectants; human STEMO cell line (32) and PHA induced blasts of PBL of an HLA-A3<sup>+</sup>, -B7<sup>+</sup>, and -Cw7<sup>+</sup> donor. PHA blasts were obtained by culturing 10<sup>6</sup> PBL with 2 µg PHA (Boehringer Mannheim, Mannheim, Germany) in 1 ml αMEM + 5% HS + 2 mM glutamin for 5 d. <sup>51</sup>Cr-labeled target cells were incubated for 4 or 6 h with the NK cells in 200 µl RPMI + 5% FCS + 2 mM glutamin. Afterwards, 50 µl of the supernatant was harvested and the radioactivity was measured in a microplate format scintillation counter (1450 Microbeta Plus, Wallac, Turku, Finland) using solid-phase scintillation (LumaPlate-96, Packard, Groningen, The Netherlands). Percent-specific lysis was calculated as [(cpm experimental well – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release)] × 100%. Spontaneous re-

**Table 1.** Listing of Human Inhibitory NK Receptors, their HLA Class I Counterparts, and the HLA Amino Acids Likely to be Recognized by KIRS

Human inhibitory NK receptors	Inhibiting HLA class I molecules	Amino acids at positions			
		77	78	79	80
NKAT1 (46)	HLA-Cw4, -Cw5, -Cw6	Asn(N)	X	X	Lys(K)
NKAT2 (46)	HLA-Cw1, -Cw3, -Cw7, -Cw8	Ser(S)	X	X	Asn(N)
NKAT3 (46)	HLA-Bw4	Ser(S)	Leu(L)	Arg(R)	Thr(T)
		Asn(N)			Ile(I)
		Asp(D)			
NKAT4 (34, 44)	HLA-A*0301	Asp(D)	Leu(L)	Gly(G)	Thr(T)
NKAT3 (this report)	HLA-G	Asn(N)	Leu(L)	Gln(Q)	Thr(T)

X, polymorphic position.



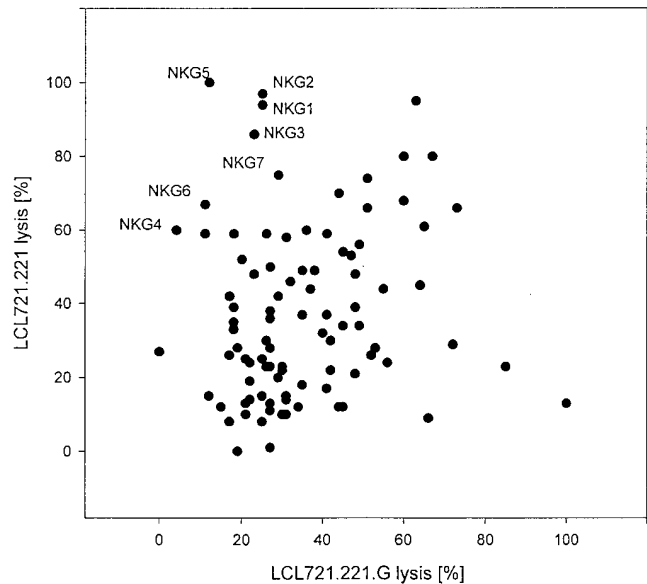
**Figure 1.**  $^{51}\text{Cr}$  release assay of  $\text{CD56}^+$  PBL versus LCL721.221 (●), as well as LCL721.221.G (○), and  $\text{CD56}^-$  PBL versus LCL721.221 (▼).

lease was determined by incubating the labeled target cells with medium; maximum release was determined by incubating the target cells in 1% Triton X-100 medium. The mAbs 5.133,  $\alpha\text{NKAT3}+4$  and HP-3E4,  $\alpha\text{NKAT1}$  were used at a final concentration of 2.5  $\mu\text{g}/\text{ml}$ .

## Results

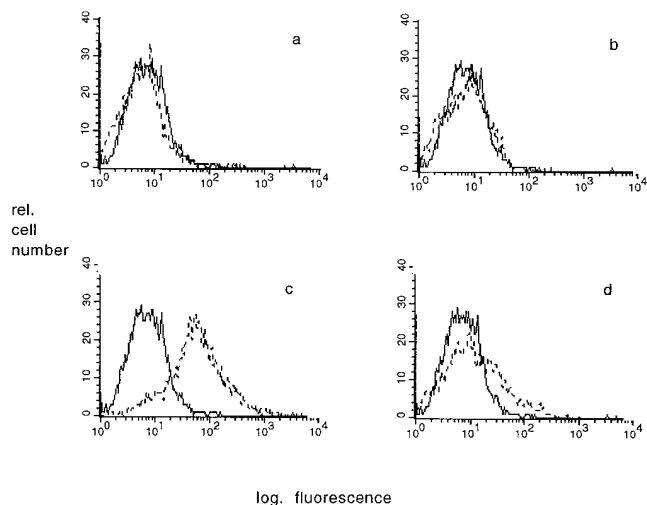
**Inhibition of  $\text{CD56}^+$  NK Bulk Cultures by Target Cells Transfected with HLA-G.** The effect of HLA-G transfection on killing by  $\text{CD56}^+$  NK cultures was investigated using the HLA-null human lymphoblastoid cell line LCL721.221 with and without HLA-G transfection as targets for positively MACS-selected  $\text{CD56}^+$  PBL of healthy donors.  $\text{CD56}^+$  PBL show the phenotype of peripheral NK cells by FACS<sup>®</sup> analysis:  $\text{CD16}^+$ ,  $\text{CD56}^{\text{dim}}$ ,  $\text{CD4}^-$  and partial  $\text{CD8}$  expression (data not shown). Our HLA-G transfectant of the LCL721.221 cell line, LCL721.221.G, expresses the non-classical MHC class I molecule with a mean fluorescence value of 45–50 (14). In a 4-h  $^{51}\text{Cr}$  release assay, the specific lysis of LCL721.221 mediated by the isolated NK population against LCL721.221 was between 70–80% at a E/T ratio of 1:1 (Fig. 1). At the same ratio, the killing of LCL721.221.G was remarkably reduced (to 40–45%; Fig. 1). In both cases, the killing decreased with lower E/T ratios. The  $\text{CD56}^-$  PBL population showed only low background killing of both cell lines (around 10% specific lysis; Fig. 1).

**Isolation of HLA-G-specific NK Lines.** Positively MACS-selected  $\text{CD56}^+$  PBL from healthy donors were diluted in 96-well plates, split into three sets of plates after 7 d, and assayed for killing of LCL721.221 and LCL721.221.G after another 7 d. 50% of the NK-containing wells showed inhibition of killing upon HLA-G transfection of the targets. Seven wells that showed 50–90% higher specific lysis of LCL721.221 compared with specific lysis of LCL721.221.G were expanded (NKG1–7; Fig. 2). Of the seven cultures, three cultures, NKG1, NKG2, and NKG7, preserved recognition of HLA-G in our culture conditions, and killing

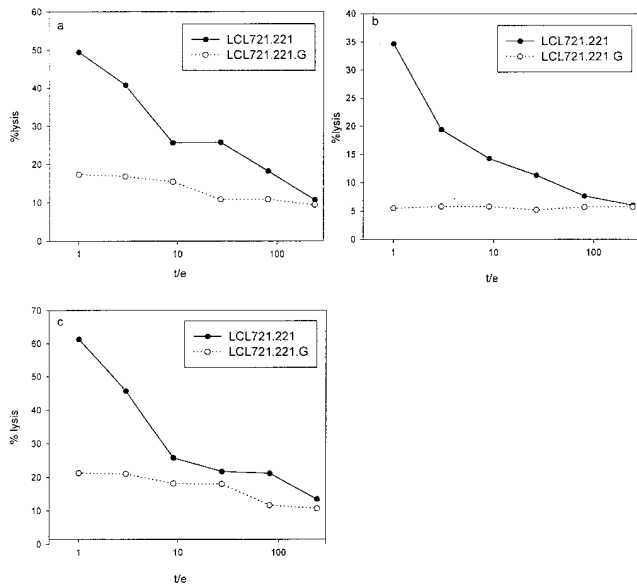


**Figure 2.** Lysis of  $\text{CD56}^+$  PBL subcultures distributed over the wells of a 96-well plate. Each dot represents the behavior of one well in a  $^{51}\text{Cr}$  release assay against LCL721.221 (y axis) and LCL721.221.G (x axis). The marked NK lines, NKG1–7, were picked and expanded.

of LCL721.221 by these lines was inhibited to background levels upon HLA-G surface expression. FACS<sup>®</sup> analysis of the three NK lines showed homogeneous surface expression of the NK surface markers  $\text{CD16}$  and  $\text{CD56}$ .  $\text{CD4}$  and  $\text{CD8}$  expression could not be detected, as shown in Fig. 3 for NKG7. NKG1 and NKG2 showed the same phenotype of tested surface markers:  $\text{CD16}^+$ ,  $\text{CD56}^{\text{dim}}$ ,  $\text{CD4}^-$ ,  $\text{CD8}^-$  (data not shown). The ratios of specific lysis of LCL721.221 to the specific lysis of LCL721.221.G for the three lines af-



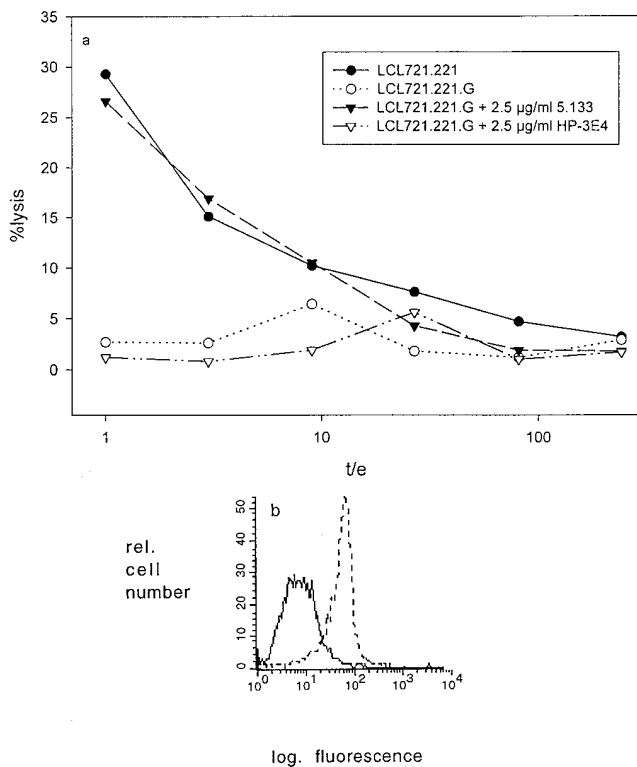
**Figure 3.** Flow cytometry analysis of the surface molecules of NKG7 (broken line) by staining of (a)  $\text{CD8}$  with a directly FITC-labeled  $\alpha\text{CD8}$  antibody; (b)  $\text{CD4}$  with a directly FITC-labeled  $\alpha\text{CD4}$  antibody; (c)  $\text{CD16}$  with the B73.1 antibody and a FITC-labeled goat  $\alpha$ -mouse antibody; (d)  $\text{CD56}$  with a directly FITC-labeled  $\alpha\text{CD56}$  antibody. The negative control represents NKG7 cells incubated with the secondary goat  $\alpha$ -mouse antibody (solid line).



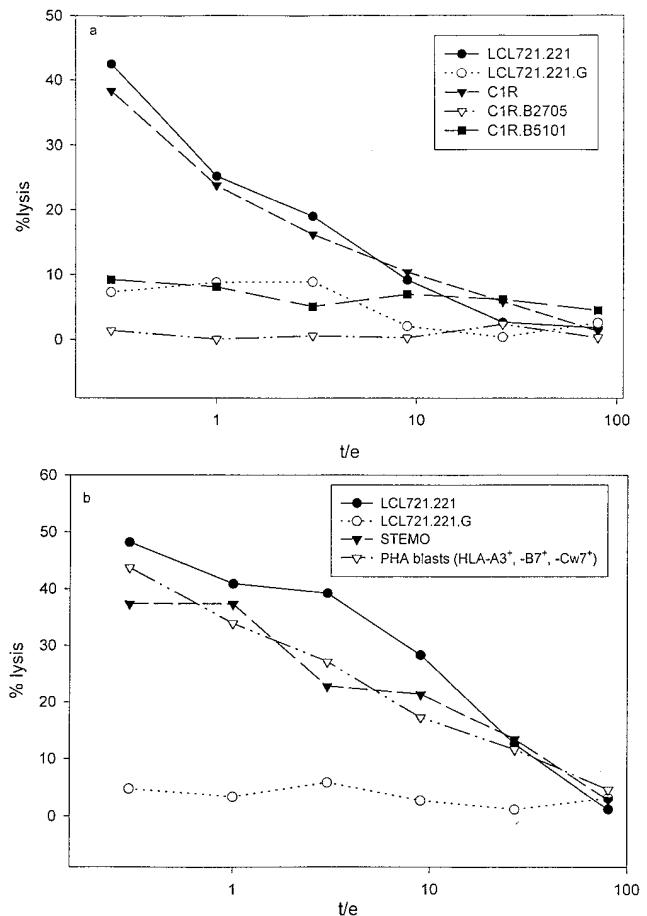
**Figure 4.**  $^{51}\text{Cr}$  release assays of NKG1 (a), NKG2 (b), and NKG7 (c) against LCL721.221 (●) and LCL721.221.G (○).

ter 4 wk in culture at a E/T ratio of 1:1 were the following: NKG1, 50/18; NKG2, 35/5; NKG7, 62/21 (Fig. 4).

*Identification of NKAT3 as the Receptor Mediating HLA-G Inhibition.* This was done in two sets of experiments.



**Figure 5.** (a)  $^{51}\text{Cr}$  release assay of NKG7 against LCL721.221 (●) and LCL721.221.G (○) in the presence of 2.5  $\mu\text{g/ml}$  5.133 (▼) or HP-3E4 (▽). (b) Flow cytometry analysis of NKAT3 and/or NKAT4 expression of NKG7. Staining with the 5.133 antibody and a goat  $\alpha$ -mouse FITC-labeled secondary antibody (broken line). Staining with the secondary antibody only served as the negative control (solid line).



**Figure 6.** (a)  $^{51}\text{Cr}$  release assay of NKG7 against LCL721.221 (●), LCL721.221.G (○), C1R (▼), C1R-B\*2705 (▽), and C1R-B\*5101 (■); (b)  $^{51}\text{Cr}$  assay of NKG7 against LCL721.221 (●), LCL721.221.G (○), STEMO (▼), and PHA blasts of PBL of a HLA-A3<sup>+</sup>, -B7<sup>+</sup> and -Cw7<sup>+</sup> donor (▽).

First, blocking of the receptor during  $^{51}\text{Cr}$  release assay with mAbs was used to prevent inhibition by HLA-G. Second, transfectants, as well as PHA blasts of typed donors, were surveyed for coinhibition of NKG1, NKG2, and NKG7. The mAbs 5.133, recognizing NKAT3 as well as NKAT4, and HP-3E4, recognizing NKAT1, were used at a final concentration of 2.5  $\mu\text{g/ml}$  in the medium during  $^{51}\text{Cr}$  release assay. Addition of 5.133 prevented inhibition of LCL721.221 lysis by HLA-G, while addition of HP-3E4 did not. This effect was observed with all three NK lines. Representative data for NKG7 are given in Fig. 5 a. 5.133 Fab fragments and HP-3E4 antibodies were previously used to restore NK lysis by blocking KIRs (33, 34). In agreement with this finding, the 5.133 antibody stains the NK lines efficiently in flow cytometry (Fig. 5 b).

To examine whether HLA-G inhibition of the NK lines is mediated versus NKAT3 or NKAT4 reactivity of these lines, targets expressing NKAT3 or NKAT4 ligands were tested. Fig. 6 shows representative data obtained with the NKG7 line for recognition of (a) HLA-B\*2705 or -B\*5101-transfected C1R cells and (b) HLA-A3-expressing cells (STEMO and HLA-A3<sup>+</sup> PHA blasts). C1R, which is reduced in

MHC class I expression, was found to be killed by the NK lines just as efficiently as LCL721.221 (Fig. 6 a). Only target cells expressing the HLA-B\*2705/-B\*5101 alleles inhibited the activity of the NK lines. This set of data suggests that HLA-G inhibition of NKG1, NKG2, and NKG7 runs parallel to inhibition by HLA-B\*2705 and -B\*5101, both of which carry the HLA-Bw4 public epitope. As shown in Table 1, HLA-Bw4 is recognized by the NKAT3 inhibitory human NK receptor, while HLA-A3 reacts with NKAT4. In conclusion, HLA-G inhibition of NKG1, NKG2, and NKG7 can be blocked by the 5.133 antibody that recognizes NKAT3 as well as NKAT4, but only NKAT3 seems to mediate the inhibition of the NK lines, because they alone can be inhibited by HLA-Bw4 but not HLA-A\*0301-bearing targets.

## Discussion

In this study, we have demonstrated that half of the NK activity of PBL can be inhibited upon HLA-G transfection and that inhibition was mediated, in part at least, by NKAT3. The fetus downregulates classical MHC class I molecules, HLA-A and HLA-B, at the feto-maternal interface (22) and, for this reason, the immune system of the mother is not able to attack the placenta by alloreactive T cells. However, the absence of HLA-A and HLA-B molecules potentially renders the fetal cytotrophoblast sensitive for NK recognition and lysis. Moreover, high numbers of CD56<sup>+</sup> large granular lymphocytes (LGL) are present in the decidua during early pregnancy; yet, even so, the trophoblast is usually not destroyed. Up to 70% of all decidual lymphocytes show this phenotype (35). Based on the two NK receptor theory (25), there are two possible explanations for nonrecognition of the fetal cytotrophoblast by maternal NK cells. One is that these cells lack activatory structures for NK cells on their surface (36) and the other postulates a substitute for classical MHC class I molecules inhibiting NK cells (27).

Lysis of LCL721.221 by CD56<sup>+</sup> NK cells can be reduced by up to 50% upon HLA-G transfection. If inhibition by HLA-G is one of the major mechanisms for protecting the fetal semiallograft from maternal NK cells, this inhibition should be close to 100%. There are three possible explanations for the only partial inhibition of NK cells by HLA-G in our experimental system. One reason could be that the CD56<sup>+</sup> NK cells present in the decidua express inhibitory NK receptors for HLA-G at higher levels, resulting in stronger inhibition. It has been observed that KIR expression on peripheral NK cells differs remarkably between donors and often a high and a low expressing population can be detected (25). A second explanation could be that in addition to HLA-G, HLA-C molecules are also expressed on first trimester trophoblast cells (19). Different HLA-C alleles have the potential to inhibit either NKAT1- or NKAT2-bearing NK cells (Table 1). This additional inhibition of NK reactivity may protect the fetus from the remaining lysis in our model system. The third reason could be that the expression level of HLA-G on our

LCL721.221 transfectant is lower than the physiological HLA-G expression. Because NK cells are highly sensitive to missing self in a density-dependent way (24), low HLA-G levels could explain the residual lysis of our transfectant. In the LCL721.221 cell line, TAP and LMP7 molecules are expressed at much lower levels compared with the fetal cytotrophoblast cells (20, 21). Because these molecules influence the peptide supply of MHC molecules inside the ER (37, 38) and HLA-G binds peptides as do other HLA class I molecules (14, 15), fetal cytotrophoblast cells are expected to express higher levels of HLA-G, which should result in a more complete inhibition of NK cells (39). In line with this explanation is the finding that externally added peptide up-regulates HLA-G expression on the LCL721.221.G cells (14).

The three NK lines, NKG1, NKG2, and NKG7 are strongly inhibited by HLA-G (Fig. 4). This inhibition is mediated by the NKAT3 receptor (Figs. 5 a and 6). The human KIR family interacts with the HLA class I molecules via the  $\alpha$ 1 domain of the MHC class I heavy chain, and especially by the three COOH-terminal turns of the  $\alpha$  helix belonging to this domain (40). The correct three-dimensional folding of the HLA class I heavy chain for interaction with the KIRs seems to be dependent on peptides present in the binding groove (41, 42). However, direct interaction of the inhibitory NK receptors with peptides seems to be unlikely, because stabilization of empty HLA-C molecules at 26°C leads to the same level of inhibition as endogenous peptide-loaded MHC class I molecules (33). NK recognition of HLA class I molecules is especially sensitive to the identity of the amino acids at positions 77 and 80 (Table 1). Resistance against NKAT1-bearing clones could be transferred to HLA-C molecules normally recognized by NKAT2 by changing S77 to N77 and N80 to K80 (9). Protection against NKAT1-bearing NK clones, as well as NKAT2-bearing NK clones, was abolished upon mutation of position 80 (9, 33). Furthermore, NKAT3 recognizes HLA-Bw4 molecules with isoleucine or threonine at position 80 with high statistical significance (40, 43) and the NKAT4 receptor interacts with HLA-A3 (34, 44). On comparing the primary structure of HLA-G to HLA-A, -B, and -C sequences, a high homology for HLA-A2 can be found (45). There is 89.9% similarity and 81.1% identity in an amino acid sequence alignment. HLA-G also assembles with  $\beta$ 2m and the bound peptides show a distinct motif similar to classical MHC class I peptide motifs (14, 15). The three-dimensional structure of HLA-G therefore is probably quite similar to the structure of classical HLA class I molecules and it can be assumed that HLA-G interacts via the same region with the inhibitory NK receptors as the HLA-A, -B, and -C molecules. Comparison of the amino acid sequence of HLA-G at positions 77–80 shows a clear homology to HLA-Bw4 molecules in this particular region (Table 1). At positions 77 and 80 in particular, HLA-Bw4 molecules possess distinct amino acids: position 77 is always occupied either by asparagine, N; aspartic acid, D; or serin, S; whereas position 80 is always threonine, T; or isoleucine, I. In the HLA-G molecule, position 77 is occupied by asparagine, N, and position 80 by threonine, T. This probably

enables the NKAT3 receptor to interact with this region and to mediate a negative signal to its NK cells, which prevents target cell lysis.

In conclusion, we have demonstrated that NK inhibition by HLA-G is, in part at least, mediated by the NKAT3 re-

ceptor. The inhibition of NK-mediated cell lysis is probably essential for the survival of the fetal semiallograft in the mother during pregnancy. Thus, deletion of HLA-G or mutations in this gene might lead to loss of the fetus in early pregnancy.

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