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This information is current as of August 9, 2022.

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*J Immunol* 2001; 166:5018-5026; ;  
doi: 10.4049/jimmunol.166.8.5018  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# HLA-G2, -G3, and -G4 Isoforms Expressed as Nonmature Cell Surface Glycoproteins Inhibit NK and Antigen-Specific CTL Cytolysis<sup>1</sup>

Béatrice Riteau,<sup>2\*</sup> Nathalie Rouas-Freiss,<sup>2\*</sup> Catherine Menier,<sup>\*</sup> Pascale Paul,<sup>\*</sup> Jean Dausset,<sup>†</sup> and Edgardo D. Carosella<sup>3\*</sup>

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 (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. *The Journal of Immunology*, 2001, 166: 5018–5026.

A feature unique to HLA-G, a nonclassical HLA class I locus, is alternative splicing of its primary transcript, resulting in at least six isoforms: four membrane-bound proteins (HLA-G1 to HLA-G4) and two soluble proteins (HLA-G5 and -G6) (1–3). The full-length transcript (HLA-G1) encodes three extracellular domains ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ), a transmembrane region, and a cytoplasmic tail (4). Three other spliced transcripts encode membrane-bound proteins that lack  $\alpha 2$  (HLA-G2),  $\alpha 3$  (HLA-G4), or both the  $\alpha 2$  and  $\alpha 3$  domains (HLA-G3). The full-length HLA-G1 isoform, which has a classical HLA class I structure (5), has been widely investigated over the past few years. First identified on extravillous cytotrophoblasts (6, 7), HLA-G is also expressed in HLA class I-positive tissues such as oocytes (8), embryos (9), amnion (10), thymic epithelial cells (11), cytokine-activated monocytes (12, 13), and some tumors (14). HLA-G1 has been identified as a key molecule in maternal-fetal tolerance (15), since it inhibits the lytic activity of both decidual and peripheral blood NK cells (16–20) as well as Ag-specific CTL (21) and allogeneic proliferative responses (22). Additional evidence that HLA-G can be expressed in heart after transplantation suggests that it may participate in graft tolerance (23).

In contrast to well-characterized HLA-G1, studies of both the expression and function of HLA-G2, -G3, and -G4 isoforms have been hampered by a lack of suitable reagent. Accordingly, we

separately transfected the cDNA of HLA-G1, -G2, -G3, and -G4 into the HLA-A-, -B-, -C-, and -E-positive M8 cell line, which we have previously described as lacking HLA-G transcription (24). Stable transfectants were then obtained for each HLA-G isoform. The original approach of this work was to investigate the biochemical and functional features of HLA-G2, -G3, and -G4 isoforms (1) without interfering with the proper folding of these truncated isoforms (i.e., untagged HLA-G isoforms), and (2) in which HLA-G is coexpressed with the other HLA class I molecules. Using flow cytometric analysis, cell surface protein biotinylation, endoglycosidase-H (Endo-H)<sup>4</sup> treatments, and pulse-chase experiments, we show here that HLA-G2, -G3, and -G4 isoforms can reach the cell surface with an immature glycosylation pattern after a 2-h chase period. Moreover, cytotoxicity experiments indicate that the transfection of each HLA-G isoform in M8 cells leads to inhibition of polyclonal NK cells and NK clone lytic activity. Furthermore, evidence is provided that each HLA-G isoform also impairs the Ag-specific CTL response. Taken together, these results strongly suggest that, like the full-length HLA-G1, the HLA-G2, -G3, and -G4 truncated isoforms are expressed at the cell surface and exhibit an important role in modulating immune responses.

## Materials and Methods

### Cell lines and transfectants

M8 is an HLA-A-, -B-, -C-, and -E-positive (HLA-A1, -A2, -B12, and -B40/male), but HLA-G-negative, melanoma cell line (24). HLA-G cDNA was obtained by RT-PCR from the HLA-G-positive JEG-3 choriocarcinoma cell line cloned into the pcDNA3-1/hygromycin expression vector (Invitrogen, San Diego, CA), and transfected by electroporation into M8 cells, as previously described (25). Stable transfectant cells were named M8-HLA-G1, M8-HLA-G2, M8-HLA-G3, M8-HLA-G4, and M8-HLA-G5, according to the HLA-G isoform cDNA transfected (25). M8 cells transfected with the pcDNA3-1 vector alone were used as a negative control cell line (M8-pcDNA). All transfectants were controlled by RT-PCR and Southern blot analysis using specific primers and probes (25). The NK-like YT2C2-PR subclone and the M58–66 influenza virus matrix peptide-specific CTL line (HC12) were obtained as previously described (21,

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Received for publication October 20, 2000. Accepted for publication February 13, 2001.

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<sup>1</sup> This work was supported by grants from the French Association pour la Recherche sur le Cancer, the Etablissement Français des Greffes, and Vaincre la Mucoviscidose.

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<sup>4</sup> Abbreviations used in this paper: Endo-H, endoglycosidase H; CHAPS, 3-[(3-choleamidopropyl)-dimethylammonio]-1-propanesulfonate; PNGase-F, peptide-N-glycosidase F; ER, endoplasmic reticulum; ILT, Ig-like transcript; LIR, leukocyte Ig-like receptor;  $\beta_2m$ ,  $\beta_2$ -microglobulin; KIR, killer-Ig-related receptor.

26). Cells were maintained in RPMI 1640 medium supplemented with 10% inactivated FCS, 2 mM L-glutamine, 1  $\mu$ g/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, Mar-seilles, France); PAG 1, a rabbit polyclonal Ab that we raised against peptide 61–83 of the HLA-G  $\alpha$ 1 domain that recognizes denatured HLA-G  $\alpha$ -chain of all HLA-G isoforms (25, 27); and B-5-1-2 anti- $\alpha$ -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 cm<sup>2</sup>). Cells were first washed twice with PBS and treated with sulfo-succinimidyl-6-(biotinamido)hexanoate-biotin (Pierce, Rockford, IL; 200  $\mu$ g/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 supernatant 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  $\mu$ l of 50% streptavidin-agarose slurry (Bio-Rad, Richmond, CA) for 1 h at 4°C. After extensive washing of streptavidin-agarose beads in 0.05% CHAPS, 0.1% SDS, 300 mM NaCl, and 10 mM Tris-HCl, pH 7.5, they were resuspended in 40  $\mu$ l of sample buffer and boiled for 5 min.

#### Enzymatic treatments

Total proteins or cell surface precipitated proteins from M8 transfectants were incubated for 16 h at 37°C in 80  $\mu$ l of 0.2% SDS, 1% 2-ME, 100 mM sodium citrate, and 0.2 M PMSF with or without 8 mU of peptide-N-glycosidase (PNGase; Roche, Indianapolis, IN) or Endo-H (Roche). Subsequently, the samples were examined by Western blot analysis.

#### Pulse-chase experiments

M8 cells were grown in RPMI 1640 medium supplemented with 10% FCS (Sigma). Radiolabeling was accomplished by incubating cells that had reached 90% confluence. Cells ( $10 \times 10^7$ ) were first starved for 30 min at 37°C in RPMI 1640-methionine/cysteine (Life Technologies, Rockville, MD) supplemented with dialyzed 5% FCS, 300 mg/ml L-glutamine, 50 mg/ml L-leucine, 200 mg/ml L-arginine, 200 mg/ml glucose, and 35 mg/ml inositol. Cells were then labeled for 20 min at 37°C with [<sup>35</sup>S]ProMix (250  $\mu$ Ci/ml; Amersham, Arlington Heights, IL). Labeled cells were extensively washed in RPMI 1640 medium containing 10% FCS and an excess of cold methionine/cysteine and incubated at 37°C in 5% CO<sub>2</sub> for 0, 2, or 4 h in fresh cell culture medium. At these different time points, cells were bio-

tinylated, and cell surface proteins were precipitated with streptavidin-agarose beads as described above. At this time, lysates were centrifuged, and both beads and supernatant were collected separately and treated in parallel as described in the following steps 1 and 2, respectively. 1) After washing, precipitated molecules were eluted from the beads in 50  $\mu$ l 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 reprecipitated 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. 2) The supernatant was reprecipitated with rabbit polyclonal anti-HLA-G, and then unbiotinylated HLA-G proteins were analyzed by SDS-PAGE.

#### Cytotoxicity assays

The cytolytic activity of PBMC from healthy adult volunteer donors (men and women, aged 30–60 years) obtained by Ficoll/Histopaque density gradient, of YT2C2-PR, or of the HC12 CTL line used as effectors was assessed in 4-h <sup>51</sup>Cr release assays in which effector cells were mixed with  $5 \times 10^3$  <sup>51</sup>Cr-labeled targets (100  $\mu$ Ci of <sup>51</sup>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% CO<sub>2</sub> incubator, 50  $\mu$ l of the supernatant 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 – cpm spontaneous release)]  $\times$  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  $\mu$ g/ml), then washed and incubated with an F(ab')<sub>2</sub> goat anti-mouse 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%.

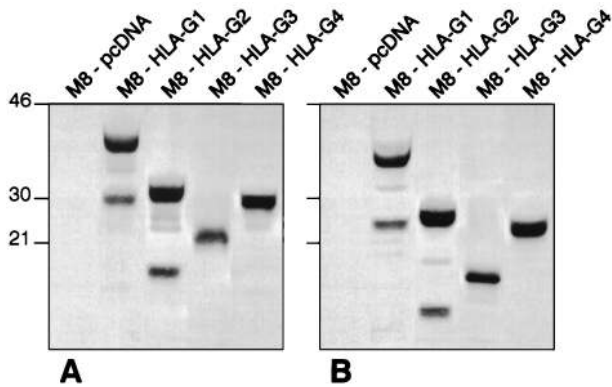
#### Statistical analysis

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  $\alpha$ 1 domain epitope common to all HLA-G isoforms (27). In addition to the expected translation of the HLA-G1 transcript into a 39- to 40-kDa protein, we found that the HLA-G2, -G3, and -G4 transcripts were, respectively, translated into 30-/31-, 22-, and 29-/30-kDa proteins, respectively (Fig. 1A). However, these molecular masses were higher than predicted, indicating that HLA-G isoforms 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,



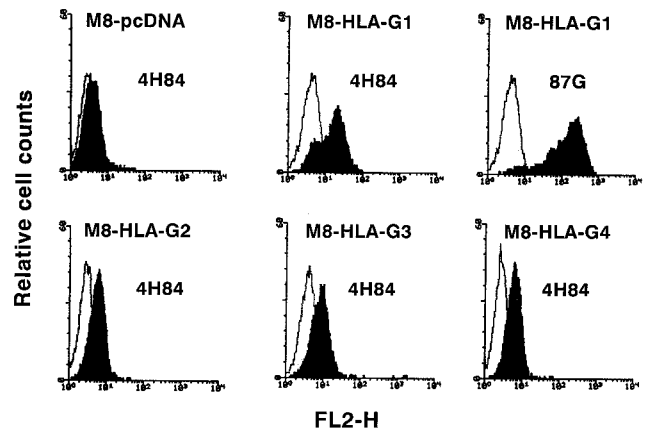
**FIGURE 1.** HLA-G1, -G2, -G3, and -G4 isoforms are translated as glycoproteins. Western blot analysis of lysates from M8 transfectants was conducted using the 4H84 mAb. Lysates from M8 transfectants were treated with PNGase F (*B*) or were not treated (*A*). Numbers at the left of the figure refer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

-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 17 kDa) in the lysate of the corresponding M8 transfectant (Fig. 1*A*). Furthermore, in other Western blot experiments, additional smaller bands were also revealed for HLA-G1 (i.e., 26 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 transfectant, 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 transfectant.

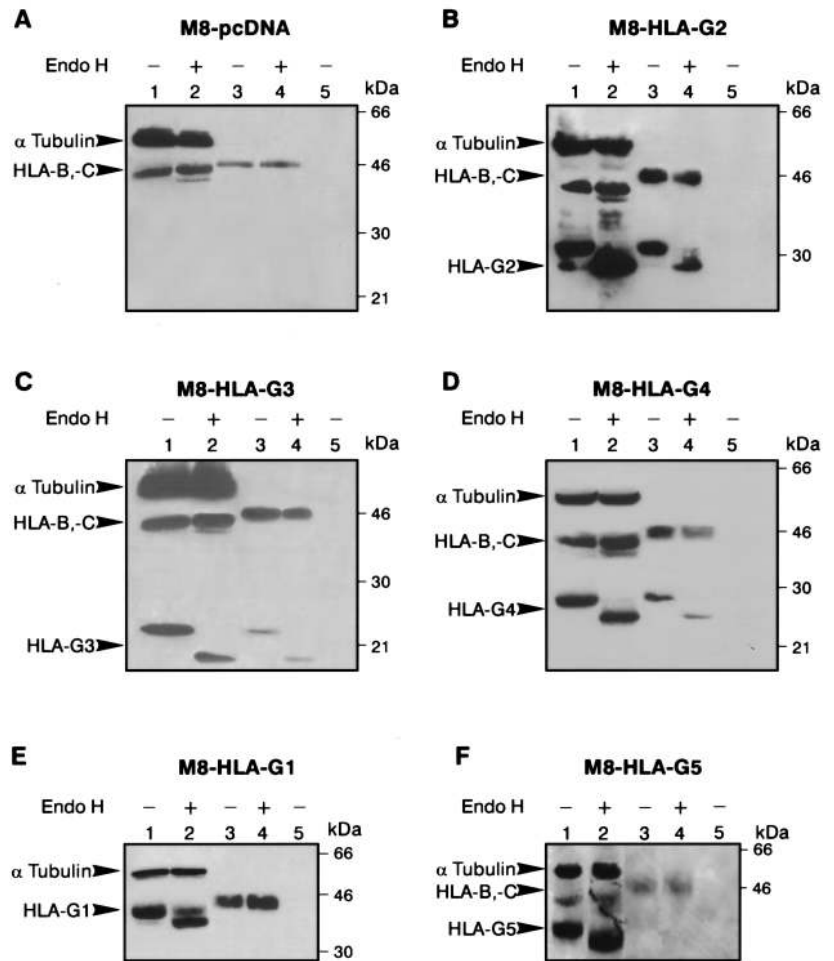
To confirm these results, we conducted cell surface protein biotinylation of the M8 transfectants, followed by immunoprecipitation with streptavidin-agarose beads. Fig. 3 shows, using 4H84 mAb, immunoblotting of HLA-G into the lysates (*lanes 1*) or after precipitation of biotinylated proteins (*lanes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



**FIGURE 2.** Detection of HLA-G2, -G3, and -G4 isoforms on the cell surface of M8-transfected cells by flow cytometric analysis. Cells were labeled by indirect immunofluorescence with the 4H84 mAb (bold profiles) and an isotype-matched control Ab (light profiles). The 87G mAb (bold profile) was used as a positive control on M8-HLA-G1 cells. After washing, cells were stained with PE-conjugated F(ab')<sub>2</sub> goat anti-mouse IgG. The 4H84 mAb was used as ascetic fluid at a 1/100 dilution, and the 87G mAb was used at 2  $\mu$ g/ml. Only viable cells were analyzed in these experiments. One of five representative experiments is shown.

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. 3*F*, *lane 3*), although a high level of HLA-G5 protein synthesis was observed in the lysate of the M8-HLA-G5 transfectant (Fig. 3*F*, *lane 1*); and 2) highly expressed intracellular protein (i.e., tubulin), which was indeed detected in all transfectants 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 transfectant, 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 transfectants 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.

**FIGURE 3.** HLA-G2, -G3, and -G4 are cell surface-expressed molecules containing immature oligosaccharides. Cell surface proteins of M8 transfectants were biotinylated (*lanes 1–4, A–F*) or not (*lanes 5, A–F*) as monolayers in tissue culture flasks with sulfo-succinimidyl-6-(biotinamido)hexanoate-biotin. Cells were then lysed, and, after immunoprecipitation of cell surface biotinylated proteins with streptavidin-agarose beads, the surface protein-depleted lysates (*lanes 1 and 2, A–F*) and the immunoprecipitated proteins (*lanes 3–5, A–F*) were analyzed by Western blot. The lysates and the immunoprecipitated proteins were incubated in the presence (+; *A–F*) or the absence (–; *A–F*) of Endo-H. Data were obtained by sequentially staining the blot with the anti-HLA-G 4H84 mAb together with the anti- $\alpha$ -tubulin, followed by Western-development (*A–F*) and then the anti-HLA-B, -HLA-C, -HC10 mAb (*A–D* and *F*). Note that the membrane E was not blotted by HC10 mAb because the Endo-H-treated HLA-B and -C molecules that are revealed by this mAb give bands at  $M_r$  matching those of HLA-G1, leading to a smear.



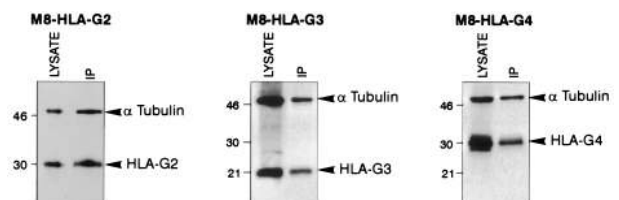
#### HLA-G2, -G3, and -G4 isoforms are immature cell surface-expressed molecules

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,

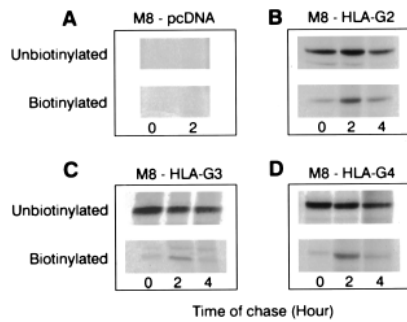
the glycosylation pattern of the truncated HLA-G2, -G3, and -G4 isoforms is altered and is consistent with their cell surface expression as immature glycoproteins.

#### 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 [ $^{35}$ S]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  $\alpha$ 1<sub>61–83</sub>



**FIGURE 4.** Tubulin and HLA-G2, -G3, and -G4 are equally biotinylated. Intracellular proteins of M8-HLA-G2, -G3, and -G4 were biotinylated and precipitated with streptavidin-agarose beads. Both precipitated proteins from biotinylated M8 cells (IP) and lysates from unbiotinylated M8-HLA-G2, -G3, and -G4 cells (Lysate) were analyzed by Western blot using anti-tubulin together with anti-HLA-G (i.e., 4H84 mAb). Numbers at the left of the figure refer to  $M_r$  in kilodaltons.



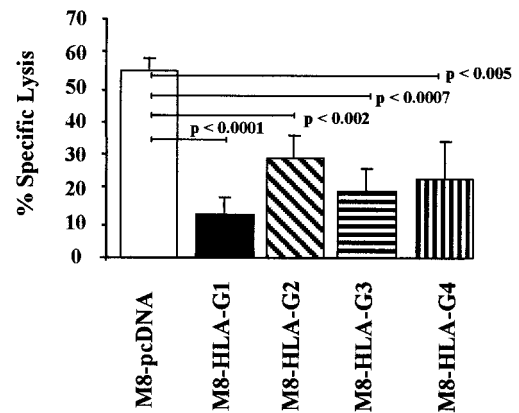
**FIGURE 5.** HLA-G2, -G3, and -G4 isoforms reach the cell surface after a 2-h chase period. Cell surface expression of HLA-G2, -G3, and -G4 isoforms was analyzed by pulse-chase analysis. M8 transfectants (A–D) were starved for 30 min and then metabolically labeled for 20 min. Labeling medium was removed 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% CO<sub>2</sub>. 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).

epitope, and analyzed by SDS-PAGE (Fig. 5, biotinylated). Concomitantly, after streptavidin-agarose precipitation, the unbiotinylated proteins were collected from the remaining supernatant, and HLA-G isoforms were reprecipitated using PAG 1 and analyzed by SDS-PAGE (Fig. 5, unbiotinylated). Results show that cell surface labeled HLA-G isoforms are detected at low levels after the pulse (Fig. 5, B–D, 0-h chase time, biotinylated), reach a maximum 2 h after the pulse (Fig. 5, B–D, 2-h chase time, biotinylated) and decreased 4 h after the pulse (Fig. 5, B–D, 4-h chase time, biotinylated). In contrast, the amounts of unbiotinylated labeled HLA-G proteins were similar at all time point (Fig. 5, B–D, unbiotinylated). No band was detected in the M8-pcDNA control cell line (Fig. 5A). Thus, we conclude that HLA-G2, -G3, and -G4 isoforms reach the cell surface within a 2-h chase period.

#### *HLA-G2, -G3, and -G4 isoforms inhibit 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 transfectant 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<sup>+</sup> 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. 6). Since all M8 transfectants exhibited similar levels of HLA-A2 cell surface expres-



**FIGURE 6.** HLA-G1, -G2, -G3, and -G4 inhibit Ag-specific CTL cytotoxicity. 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 <sup>51</sup>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.

sion (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.

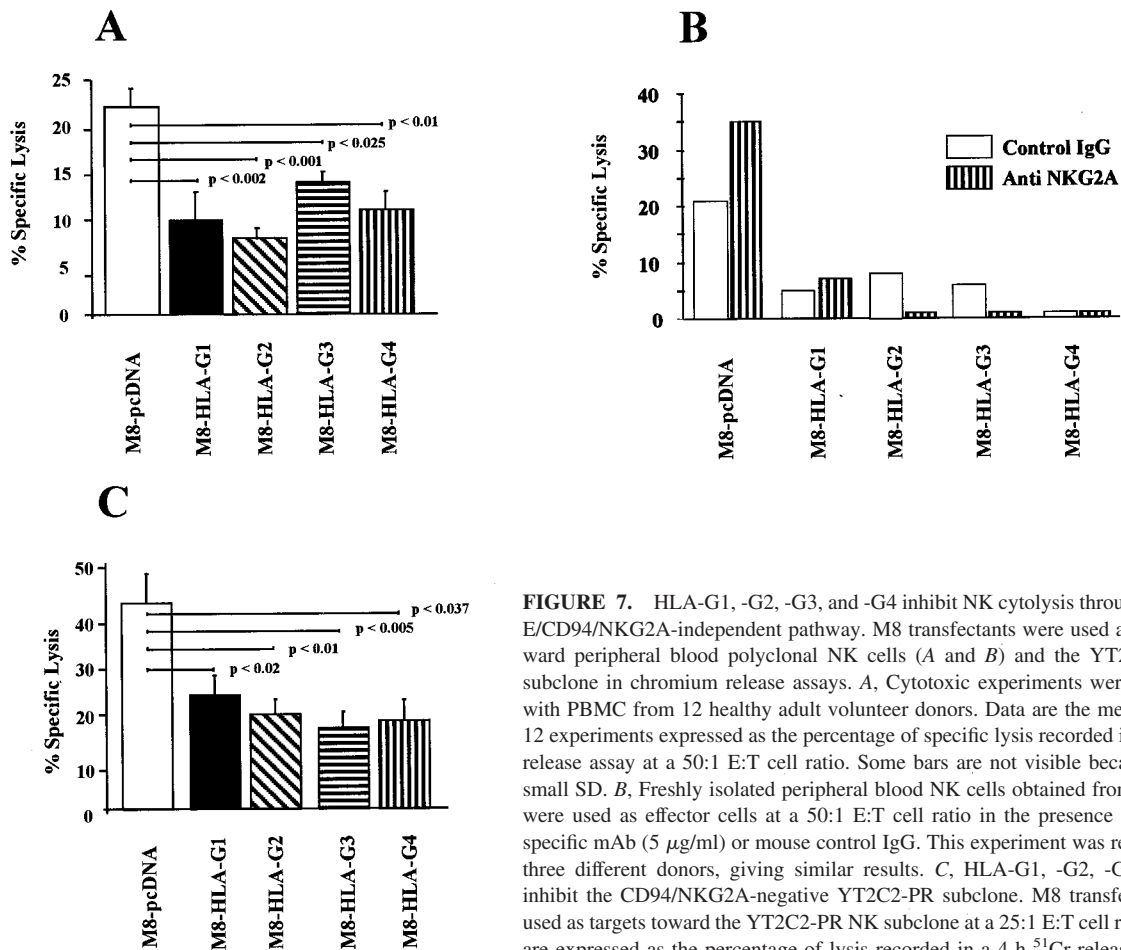
#### *HLA-G2, -G3, and -G4 isoforms inhibit the NK lytic activity of peripheral blood polyclonal NK cells*

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-E up-regulation. To investigate this point, we conducted chromium release assays by 1) blocking the HLA-E inhibitory receptor CD94/NKG2A present on polyclonal NK cells, and 2) using the YT2C2-PR NK subclone, which does not express the CD94/NKG2A receptor (26).

#### *The NK lysis inhibition of HLA-G2, -G3, and -G4 isoforms does not occur through an HLA-E/CD94NKG2A 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. Fig. 7B shows



**FIGURE 7.** HLA-G1, -G2, -G3, and -G4 inhibit NK cytolysis through an HLA-E/CD94/NKG2A-independent pathway. M8 transfectants were used as targets toward peripheral blood polyclonal NK cells (A and B) and the YT2C2-PR NK subclone in chromium release assays. A, Cytotoxic experiments were conducted with PBMC from 12 healthy adult volunteer donors. Data are the means of these 12 experiments expressed as the percentage of specific lysis recorded in a 4-h  $^{51}\text{Cr}$  release assay at a 50:1 E:T cell ratio. Some bars are not visible because of very small SD. B, Freshly isolated peripheral blood NK cells obtained from one donor were used as effector cells at a 50:1 E:T cell ratio in the presence of NKG2A-specific mAb (5  $\mu\text{g}/\text{ml}$ ) or mouse control IgG. This experiment was repeated with three different donors, giving similar results. C, HLA-G1, -G2, -G3, and -G4 inhibit the CD94/NKG2A-negative YT2C2-PR subclone. M8 transfectants were used as targets toward the YT2C2-PR NK subclone at a 25:1 E:T cell ratio. Results are expressed as the percentage of lysis recorded in a 4-h  $^{51}\text{Cr}$  release assay.

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.

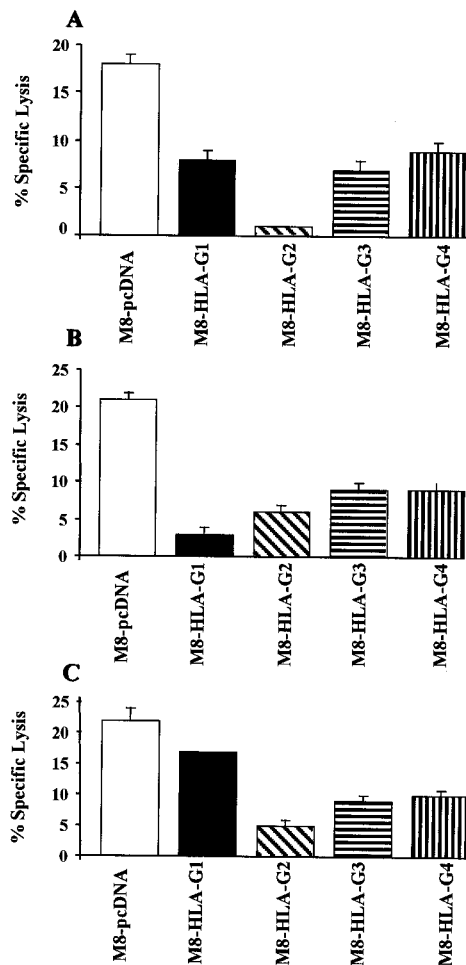
To confirm that the HLA-G isoforms can inhibit NK cytolysis through an HLA-E-independent pathway, we tested the ability of the CD94-negative YT2C2-C2 NK clone to be inhibited by each HLA-G isoform expressed on M8 cells. We have previously reported that the lytic activity of the YT2C2-PR NK clone was 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. 7C, while the M8-pcDNA control cell line was efficiently lysed by YT2C2-PR, lysis of M8-HLA-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  $\alpha 1$  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 targets (Fig. 8C;  $p < 0.02$ ). This result is in good agreement with the specificity of 87G, which reacts only with the full-length HLA-G1 isoform and consequently has no effect on the function of HLA-G2, -G3, and -G4. In contrast, although the 4H84 mAb reacts with HLA-G1, -G2, -G3, and -G4, it does not restore lysis of all M8-HLA-G transfectants (Fig. 8B). Similar results were obtained using the YT2C2-PR subclone as



**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  $\pm$  SD of the mean of triplicate determinations from one of three representative experiments, measured in a 4-h  $^{51}\text{Cr}$  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

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). Similarly, the cell surface expression of HLA-G isoforms may be due to association of the truncated HLA-G isoforms with a chaperoning protein, such as classical HLA class I molecules, allowing their escape from ER retention. In this regard, mouse nonclassical Qa-1 molecules form heterodimers with mouse classical H-2L<sup>d</sup> molecules (37).

Previous reports in discrepancy with our results show that only HLA-G1, but not the other alternative forms of HLA-G, is expressed at the cell surface of transfected cells (33, 34). In these studies the authors used HLA-G cDNA linked to a tag molecule that can be detected with a specific Ab to track HLA-G isoform expression. Several explanations may account for such an inability to detect cell surface expression of HLA-G2, -G3, and -G4 proteins. 1) Since it is well established that the addition of a tag molecule to proteins can affect protein structure, we may expect that the tagged HLA-G2, -G3, and -G4 isoforms exhibit a misfolded protein structure. In this case, energy-dependent cytoplasmic proteases will eliminate the misfolded proteins, thus compromising their forward transport to the cell surface. The fact that tagged HLA-G protein may behave differently from its untagged HLA-G counterpart is supported by the absence of HLA-G5 protein in the lysate of tagged HLA-G5 transfectants (33), while it is well described that untagged HLA-G5 protein is easily detectable in transfected cell lysates (5), as also reported in our study. 2) The putative association between the short HLA-G isoforms and a chaperoning protein required for their cell surface expression may be prevented by the conformation adopted by tagged HLA-G isoforms. 3) The cell lines used for HLA-G transfection (i.e., HLA class I-negative JAR, J26, and C1R) may not express the chaperoning molecule to which HLA-G2, -G3, and -G4 would be associated. Accordingly, the cell surface expression of the short HLA-G isoforms will depend on the cell type in which they are expressed.

In view of the presence of HLA-G isoforms at the cell surface, our further efforts were concentrated on the functional role of these truncated HLA-G2, -G3, and -G4 isoforms. Our results show that all HLA-G isoforms inhibit both acquired (Ag-specific CTL) and innate (NK cells) immune effector cells. Indeed, we found that HLA-G1, -G2, -G3, and -G4 isoforms impair the cytolytic activity of Ag-specific CTL. This may provide new insight into the ability of a nonclassical HLA ligand to inhibit virus-specific CTL activation through inhibitory receptors normally found on NK cells, resulting in a less efficient immune control of viral replication.

Using freshly isolated polyclonal NK cells, we have also demonstrated that transfection of the cDNA corresponding to HLA-G2,



-G3, and -G4 isoforms significantly inhibits the NK cytotoxicity 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 cytotoxicity. 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  $\alpha 1$  domain common to all HLA-G isoforms. This conjecture is supported by the ability of the HLA-G3 isoform, which consists only of the  $\alpha 1$  domain, to inhibit the KIR2DL4-positive YT2C2-PR NK subclone cytotoxicity. It should be noted that in the classical HLA class I  $\alpha 1$  domain, residues 77–80 have been described as an important KIR recognition site (38). In this region, Met<sup>76</sup> and Glu<sup>79</sup> are unique to HLA-G (in all alleles described to date) (39) and might be involved in KIR2DL4 recognition. However, since the KIR2DL4 antiserum 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 cytotoxicity through interaction with this KIR, since a recent report showed that ILT2 (LIR-1) interacts with the  $\alpha 3$  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 cytotoxicity 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  $\alpha 1$  domain) on HLA-G1 is not involved in HLA-G1-mediated NK

lysis inhibition. Furthermore, while 87G recognizes the  $\beta_2$ -microglobulin ( $\beta_2m$ )-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  $\alpha$ -chain/ $\beta_2m$ /peptide complex, is TAP and  $\beta_2m$  dependent (5, 42). In this regard, in homozygous 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-G 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 homozygous 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 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 down-regulated 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 only  $\alpha 1$  monomorphic domain.

## Acknowledgments

We are grateful to Drs. S. Fisher, D. Geraghty, J.-G. Guillet, E. Long, M. McMaster, and H. Ploegh for providing us with Abs and T cell lines. We thank Dr. I. Khalil-Daher for her useful advises in biochemistry, Drs. S. Amigorena and J.-Y. Thuret for helpful discussions, and M. O'Brien for rereading the manuscript.

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