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Magali Le Discorde, Caroline Le Danff, Philippe Moreau, Nathalie Rouas-Freiss, Edgardo D Carosella

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*HLA-G*0105N* Null Allele Encodes Functional HLA-G Isoforms[1]

Magali Le Discorde,[2] Caroline Le Danff, Philippe Moreau, Nathalie Rouas-Freiss, and Edgardo D. Carosella

Service de Recherches en Hémato-Immunologie, CEA-DSV-DRM, Institut d'Hématologie, Hôpital Saint-Louis, Paris 75010, France

ABSTRACT

Expression of the nonclassical HLA class I antigen, *HLA-G*, is associated with immune tolerance in view of its role in maintaining the fetus in utero, allowing tumor escape, and favoring graft acceptance. Expressed on invasive trophoblast cells, HLA-G molecules bind inhibitory receptors on maternal T lymphocytes and NK cells, thereby blocking their cytolytic activities and protecting the fetus from maternal immune system attack. The *HLA-G* gene consists of 15 alleles, including a null allele, *HLA-G*0105N. HLA-G*0105N* presents a single base deletion, preventing translation of both membrane-bound (HLA-G1) and full-length soluble isoforms (HLA-G5) as well as of the spliced HLA-G4 isoform. The identification of healthy subjects homozygous for this *HLA-G* null allele suggests that the *HLA-G*0105N* allele may generate other HLA-G isoforms, such as membrane-bound HLA-G2 and -G3 and the soluble HLA-G6 and -G7 proteins, which may substitute for HLA-G1 and -G5, thus assuming the immune tolerogeneic function of HLA-G. To investigate this point, we cloned genomic *HLA-G*0105N* DNA and transfected it into an HLA-class I-positive human cell line. The results obtained indicated that HLA-G proteins were indeed present in *HLA-G*0105N*-transfected cells and were able to protect against NK cell lysis. These findings emphasize the role of the other HLA-G isoforms as immune tolerogeneic molecules that may also contribute to maternal tolerance of the semiallogenic fetus as well as tumor escape and other types of allogeneic tissue acceptance.

embryo, gene regulation, immunology, implantation, pregnancy

INTRODUCTION

Protection of the semiallogenic fetus against maternal immune recognition and attack has been attributed principally to the high and almost unique expression of human leukocyte antigen-G (*HLA-G*) on trophoblast cells at the fetal-maternal interface [1]. The nonclassical *HLA-G* gene is characterized by alternative splicing that yields seven proteins, four membrane bound (HLA-G1 through -G4) and three soluble (HLA-G5 through -G7). Full-length *HLA-G* mRNA encodes the HLA-G1 protein, which has three extracellular globular domains, one membrane-anchored domain, and one intracytoplasmic domain. Exons 3 and/or 4 may be deleted from the primary transcript, yielding the alternative mRNA HLA-G2, HLA-G3, and HLA-G4 forms. In addition, the insertion of introns 2 or 4 may generate soluble isoforms, such as HLA-G5 (the soluble full-length HLA-G1 counterpart), HLA-G6 (the soluble HLA-G2 counterpart), and HLA-G7 (the soluble HLA-G3 counterpart) (reviewed in [2]). The structures of both membrane-bound HLA-G1 and soluble HLA-G5 proteins are similar to those of classical class I proteins, consisting of three extracellular domains linked to β2-microglobulin (β2m).

Functional assays have demonstrated that both membrane-bound and soluble HLA-G proteins are able to inhibit NK cell and antigen-specific T-cell cytotoxicity [3, 4] and the proliferation of allogeneic T cells [5–7]. Moreover, soluble HLA-G is able to induce apoptosis of both activated $CD8^+$ T and NK cells [8, 9].

The identification of these seven HLA-G protein isoforms suggested that HLA-G provides multiple functions at the immune-privileged sites where they are expressed, such as the fetal-maternal interface, the thymus, and the cornea [10–12]. On the other hand, in the absence of a given HLA-G isoform, this redundancy may serve to maintain the immune tolerogenic function provided by one or more of the other HLA-G isoforms.

The HLA-G gene consists of 15 alleles, including the *HLA-G*0105N* null allele, which is characterized by a single base-pair deletion in exon 3 [13]. This deletion of a single cytosine at codon 130 results in a gap in the open reading frame, causing a premature stop at either codon 189 (TGA) near the beginning of exon 4, which blocks translation of HLA-G1 and -G5, or codon 297 (TAG) in exon 5, blocking the translation of HLA-G4. However, *HLA-G*0105N* is able to maintain translation of both the membrane-bound HLA-G2 and -G3 proteins and the soluble HLA-G6 and -G7 proteins, in all of which exon 3, containing the deletion, is removed by alternative splicing (Fig. 1).

The *HLA-G*0105N* null allele has been described in healthy adults whose own gestations and deliveries were normal (without complications). The detection of individuals who are genetically homozygous for the *HLA-G*0105N* allele suggests that the HLA-G isoforms encoded by this allele possess functions able to compensate for the absence of both the HLA-G1 and -G5 proteins and to maintain the immune privileged status of the fetal-maternal interface [14, 15].

Based on the previously mentioned observations and on a previous study showing that selection may have increased the frequency of the *HLA-G*0105N* allele [16], the goal of the present study was to demonstrate that in the absence of the HLA-G1, -G5, and -G4 isoforms, the other HLA-G isoforms present in *HLA-G*0105N* can indeed provide a protective function.

[1]Supported by the Commissariat à l'Energie Atomique.
[2]Correspondence: Magali Le Discorde, Service de Recherches en Hémato-Immunologie, CEA-DSV-DRM, Institut d'Hématologie, Hôpital Saint-Louis, Paris 75010, France. FAX: 33 1 48 03 19 60;
e-mail: teyssier@dsvidf.cea.fr

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In the present work, we investigate the structural and functional aspects of *HLA-G*0105N* proteins. We constructed the *HLA-G*0105N* null allele genomic DNA in the p*cDNA3.1* expression vector, which enabled us to study the expression and function of its proteins. The aim of this work was to acquire more data on spliced HLA-G isoforms in general and to understand how they could compensate for the absence of full-length HLA-G proteins.

MATERIALS AND METHODS

In Vitro Site-Directed Mutagenesis

In vitro site-directed mutagenesis was carried out using the Stratagene QuickChange XL site-directed mutagenesis kit, which allows site-specific mutation in double-stranded plasmids. The plasmid DNA template (50 ng) *HLA-G*010102*-pcDNA3.1, isolated from the dam$^+$ JM109 *Escherichia coli* strain, was replicated by PfuTurbo DNA polymerase (2.5 U) using two synthetic primers, G.Null S and G.Null AS (125 ng each), consisting of 15 bases on both sides of the ΔC mutation. The oligonucleotide primers, each complementary to opposite strands of the *HLA-G*010102* insert, were extended during temperature cycling (95°C for 1 min; 18 cycles at 95°C for 50 sec, 60°C for 50 sec, 68°C for 15 min) and terminated by 7 min at 68°C. Following temperature cycling, amplification reactions were cooled and digested for 1 h at 37°C using *Dpn I* restriction enzyme to eliminate the nonmutated parental supercoiled double-stranded DNA. At this step, genomic *HLA-G*010102*-p*cDNA3.1* was replaced by genomic *HLA-G*0105N*-p*cDNA3.1*. The mutated DNA was then transformed in XL10-Gold ultracompetent cells.

Cell Lines, Transfection, and Cultures

Melanoma M8 (kindly provided by F. Jotereau, INSERM U211, Nantes, France) and histiocytic lymphoma U937 (American Type Culture Collection) human cell lines are HLA-class II negative; HLA-A, -B, -C, and -E positive (HLA-A1, -A2, -B12, and B40/male); but HLA-G negative. The NKL NK cell line (kindly provided by E.H. Weiss, Department of Anthropology and Human Genetics, Munich, Germany) [17, 18] and the YT2C2 NK [19, 20] subclone (kindly provided by P. Paul, Hospital Saint-Louis, Paris, France) have been previously described. All cells were maintained in RPMI 1640 plus Glutamax medium supplemented with 1 μg/ml gentamicin, fungizone, and 20% inactivated fetal calf serum. NKL cells were concurrently cultured in the presence of 50 U/ml of interleukin-2 (IL2) (Sigma-Aldrich, St. Quentin Fallavier, France) and boosted to 100 U/ml IL2 the day before the cytotoxicity assays. M8 and U937 transfectants were selected using 200 mU/ml hygromycin B (Invitrogen, Cergy Pontoise, France). *HLA-G*0105N* inserted into the p*cDNA3.1*/Hygro (-) expression vector was transfected into M8 and U937 cells. We worked with bulk transfectants since absence of the HLA-G1 membrane-bound isoform prevents the selection of positive cloned cells by cell sorting. The other transfectants, M8-HLA-G1 through -G4 and M8-*HLA-G*010102*, have been previously characterized [21, 22].

Classical and Real-Time RT-PCR Analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to validate *HLA-G* expression and to determine which isoforms were expressed in our new M8-*HLA-G*0105N* cell line. Total RNA was purified by RNA-WIZ reagent (Ambion, Huntingdon, U.K.) according to the manufacturer's instructions. The cDNA was synthesized on 5 μg RNA using oligo (dT) primers (Invitrogen) and MMLV-reverse transcriptase (Life Technologies, Cergy-Pontoise, France) for 1 h at 42°C. Heating for 8 min at 95°C stopped the reaction.

Real-time PCR (ABI PRISM 7000 SDS) was used to determine the quantities of *HLA-G* transcripts in transfected cell lines. Duplex PCR was carried out for 40 amplification rounds in the presence of TaqMan Universal PCR Master Mix, using the TaqMan Assay Reagent and GAPDH as an endogenous control (probe with VIC reporter and TAMRA quencher), *HLA-G*-specific probe (200 nM, FAM reporter, and TAMRA quencher), and *HLA-G*-specific primers (300 mM, Q biogen).

Classical PCR was carried out according to the 13th HLA Workshop [21] procedure, using a Perkin-Elmer DNA thermal cycler in a total volume of 100 μl containing 2 μl of the RT reaction product, 200 μM of each *dNTP* (Invitrogen), 100 ng of each primer, 10 μl of 10× Taq buffer, and 2.5 U of Taq polymerase (Applied Biosystems, Courtabœuf, France). All *HLA-G* isoforms were amplified with G.257F/G.1004R primers, *HLA-G2* with G.-3/G.1216 primers, *HLA-G3* with G.-3-4/G.1216 primers, and *HLA-G5* with G.257/G.i4. These PCR products were revealed by radioactive hybridization with G.927 oligonucleotide, except for *HLA-G5* amplification, which was revealed using the G.R probe.

Vector Construction and Oligonucleotides

In vitro site-directed mutagenesis was accomplished from the genomic *HLA-G*010102* sequence inserted into p*cDNA3.1*/Hygro (-) expression vector (Invitrogen). The *HLA-G*010102* sequence begins in the promoter at the *Xba*I site (460 nucleotides upstream from ATG) and continues to the *Eco*RV site in the 3′UT [21]. The cytosine deletion was introduced using two primers, G.Null S (5′-GCCCTGAACGAGGACTGCGCTCCT GGACCG) and G.Null AS (5′-CGGTCCAGGAGCGCAGTCCTCGTTC AGGGC).

Real-time PCR was carried out with the G.948 forward primer located in exon 5 (5′-CTGGTTGTCCTTGCAGCTGTAG), the G.1002 reverse primer located in both sides of exon 5 and exon 6 (5′-CCTTTTCAATCTG AGCTCTTCTTTCT), the G.971F internal probe (5′-CACTGGAGCTGC GGTCGCTGCT), the FAM reporter, and the TAMRA quencher.

For classical PCR, all *HLA-G* transcripts were amplified from exon 2 by G.257 forward (5′-GGAAGAGGAGACACGGAACA) to exon 5/6 by G.1004 reverse (5′-CCTTTTCAATCTGAGCTCTTCTTT). *HLA-G2* and *HLA-G3* transcripts were amplified, respectively, from exons 2/4 by G.-3 forward (5′-ACCAGAGCGAGGCCAACCCC) and from exons 2/5 by G.-3-4 forward (5′-ACCAGAGCGAGGCCAAGCAG) to the 3′UT sequence by G.1216 reverse (5′-GACGGAGACATCCCAGCCCC). Transcripts corresponding to soluble forms were amplified from exon 2 by G.257 up to intron 4 by G.i4 reverse (5′- CTGGGAAAGGAGGTGAAGGT). Internal probes were localized in exon 5 with G.927 reverse (5′-CCAGCAAC-GATACCCATGAT) or in exon 2 with G.R (5′-GGTCTGCAGGTT-CATTCTGTC).

Monoclonal Antibodies

The following murine antibodies were used: 4H84, IgG1 recognizing α1 domain common to all HLA-G isoforms (provided by Michael McMaster [23]); MEM-G/04, IgG1 recognizing the denatured form of HLA-G1, -G2, -G5, and -G6 (provided by Vaclav Horejsi, Prague, Czech Republic); MEM-G/09, IgG1 recognizing the HLA-G molecules associated with beta2-microglobulin (HLA-G1 and -G5) [24] (Exbio, Prague, Czech Republic); 5A6G7, IgG1 antibody against intron 4 of soluble HLA-G5 and -G6 isoforms made in our laboratory [25].

Immunocytochemical Analysis

Cells were grown on LabTech slides (Dutscher, Brumath, France), fixed in acetone, and stored at −80°C until evaluation of the expression of *HLA-G* and class I protein by immunohistochemistry, using the Ultra-Tech HRP Streptavidin-Biotin Universal Detection System (Immunotech Coulter, Roissy, France) as previously described [12]. Briefly, endogenous peroxidase was blocked with 3% H_2O_2, and antibodies were diluted in buffer containing 0.1% saponin.

Flow Cytometry Assays

The flow cytometry technique was used to analyze cell-surface HLA-G protein expression. Transfected cells were phenotyped according to standard procedure by incubating them with the primary anti-HLA-G antibody MEM-G/09 in PBS 2% heat-inactivated fetal calf serum for 30 min at 4°C. Cells were subsequently stained with an $F(ab')_2$ goat anti-mouse IgG antibody conjugated with phycoerythrin (Immunotech Coulter).

Cell-Surface Protein Biotinylation

Cell-surface proteins of viable M8 transfected cells were biotinylated as previously described [4]. Cells were first washed twice with PBS and treated with sulfo-NHS-LC-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 (nonadherent) cells were removed by washing. Cells were then detached and collected in tubes, then washed in PBS five times at 4°C. Cells in the last pellet were readied for analysis by immunoprecipitation.

Immunoprecipitation of Cell-Surface Proteins

Cells were lysed in 1 ml of lysis buffer (0.5% CHAPS, 50 mM Tris-HCl). After centrifugation at 14 000 rpm for 30 min at 4°C, biotinylated

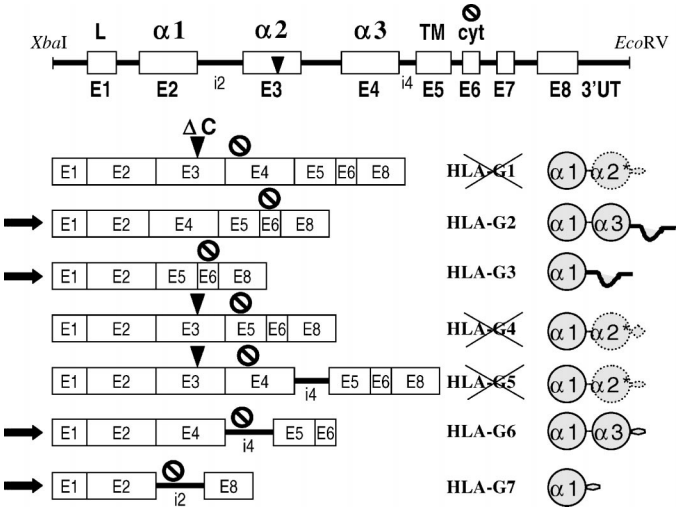

FIG. 1. Schematic drawing of the *Xba*I - *Eco*RV genomic DNA sequence of *HLA-G*. The *HLA-G*0105N* sequence corresponds to that of *HLA-G*010102,* except for the deletion of cytosine, indicated by an arrow in exon 3 (ΔC). The *HLA-G* gene consists of eight exons (E1–E8), corresponding, respectively, to the peptide leader domains (L), α1, α2, and α3, the transmembrane domain (TM), and the intracytoplasmic domain (cyt). Exons were separated by introns (i). The *HLA-G*0105N* gene putatively encodes the membrane-bound HLA-G2, -G3, and soluble HLA-G6 and -G7 proteins, in all of which exon 3, which contains the deletion, is deleted. HLA-G1, -G4, and -G5 might consist of the leader peptide, the complete α1 domain, the first half of the α2 domain, and a premature end in the following exon.

proteins were precipitated from the supernatant by precipitation with 100 μl of 50% streptavidin-agarose beads (Bio-Rad, Marnes la Coquette, France) for 1 h at 4°C. Nonbiotinylated proteins in the supernatant of the first centrifugation were taken to be the intracellular material and retained for Western blot analysis. Bead-bound biotinylated proteins were washed in three buffers (buffer 1: 0.1% CHAPS, 150 mM NaCl 40 mM, 0.05% NaN_3, and 20 mM Tris-HCl pH7.5; buffer 2: 0.05% CHAPS, 0.1% SDS, 300 mM NaCl, and 10 mM Tris-HCl pH 8; buffer 3: 0.1% CHAPS and 20 mM Tris-HCl pH 7.4). Biotinylated proteins were then resuspended in 60 μl Laemmli buffer 2×, and 40 μl of the nonbiotinylated proteins were mixed with 20 μl Laemmli buffer 6×. These two samples were boiled for 5 min and analyzed by Western blot.

Deglycosylation Treatment

Forty microliters of M8 transfectants lysed in 0.5% CHAPS; 50 mM Tris-HCl pH 7.5 were deglycosylated, using peptide-N-glycosidase F from flavobacterium, according to Sigma's instructions (Sigma-Aldrich). The deglycosylated proteins were examined by Western blot analysis.

Western Blot Analysis

Proteins were loaded and separated on 12% Tris-glycine-SDS polyacrylamide gels and electroblotted to nitrocellulose membranes (Hybond, Amersham Biosciences, Orsay, France). The membranes were probed with an HLA-G-specific antibody (4H84, MEM-G/04), then revealed with peroxidase-conjugated sheep anti-mouse IgG Ab (Sigma-Aldrich). Western blots were developed by chemiluminescence (Amersham).

Cytotoxicity Assays

The cytolytic activity of the NKL cells and YT2C2 cells used as effectors (E) was assessed in 4-h ^{51}Cr release assays in which the effector cells were mixed with 5×10^3 ^{51}Cr-labeled transfected-M8 or U937 (T) (100 μCi ^{51}Cr sodium chromate; 1 Ci = 37 Gbq; Amersham) at various E:T ratios, as previously described [4]. The percentage specific lysis was calculated as follows: % specific lysis = [(cpm experimental − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)] × 100. Spontaneous release was determined by incubation of labeled target cells in RPMI 1640 medium supplemented with 10% FCS. Maximum release was determined by solubilizing target cells in 0.1 N HCl. In all experiments, spontaneous release was less than 10% of maximum release.

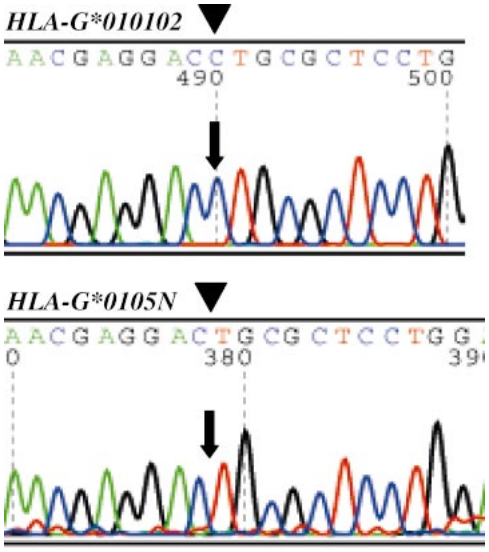

FIG. 2. DNA sequencing electropherogram of the *HLA-G*010102* allele (**A**) and before deletion of the cytosine in exon 3 and after directed mutagenesis, yielding the *HLA-G*0105N* allele (**B**).

RESULTS

*Construction of HLA-G*0105N Null Allele Genomic DNA*

To study proteins encoded by the *HLA-G* null allele, we generated a DNA sequence identical to *HLA-G*0105N* in the p*cDNA 3.1* expression vector. In vitro site-directed mutagenesis of *HLA-G*010102* genomic DNA allowed us to delete the cytosine at position 815 from ATG (exon 1), yielding the genomic DNA of the *HLA-G*0105N* allele. We used the *HLA-G*010102* rather than the *HLA-G*010101* reference sequence since *HLA-G*010102* and *-G*0105N* have identical DNA sequences with the exception of the cytosine deletion at codon 130 [14]. The *Xba*I-*Eco*RV *HLA-G*010102* fragment linked into the p*cDNA3.1* vector used as the template is represented in Figure 1, and the electropherogram of DNA sequences before and after directed mutagenesis demonstrating deletion of the cytosine in the newly synthesized DNA is shown Figure 2, the remaining sequence being identical. The genomic *HLA-G*0105N* DNA presented a deletion at codon 130 (CTG → TGC), which generated a stop codon 171 nucleotides further on at codon 189 (GTG → TGA) in exon 4 or 495 nucleotides further on at codon 297 (GTA → TAG) in exon 5. At this step, we had obtained a copy of the *HLA-G*0105N* allele of 3932 nucleotides, beginning 460 bp upstream from exon 1 and ending 541 bp downstream from exon 8.

*Pattern of mRNA Expression in HLA-G*0105N-Transfected Cells*

Real-time RT-PCR was carried out on mRNA prepared from M8 cells transfected with both genomic *HLA-G*0105N* and *HLA-G*010102* as well as mRNA from JEG-3 cells (Fig. 3). These results revealed that higher levels of HLA-G transcripts were produced in both M8-*HLA-G*0105N* and M8-*HLA-G*010102* than in the JEG-3 cells (JEG-3 is taken as a reference since it is one of the rare cell lines that constitutively expresses HLA-G proteins). HLA-G transcript levels measured in the M8-transfected cells varied according to the particular HLA-G sequence

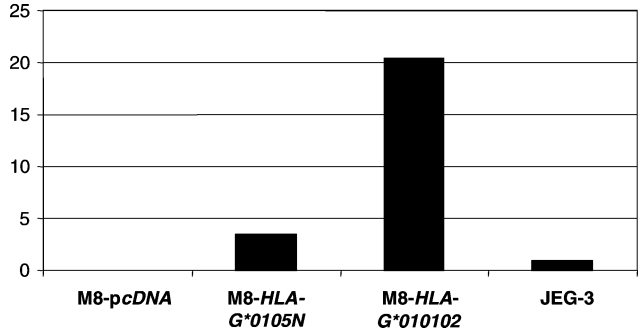

FIG. 3. Results of real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis showing relative quantities of HLA-G transcripts in M8-*HLA-G*0105N,* M8-*HLA-G*010102,* and M8-p*cDNA* compared with that of JEG-3 cells (assigned a value of 1).

transfected, lower levels being found for M8-*HLA-G*0105N* than for M8-*HLA-G*010102* (Fig. 4). To determine the mRNA pattern of M8-*HLA-G*0105N*, we carried out HLA-G-specific RT-PCR and radioactive hybridization reactions comparing M8-*HLA-G*0105N* with M8-*HLA-G*010102*. The specific G.927 probe, located in exon 5, was used to hybridize all PCR products except those of G.257/G.i4, which were hybridized using the G.R probe, located in exon 2. The G.257F/G.1004R primers disclosed four bands, corresponding to the principal mRNAs: *HLA-G5* (889 bp), *-G1* (767 bp), *-G2* and *-G4* (491 bp), and *-G3* (215 bp) (Fig. 4a). *HLA-G2* was amplified using the G.-3/G.1216 primers (627 bp) (Fig. 4b) and *HLA-G3* with the G.-3-4/G.1216 primers (341 bp) (Fig. 4c). The G.257/G.i4 primers allowed amplification of *HLA-G5* (765 bp) and *HLA-G6* (489 bp) (Fig. 4d) . We thus confirmed that all HLA-G mRNA had been transcribed in M8-*HLA-G*0105N* cell line.

*HLA-G Proteins Are Translated in HLA-G*0105N-Transfected Cells*

The proteins synthesized by the *HLA-G*0105N* allele have been deduced from its nucleotide sequence [14]. According to these authors, HLA-G isoform amino-acid sequences deduced from the *HLA-G*0105N* DNA sequence would yield the complete membrane-bound HLA-G2 and -G3, as well as the soluble HLA-G6 and -G7 isoforms. HLA-G1, -G4, and -G5 undergo a reading frame shift, and the corresponding proteins are prematurely truncated in the null allele.

To detect HLA-G proteins that were expressed by M8-*HLA-G*0105N* in our experiments, we carried out immunocytochemistry assays on permeabilized cells. As may be seen in Figure 5, M8-*HLA-G*0105N* cells were positively stained by 4H84, an antibody that targets the α1 domain present in all HLA-G isoforms (Fig. 5b); by MEM-G/04, an antibody that recognizes the α3 domain (Fig. 5f); and by 5A6G7, an antisoluble antibody against HLA-G5 and -G6) (Fig. 5n). As expected, the presence of HLA-G1 or -G5 was not detected in M8-*HLA-G*0105N* with the MEM-G/09 antibody (Fig. 5j). All these anti-HLA-G antibodies had positively bound M8-*HLA-G*010102*, a genomic HLA-G sequence in which HLA-G1 is predominantly expressed, but one would expect the other isoforms to be present (Fig. 5, c, g, k, and o). The HLA-G2 isoform was stained by both the 4H84 and the MEM-G/04 antibodies in M8-*HLA-G2* cells (Fig. 5, d and h). Collectively, these immunocytochemical analyses revealed the presence of HLA-G2 and -G6 isoforms in M8-*HLA-G*0105N*.

Using flow cytometry and the MEM-G/09 antibody, we confirmed that whereas M8-*HLA-G*0105N* did not express HLA-G1 protein at the cell surface, M8-*HLA-G*010102* did (Fig. 6). Because of the unavailability of antibodies able to bind HLA-G2 and HLA-G3 in the flow cytometry assay, we were unable to identify these membrane-bound isoforms, which could have been translated by M8-*HLA-G*0105N* cells.

*Characterization of HLA-G Proteins in HLA-G*0105N-Transfected Cells*

In succeeding experiments, we attempted to characterize the HLA-G proteins that had been translated in M8-*HLA-G*0105N* cells, using M8-*HLA-G*010102* and M8-*HLA-G2* as positive controls. We used immunoprecipitation of biotinylated cell-surface proteins to achieve separation of membrane-bound and intracytoplasmic proteins. The results revealed membrane-bound HLA-G2 protein on M8-*HLA-G2* (bands at 30 and 27 kDa) and HLA-G1 protein on M8-*HLA-G*010102* (one band at 43 kDa), but no HLA-G molecules could be detected in M8-*HLA-G*0105N* by this method (Fig. 7A). However, study of the intracellular pro-

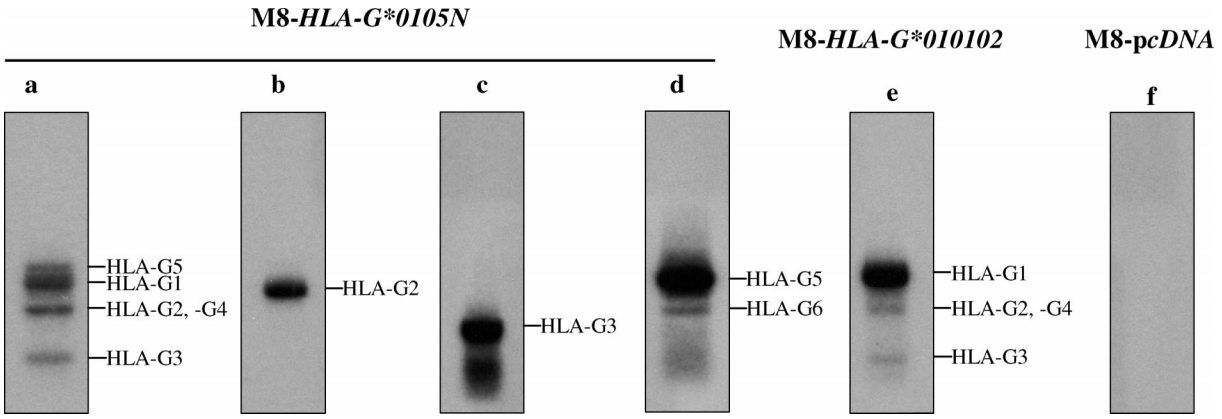

FIG. 4. Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot characterization of *HLA-G* mRNA isoforms in M8-*HLA-G*0105N* (**a**–**d**). PCR amplifications are carried out using pan-*HLA-G* primers G.257F/G.1004R (**a**, **e**, **f**); primer set G.-3F/G.1216R, specific for *HLA-G2* (**b**); primer set G.-3–4F/G.1216R, specific for *HLA-G3* (**c**); and primer set G.257F / G.i4R, specific for both *HLA-G5* and *-G6* (**d**). M8-*HLA-G*010102* (**e**) and M8-p*cDNA* (**f**) are, respectively, the positive and negative controls. Southern blots **a**, **b**, **c**, **e**, and **f** were hybridized with G.927R (exon 5), and blot **d** was hybridized with G.R (exon 2) probes.

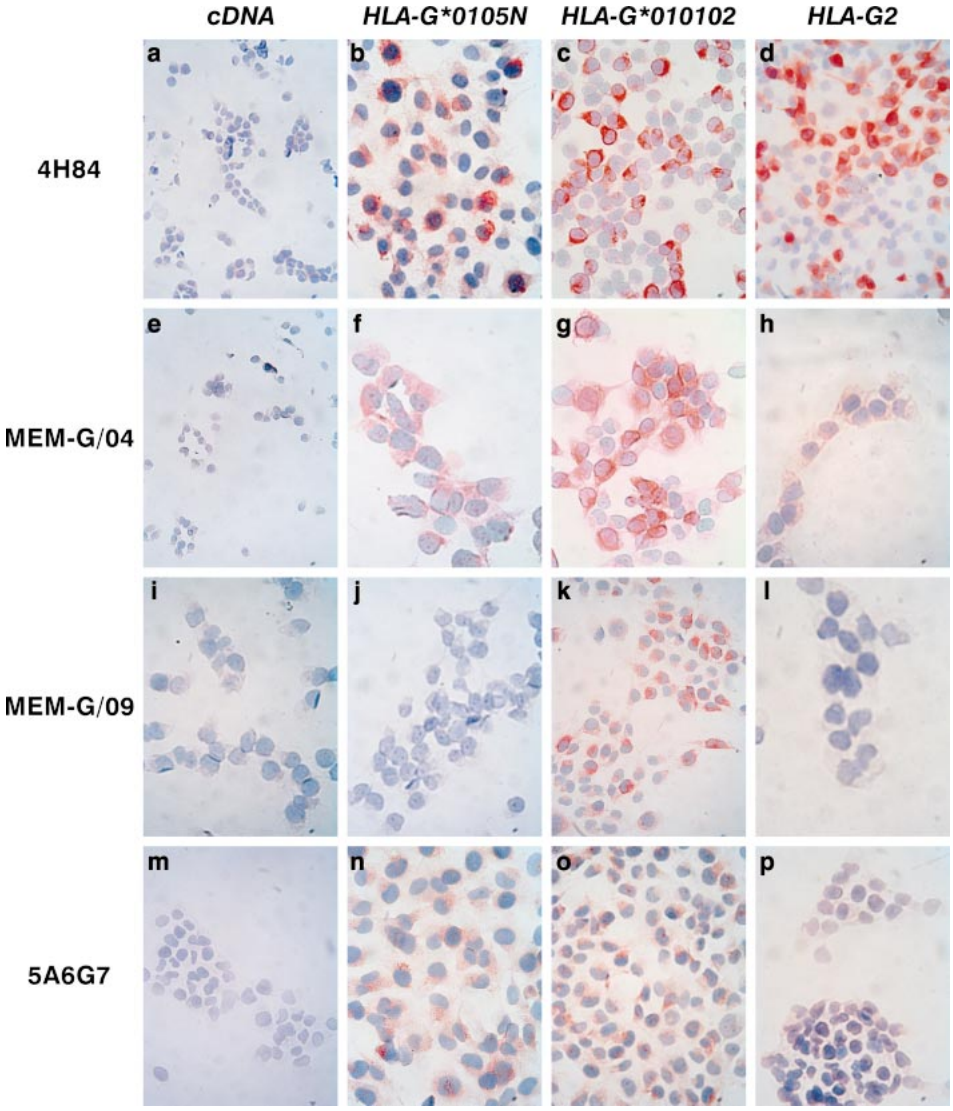

FIG. 5. Immunocytostaining of M8 transfectants with anti-HLA-G antibodies 4H84 (**a**–**d**), MEM-G/04 (**e**–**h**), MEM-G/09 (**i**–**l**), and 5A6G7 (**m**–**p**). All antibodies are negative in the control M8-p*cDNA* (**a**, **e**, **i**, **m**). The anti-HLA-G1, -G2, -G5, -G6 antibody MEM-G/04 is positive in all *HLA-G* transfected cells (**f**–**h**). The anti-HLA-G1, -G5 antibody MEM-G/09 stained M8-*HLA-G*010102* (**k**) but not M8-*HLA-G*0105N* (**j**) or M8-*HLA-G2* (**l**). The anti-HLA-G5, -G6 antibody 5A6G7 stained M8-*HLA-G*0105N* (**n**) and M8-*HLA-G*010102* (**o**) but not M8-*HLA-G2* (**p**). Original magnification ×200.

teins revealed the presence of HLA-G isoforms. We obtained three bands, one at 30 kDa and one at 27 kDa, that were identical to those produced by M8-*HLA-G2*, plus one located above them, at 35 kDa, that has never been characterized (Fig. 7B). To determine whether this latter band corresponded to hyperglycosylated HLA-G2, we treated the M8-*HLA-G*0105N* cells with PNGase F, an enzyme that catalyzes the removal of N-linked oligosaccharide chains from glycoproteins (Fig. 8). This enzyme treatment revealed a smaller band just below that of the mature protein, corresponding to the deglycosylated protein. This finding indicated that M8-*HLA-G*0105N* had indeed translated some glycoproteins, but the molecular weight obtained for the 35-kDa protein did not correspond to any known HLA-G isoform. Staining with 4H84 and MEM-G/04 antibodies allowed us to determine that at least the α1 and α3 domains were present in this protein, which remains to be characterized.

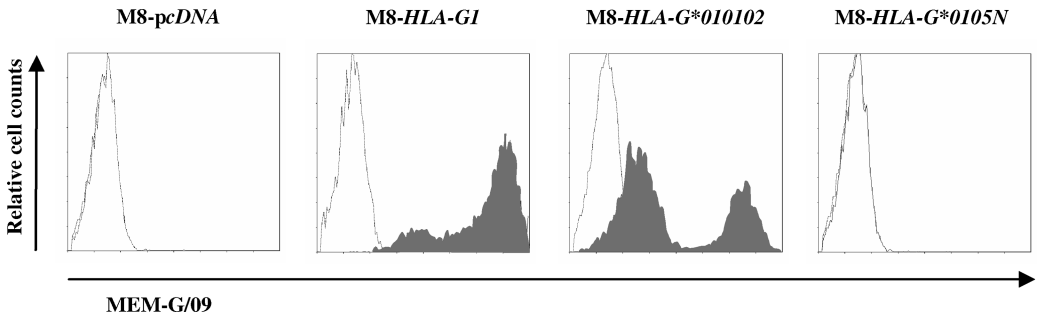

FIG. 6. Detection by flow cytometry analysis of the HLA-G1 isoform on M8-*HLA-G*010102* but not on M8-*HLA-G*0105N*. Cells were labeled by indirect immunofluorescence with MEM-G/09 mAb (bold profiles) and an isotope-matched control Ab (light profiles). M8-p*cDNA* and M8-*HLA-G1* cells were used as controls.

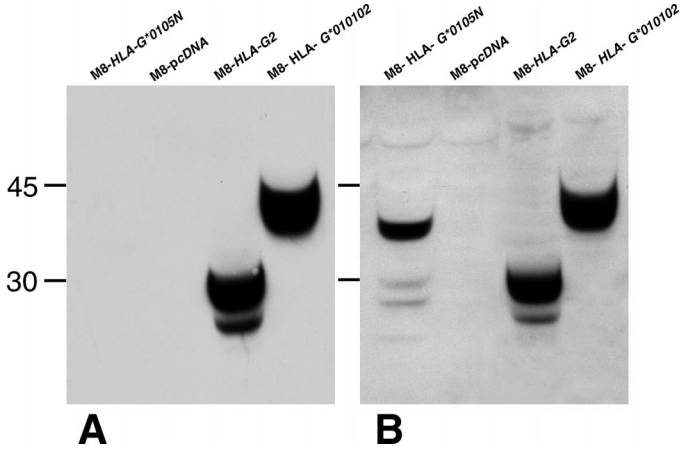

FIG. 7. The *HLA-G*0105N* allele produced intracytoplasmic HLA-G proteins. Cell-surface proteins are biotinylated and separated from the nonbiotinylated inside proteins. Immunostaining with both 4H84 and MEM-G/04 mAbs revealed the intracellular presence of HLA-G proteins (**B**) but not on the cell surface (**A**). The mechanism providing the additional smaller band for HLA-G2 (i.e., band at 27 kDa) remains to be determined.

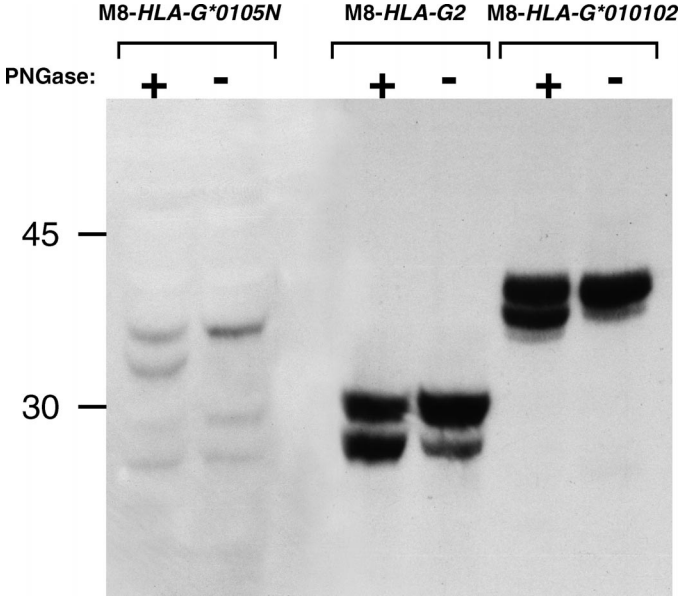

FIG. 8. *HLA-G*0105N* isoforms are translated as glycoproteins. Lysate from M8 transfectants were treated with PNGase F allowing visualization of mature and deglycosylated proteins. Western blots were stained with 4H84 mAb. PNGase F treatment removed oligosaccharides from the glycoproteins. Revelation of two bands (glycosylated and not) indicated that this treatment was not complete. M8-HLA-G*0105N presents two weak bands, at 30 and 27 kDa (representing glycosylated and nonglycolysated HLA-G2) and one unidentified band at 35 kDa, which migrated at 31 kDa after PNGase F treatment.

*HLA-G*0105N-Transfected Cells Are Protected from NK Cell-Mediated Cytotoxicity*

To determine whether the *HLA-G*0105N* allele translated functional HLA-G isoforms, cytotoxic assays were carried out confronting either the NKL or the YT2C2 cell line [17, 19] used as the effector with the M8 or U937 transfectant cells used as the target (Fig. 9). When stimulated with interleukin-2, the NKL human NK cell line retained most of the original features of NK cells, including very similar proliferative responses and natural killing function. While YT2C2 express only KIR2DL4, NKL cells express both ILT2 and KIR2DL4 inhibitory receptors [18, 26], which are implicated in the NK-cell cytotoxicity pathways, by binding to HLA-G molecules. The lytic activity of NKL against M8-*HLA-G*0105N* was compared with its lytic activity against M8-*HLA-G*010102* cells, which at least express HLA-G1, as well as against M8-*HLA-G2* cells, which express only the HLA-G2 isoform, and against M8-p*cDNA*, used as the HLA-G-negative control. Cytotoxicity experiments showed that in addition to M8-*HLA-G2*, both genomic M8-*HLA-G*0105N* and M8-*HLA-G*010102* were protected from NKL lysis. Lysis of M8-transfectants was reduced by 50% compared with that of the M8-p*cDNA* control cell line. Inhibition of cytotoxicity was also obtained using YT2C2 cells, which only express the HLA-G-specific receptor against U937-transfectants.

DISCUSSION

After our study of the tolerogeneic role of HLA-G in maternal-fetal tolerance [27, 28], we designed experiments to identify the structural and functional characteristics of proteins generated by the *HLA-G*0105N* allele. First, we used site-directed mutagenesis to construct the *HLA-G*0105N* from the nucleotide sequence of *HLA-G*010102* and transfected it into the M8 human cell line. Then we characterized the transcripts and proteins generated by the *HLA-G*0105N* gene before analyzing the functional capacity of the isoforms produced.

Although the deletion of one base in exon 3 of *HLA-G*0105N* disrupts the reading frame, it should have no effect on transcription of the primary transcript or on its alternative splicing [14]. Indeed, in our RT-PCR analysis, the *HLA-G* mRNA profile was the same in both M8-*HLA-G*0105N*- and M8-*HLA-G*010102*-transfected cells, indicating the presence of all *HLA-G* mRNA transcripts normally found. Thus, deletion of the cytosine in codon 130 did not block the transcription and splicing mechanisms in the null allele. Nevertheless, real-time and classical RT-PCR showed that *HLA-G* mRNA levels were lower in M8-*HLA-G*0105N* than in M8-*HLA-G*010102* cells, and classical PCR revealed that the *HLA-G1*/*HLA-G2* mRNA ratio was lower in M8-*HLA-G*0105N* than in M8-*HLA-G*010102*. This discrepancy in the expression level of HLA-G genotypes and isoforms has already been reported for alleles other than the null allele in pathological pregnancies in which altered *HLA-G* transcription was associated with certain *HLA-G* genotypes [29, 30] as well as in a study of the role of *HLA-G* alleles in determining the soluble HLA-G protein plasma level [31]. Another explanation is that nonsense-mediated decay, a eukaryotic regulatory process that degrades mRNA with premature termination codons, could be responsible for the higher degradation of HLA-G1 transcripts in cells expressing the null allele compared with other alleles [32].

Furthermore, the higher levels of mRNA in transfected cells compared with JEG-3 cells could be explained by the fact that 1) in transfected cells, *HLA-G* is ligated in the p*cDNA3.1* plasmid, which possesses the CMV promoter and is thus able to influence and perhaps increase *HLA-G* mRNA expression, and 2) *HLA-G*010102* has been described as a much higher ''HLA-G secreting'' allele than either *HLA-G*0105N* or *HLA-G*010103* (carried by JEG-3 cells [33]) [31].

Finally, although mRNA levels are higher in M8-transfected cells than in JEG-3 cells, this cannot be correlated with either the quality or the quantity of the corresponding

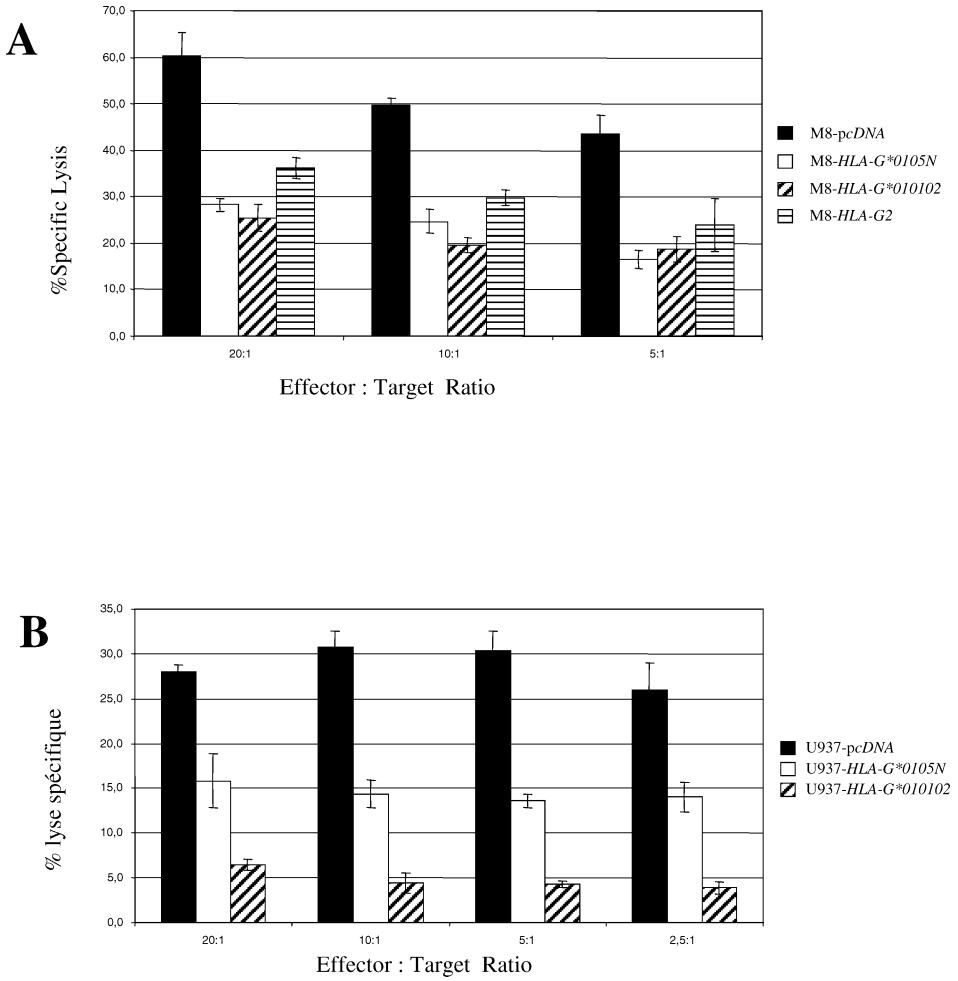

FIG. 9. M8-*HLA-G*0105N* is able to inhibit NKL cell cytolytic activity. Chromium release assays were carried out using M8- or U937-p*cDNA,* M8- or U937-*HLA-G*0105N,* M8- or U937-*HLA-G*010102,* or M8-*HLA-G2* as target cells and NKL cell line (**A**) or YT2C2 cell line (**B**) as effectors. Results are expressed as the percentage lysis recorded in a 4-h ^{51}Cr-release assay. Values represent means of triplicate ± SD. This experiment is representative of five distinct experiments.

translated isoform proteins. Thus, the mRNA level is lower in M8-*HLA-G*0105N* cells than in M8-HLA-G010102 cells (Fig. 3), and both M8 transfectants are equally protected from NKL-mediated cytolysis (Fig. 9A).

Since the presence of *HLA-G* mRNA had been demonstrated in M8-*HLA-G*0105N,* we also looked for HLA-G proteins in it. For this purpose, *HLA-G*0105N*-transfected cells were immunostained with specific HLA-G antibodies targeting either all HLA-G proteins or specific isoforms. As expected from the deletion in *HLA-G*0105N,* neither the HLA-G1 nor the HLA-G5 isoform was detected in M8-*HLA-G*0105N* since these cells were not stained by the MEM-G/09 mAb, which is specific for HLA-G1 and HLA-G5. The absence of HLA-G5 and of shed HLA-G1 was confirmed by a specific ELISA (data not shown). Interestingly, the other mAbs that recognized either all HLA-G isoforms (4H84), both HLA-G5 and -G6 (5A6G7), or HLA-G1, -G2, -G5, and -G6 (MEM-G/04) were positive in M8-*HLA-G*0105N,* demonstrating that in the absence of both HLA-G1 and -G5, other HLA-G isoforms are produced. These isoforms may correspond to at least the HLA-G2 and/or HLA-G6 proteins. In support of this observation, previous immunohistochemical analyses of placentas homozygous for *HLA-G*0105N* presented negative staining with an anti-HLA-G1, -G5 antibody (87G) and positive staining with an anti-HLA-G1, -G2 antibody (15C6) [15]. These results confirm that HLA-G proteins are indeed translated in *HLA-G*0105N* cells.

To further characterize the HLA-G isoforms produced by M8-*HLA-G*0105N* cells, we carried out immunoprecipitation and Western blot experiments. Under our experimental conditions, proteins at 35 and 30 kDa were detected within such cells but not on the cell surface. However, we cannot exclude that HLA-G proteins are secreted by these cells and that they exercise biological effects. To address this point, we carried out experiments aimed at analyzing the role of the HLA-G proteins generated by M8-*HLA-G*0105N* with respect to NK function and tested their capacity to inhibit NK cell lysis.

Considered together, these results demonstrate that non-HLA-G1/-G5 isoforms of the *HLA-G*0105N* allele participate in the inhibition of NK cells. This inhibition is perhaps not mediated by HLA-E or other HLA class I molecules, since *HLA-G*0105N*-transfected cells were protected from lysis by the NK-like clone Y2T2C2. Indeed Y2T2C2 expresses the HLA-G-specific receptor KIR2DL4, but not HLA-A-, -B-, -C-, and -E-specific KIRs.

The *HLA-G*0105N* allele is estimated to have appeared 18 000 yr ago, leading to the conclusion that it had been positively selected during evolutionary history [16]. A high incidence of the null allele has notably been reported in Africa [34]. A possible explanation for this selection is that it may be due to the high incidence of intrauterine pathogens among the Africa population, which therefore has to maintain an efficient immune system to eliminate uterine infections [16]. One would expect that the absence of HLA-G1 and -G5 in *HLA-G*0105N* individuals would serve to maintain the immunocompetence of maternal NK and T cells at the maternal-fetal interface [15, 16]. Weighing the trade-off between increased risk of miscarriage due to the

absence of HLA-G1 and -G5 but low risk of uterine infection and successful outcome of pregnancy but high risk of womb infection, *HLA-G*0105N* was selected over HLA-G1, -G5, thus eradicating intrauterine pathogen contamination despite the fact that the null genotype is a contributing factor in spontaneous abortion [35, 36]. Our results suggest that *HLA-G*0105N* would be of interest for purposes other than merely preventing interactions between HLA-G1, -G5 and inhibitory NK receptors since HLA-G2 has maintained that function. The advantages of the selection of this allele must be further investigated.

The emergence of the *HLA-G*0105N* homozygous gene is not the only condition under which HLA-G protein expression has been found to be impaired. As for classical HLA-class I molecules, both HLA-G1 and -G5 contain three globular domains associated with β2m and present peptides. Their conformation renders these HLA molecules TAP and β2m dependent. Therefore, in TAP- or β2m-deficient individuals, NK-mediated reactions may still occur, and NK cells may retain the ability to reject allogeneic and autologous HLA-class I-negative cells. However, studies of such individuals showed that HLA-class I-deficient cells remain protected from NK lysis. Notably, there are other situations in which expression of both HLA-G1 and -G5 isoforms would also be altered without negatively affecting the outcome of pregnancy. One example is the survival of homozygous TAP-negative children in whom one would expect HLA-G1/-G5 placental expression to be impaired. Indeed, expression of these two isoforms as a trimolecular α-chain/β2m/peptide complex is TAP dependent. In the fetuses of such TAP-negative children, the other HLA-G isoforms, whose expression may be independent of peptide loading via TAP, may substitute for the loss of HLA-G1/-G5, thus contributing to the survival of these fetuses [37].

In another study, functional analyses indicated that $TAP^{-/-}$ NK cells were unable to kill autologous class I-negative cells. This inhibition was attributed to an unknown inhibitory receptor capable of binding ligand expressed by targeted autologous class I-negative cells, thereby down-regulating NK cell cytotoxicity [38]. Considered together, these studies suggest that still-unknown mechanisms prevent an attack against autologous normal cells that express insufficient quantities of HLA-class I molecules. Therefore, the HLA-G2, -G3, -G6, and -G7 isoforms, in which cell processing and transport are probably peptide independent, might be candidate molecules to compensate for the loss of HLA class I antigens, including HLA-G1 and -G5. The fact that $TAP^{-/-}$ NK cells express an unidentified receptor suggests that this unidentified inhibitory receptor is specific for spliced HLA-G isoforms. These interpretations could be extrapolated to tumor cells, which often present down-regulation of classical and nonclassical HLA class I molecules. Expression of the TAP and β2m-independent spliced HLA-G isoforms (HLA-G2 through -G7) observed in some tumors may constitute a new escape mechanism from NK cell-mediated lysis [39].

Finally, our study provided information indicating that *HLA-G*0105N* does not encode the complete HLA-G1 or HLA-G5 isoforms but does encode functional HLA-G proteins able to inhibit NK cell cytolysis. Thus, although the biological functions of HLA-G1 and HLA-G5 proteins are abrogated, the other isoforms can assume their roles. Two different effectors were used against M8-transfectant cells: the NKL and YT2C2 cells. Since NKL cells expressed ILT2, KIR2DL4, and CD94/NKG2A receptors, lysis of *HLA-G*0105N* cells could be due to a direct interaction between HLA-G and ILT2 or KIR2DL4 receptors and/or to a indirect effect through HLA-E (whose surface expression is up-regulated in presence of HLA-G leader peptide) and CD94/NKG2A. Nevertheless, results obtained with the YT2C2 effector cells demonstrate that at least HLA-G proteins expressed by *HLA-G*0105N* inhibit NK lysis through direct interaction with KIR2DL4. Our results showing that *HLA-G*0105N* cells retained the capacity to inhibit NK cytotoxicity activity agree with the previously described inhibitory role played by the HLA-G2 and -G3 [4]. In support of a biological role for other HLA-G isoforms than -G1 and -G5, two recent reports found that 1) HLA-G2 and -G6 isoforms are expressed by specific subpopulations of trophoblast cells [40] and that 2) recombinant HLA-G6 induces TGF-β production by monocytic lineage cells [41].

Most classical HLA-class I genes are translated into one isoform. Therefore, a mutation in the gene yields a null allele, which results in the disappearance of this HLA molecule. A feature of the HLA-G gene is that it encodes seven isoforms, including some that are not altered in the null genotype (HLA-G2/-G3/-G6/-G7). Thus, *HLA-G*0105N* could maintain the presence of functional HLA-G proteins. In this respect, it would be important to determine the function of each HLA-G isoform and whether a specific role may be attributed to each of them in pregnancy as well as in tumors and transplants.

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