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HLA-G1 co-expression boosts the HLA class I-mediated NK lysis inhibition

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Abstract

It is now acknowledged that the pattern of HLA-G expression is not restricted to extravillous cytotrophoblast cells, as several studies described HLA-G in HLA class I^+ cells, such as thymic epithelial cells, cytokine-activated monocytes and some tumors. In these situations, HLA-G may provide an additional inhibitory signal to escape from NK cell-mediated cytotoxicity. Accordingly, the aim of this study was to define the behavior of HLA-G once it is co-expressed into an HLA-A, -B, -C and -E^+ cell line. For this purpose, HLA-G1 cDNA was transfected into an HLA class I^+ melanoma cell line which was used as a target towards freshly isolated peripheral blood NK cells. Cytotoxic experiments using either anti-HLA-G1 or anti-HLA-G1 inhibitory receptor mAb show that HLA-G1 boosts the HLA class I-mediated inhibition of polyclonal NK cells through interaction with ILT-2, which appears as the major HLA-G1 inhibitory receptor involved. Nevertheless, HLA-G1 is also able to inhibit the cytolytic activity of an ILT-2^- NK clone which otherwise expresses another HLA-G1 inhibitory receptor belonging to the KIR103 gene family. In order to more precisely define the relative role exerted by HLA-G1 versus -E on polyclonal NK cells, antibody-blocking assays were carried out using either anti-HLA class I or anti-CD94/NKG2A. Results demonstrate that in the absence of HLA-G1, the naturally expressed HLA class I-mediated NK inhibition is predominantly exerted by HLA-E through binding with CD94/NKG2A. In contrast, once HLA-G1 is expressed, it becomes the major NK inhibitory ligand.

Introduction

The non-classical MHC class I molecule HLA-G is a quasi-monomorphic HLA antigen mainly expressed on extravillous cytotrophoblast cells (1) where it may contribute to immunological tolerance of the fetal semi-allograft facing maternal immune system (2). In this regard, all previous HLA-G functional studies were carried out by using HLA class I^- cell lines, such as LCL 721.221 or K562 that became protected against NK cell-mediated cytolysis when transfected with HLA-G (3–6). However, recent reports have shown that the non-classical HLA-E class I antigen, which is synthesized by LCL 721.221 cells, binds leader sequence peptides from several classical and non-classical molecules (7–9), including HLA-G (10). This binding leads to both HLA-E cell surface expression and interaction with the C-type lectin inhibitory receptor CD94/NKG2A which provides a way to inhibit NK cytolysis. Consequently, it was unclear whether the protective role of HLA-G facing the NK immune system was direct or indirect through HLA-E expression. There is now increasing evidence that the full-length HLA-G1 molecule is able to inhibit NK cytolysis by itself when expressed on classical HLA class I^- cells (11–13).

In contrast, no data is available concerning the role of HLA-G1 when it is co-expressed with the other HLA-A, -B, -C and -E inhibitory molecules towards NK cell cytolysis. This information is of particular interest since, beside the selective placental expression of HLA-G, it is also found in HLA class I^+ cells, as reported in thymic epithelial cells (14), cytokine-activated monocytes (15,16) and some tumors (17). Thus, the aim of this study was to define the behavior of HLA-G once it is co-expressed into an HLA-A, -B, -C and -E^+ cell line. For this purpose, we have constructed an *in vitro* model of transfectants expressing HLA-G1, -E and the classical HLA-A, -B, and -C class I molecules that were used as targets towards freshly isolated peripheral blood NK cells and NK

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clone. We provide evidence here that in the absence of HLA-G1, NK cytolysis is principally inhibited through direct engagement of HLA-E with CD94/NKG2A. In contrast, when HLA-G1 is expressed, it inhibits NK cytolysis by itself and constitutes the predominant inhibitory molecule conferring cells protection from NK lysis. To date, three NK cell Ig-like receptors (KIR) that recognize HLA-G1, i.e. ILT-2 (18,19), p49 (20) and KIR2DL4 (21), have been identified. While ILT-2 belongs to the Ig-like transcript family (ILT or LIR), p49 and KIR2DL4 belong to the KIR103 gene family (22), and are respectively encoded by two distinct cDNA (i.e. 15.212 and KIR103AS). Among these receptors, our results show that ILT-2 is the major inhibitory receptor involved in HLA-G1-mediated inhibition of fresh NK cells. However, HLA-G1 is still able to inhibit the lytic activity of the YT2C2-PR NK clone which does not express ILT-2 but an inhibitory receptor belonging to the KIR103 family. Thus, our results strongly suggest that in the context of naturally expressed HLA-E and classical HLA class I molecules, the efficient HLA-G1-mediated NK inhibition involves principally the ILT-2 receptor. The other HLA-G1 receptors may otherwise play an important role when NK cells bearing only those receptors are recruited under certain conditions in tissues that express HLA-G.

Methods

Cells

The M8, HLA class I^+ melanoma cell line (23), the K562 human erythroleukemia cell line (HLA A, -B, -C and $-E^-$; ATCC, Rockville, MD), and the NK-like YT2C2-PR subclone (3) were maintained in RPMI medium supplemented with 10% inactivated FSC, 1 μg/ml gentamicin and fungizone (Sigma, St Louis, MO). The TER-1 cytotoxic T lymphocyte clone was cultured as described (24). HLA-G transfectants were obtained as previously described (3,25), and selected in media containing 1 mg/ml geneticin for M8-RSV (transfected with the control vector alone) and M8-HLA-G1 (transfected with the vector containing the HLA-G1 cDNA) or with 100 μg/ml hygromicin for K562-pcDNA (transfected with the control vector alone) or K562-HLA-G1 (transfected with the vector containing the HLA-G1 cDNA). The cells used were routinely tested for, and found to be free of, mycoplasma.

Antibodies and flow cytometry analysis

In this study, we used the following antibodies: W6/32, IgG2a anti-HLA class I heavy chain associated with β_2-microglobulin (Sigma); 87G, IgG2a, anti-HLA-G1 (kindly provided by D. Geraghty, Fred Hutchinson Cancer Research, Seattle, WA) (26); and TP25.99, IgG1 anti-HLA-A, -B, -C and -E (kindly provided by S. Ferrone, Roswell Park Cancer Institute, Buffalo, NY) (27). Rabbit antisera to the N-terminal region of KIR2DL4 (kindly provided by E. Long, NIH, Bethesda, MD) and GHI/75, IgG ILT-2-specific mAb (kindly provided by M. Colonna, Basel Institute for Immunology, Basel, Switzerland) were raised as previously described (21,28).

Flow cytometry assays were carried out as previously described (3). The enriched NK cell populations were isolated from peripheral blood mononuclear cells (PBMC) by carrying out $CD3^+$ cell depletion using anti-CD3-coated Dynabeads (Dynal, Oslo, Norway), as previously described (3).

NK cell cytotoxicity assays

Human PBMC were isolated from healthy adult volunteer donors by standard Ficoll-Hypaque density centrifugation (Sigma). The fresh PBMC were used immediately in cytotoxicity assays upon isolation. The cytolytic activity of either PBMC, YT2C2-PR or TER-1, used as effectors was assessed in 4 h ^{51}Cr-release assays in which effector cells were mixed with 5×10^3 ^{51}Cr-labeled targets (100 μCi [^{51}Cr]sodium chromate; 1 Ci = 37 GBq; Amersham) at various E:T ratios in U-bottomed microtiter plates, as previously described (3). After 4 h at 37°C in a humidified 5% CO_2 incubator, 50 μl of the supernatant was collected for scintillation counting (Wallac 1450 Microbeta; EGG Instruments, Evry, France). The percentage of specific lysis was calculated as follows: percent specific lysis = [(c.p.m. experimental well – c.p.m. spontaneous release)/(c.p.m. maximum release – c.p.m. spontaneous release)]$\times$100. Spontaneous release was determined by incubation of labeled target cells with medium alone. Maximum release was measured by treatment of target cells with 0.1 M HCl. In all experiments, the spontaneous release was $<$10% of maximum release. Results are presented as the means of triplicate samples.

Antibody-blocking assays

In experiments in which mAb were used to block HLA class I antigens, target cells were incubated with the corresponding mAb, then washed and incubated with an F(ab′)2 goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) to prevent antibody-dependent cell cytotoxicity by interaction of NK cell Fc receptors with the mAb used. In experiments in which mAb were used to block NK inhibitory receptors, effector NK cells were firstly pre-incubated for 15 min at room temperature in culture medium containing 10% human AB serum and secondly pre-incubated for 15 min at room temperature with either GHI/75 (mouse ILT-2 specific mAb) (21,28) or HP-3B1 (mouse anti-human CD94; Immunotech, Marseille, France), and Z199 (mouse anti-human NKG2A; Immunotech) or EB6 (mouse anti-human p58.1; Immunotech), GL183 (mouse anti-human p58.2; Immunotech) and Z27 (mouse anti-human p70; Immunotech) before NK cell cytotoxicity assay. The mAb were present in the culture media during the entire assay period. mAb toxicity assays were checked in each assay and were always $<$3%.

Statistical analysis

The statistical significance of cytotoxic assays ($P < 0.05$) was analyzed using Student's *t*-test to compare percentage lysis of M8-HLA-G1 to M8-RSV. Assays were carried out in triplicate for each experiment and the SD of the mean triplicates was $<$5%.

Results

Expression of HLA class I molecules on HLA-G-transfected cell lines

As a necessary step towards understanding the inhibitory role exerted by HLA-G1 co-expressed with HLA-A, -B, -C

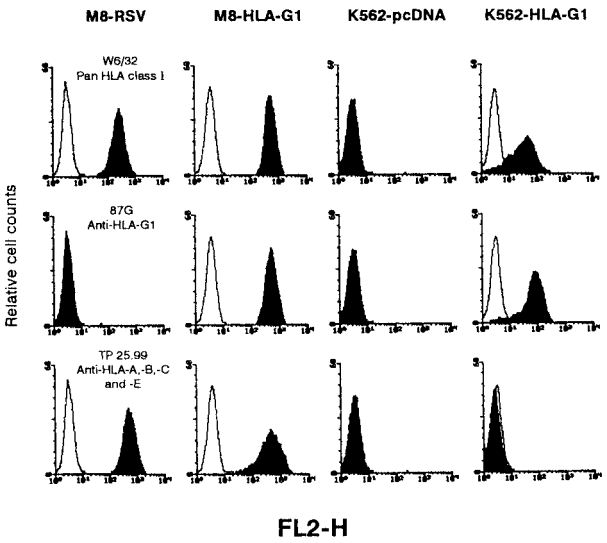

Fig. 1. Cell-surface expression of HLA-A, -B, -C and -G molecules on transfectants detected by cytofluorometry. K562 cells transfected with either the vector alone (K562-pCDNA) or with HLA-G1 cDNA (K562-HLA-G1), and M8 cells transfected with either the vector alone (M8-RSV) or HLA-G1 cDNA (M8-HLA-G1), were labeled by indirect immunofluorescence with the following primary mAb (bold profiles): W6/32 (monomorphic anti-class I), 87G (anti-HLA-G1) and TP25-99 (anti-HLA-A, -B, -C and -E). Controls were the same cells stained with an isotypic control antibody (light profiles). After washing, cells were stained with phycoerythrin-conjugated goat anti-mouse IgG.

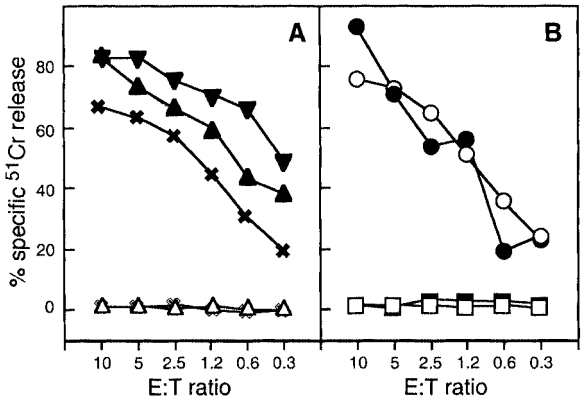

Fig. 2. Cytotoxic reactivity of class I leader sequence-derived peptide-specific HLA-E-restricted mouse cytotoxic T lymphocyte clone (TER-1) on HLA-G1 transfectants. Cytotoxic reactivity of the TER-1 clone was tested in a 4 h ^{51}Cr-release assay, using the following targets: (A) concanavalin A blasts from HLA-E transgenic mice expressing HLA-E molecules (EM, ×) or from transgenic mice expressing human β_2-microglobulin (M,), human homozygous EBV-transformed cells (HLA-A3; B7; Cw7) carrying EG (SCHU, ▼) or ER (EA, ▲) alleles, and HLA class I$^-$ cell line (Daudi, △), and (B) M8 (circles) and K562 (squares) cells transfected with either the vector alone (open symbols) or HLA-G1-cDNA (filled symbols).

and -E molecules, HLA-G1 cDNA was transfected into the M8 cell line that we have previously described as negative for HLA-G transcription and protein expression (23). While M8-RSV (transfected with the vector alone) did not react with the HLA-G1-specific 87G mAb, M8-HLA-G1 (transfected with the vector containing the HLA-G1 cDNA) was positively stained, showing a high level of HLA-G1 cell-surface expression. Both M8-RSV and M8-HLA-G1 expressed HLA class I molecules, as revealed by their staining with the pan-HLA class I, W6/32 and the HLA-A, -B, -C and -E, TP 25.99 mAb (Fig. 1). It is noteworthy that both M8-RSV and M8-HLA-G1 exhibit similar HLA-A, -B and -C expression levels, as both transfectants were similarly stained by HLA-A-, -B- or -C-specific mAb (data not shown). K562-pcDNA, used as HLA class I$^-$ control, was indeed not stained by W6/32 and TP25.99 mAb, while K562-HLA-G1, used as an HLA-G1$^+$ control, was stained by W6/32 and 87G but not TP25.99 mAb (Fig. 1).

In order to check for HLA-E cell-surface expression on M8 cells, we used a cytotoxic mouse T cell clone (TER-1), a very potent and highly specific HLA-E cell probe (24). The TER-1 clone has been shown to bear an $\alpha\beta$ TCR that specifically recognizes HLA-E complexed with MHC class I signal sequence-derived peptides. As shown in Fig. 2(A), very efficient killing was observed towards mouse target cells expressing HLA-E molecules and human Epstein–Barr virus (EBV)-transformed cells. Similarly, TER-1 efficiently lysed both M8 transfectants attesting to their HLA-E cell-surface expression (Fig. 2B). It is noteworthy that both M8 transfectants are similarly lysed by TER-1, demonstrating that HLA-G1 transfection into this naturally membrane-bound HLA-E$^+$ cell line does not quantitatively enhance HLA-E cell surface expression. This was confirmed by flow cytometry analysis with TP25.99 mAb which similarly stains both M8 transfectants. It is worth mentioning that the HLA-E-specific TER-1 clone equally lysed human EBV-transfected cells carrying HLA-EG (SCHU line) or HLA-ER (EA line) alleles, indicating that HLA-E allelic changes at position 107 (Gly → Arg) did not affect recognition by this clone (Fig. 2A). None of the K562-pcDNA or K562-HLA-G1 transfectant, used as HLA-E$^-$ controls, were lysed by the TER-1 clone (Fig. 2B) or stained by TP25.99 mAb (Fig. 1), which confirms our previous results demonstrating that HLA-E expression is not induced after HLA-G1 transfection into the K562 cell line (11).

HLA-G1 co-expression with naturally expressed HLA-A, -B, -C and -E molecules acts as a powerful inhibitory mechanism protecting cells from peripheral blood polyclonal NK cytolysis

In order to study the inhibitory status of HLA-G1 concomitantly expressed with HLA-A, -B, -C and -E molecules, cytotoxicity assays were carried out using as effectors, polyclonal NK cells present among PBMC obtained from healthy adult blood donors against M8 transfectants used as targets. Twenty experiments were carried out, each recorded with polyclonal NK cells from a different donor. Three representative experiments are presented in Fig. 3. As expected, the HLA class I molecules expressed on M8-RSV and M8-HLA-G1 already inhibited polyclonal NK lytic activity, as demonstrated by the pretreatment of both M8 targets with the pan-class I W6/32 mAb, leading to enhanced NK lysis (Fig. 3A). More interestingly, HLA-G1 molecules expressed on M8-HLA-G1 were found to further inhibit polyclonal NK cytolysis (Fig. 3A). Although absolute levels of NK lysis varied among the 20 donors tested, the percentage of HLA-G1-mediated inhibition ranged between 40 and 70% ($P < 0.002$). In order to confirm

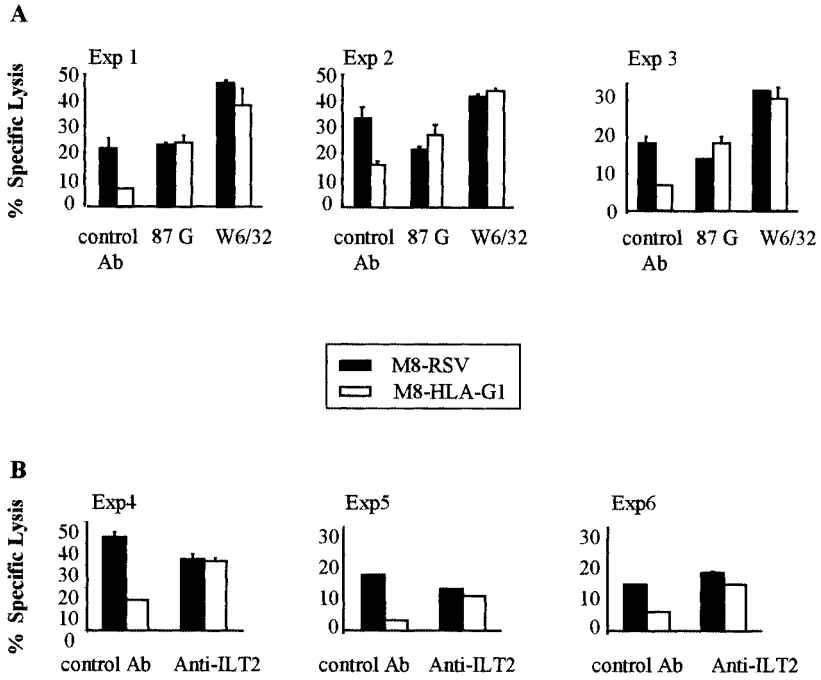

Fig. 3. Effect of HLA-G1 expression on the cytolytic activity of peripheral blood polyclonal NK cells. M8 cells transfected with either the vector alone (M8-RSV, black) or HLA-G1 (M8-HLA-G1, white) were used as targets. (A) These targets were preincubated with either 87G, W6/32 or a control antibody at 20 μg/ml. B-PBMC were preincubated with either ILT-2 specific antibody or an isotype control. Freshly isolated PBMC from healthy donors were used as effectors at a 25:1 E:T ratio. Results are expressed as the percentage lysis recorded in a 4 h ^{51}Cr-release assay. Values represent means of triplicates ± SD. Some bars are not visible because of the very small SD.

that the protection of M8-HLA-G1 was mediated directly by the expression of HLA-G1, antibody-blocking assays were performed using the HLA-G1-specific mAb, 87G. In this case, NK lysis of M8-HLA-G1 was restored to a level similar to that of M8-RSV (Fig. 3A), whatever the E:T ratio tested (data not shown). In all antibody-blocking assays, the Fc portion of the mAb used was blocked with an F(ab′)$_2$ goat anti-mouse IgG to prevent antibody-dependent cell cytotoxicity occurring. Hence, HLA-G1 co-expression with HLA-A, -B, -C and -E molecules further protects target cells from polyclonal NK lysis, through direct interaction with NK cells whose lytic activity is not already inhibited.

The additional HLA-G1-mediated inhibition of polyclonal NK cells occurs through interaction with the ILT-2 inhibitory receptor

To date, among the HLA-G1 inhibitory receptors described on NK cells, only antibody directed against the ILT-2 receptor is available and can be used in blocking experiments. In this regard, we evaluated the contribution of ILT-2 to the HLA-G1-mediated inhibition by carrying out experiments in the presence or absence of ILT-2-specific mAb. Figure 3(B) shows data from three different representative experiments where the HLA-G1-mediated inhibition is fully reversed by blocking ILT-2 with specific mAb, suggesting that ILT-2 has a major contribution to such HLA-G1-mediated NK inhibition. Hence, co-expression of HLA-G1 on HLA class I$^+$ cells constitutes an additional inhibitory mechanism leading to further protection from polyclonal NK cytolysis through interaction with ILT-2 present on NK cells.

HLA-E prevents NK cell-mediated lysis of an HLA-A, -B, -C and -E$^+$ cell line

To define the relative role played by both classical HLA class I molecules and HLA-E expressed on M8-RSV cells in preventing their NK cytolysis, we used the HLA class I$^+$ M8-RSV cell line as target towards peripheral blood polyclonal NK cells as effectors. For this purpose, we analysed the effect of mAb recognizing HLA-A, -B, -C and -E molecules on targets (i.e. W6/32) or the HLA-E inhibitory receptor on NK effectors (i.e. anti-CD94/NKG2A). As shown in Fig. 4(A), the protection from NK lysis conferred by all HLA class I molecules present on M8-RSV cells was, as expected, reversed by blocking these HLA molecules using saturating W6/32 mAb concentrations. Blockage of the HLA-E-specific inhibitory receptor, CD94/NKG2A, on NK effectors using specific mAb shows that HLA-E molecules are able to inhibit NK lysis. Strikingly, anti-CD94/NKG2A treatment is sufficient to completely restore NK cytotoxicity to the level obtained with W6/32-treated M8-RSV target cells. These results show that abrogation of the HLA-E-mediated protection leads to a maximal NK lysis response suggesting that in an HLA-A, -B, -C and -E$^+$ cell line, HLA-E exerts a major role in inhibiting NK cytolysis.

HLA-G1 plays a major role in preventing NK cell-mediated lysis of an HLA-A, -B, -C, -G1 and -E$^+$ cell line

Since we showed that HLA-G1 was able to further inhibit NK cytolysis in the presence of HLA-A, -B, -C and -E molecules, we then questioned about the relative inhibitory role of HLA-E in presence of HLA-G1. To answer this, we performed

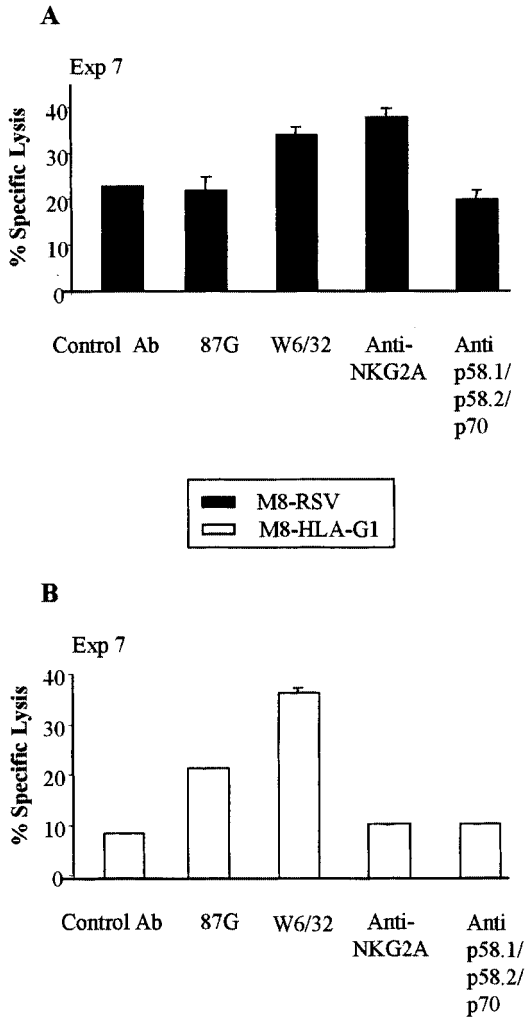

Fig. 4. Part of HLA class I molecules expressed on M8 transfectants in NK lysis inhibition. Freshly isolated peripheral blood lymphocytes were used as effector cells at a 100:1 E:T ratio against M8-RSV (A) or M8-HLA-G1 (B) in the presence of anti-CD94 and anti-NKG2A or anti-p58.1, anti-p58.2 and anti-p70 specific mAb (5 μg/ml) or mouse control IgG. The targets were preincubated with either 87G, W6/32 mAb or a control antibody at 20 μg/ml. Results are expressed as the percentage lysis recorded in a 4 h ^{51}Cr-release assay. Values represent means of triplicates ± SD. Some bars are not visible because of the very small SD.

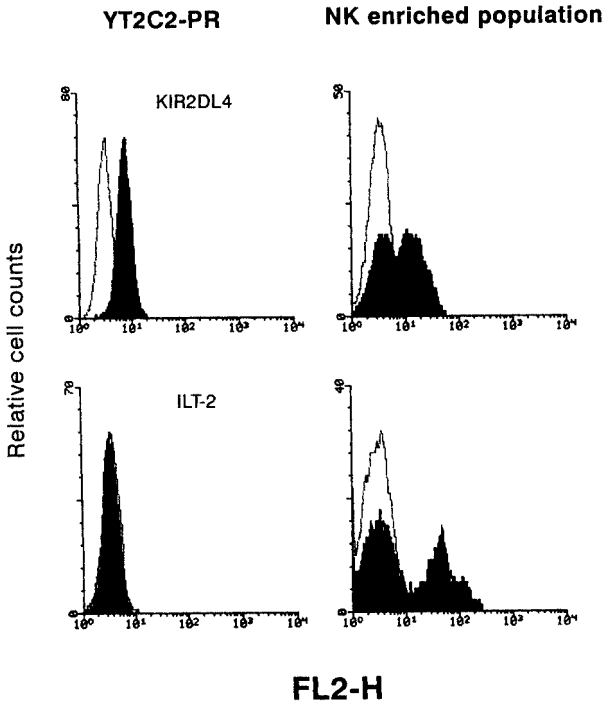

Fig. 5. KIR expression on the YT2C2-PR subclone and NK-enriched population. Cells were labeled by indirect immunofluorescence with either rabbit anti-KIR2DL4 or murine anti-ILT-2 antibody. After washing, cells were stained with phycoerythrin-conjugated pig anti-rabbit or goat anti-mouse IgG respectively.

cytotoxicity experiments using as target cells M8-HLA-G1 towards polyclonal NK cells treated with CD94/NKG2A-specific mAb. Although the HLA-A, -B, -C, -E and -G1-mediated inhibition could be reversed using W6/32-treated M8-HLA-G1, addition of the CD94/NKG2A-specific mAb to NK effectors had no effect (Fig. 4B). These results show that the HLA-G1-mediated inhibition is not affected by blocking CD94/NKG2A and that HLA-G1 is still able to inhibit polyclonal NK cells even so HLA-E/CD94/NKG2A interactions are blocked. This points out that the NK cell population, which is inhibited by HLA-E, can also be inhibited by HLA-G1.

Then, in order to evaluate the contribution of the classical HLA class I molecules, we carried out antibody-blocking assays using as effectors, polyclonal NK cells in which classical HLA class I-specific inhibitory receptors, i.e. p58.1, p58.2 and p70, have been blocked with specific mAb. As shown in Fig. 4, these antibodies do not restore lysis of both M8-RSV and M8-HLA-G1 targets. This result confirms that classical HLA class I–KIR interactions do not protect M8 targets from polyclonal NK cytolysis. In contrast, HLA-G1-mediated NK lysis inhibition can be reversed by blocking HLA-G1 with a specific mAb, 87G (Fig. 4B). Altogether, these results highlight that HLA-G1 protects target cells from (i) the lytic activity of NK cells that are inhibited by the other HLA class I molecules and (ii) additional NK cells. Thus, HLA-G1 appears as a major molecule inhibiting polyclonal NK cytolysis.

The lytic activity of the KIR2DL4$^+$ YT2C2-PR subclone is inhibited by HLA-G1

Since, the additional HLA-G1-mediated inhibition of polyclonal NK cells occurs through ILT-2, we then asked whether HLA-G1 could mediate NK inhibition through another KIR. For this purpose, the YT2C2-PR NK subclone was tested for HLA-G receptor cell-surface expression and used as effector against both M8 transfectants. We carried out immunofluorescence analysis using either an ILT-2-specific mAb or a rabbit anti-serum directed against a peptide corresponding to the N-terminal sequence of KIR2DL4 which does not discriminate between p49 and KIR2DL4 receptors (both belonging to the KIR103 gene family). Figure 5 shows that YT2C2-PR is an ILT-2$^-$, KIR103$^+$ NK subclone. CD3-depleted peripheral blood lymphocyte population (enriched in NK cells) were stained

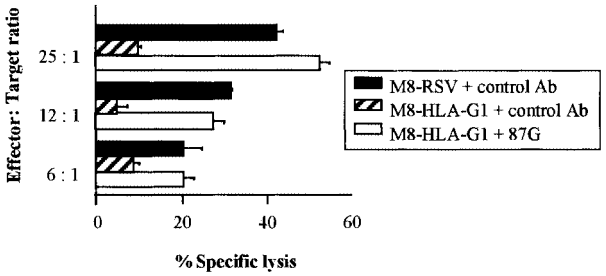

Fig. 6. Effect of HLA-G1 expression on the cytolytic activity of the YT2C2-PR subclone. M8 cells transfected with either the vector alone (M8-RSV) or HLA-G1 (M8-HLA-G1) were used as targets. These targets were preincubated with either the 87G mAb or an isotype-matched control antibody at 20 μg/ml. Results are expressed as the percentage lysis of triplicate recorded in a 4 h ^{51}Cr-release assay. This experiment is representative of at least 10 experiments. Values represent means of triplicates ± SD.

by both ILT-2 and KIR2DL4 specific antibodies (Fig. 5) while these antibodies did not stain the K562 cell line, used as a negative control (data not shown).

As shown in Fig. 6, the YT2C2-PR lytic activity was inhibited by HLA-G1 molecules expressed on M8-HLA-G1 cells and this lytic activity was restored when cytolytic assays were performed in the presence of HLA-G1-specific mAb (87G). These results demonstrate the direct implication of HLA-G1 in inhibiting the YT2C2-PR cytolysis through an ILT-2-independent pathway.

Unfortunately, the KIR2DL4 antiserum is not functional in cytotoxicity experiments (S. Rajagopalan, pers. commun.), which enable us to directly implicate the KIR103 receptor as the inhibitory receptor involved in HLA-G1-mediated inhibition. However, since this NK subclone bears, as the only known inhibitory receptor, the KIR103 receptor, neither CD94/NKG2A, nor p58, p70 and p140 are detected on this clone (3), our results suggest that the KIR103 might be involved in the inhibition observed.

Discussion

In this study, we have transfected the cDNA encoding the full-length HLA-G1 membrane-bound isoform into an HLA class I$^+$ cell line, i.e. M8, to question the inhibitory status of HLA-G1 on NK cells when concomitantly expressed with the classical HLA-A, -B, -C and HLA-E molecules. Although NK clones are useful tools to characterize HLA–KIR interactions, freshly isolated peripheral blood NK cells permit a more precise evaluation of the biological relevance of these interactions. We took advantage here of both approaches to study the biological role of HLA-G on *ex vivo* polyclonal NK cells and to investigate HLA-G-specific inhibitory receptors on NK clone.

We demonstrate here that, as reported in a HLA class I$^-$ context (11,13,29), HLA-G1 molecules co-expressed with HLA-A, -B, -C and -E are able to act as a powerful inhibitory mechanism capable of protecting target cells from the lytic activities of both peripheral blood polyclonal NK cells and NK clone. Previous studies have shown that HLA-E molecules are up-regulated upon acquisition of peptides derived from the leader sequence of other MHC class I molecules, including HLA-G1, leading to NK lysis inhibition through interaction between HLA-E and CD94/NKG2A (7–9). In this context, HLA-G1 could mediate NK inhibition through an indirect pathway via HLA-E cell-surface up-regulation or/and a direct pathway via interaction with HLA-G1-specific KIR(s). However, evidence is provided here that in the context of naturally expressed HLA class I molecules, HLA-G1 is able to inhibit NK cytolysis by itself. First, we demonstrate that HLA-E expression is not increased after HLA-G1 transfection. Indeed, cytofluorometry analysis using the HLA-A, -B, -C and -E TP 25.99 mAb, shows that, apart from HLA-G1, both M8-RSV and M8-HLA-G1 cells express a similar level of HLA class I antigens. More relevant, the TER-1-specific HLA-E cell probe similarly lyses both M8-RSV and M8-HLA-G1 cells, demonstrating that HLA-G1 transfection does not enhance HLA-E cell-surface expression. Since the M8 cell line carries both HLA-A1 and -A2 alleles that contain nonamers within their leader sequences able to stabilize HLA-E cell-surface expression (7–9), we suggest that HLA-E is already expressed at the cell surface at its optimal level. Moreover, since the specific HLA-G1 87G mAb leads to full restoration of cytolysis against M8-HLA-G1 cells (compared to M8-RSV cells), we further confirm the direct implication of HLA-G1 in mediating polyclonal NK lysis inhibition. It is noteworthy that HLA-G1 inhibits polyclonal NK lytic activity from all 20 donors tested, although their KIR phenotype and NK functional status are heterogeneous. This is in good agreement with a large percentage of NK cells carrying inhibitory receptors for HLA-G1 (21). However, the variation in the NK subpopulation among different persons may explain the variation in percentage of HLA-G1-mediated inhibition observed.

Blockage of HLA class I molecules on target cells leads to enhancement of NK cytolysis. Thus, the HLA class I-mediated NK inhibition may occur through interaction between HLA-A, -B, -C and -E and their inhibitory receptor counterparts (i.e. ILT-2 or p140, p70, p58 and CD94/NKG2A) (30). However, the lytic activity of freshly obtained polyclonal NK cells is principally inhibited by HLA-E, thus acting as the major inhibitory molecule, expressed on M8 cells. Indeed, as shown in Table 1, after blocking the specific HLA-E receptor with CD94/NKG2A-specific mAb, the HLA-E-mediated NK inhibition is comparable to the 'total' HLA class I-mediated NK inhibition. These results show that both HLA-G and HLA-E are able to protect cells from polyclonal NK lysis, while classical HLA class I molecules are not, certainly due to the low percentage of NK cells carrying receptors specific for a given classical HLA class I molecule (30). This is consistent with our observation that blockage of KIR specific for particular HLA class I alleles (i.e. p58.1, p58.2 and p70) does not have any effect on polyclonal NK cytolysis against M8 HLA class I$^+$ transfectants. This result is confirmatory of previous ones, showing that classical HLA class I alleles could not protect target cells from polyclonal NK cells (13).

Interestingly, in the presence of HLA-G1, anti-CD94/NKG2A treatment does not significantly affect the HLA class I-mediated NK inhibition (HLA-E-mediated NK lysis inhibition of 0 versus 80% for the total HLA class I-mediated NK lysis inhibition, as shown in Table 1). Since NK cells expressing both ILT-2 and CD94/NKG2A lyse HLA-E and -G$^+$ target in

Table 1. Part of HLA class I molecules expressed on M8 transfectants in inhibiting NK lysis

M8-RSV		M8-HLA-G1	
HLA class I-mediated NK lysis inhibition (%)[a]	HLA-E-mediated NK lysis inhibition (%)[b]	HLA class I-mediated NK lysis inhibition (%)[c]	HLA-E-mediated NK lysis inhibition (%)[d]
50	50	80	0

[a]100 – [(% specific lysis of M8-RSV treated with control antibody)/(% specific lysis of M8-RSV treated with $M^6/32$)×100].
[b]100 – [(% specific lysis of M8-RSV treated with anti-CD94/NKG2A)/(% specific lysis of M8-RSV treated with $W^6/32$)×100].
[c]100–[(% specific lysis of M8-HLA-G1 treated with control Ab)/(% specific lysis of M8-HLA-G1 treated with $W^6/32$ × 100%.
[d]100–[(% specific lysis of M8-HLA-G1 treated with anti-CD54/NKG2A)/(% specific lysis of M8-HLA-G1 treated with $W^6/32$) × 100

the presence of the combination of anti-ILT-2 and anti-CD94/NKG2A but not in the presence of either antibody alone (12 and data not shown), we could postulate that in the context of polyclonal NK cells: (i) NK cells inhibited by HLA-E (i.e. CD94/NKG2A$^+$ NK cells) are also inhibited by HLA-G1, and thus bear both CD94/NKG2A and HLA-G-specific inhibitory receptors, and (ii) the additional HLA-G1-mediated inhibition of polyclonal NK cells results from inhibition of NK cells which are not already inhibited by HLA-A, -B, -C and -E molecules. Another explanation is that in the presence of HLA-G1, HLA-E may form multimeric complexes with HLA-G1 that lead to NK cytolysis inhibition that cannot be reversed neither by anti-HLA-G1 mAb nor by CD94/NKG2A-specific mAb, as also suggested by Navarro and colleagues (12).

Altogether, our results point out that HLA-G1 may play a fundamental role in modulating immune response, by altering NK function. Such a protective effect is at least due to the direct interaction with KIR (31). To date, two structurally distinct families of KIR which interact with HLA-G1 have been identified. First, the killer cell Ig-like receptor family which contains KIR(s), such as p49 and KIR2DL4 also called KIR103AS (22,32), that bind either HLA-A, -B, and -G1 molecules or only HLA-G1, respectively (20,21). Second, the Ig-like transcript molecules (ILT, also called LIR), including ILT-2 (18,19) and ILT-4 (33), which both bind classical HLA class I and HLA-G molecules. However, only KIR2DL4 and ILT-2 have been described on peripheral blood NK cells. Thus, we investigate whether HLA-G1 interacts with the known KIR present on NK cells. ILT-2 was clearly defined as interacting with HLA-G1 and our results provide further information on its implication in HLA-G1-mediated inhibition towards NK cells. Indeed, ILT-2 appears as the major inhibitory receptor contributing to HLA-G1-mediated inhibition of peripheral blood NK cells. Thus, the additional HLA-G1 inhibitory mechanism may engage NK cells bearing ILT-2 and whose lytic activity is not inhibited by the other HLA class I molecules.

On the other hand, our results also show that HLA-G transfected into M8 cells protect such targets from the lysis by YT2C2-PR NK subclone which does not express ILT-2. Thus, the YT2C2-PR-mediated cytolysis is inhibited by HLA-G1 through an ILT-2-independent pathway. Interestingly, this NK subclone is stained by antisera raised against a synthetic peptide corresponding to the N-terminal sequence of KIR2DL4, consistent with expression of KIR103 AS transcripts in the original YT cell line, as previously described by Selvakumar *et al.* (34). Since a variant of this synthetic peptide is found in other classical HLA class I specific KIR, this antisera could be reactive with other KIR as well. However, none of the HLA-A, -B, -C and E-specific KIRs are expressed on YT2C2-PR (35), strongly suggesting that the antisera recognizes a KIR103 receptor expressed at the cell surface of YT2C2-PR. Moreover, this synthetic peptide (VGGQDKPFL) is present in p49 sequence. Thus, we can not precisely define wether p49 or KIR2DL4 (both belonging to the KIR103 gene family) is expressed on YT2C2-PR. Nevertheless, p49 has been described as being exclusively present on decidua NK cells during pregnancy by using a specific p49 antibody (36). Accordingly, we hypothesize that KIR2DL4 is the KIR103 receptor present on YT2C2-PR. We previously reported that the sole expression of HLA-G1 in an HLA class I$^-$ target cell line (i.e. K562) inhibits the YT2C2-PR cytolysis (3). Altogether, these data support the involvement of KIR2DL4 in the HLA-G1-mediated inhibition of the ILT-2$^-$ NK clone. Allan *et al.* have recently published that HLA-G1 tetramers do not bind to KIR2DL4-transfected cells (37). Several reasons could explain their results: (i) KIR2DL4/HLA-G1 interactions may be glycosylation dependent while HLA-G1 tetramers expressed in *Escherichia coli* are devoid of glycosylations or (ii) KIR2DL4 binds HLA-G1 with low affinity, rending the binding of HLA-G1 tetramers to KIR2DL4 undetectable by flow cytometry analysis. The existence of NK cells bearing only such HLA-G1-specific inhibitory receptor, as described here for YT2C2-PR, is of particular interest since they could be recruited in tissues that express HLA-G, such as trophoblast or HLA-G$^+$ tumors. It is of note that recent studies described that decidua-associated NK cells acquire the KIR103 family receptor, p49, during pregnancy (36). We can thus postulate that under certain conditions, such as inflammation or tumor-associated events, expression of HLA-G-specific KIR can be up-regulated, thus enhancing the biological relevance of HLA-G.

Altogether, our functional study reveals that HLA-G1 may play a relevant role in at least two situations. Firstly, during pregnancy where the survival of the semi-allogeneic fetus is an immunological paradox, our study supports that HLA-G expression on embryonic cytotrophoblasts is an important mechanism that protects the fetus from maternal NK rejection. Interestingly, upon human cytomegalovirus (HCMV) infection, while the surface expression of HLA-E is up-regulated by the leader sequence of the glycoprotein UL40 (gpUL40) (38,39), HLA-G expression is down-regulated (40). Strikingly, it is well established that HCMV infection during pregnancy is associated with spontaneous pregnancy loss (41,42), supporting that HLA-G is the major HLA class I molecule involved in maternal fetal tolerance. Secondly, in cases where HLA-

G1 is expressed together with naturally expressed HLA class I molecules, as reported in thymic epithelial cells (14), cytokine-activated monocytes (15,16), as well as in some tumors (17), HLA-G1 would act as a powerful HLA checkpoint for NK cells (43). Finally, the capacity of HLA-G1 to inhibit NK cytolysis could be invaluable during immune reactions following transplantation, when the NK cells are the first lymphoid cells to be involved in rejection. Indeed, HLA-G expression was recently described in heart transplant patients and associated to a better heart graft acceptance (44).

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Abbreviations

EBV	Epstein–Barr virus
HCMV	human cytomegalovirus
KIR	killer cell Ig-like receptor
PBMC	peripheral blood mononuclear cells

References

1 Kovats, S., Main, E. K., Librach, C., Stubblebine, M., Fisher, S. J. and DeMars, R. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 248:220.
2 Rouas-Freiss, N., Marchal-Bras Goncalves, R., Menier, C., Dausset, J. and Carosella, E. D. 1997. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc. Natl Acad. Sci. USA* 94:11520.
3 Rouas-Freiss, N., Marchal, R., Kirszenbaum, M., Dausset, J. and Carosella, E. D. 1997. The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors? *Proc. Natl Acad. Sci. USA* 94:5249.
4 Söderström, K., Corliss, B., Lanier, L. L. and Phillips, J. H. 1997. CD94/NKG2 is the predominant inhibitory receptor involved in recognition of HLA-G by decidual and peripheral blood NK cells. *J. Immunol.* 159:1072.
5 Perez-Villar, J. J., Melero, I., Navarro, F., Carretero, M., Bellon, T., Llano, M., Colonna, M., Geraghty, D. E. and Lopez-Botet, M. 1997. The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1. *J. Immunol.* 158:5736.
6 Pende, D., Sivori, S., Accame, L., Pareti, L., Falco, M., Geraghty, D., Le Bouteiller, P., Moretta, L. and Moretta A. 1997. HLA-G recognition by human natural killer cells. Involvement of CD94 both as inhibitory and as activating receptor complex. *Eur. J. Immunol.* 27:1875.
7 Braud, V. M., Allan, D. S., O'Callaghan, C. A., Soderstrom, K., D'Andrea, A.,. Ogg, G. S, Lazetic, S., Young, N. T., Bell, J. I., Phillips, J. H., Lanier, L. L. and McMichael, A. J. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391:795.
8 Lee, N., Llano, M., Carretero, M., Ishitani, A., Navarro, F., Lopez-Botet, M. and Geraghty, D. E. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl Acad. Sci. USA* 95:5199.
9 Borrego, F., Ulbrecht, M., Weiss, E. H., Coligan, J. E. and Brooks, A. G. 1998. Recognition of human histocompatibility leucocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187:813.
10 Lee, N., Goodlett, D. R., Ishitani, A., Marquardt, H. and Geraghty, D. E. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* 160:4951.
11 Khalil-Daher, I., Riteau, B., Menier, C., Sedlik, C., Paul, P., Dausset, J., Carosella, E. D. and Rouas-Freiss N. 1999. Role of HLA-G versus HLA-E on NK function: HLA-G is able to inhibit NK cytolysis by itself. *J. Reprod. Immunol.* 43:175.
12 Navarro, F., Llano, M., Bellon, T., Colonna, M., Geraghty, D. E. and Lopez-Botet, M. 1999. The ILT-2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *Eur. J. Immunol.* 29:277.
13 Sasaki, H., Xu, X.-C. and Mohanakumar, T. 1999. HLA-E and HLA-G expression on porcine endothelial cells inhibit xenoreactive human NK cells through CD94/NKG2A-dependent and -independent pathways. *J. Immunol.* 163:6301.
14 Crisa, L., McMaster, M. T., Ishii, J. K., Fisher, S. J. and Salomon, D. R. 1997. Identification of a thymic epithelial cell subset sharing expression of the class Ib HLA-G molecule with fetal trophoblasts. *J. Exp. Med.* 186:289.
15 Yang, Y., Chu, W., Geraghty, D. E. and Hunt, J. S. 1996. Expression of HLA-G in human mononuclear phagocytes and selective induction by IFN-γ. *J. Immunol.* 156:4224.
16 Moreau, P., Adrian-Cabestre, F., Menier, C., Guiard, V., Gourand, L., Dausset, J., Carosella, E. D. and Paul, P. 1999. IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int. Immunol.* 11:803.
17 Paul, P., Adrian Cabestre, F. Le Gal, F.-A. Khalil-Daher, I. Le Danff, C. Schmid, M. Mercier, S. Avril, M.-F., Dausset, J., Guillet, J.-G. and Carosella, E. D. 1999. Heterogeneity of HLA-G gene transcription and protein expression in malignant melanoma biopsies. *Cancer Res.* 59:1954.
18 Colonna, M., Navarro, F., Bellon, T., Llano, M., Samaridis, J., Angman, L., Cella, M. and Lopez-Botet, M.. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.* 186:1809.
19 Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L. and Hsu, M.-L. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* 7:273.
20 Cantoni, C., Verdiani, S., Falco, M., Pessino, A., Cilli, M., Conte, R., Pende, D., Ponte, M., Mikaelsson, M. S., Moretta, L. and Biassoni, R. 1998. p49, a putative HLA class I-specific inhibitory NK receptor belonging to the Ig superfamily. *Eur. J. Immunol.* 28:1980.
21 Rajagopalan, S. and Long, E. O. 1999. A human histocompatibility leucocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.* 189:1093.
22 Steffens, U., Vyas, Y., Dupont, B. and Selvakumar, A. 1998. Nucleotide and amino acid sequence alignment for human killer cell inhibitory receptors (KIR), 1998. *Tissue Antigens* 51:398.
23 Paul, P., Rouas-Freiss, N., Khalil-Daher, I., Moreau, P., Riteau, B., Anne Le Gal, F., F. Avril M., Dausset, J., Guillet, J. G. and Carosella, E. D. 1998. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc. Natl Acad. Sci. USA* 95:4510.
24 Martinozzi, S., Pacasova, R., Boulouis, H. J., Ulbrecht, M., Weiss, E. H., Sigaux, F. and Pla, M. 1999. Requirement of class I signal sequence-derived peptides for HLA-E recognition by a mouse cytotoxic T cell clone. *J. Immunol.* 162:5662.
25 Le Gal, F.-A., Riteau, B., Sedlik, C., Khalil-Daher, I., Menier, C., Dausset, J., Guillet, J.-G., Carosella, E. D. and Rouas-Freiss, N. 1999. HLA-G mediated-inhibition of antigen-specific cytotoxic T lymphocytes. *Int. Immunol.* 11:1351.
26 Lee, N., Malacko, A. R., Ishitani, A., Chen, M. C., Bajorath, J., Marquardt, H. and Geraghty, D. E. 1995. The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous

peptides but differ with respect to TAP association. *Immunity* 3:591.
27 Rebmann, V., Pfeiffer, K., Passler, M., Ferrone, S., Maier, S., Weiss, E. and Grosse-Wilde, H. 1999. Detection of soluble HLA-G molecules in plasma and amniotic fluid. *Tissue Antigens* 53:14.
28 Banham, A. H., Colonna, M., Cella, M., Micklem, K. J., Pulford, K., Willis, A. C. and Mason, D. Y. 1999. Identification of the CD85 antigen as ILT-2, an inhibitory MHC class I receptor of the immunoglobulin superfamily. *J. Leuk. Biol.* 65:841.
29 Sasaki, H., Xu, X.-C., Smith, D. M., Howard, T. and Mohanakumar, T. 1999. HLA-G expression protects porcine endothelial cells against natural killer-mediated xenogeneic cytotoxicity. *Transplantation* 57:31.
30 Lanier, L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359.
31 Carosella, E. D., Rouas-Freiss, N., Paul, P. and Dausset, J. 1999. HLA-G: a tolerance molecule from the major histocompatibility complex. *Immunol. Today* 20:60.
32 Valliante, N. M., Uhrberg, M., Shilling, H. G., Lienert-Weidenbach, K., Arnett, K. L., D'Andrea, A., Phillips, J. H., Lanier, L. L. and Parham, P. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity* 7:739.
33 Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R. L., O'Callaghan, C., Dunbar, R., Ogg, G. S., Cerundolo, V. and Rolink, A. 1998. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.* 160:3096.
34 Selvakumar, A., Steffens, U. and Dupont, B. 1996. NK cell receptor gene of the KIR family with two Ig domains but highest homology to KIR receptors with three Ig domains. *Tissue Antigens* 48:285.
35 Rouas-Freiss, N., Khalil-Daher, I., Riteau, B., Menier, C., Paul, P., Dausset J. and Carosella, E. D. 1999. The immunotolerance role of HLA-G. *Semin. Cancer Biol.* 9:3.
36 Ponte, M., Cantoni, C., Biassoni, R., Tradori-Cappai, A., Bentivoglio, G., Vitale, C., Bertone, S., Moretta, A., Moretta, L. and Mingari, M. C. 1999. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc. Natl Acad. Sci. USA* 96:5674.
37 Allan, D. S. J., Colonna, M., Lanier, L., Churakova, T., Abrams, J. S., Ellis, S. A., McMichael, A. J. and Braud, V. M. 1999. Tetrameric complexes of human histocompatibility leucocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *J. Exp. Med.* 189:1149.
38 Ulbrecht, M., Martinozzi, S., Grzeschik, M, Hengel, H., Ellwart, J.W., Pla, M. and Weiss, E. H. 2000. The human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J. Immunol.* 164:5019.
39 Tomasec, P., Braud, V. M., Rickards, C., Powell, M. B., McSharry, B. P., Gadola, S., Cerundolo, V., Borysiewicz, L. K., McMichael, A. J. and Wilkinson, G. W. G. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287:1031.
40 Jun, Y., Kim, E., Jin, M., Sung, H. C., Han, H., Geraghty, D. E. and Ahn, K. 2000. Human cytomegalovirus gene products US3 and US6 down-regulate trophoblast class I MHC molecules. *J. Immunol.* 164:805.
41 Altshuler, G. 1974. Immunologic competence of the immature human fetus: morphologic evidence from intrauterine cytomegalovirus infection. *Obstet. Gynecol.* 43:811.
42 Kriel, R. L., Gates, G. A., Wuff, H., Powell, N., Poland, J. D. and Chin, T. D. 1970. Cytomegalovirus isolations associated with pregnancy wastage. *Am. J. Obstet. Gynecol.* 106:885.
43 Carosella, E. D., Dausset, J. and Rouas-Freiss, N. 1999. Transplacental transmission of natural-killer-cell lymphoma. *N. Engl. J. Med.* 341:1937.
44 Lila, N., Carpentier, A., Amrein, C., Khalil-Daher, I., Dausset, J. and Carosella, E. D. 2000. Implication of HLA-G molecules in hear graft acceptance. *Lancet* 355: 2138.