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HLA-G2, -G3, and -G4 Isoforms Expressed as Nonmature Cell Surface Glycoproteins Inhibit NK and Antigen-Specific CTL Cytolysis

Béatrice Riteau, Nathalie Rouas-Freiss, Catherine Menier, Pascale Paul, Jean Dausset, and Edgardo D. Carosella

HLA-G is a nonclassical MHC class I molecule that plays a major role in maternal-fetal tolerance. Four membrane-bound (HLA-G1 to -G4) and two soluble proteins (HLA-G5 and -G6) proteins are generated by alternative splicing. Only HLA-G1 has been extensively studied in terms of both expression and function. We provide evidence here that HLA-G2, -G3, and -G4 truncated isoforms reach the cell surface of transfected cells, as endoglycosidase-H-sensitive glycoproteins, after a 2-h chase period. Moreover, cytotoxicity experiments show that these transfected cells are protected from the lytic activity of both innate (NK cells) and acquired (CTL) effectors. These findings highlight the immunomodulatory role that HLA-G2, -G3, and -G4 proteins will assume during physiologic or pathologic processes in which HLA-G1 expression is altered.


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2 B.R and N.R.F. contributed equally to this work.

3 Abbreviations used in this paper: Endo-H, endoglycosidase-H; CHAPS, 3-[(3-cholamidopropyl)dimethy lammonio]-1-propanesulfonate; PNGase-F, peptide-N-glycosidase-F; ER, endoplasmic reticulum; ILT, Ig-like transcript; LIR, leukocyte Ig-like receptor; β2m, β2-microglobulin; KIR, killer-Ig-related receptor.
Cells were maintained in RPMI 1640 medium supplemented with 10% inactivated FCS, 2 mM l-glutamine, 1 μg/ml gentamicin, and fungizone. HLA-G transfectants were selected in medium containing hygromycin (Sigma, St. Louis, MO).

Monoclonal Abs and flow cytometric analysis

The following mAbs were used: 4H84, IgG1 anti-denatured HLA-G heavy chain (provided by S. Fisher and M. McMaster); HC10, IgG2a anti-HLA-B and -C (provided by H. Ploegh); 87G, IgG2a anti-HLA-G1 and -G5 (provided by D. Geraghty); Z199, IgG2b anti-NKG2A (Immunotech, Marseille, France); PAG 1, a rabbit polyclonal Ab that we raised against peptide 61–83 of the HLA-G α1 domain that recognizes denatured HLA-G α-chain of all HLA-G isoforms (25, 27); and B-5-1-2 anti-α-tubulin (Sigma, Aldrich reagent). Flow cytometry assays were conducted as previously described (26). In addition, we performed propidium iodide staining of dying or dead cells, which allowed us to gate only nonpermeabilized cells.

Western blot analysis

Aliquots of total proteins from M8 transfectants (lysates) or precipitated proteins from M8 transfectants were separated in 12% SDS-PAGE. The gels were blotted onto nitrocellulose membranes (Hybond, Amersham, Buckinghamshire, U.K.), and the membranes were blocked by incubation with PBS containing 0.2% Tween 20 and 5% nonfat dry milk. The membranes were then probed with the corresponding Ab overnight at 4°C and washed in PBS containing 0.2% Tween 20. In biotinylation experiments, membranes were first incubated with the 4H84 mAb together with the anti-tubulin, and a second revelation was conducted by incubating membranes with the HC10 mAb. Then, the membranes were subsequently incubated for 30 min at room temperature with peroxidase-conjugated sheep anti-mouse IgG Ab (Sigma), washed thoroughly, stained with enhanced chemiluminescence reagent (Amersham), and exposed to x-ray film.

Cell surface protein biotinylation

Cell surface proteins of viable M8 transfected cells were biotinylated as monolayers in tissue culture flasks (75 cm²). Cells were first washed twice with PBS and treated with sulfo-succinimidyl-6-biotin (Pierce, Rockford, IL; 200 μg/ml of PBS) for 4 min at room temperature. The cells were washed twice with PBS, treated with 50 mM glycine for 5 min, and again washed twice with PBS. Dead or dying cells (nonadherent cells) were thus removed by washing the cells. Cells were then collected in tubes, recovered with PBS, and washed five times at 4°C. Biotinylation experiments in which intracellular proteins were voluntarily biotinylated, cells were collected in tubes and treated with 1 mg/ml of biotin for 2 h at room temperature. Cells were then centrifuged, and the supernant was removed before cell lysis.

Immunoprecipitation of cell surface protein

Cells were lysed in 1 ml of lysis buffer containing 0.5% 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS; Sigma). Insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. Biotinylated proteins were then precipitated with 100 μl of 50% streptavidin-agarose beads slurry (Bio-Rad, Richmond, CA) for 1 h at 4°C. After washing, precipitated molecules were eluted from the beads in 50 μl of 2% SDS in lysis buffer at 95°C for 5 min and overnight at 37°C. The proteins were diluted in 1 ml of lysis buffer and precipitated with the anti-HLA-G, PAG 1 rabbit polyclonal Ab for 1 h at 4°C. Surface HLA-G proteins were precipitated with protein A-Sepharose beads and analyzed by SDS-PAGE. The supernant was reprecipitated with rabbit polyclonal anti-HLA-G, and then unbiotinylated HLA-G proteins were analyzed by SDS-PAGE.

Cytotoxic assays

The cytolytic activity of PBMC from healthy adult volunteer donors (men and women, aged 30–60 years) was obtained by Ficoll/Histopaque density gradient, of YT2C2-PR, or of the HC12 CTL line used as effectors was assessed in 4-h 51Cr release assays in which effector cells were mixed with 5 × 10⁵ 125I-Cr-labeled targets (100 μCi of 125I-Cr sodium chromate; 1 Ci = 37 GBq; Amersham) at various E/T cell ratios in U-bottom microtiter plates, as previously described (26). For cytotoxicity assays in which the HC12 CTL line was used as the effector, targets were pulsed with M58–66 peptide for 1.5 h. After 4 h at 37°C in a humidified 5% CO₂ incubator, 50 μl of the supernant was collected for scintillation counting (Wallac 1450 Microbeta; Pharmacia, Piscataway, NJ). The percentage of specific lysis was calculated as follows: % specific lysis = [cpm experimental well – cpm spontaneous release]/cpm maximum release × 100. Spontaneous release was determined by incubation of labeled target cells with medium. Maximum release was determined by solubilizing target cells in 0.1 M HCl. In all experiments spontaneous release was <10% of maximum release.

Ab blocking assays

In experiments in which anti-NKG2A mAb was used to block the HLA-E inhibitory receptor CD94/NKG2A, effector cells were firstly preincubated for 15 min at room temperature in culture medium containing 10% human AB serum and then preincubated for 15 min at room temperature with Z199 (mouse anti-human NKG2A; Immunotech, Westbrook, ME) before the NK cell cytotoxicity assay. The mAb was present in the culture medium during the entire assay period. In experiments in which mAbs were used in an attempt to block HLA-G-NK interaction, target cells were incubated with the optimal concentration of either the 4H84 (1/100) or the 87G mAb (2 μg/ml), then washed and incubated with 1Fb 1β anti-glycosylated IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) to prevent Ab-dependent cell cytotoxicity by interaction of NK cells with the first mAb used. mAb toxicities were checked in each assay and were always <3%.

Statistical analysis

The statistical significance (p < 0.05) of cytotoxic assays was analyzed using Student’s t test to compare the percentage of specific lysis of M8-HLA-G transfectants to that of the M8-pcDNA control cell line. Assays were conducted in triplicate for each experiment, and the SD of the mean triplicate value was <5%. Note that all HLA-G-positive M8 transfectants inhibit effector lysis, whatever the E:T cell ratio.

Results

HLA-G2,-G3, and -G4 isoforms are translated as glycoproteins

To determine whether the HLA-G2, -G3, and -G4 transcripts are translated into proteins, we transfected the corresponding cDNA into the M8 HLA class I-positive melanoma cell line. Western blot analysis of all HLA-G transfectants was then conducted using the 4H84 mAb, which specifically detects denatured HLA-G via the 1ό chain of all HLA-G isoforms (25, 27); and B-5-1-2 anti-α-chain of HLA-B (25). However, these molecular sizes may have been glycosylated. After PNGase F treatment of cell lysates obtained from M8 transfectants, the 4H84 mAb revealed bands that matched the predicted molecular masses (36, 27, 17, and 25 kDa, respectively, for HLA-G1, -G2, -G3, and -G4; Fig. 1B). These results demonstrate that, like HLA-G1, the HLA-G2, -G3, and -G4 isoforms are translated as glycoproteins.
-G3, and -G4 isoforms are indeed translated as glycoproteins. Additional smaller bands corresponding to glycosylated proteins were observed for HLA-G1 (i.e., band at 30 kDa) and HLA-G2 (i.e., band at 28 kDa) as well as for HLA-G3 (i.e., 17 kDa) and HLA-G4 (i.e., 26 kDa; data not shown). Whether these smaller migrating bands are due to in vivo protein degradation remains to be determined.

HLA-G2, -G3, and -G4 isoforms are expressed at the cell surface of transfected cells

Although the HLA-G2, -G3, and -G4 proteins were observed in the corresponding M8 transfecant, they were undetectable on the cell surface by flow cytometry with the currently available specific anti-HLA-G1 87G (5) and 01G (25) mAbs that otherwise positively stain HLA-G1 at the cell surface of M8-HLA-G1 (data not shown). Failure to detect these smaller isoforms could have been due to their inability to form the conformational epitope recognized by these mAbs. We then asked whether the anti-HLA-G heavy chain, 4H84 mAb, could detect the HLA-G isoforms at the cell surface. To exclude intracellular HLA-G staining by 4H84 mAb, we gated on nonpermeabilized cells, as revealed by negative propidium iodide staining. As shown in Fig. 2, M8-HLA-G1 cells are positively stained by both 87G and 4H84 mAbs. All M8-HLA-G1 cells are stained by 87G, but not by 4H84, which may be due to the distinct affinity and/or specificity between these mAbs. Interestingly, M8-G2, -G3, and -G4 are positively stained by the 4H84 mAb, showing that all HLA-G isoforms are expressed on the cell surface of the corresponding M8 transfecant.

To confirm these results, we conducted cell surface protein biotinylation of the M8 transfecants, followed by immunoprecipitation with streptavidin-agarose beads. Fig. 3 shows, using 4H84 mAb, immunoprecipitation of HLA-G into the lysates (lanes 1) or after precipitation of biotinylated proteins (lanes 3). As expected, while no HLA-G protein was detected on the M8-pcDNA control cell line (Fig. 3A, lane 3), a band corresponding to surface HLA-G1 was revealed on M8-HLA-G1 transfected cells (Fig. 3E, lane 3). Under similar experimental conditions, bands corresponding to HLA-G2, -G3, and -G4 isoforms were also revealed on the corresponding transfecant (Fig. 3, B–D, lanes 3), showing that these isoforms reach the cell surface.

FIGURE 1. HLA-G1, -G2, -G3, and -G4 isoforms are translated as glycoproteins. Western blot analysis of lysates from M8 transfecants was conducted using the 4H84 mAb. Lysates from M8 transfecants were treated with PNGase F (B) or were not treated (A). Numbers at the left of the figure refer to Mr, in kilodaltons.

To confirm that under our experimental conditions, only cell surface proteins have been biotinylated, we used as controls 1) the M8-HLA-G5 transfected cells. HLA-G5 is a secreted HLA-G form encoded by alternatively spliced transcript missing the transmembrane and cytoplasmic domains. This soluble form could not be detected by cytofluorometric analysis using anti-HLA-G5-specific mAb (data not shown) or by our biotinylation experiments (Fig. 3F, lane 3), although a high level of HLA-G5 protein synthesis was observed in the lysate of the M8-HLA-G5 transfecant (Fig. 3F, lane 1); and 2) highly expressed intracellular protein (i.e., tubulin), which was indeed detected in all transfecants lysates (Fig. 3, lanes 1), but was not precipitated by streptavidin after biotinylation (Fig. 3, lanes 3). Furthermore, we have compared the abilities of HLA-G2, -G3, and -G4 and tubulin to be biotinylated. For this purpose we have conducted similar experiments in which intracellular proteins from M8-HLA-G2, -G3, and -G4 cells were voluntarily biotinylated and precipitated by streptavidin-agarose beads. These precipitated proteins were compared with lysates from unbiotinylated cells after Western blot analysis using both anti-tubulin and anti-HLA-G (i.e., 4H84 mAb) Abs. As shown in Fig. 4, both tubulin and HLA-G2, -G3, or -G4 are equally biotinylated, while in the lysate of the corresponding M8 transfecant, the tubulin is either equally revealed compared with HLA-G2 and -G3 or expressed less compared with HLA-G4. Altogether, these results highlight that tubulin exhibits a similar capacity for biotinylation as HLA-G2, -G3, and -G4, leading to the conclusion that in our experiments, we may underestimate the amounts of HLA-G2, -G3, and -G4 surface protein. Furthermore, experiments performed on unbiotinylated M8 transfecants also confirmed that streptavidin-agarose beads did not precipitate any nonspecific proteins (Fig. 3, A–F, lanes 5). Taken together these results show that under our conditions biotinylated proteins correspond to membrane-bound proteins, leading to the conclusion that HLA-G2, -G3, and -G4 isoforms can reach the cell surface as membrane-anchored glycoproteins.
HLA-G2, -G3, and -G4 isoforms are immature cell surface-expressed molecules

Having demonstrated that HLA-G2, -G3, and -G4 are expressed at the cell surface, we then asked whether they are expressed as mature glycoproteins. The acquisition of complex N-linked sugar during maturation of HLA class I glycoproteins occurs when the proteins pass through the medial Golgi, at which point they become resistant to digestion with Endo-H. Thus, the presence of Endo-H resistance indicates that protein maturation has proceeded past the endoplasmic reticulum (ER). To further examine whether the N-linked carbohydrate side chains of the membrane-bound HLA-G2, -G3, and -G4 glycoproteins are modified, both lysates and surface proteins (i.e., biotinylated proteins) were subjected to Endo-H digestion, followed by SDS-PAGE analysis. By using the anti-HLA-G 4H84 mAb, intracellular HLA-G1 molecules contain oligosaccharides both sensitive (immature HLA) and resistant (mature HLA) to Endo-H (Fig. 3E, lane 2), while biotinylated HLA-G1 molecules are totally Endo-H resistant (Fig. 3E, lane 4). In contrast, HLA-G2, -G3, and -G4 isoforms, whether they are expressed (i.e., biotinylated) or not at the cell surface remain completely sensitive to Endo-H (Fig. 3, B–D, lanes 4 and 2, respectively). As a control, the same membrane was rehybridized with the anti-HLA-B and -C HC10 mAb, showing that, as expected, the HLA-B and -C biotinylated molecules are totally resistant to Endo-H treatment (Fig. 3, A–D and F, lanes 4). This attests that newly synthesized proteins are undetectable in our biotinylation experiments. Taken together, these results indicate that in contrast to classical HLA class I molecules, and to the full-length HLA-G1, the glycosylation pattern of the truncated HLA-G2, -G3, and -G4 isoforms is altered and is consistent with their cell surface expression as immature glycoproteins.

HLA-G2, -G3, and -G4 isoforms are transported to the cell surface after a 2-h chase period

To investigate the time during which HLA-G isoforms reach the cell surface, cell surface protein biotinylation experiments of [35S]methionine/cysteine pulse-labeled M8-HLA-G2, -G3, and -G4 cells were conducted at various chase times. Surface biotinylated molecules were then precipitated with streptavidin-agarose beads, and HLA-G isoforms were reprecipitated using a rabbit Ab, namely PAG 1, which is directed against the HLA-G α1 61–83.
HLA-G2, -G3, and -G4 isoforms impair the lytic activity of Ag-specific CTL

Previous data have shown that the full-length HLA-G1 isoform impairs the lytic activities of both NK and CTL cytotoxic effectors (21, 28). To determine whether the truncated HLA-G isoforms, like HLA-G1, were functional molecules with respect to CTL and NK effectors, we conducted chromium release assays using M8 transfected cells as targets.

To test whether HLA-G2, -G3, and -G4 isoforms impaired T cell cytolytic function, we used an in vitro model of the T cell anti-virus response. This model consists of CD8+ CTL cells that are specific for the influenza virus matrix epitope, M58–66, presented by HLA-A2 (21). These CTL effectors were induced in vitro from the PBMC of a healthy donor by incubation for 1 wk with the HLA-A2.1-restricted M58–66 peptide, giving rise to the HC12 cell line. Carrying out CTL cytotoxicity assays using HLA-A2-positive M8 transfectants sensitized with M58–66 as the target cells, we found that lysis of M8 HLA-G1, -G2, -G3, and -G4 targets by peptide-specific CTL was significantly reduced compared with lysis of M8-pcDNA cells (Fig. 5). Since all M8 transfectants exhibited similar levels of HLA-A2 cell surface expression (data not shown), we conclude that the CTL inhibitory effect was not due to decreased peptide presentation, but to HLA-G surface expression on the target cells. Considered together, these results suggest that all HLA-G isoforms have the capacity to inhibit CTL lysis, thus counteracting the positive CTL lytic signal resulting from TCR recognition of the HLA class I/peptide complex.

HLA-G2, -G3, and -G4 isoforms inhibit the NK lytic activity of peripheral blood polyclonal NK cells

To determine whether the truncated HLA-G isoforms were functional molecules with respect to NK cells, cytotoxicity assays were conducted using freshly isolated polyclonal NK cells obtained from PBMC as the effectors against M8 transfectants, which were used as targets. Twelve experiments were conducted, each recorded with polyclonal NK cells from a different donor. We observed that lysis of M8 HLA-G1, -G2, -G3, and -G4 targets by fresh NK cells obtained from all 12 donors tested was significantly reduced compared with lysis of the M8-pcDNA control cell line (Fig. 7A). Taken together, these results show that all HLA-G isoforms are able to inhibit the lytic activity of ex vivo-isolated polyclonal NK cells.

HLA-E molecules are stabilized on the cell surface by the leader sequence peptides of certain HLA molecules, including HLA-G, leading to NK lysis inhibition through interaction with the CD94/NKG2A inhibitory receptor (29, 30). Accordingly, we further asked whether the inhibition of NK lysis mediated by the HLA-G isoforms was indirectly due to HLA-G surface expression on the target cells. Considered together, these results show that all HLA-G isoforms have the capacity to inhibit CTL lysis, thus counteracting the positive CTL lytic signal resulting from TCR recognition of the HLA class I/peptide complex.

The NK lysis inhibition of HLA-G2, -G3, and -G4 isoforms does not occur through an HLA-E/CD94/NKG2A pathway

To question the implication of HLA-E in the HLA-G isoform-mediated NK lysis inhibition, we conducted cytotoxicity experiments using as target cells the M8 transfectants facing polyclonal NK cells treated with an mAb specific for NKG2A.

FIGURE 5. HLA-G2, -G3, and -G4 isoforms reach the cell surface after a 2-h chase period. Cell surface expression of HLA-G2, -G3, and -G4 isoforms was analyzed by pulse-chase analysis. M8 transfectants (A–D) were starved for 30 min and then metabolically labeled for 20 min. Labeling medium was removed and replaced with chase medium containing an excess of cold methionine/cysteine. Cells were further incubated for 0, 2, or 4 h at 37°C in 5% CO2. At each time point, cells were biotinylated, and cell surface proteins were precipitated with streptavidin-agarose beads as described in Materials and Methods. At this time, lysates were centrifuged, and both beads and supernatant were collected separately and treated in parallel as follows. After washing, precipitated molecules were eluted from the beads and reprecipitated with the rabbit polyclonal anti-HLA-G, PAG 1. The surface HLA-G proteins were then analyzed by SDS-PAGE (biotinylated). The nonbiotinylated HLA-G proteins present in the supernatant were reprecipitated with rabbit polyclonal anti-HLA-G and then analyzed by SDS-PAGE (unbiotinylated).

FIGURE 6. HLA-G1, -G2, -G3, and -G4 inhibit Ag-specific CTL cytolysis. M8 transfectants were incubated with the M58–66 peptide at a final concentration of 0.01 μg/ml and were used in a cytotoxicity assay with the HC12 cell line, an HLA-A2-restricted CTL line specific for the M58–66 viral peptide, at a E:T cell ratio of 15:1. These results are expressed as the percentage of specific lysis ± SD of the mean of three separated experiments measured in a 4-h 51 Cr release assay. The percentage of specific lysis measured after incubating M8 transfectants without the M58–66 peptide was <5%. Note that all HLA-G-positive M8 transfectants inhibit M58–66-specific CTL lysis, whatever the E:T cell ratio tested.
that NK lysis of M8-pcDNA is enhanced by blocking NKG2A on polyclonal NK cells, showing that cell surface HLA-E molecules interact with CD94/NKG2A and act as NK inhibitory molecules. In contrast, HLA-G isoform-mediated NK lysis inhibition is not enhanced by blocking the CD94/NKG2A receptor. These results show that the HLA-G isoform-mediated NK lysis inhibition does not occur through HLA-E/CD94/NKG2A interactions.

To confirm that the HLA-G isoforms can inhibit NK cytolysis through an HLA-E-independent pathway, we tested the ability of the CD94-negative YT2C2-C2 NK clone to be inhibited by each HLA-G isoform expressed on M8 cells. We have previously reported that the lytic activity of the YT2C2-PR NK clone was inhibited by HLA-G1 through an unknown killer-Ig-like receptor (KIR) (26), but not inhibited by the nonclassical HLA-E and by the classical HLA-A, -B, and -C class I molecules (31). This was prompted by our observation that neither CD94/NKG2A, nor p140, p70, p58, and Ig-like transcript 2 (ILT2) or leukocyte Ig-like receptor-1 (LIR-1) receptors are detected on this clone (31). As shown in Fig. 7, while the M8-pcDNA control cell line was efficiently lysed by YT2C2-PR, lysis of M8-HLA-G1, -G2, -G3, and -G4 transfectants was significantly reduced. Thus, like HLA-G1, the other membrane-bound isoforms also have an inhibitory effect on this CD94/NKG2A-negative NK subclone. This result confirms that such HLA-G-mediated inhibition occurs through a CD94/NKG2A-independent pathway.

Interestingly, we recently found that among the HLA class I inhibitory receptors characterized to date, the only receptor that could be detected at the cell surface of YT2C2-PR is an HLA-G1-specific receptor belonging to the KIR103 gene family (data not shown), namely KIR2DL4 (18, 32). Unfortunately, the KIR2DL4 antiserum is not functional in cytotoxicity experiments, which leaves us unable to directly implicate this receptor as the inhibitory receptor involved in HLA-G-mediated inhibition. However, since KIR2DL4 is the only inhibitory receptor expressed on this NK cell, we suggest that the HLA-G inhibitory effect is most likely manifested through interaction between KIR2DL4 and the HLA-G isoforms, probably via their common extracellular α1 domain.

The NK lysis of M8-HLA-G2, -G3, and -G4 cells is not restored by anti-HLA-G mAbs

Since the 87G mAb reacts specifically with HLA-G1 and the 4H84 mAb reacts with all the HLA-G protein isoforms, we next asked whether these Abs could restore NK lysis of M8-HLA-G1, -G2, -G3, and -G4 target cells. For this purpose, Ab blocking assays were conducted using as effectors, polyclonal NK cells in the presence of target cells preincubated, or not, with 4H84 or 87G mAb (Fig. 8). The results show that, as described above, M8-HLA-G1, -G2, -G3, and -G4 are protected from lysis of polyclonal NK cells in the absence of Ab (Fig. 8A; p < 0.02). By using mAb-treated targets it was found that while the 87G mAb restores NK lysis of the M8-HLA-G1 cells (p > 0.4), it does not restore lysis of M8-HLA-G2, -G3, and -G4 protected from lysis of polyclonal NK cells in the absence of Ab (Fig. 8A; p < 0.02). By using mAb-treated targets it was found that while the 87G mAb restores NK lysis of the M8-HLA-G1 cells (p > 0.4), it does not restore lysis of M8-HLA-G2, -G3, and -G4 protected from lysis of polyclonal NK cells in the absence of Ab (Fig. 8C; p = 0.037). The results confirm that such HLA-G-mediated inhibition occurs through a CD94/NKG2A-independent pathway.

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particular interest, since it mimics a situation in which HLA-G is coexpressed with HLA class I molecules, while all previous studies were conducted using HLA class I-deficient cell lines transfected with HLA-G (16, 17, 33, 34).

As a first question, we asked whether these truncated HLA-G2, -G3, and -G4 isoforms, the sequences of which retain exons encoding a transmembrane region and a cytoplasmic tail, could be expressed at the cell surface as proteins formed by one or two extracellular domains. By carrying out cell surface flow cytometry, biotinylation, and pulse-chase experiments, our results provide evidence that HLA-G2, -G3, and -G4 isoforms are able to reach the cell surface within 2 h after synthesis, as Endo-H-sensitive glycoproteins. In contrast, HLA-G1 and classical HLA class I molecules are well defined as Endo-H-resistant, cell surface glycoproteins. Interestingly, although this feature of proteins containing immature oligosaccharides that can reach the cell surface is unusual, it has been reported in the literature for other proteins, such as the HLA class I-like molecule, CD1d (35). An escape from ER retention has also been described for the mouse CMV glycoprotein, gp34 which associates with folded class I MHC molecules and bears two oligosaccharides that are Endo-H sensitive at the cell surface (36).

FIGURE 8. HLA-G2-, -G3-, and -G4-mediated NK lysis inhibition is not reversed by 87G and 4H84 mAbs. M8 transfectants were used as targets of peripheral blood polyclonal NK cells in chromium release assays at a 100:1 E:T cell ratio. M8-HLA-G2, -G3, and -G4 were incubated, or not (A), with 4H84 mAb (B) or 87G mAb (C) as described in Materials and Methods. Results are expressed as the percentage of specific lysis ± SD of the mean of triplicate determinations from one of three representative experiments, measured in a 4-h 51Cr release assay.

effector (data not shown). Taken together, these data show that HLA-G2, -G3, and -G4 isoforms are able to inhibit NK lytic activity, although neither 87G nor 4H84 mAb are able to restore such inhibition.

Discussion

A specific characteristic between HLA-G and other HLA class I molecules is the extent to which HLA-G pre-mRNA is alternatively spliced into multiple variants, leading to several isoforms, including four membrane-bound (i.e., HLA-G1 to -G4) and two soluble isoforms (HLA-G5 and HLA-G6) (1–3). Among the membrane-bound HLA-G isoforms, the full-length HLA-G1 was widely investigated and described as a cell surface immunotolerant molecule playing an important role, particularly in maternal-fetal tolerance (4). The aim of the present study was to investigate both the biochemical and functional aspects of the other putative membrane-bound HLA-G isoforms compared with those of HLA-G1. For this purpose, vectors containing the cDNA corresponding to HLA-G1, -G2, -G3, and -G4 isoforms were transfected into an HLA class I-positive cell line named M8. This in vitro model is of particular interest, since it mimics a situation in which HLA-G is
-G3, and -G4 isoforms significantly inhibits the NK cytolysis of M8 cells. These results show that expression of even one HLA-G isoform on HLA class I-positive targets constitutes an additional protective mechanism against NK cytolysis. To control the implication of the other HLA class I molecules present on the M8 transfectants in the inhibitions observed, we checked by flow cytometry that all the M8 transfectants exhibited a similar level of HLA-A, -B, -C, and -E expression, as attested by their similar staining using anti-HLA-A, -B, -C, and -E mAbs (data not shown). Furthermore, previous reports show that HLA-G1-mediated NK inhibition can be indirectly due to HLA-E cell surface up-regulation and its interaction with the CD94/NKG2A inhibitory receptor (29, 30). However, we show here that the HLA-G isoform-mediated NK lysis inhibition does not occur through HLA-E, since 1) M8-HLA-G2, -G3, and -G4 are still protected from polyclonal NK lysis despite the blockage of HLA-E/CD94/NKG2A interactions; and 2) the lytic activity of the YT2C2-PR, which does not express CD94/ NKG2A, is inhibited by each HLA-G isoform. Our recent evidence that the HLA-G1-specific inhibitory receptor, namely KIR2DL4, is expressed on YT2C2-PR strongly suggests that HLA-G isoform-mediated inhibition occurs through KIR2DL4 and more probably through the α1 domain common to all HLA-G isoforms. This conjecture is supported by the ability of the HLA-G3 isoform, which consists only of the α1 domain, to inhibit the KIR2DL4-positive YT2C2-PR NK subclone cytolysis. It should be noted that in the classical HLA class I α1 domain, residues 77–80 have been described as an important KIR recognition site (38). In this region, Met76 and Glu78 are unique to HLA-G (in all alleles described to date) (39) and might be involved in KIR2DL4 recognition. However, since the KIR2DL4 antisera is not functional in cytotoxicity experiments, we cannot rule out that other inhibitory receptors may be implicated in this HLA-G-mediated inhibition. Besides KIR2DL4, another described HLA-G inhibitory receptor present on peripheral blood NK cells is ILT2 (or LIR-1) (16, 19, 20). However, it is unlikely that HLA-G3 and -G4 isoforms inhibit NK cytolysis through interaction with this KIR, since a recent report showed that ILT2 (LIR-1) interacts with the α1 domain of the HLA class I ligand (40), which is not found in HLA-G3 and -G4 isoforms. Moreover, we cannot exclude that HLA-G isoforms mediate NK and CTL cytolysis inhibition through another indirect pathway distinct from HLA-E. Indeed, in an attempt to provide direct evidence that the inhibition of NK lysis was due to the presence of HLA-G2, -G3, and -G4 molecules on the cell surface of M8 cells, we conducted cytotoxicity experiments using as effectors polyclonal NK cells or the YT2C2-PR subclone against the M8-HLA-G1, -G2, -G3, and -G4 targets that had been previously treated, or not, with 87G (which reacts specifically with the HLA-G1 isoform) or 4H84 mAb (which reacts with all HLA-G isoforms). Unfortunately, while the 87G mAb restores NK lysis of the M8-HLA-G1 cells, neither 87G nor 4H84 mAbs restore lysis of M8-HLA-G2, -G3, and -G4 targets. Thus, although the 4H84 mAb recognizes HLA-G1, -G2, -G3, and -G4, it does not reverse their NK inhibitory function. Interestingly, a distinct behavior between 87G and 4H84 is also observed in flow cytometry experiments, in which all M8-HLA-G1 cells are stained by 87G mAb, but not by 4H84 mAb. Taking these findings together, one could postulate that the distinct ability of 87G and 4H84 mAbs to react with M8-HLA-G1 and to restore M8-HLA-G1 lysis may be explained by 1) mAb affinity: the 4H84 mAb presents a lower affinity than the 87G mAb to detect HLA-G1 cell surface molecules; and/or 2) mAb specificity: the epitope recognized by the 4H84 mAb (i.e., the sequence 61–83 of the HLA-G α1 domain) on HLA-G1 is not involved in HLA-G1-mediated NK lysis inhibition. Furthermore, while 87G recognizes the β2-microglobulin (β2m)-associated form, 4H84 may not. Thus, 87G and 4H84 mAbs may not recognize the same form of cell surface HLA-G1 molecules (i.e., native vs denatured form), leading to the conclusion that conformational forms recognized by 87G are functional, while denatured forms recognized by 4H84 are not. As for HLA-G1, similar conclusions can be extrapolated for HLA-G2, -G3, and -G4 concerning the 4H84 mAb.

In HLA-A- and -B-negative trophoblast tissue, HLA-G is the predominant HLA class I molecule expressed (7). The placental expression of HLA-G2, -G3, and -G4 (41) would be particularly important in situations in which HLA-G1 expression is altered, such as mutation of the HLA-G gene or TAP deficiencies. Indeed, the cell surface expression of HLA-G1, as a trimolecular α-chain/ β2m/peptide complex, is TAP and β2m dependent (5, 42). In this regard, in homzygous TAP-negative fetuses (43) HLA-G1 placental expression should be impaired by a lack of peptide supply in the ER of fetal tissue. Therefore, HLA-G2, -G3, and -G4 isoforms, whose expression at the cell surface is probably independent of peptide loading, may substitute for the loss of HLA-G1 surface expression and thus contribute to the survival of these fetuses. Consistently, intracellular transport of HLA-G1 was previously found to be impaired upon ICP47 transfection, which blocks peptide transport from the cytosol to the endoplasmic reticulum via TAP (44). Instead, a small 18-kDa molecule, which may correspond to the HLA-G3 isoform, is efficiently expressed, is transported to the cell surface, and is capable of protecting the HLA-G1 transfectant from NK lysis. Furthermore, the survival of fetuses homozygous for the deleted HLA-G*0105N null allele, which is predicted to prevent HLA-G1 expression (45), may also be due to the expression of truncated HLA-G forms assuming the protective HLA-G function. Consistently, it has been suggested that in homzygous HLA-G*0105N individuals, HLA-G2 may be expressed at both mRNA and protein levels (45, 46). Moreover, by performing blocking experiments using anti-HLA class I Abs, previous studies have concluded that HLA class I molecules present on trophoblastic tissue do not protect it from decidua NK lysis (47, 48). However, the fact that trophoblastic cells are still protected from NK lysis in the presence of anti-HLA class I Abs did not take into account the possible cell surface expression of the truncated HLA-G isoforms, which can still assume protection of trophoblast cells.

Besides its selective physiological placental expression, HLA-G has also been found in some other HLA class I-positive tumors, such as melanoma (14, 24), colon (49), and lung carcinoma (50). Such expression in tumors would favor malignant progression by allowing HLA-G-positive tumors to escape from both NK and CTL immunosurveillance. As mentioned above in pregnancy disorders in which the smaller HLA-G isoforms can substitute for the loss of HLA-G1 expression, such substitution may also be extrapolated to tumor situations. Indeed, when HLA-A, -B, -C, and -E are downregulated in tumor cells as the result of a transport defect (51), HLA-G1 expression should also be impaired. In such deficient tumors, which become good targets for NK lysis, expression of the other membrane-bound HLA-G isoforms might still occur, thus facilitating the immune escape of HLA-G-positive tumors from NK immunosurveillance.

Taken together, our results strongly support the idea that expression of at least one HLA-G isoform on the cell surface may favor immune tolerance by inhibiting both NK and CTL effectors during pregnancy, tumor progression, or transplantation. This is particularly interesting for HLA-G3, which contains the expected functional epitope in its own α1 monomorphic domain.
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