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Linking Two Immuno-Suppressive Molecules: Indoleamine 2,3 Dioxygenase Can Modify HLA-G Cell-Surface Expression[1]

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ABSTRACT

Nonclassical human leukocyte antigen (HLA) class I molecule HLA-G and indoleamine 2,3 dioxygenase (INDO) in humans and mice, respectively, have been shown to play crucial immunosuppressive roles in fetal-maternal tolerance. HLA-G inhibits natural killer and T cell function by high-affinity interaction with inhibitory receptors, and INDO acts by depleting the surrounding microenvironment of the essential amino acid tryptophan, thus inhibiting T cell proliferation. We investigated whether HLA-G expression and INDO function were linked. Working with antigen-presenting cell (APC) lines and monocytes, we found that functional inhibition of INDO by 1-methyl-tryptophan induced cell surface expression of HLA-G1 by HLA-G1-negative APCs that were originally cell-surface negative, and that in reverse, the functional boost of INDO by high concentrations of tryptophan induced a complete loss of HLA-G1 cell surface expression by APCs that were originally cell-surface HLA-G1-positive. This mechanism was shown to be posttranslational because HLA-G protein cell contents remained unaffected by the treatments used. Furthermore, HLA-G cell surface expression regulation by INDO seems to relate to INDO function, but not to tryptophan catabolism itself. Potential implications in fetal-maternal tolerance are discussed.

embryo, immunology, trophoblast

INTRODUCTION

Human reproduction begins by the uniting of paternal and maternal gametes, yielding a fetus that is semiallogeneic with respect to the maternal body in which it will develop. The immunogenetically different fetus should elicit immune responses by the potentially hostile maternal organism. In fact, pregnancy is a prime example of immunotolerance. Although at first glance this maternal tolerance to the semiallogeneic fetus appears to transgress the classical rules of immunological responses, when fetal cells are confronted with maternal immunity, classical immune responses are subdued or suppressed. Several mechanisms have been put forth to explain fetal-maternal tolerance, among which are expression of immunosuppressive nonclassical human leukocyte antigen (HLA) class I molecule G (HLA-G) by fetal tissues, and expression of the tryptophan-degrading enzyme indoleamine 2,3 dioxygenase (INDO).

HLA-G is a nonclassical class I HLA whose expression in nonpathological situations is tissue-restricted to extravillous cytotrophoblast, thymic epithelial cells, and cornea [1–3]. *HLA-G* is characterized by a low genomic polymorphism of 15 alleles, as well as by 4 membrane-bound and 3 soluble isoforms (HLA-G1 to HLA-G4, and HLA-G5 to HLA-G7, respectively) that are generated by alternative splicing of a unique primary transcript [4–8]. HLA-G1 is the HLA-G isoform that has been investigated the most. HLA-G1 has a structure similar to that of classical HLA class I molecules: a heavy chain constituted of three extracellular globular domains noncovalently associated with beta-2-microglobulin, and a nonameric peptide. Functional assays demonstrated that both soluble and membrane-bound HLA-G isoforms were able to inhibit allogeneic proliferation of T cells [9, 10], natural killer (NK) cell cytotoxicity [11–15], and antigen-specific T cell cytotoxicity [16, 17]. HLA-G exerts its inhibitory functions via interaction with three inhibitory receptors: LILRB1 (ILT2/CD85j), LILRB2 (ILT4/CD85d), and KIR2DL4 (CD158d) [18–21]. LILRB1 is broadly expressed by lymphoid and myeloid cells, whereas expression of LILRB2 is myeloid-specific, and expression of KIR2DL4 is restricted primarily to NK cells. LILRB1 and LILRB2 can interact with classical HLA class I molecules, but they have a higher affinity for HLA-G [22], whereas HLA-G is the only known ligand of KIR2DL4.

HLA-G has been associated with fetal-maternal tolerance in humans. HLA-G is expressed as early as the blastocyst stage and is crucial for implantation and early fetal-maternal tolerance [12, 23]. Indeed, studies performed in embryos following in vitro fertilization have demonstrated that clinical pregnancy is obtained only if the growing embryos can produce soluble HLA-G [24]. Furthermore, 1) recurrent spontaneous abortion and preeclampsia have been associated with decreased expression/secretion of HLA-G by fetal tissues [25–28]; and 2) *Herpes simplex* virus infection, which is linked to spontaneous abortion, has been shown to block HLA-G intracellular transport to the cell surface [29]. HLA-G expression by fetal tissues persists throughout human pregnancy, but its expression by extravillous cytotrophoblast is highest during the first trimester of development, and might decline thereafter [30].

INDO is an inducible enzyme that catalyzes tryptophan degradation to formyl-kynurenine, which is then rapidly metabolized into kynurenine [31]. Until recently, this enzyme has been described as part of innate immunity, par-

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ticipating in antimicrobial defense by limiting the growth of parasites, bacteria, and viruses [32]. By catalyzing tryptophan degradation, INDO depletes the local microenvironment of this essential amino acid form and limits the growth of these pathogens. Studies by Munn et al. [33] have shown that INDO plays an important immunosuppressive role in mice. The blockade of this enzyme with the competitive inhibitor 1-methyl-tryptophan (1MT) during murine pregnancy results in maternal T cell-mediated rejection of allogeneic but not syngeneic conceptuses at the ninth day, but not at the sixth day, of murine pregnancy. The fetal allograft rejection caused by INDO inhibition is related to stimulation of innate immunity [34]. It is interesting to note that although INDO inhibition causes abortion in mice, an INDO-knockout mouse has been generated and does not differ from controls with respect to pregnancy success, leading to the hypothesis that other immunosuppressive mechanisms came into play [35]. The relationship between pregnancy and INDO activity has not been clearly established in humans, but some data suggest it can play a role in fetal-maternal tolerance. Indeed, INDO is expressed at very low levels in blastocysts [36], but its expression by placenta increases over time from the first to the third trimester of pregnancy [37]. Using two different antibodies, INDO expression was evidenced by two independent studies in syncytiotrophoblast, and one of these antibodies also stained in invasive extravillous cytotrophoblast [38]. Furthermore, reduced INDO expression levels have been associated with preeclampsia [39], and INDO transcription has been found in monocytes from pregnant women but not in normal controls [40]. Studies in a murine model have shown that INDO immunosuppressive function can be used by tumor cells to evade antitumoral immunity [41]. However, the mechanisms by which INDO exerts its immunoregulatory functions seem to extend beyond a mere nutrient depletion, because tryptophan degradation metabolites also play a role in the regulation of immune function, probably through proapoptotic mechanisms. Indeed, it has been shown that kynurenine and 3-hydroxyanthranilic acid [42] can cause apoptosis of TH2 cells through a process that seems to be mediated by oxygen free radicals.

We investigated whether HLA-G expression and INDO function were linked. We chose to work with monocytes and monocytic cell lines because these are known to strongly express INDO after recombinant human interferon-γ (IFNG) treatment [43] and because among easily accessible cells, monocytes are the subset in which HLA-G seems to be the most likely to be expressed. Indeed, 1) cell surface HLA-G expression by 4%–10% of monocytes from healthy donors has been reported, although this is still controversial; 2) HLA-G expression in monocytes could be up-regulated in vitro by IFNG, interleukin-10, and glucocorticoids; and 3) in vivo, HLA-G is expressed by monocytes that infiltrate tumors and transplants during the course of autoimmune diseases, and after viral infections (reviewed in [23]). We found that inhibition of INDO function by 1MT induced HLA-G cell surface expression in monocytes and antigen-presenting cell (APC) lines. On the other hand, INDO functional boost in cell surface HLA-G-positive monocytes and APC lines induced a complete loss of this HLA-G cell surface expression. INDO-related alterations of HLA-G cell surface expression occurred without changes in total HLA-G cellular content, thus arguing in favor of a posttranslational mechanism. Finally, INDO function, but not tryptophan catabolism, seems to be responsible for HLA-G cell surface expression regulation.

MATERIALS AND METHODS

Cells and Cell Lines

The following human cell lines were used: U937 histiocytic lymphoma (American Type Culture Collection, Manassas, VA), THP-1 acute monocytic leukemia (American Type Culture Collection), HLA-G1 and HLA-G5-transfected melanoma lines M8-HLA-G1 and M8-HLA-G5 [44], naturally HLA-G-expressing melanoma line FON, and HLA-G-expressing choriocarcinoma line JEG-3 (American Type Culture Collection). HLA-G-positive and HLA-G-negative U937 and THP-1 cells arose from bulk culture and were sorted according to cell surface HLA-G expression using anti-HLA-G-coated magnetic beads (Dynal Biotech France, Compiegne, France). Differentiation of THP-1 cells into macrophages was achieved by treatment with phorbol ester miristate during 72 h as previously described [45]. For purification, monocytes and peripheral blood mononuclear cells were first isolated on Ficoll Hypaque (Sigma, St. Louis, MO), and monocytes were separated after adherence on plastic plates.

Cell Culture and Treatments

All cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 1 μg/ml gentamicin and fungizone (Sigma). When indicated, cells were treated with 500 IU IFNG (Sigma), 10 μg/ml LPS (Sigma), 1 mM tryptophan (Sigma), 1 mM 1MT, 1 mM kynurenine (Sigma), or a combination of these. Treatment of APCs with IFNG boosts INDO expression as well as INDO function, and even more so if IFNG is combined with lipopolysaccharide (LPS) treatment [43, 46, 47]. Similarly, increasing tryptophan concentrations in the culture medium increases INDO function and production of kynurenine [48]. 1MT is a competitive inhibitor of tryptophan with respect to INDO function and blocks this metabolic pathway [49].

Antibodies, Flow Cytometry, and Immunohistochemistry

For flow cytometry and immunohistochemistry we used the following mouse monoclonal antibodies: biotinylated anti-pan HLA class I W6/32 (Leinco Technologies, St Louis, MO), anti-HLA-A,-B,-C,-E TP25.99 (a kind gift from Dr. S. Ferrone, Roswell Park Cancer Institute, Buffalo, NY), anti-HLA-G1/HLA-G5 MEMG/09 (Exbio, Praha, Czech Republic), anti-pan HLA-G 4H84 (a kind gift from Dr. McMaster, University of California at San Francisco, San Francisco, CA), anti-HLA-G5,-G6 5A6G7B12 [50], anti-INDO (Chemicon, Temecula, CA), and anti-beta-2-microglobulin (Boehringer-Mannheim, Mannheim, Germany) in combination with secondary antibodies phycoerythrin-conjugated goat anti-mouse immunoglobulin G (IgG; Immunotech, Marseille, France) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (DAKO, Hamburg, Germany). For flow cytometry analyses, Fc receptors were blocked by a 30-min incubation in 25% human serum, and isotypic controls were systematically included. Analyses were carried on an EPICS XL cytometer (Beckman Coulter, Krefeld, Germany) using the Expo32 software (Beckman Coulter). Analysis of HLA-G and INDO expression by immunohistochemistry was carried out on cytospins (Labonord, Templemars, France). The methodology for performing HLA-G immunohistochemistry is described elsewhere [51]. The positive control was M8-HLA-G5 cells. Immunohistochemistry for INDO was performed on cytospin slides using Ultratech HRP (Immunotech). Slides were rehydrated for 5 min in PBS containing 0.1% Triton X-100. Nonspecific binding was prevented by applying protein blocking agent for 10 min before staining with anti-INDO monoclonal antibody (mAb) at a dilution of 1:150 for 30 min at room temperature. An isotype-matched antibody was used under similar conditions to control nonspecific staining. Cell immunostaining was evaluated on tissues cells using the DAKO EnVision + System, peroxidase (AEC; DAKO).

Enzyme-Linked Immunosorbent Assay

The presence of soluble HLA-G was evaluated using two different ELISA methodologies specific for either secreted HLA-G5 and shed HLA-G1, or for HLA-G5 only. An ELISA specific for HLA-G5 and shed HLA-G1 used anti-HLA-G MEM-G/09 as the capture antibody and anti-beta-2-microglobulin as the secondary antibody, and has been described elsewhere [50]. An ELISA specific for HLA-G5 used anti-HLA-G5 5A6G7B12 mAb as the capture antibody, biotinylated anti-pan class I W6/32 mAb as the secondary antibody, and streptavidin-HRP for revelation. The methodology for this ELISA has been published elsewhere [50].

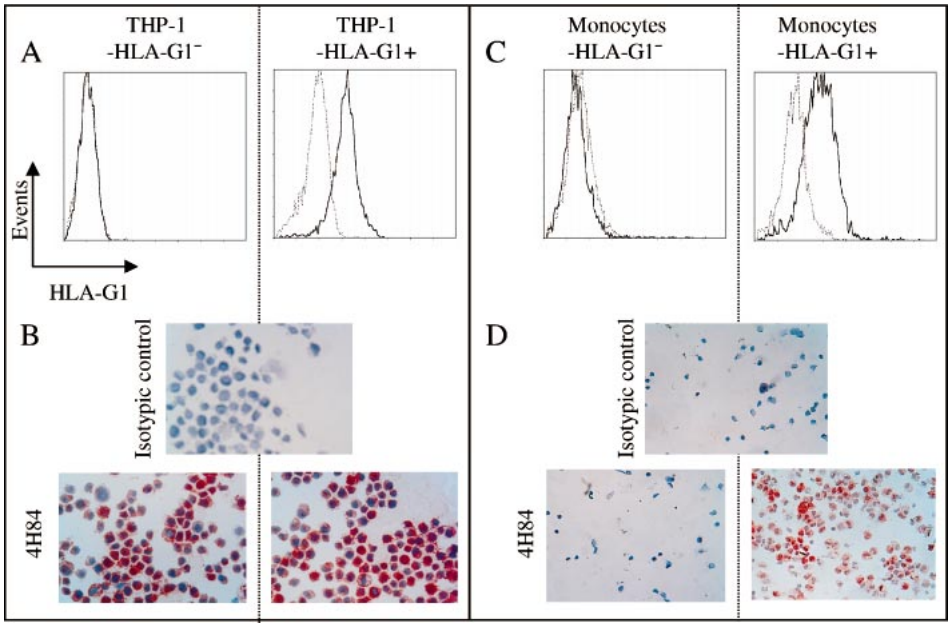

FIG. 1. HLA-G expression by cell surface HLA-G1-positive THP-1-HLA-G1$^+$ cells and monocytes-HLA-G1$^+$, and by cell surface HLA-G1-negative THP-1-HLA-G1$^-$ cells and monocytes-HLA-G1$^-$ cells. **A**) Cell surface of HLA-G1 in THP-1-HLA-G1$^-$ and THP-1-HLA-G1$^+$ cells by flow cytometry using anti-HLA-G1 MEM-G/09 mAb (plain lines) or isotypic control mAb (dotted lines). **B**) Expression of all HLA-G isoforms in THP-1-HLA-G1$^-$ or THP-1-HLA-G1$^+$ cells by immunohistochemistry using anti-pan-HLA-G mAb 4H84 (red staining). **C**) Cell surface of HLA-G1 in monocytes-HLA-G1$^-$ and monocytes-HLA-G1$^+$ cells by flow cytometry using anti-HLA-G1 MEM-G/09 mAb (dark lines) or isotypic control mAb (dotted lines). **D**) Expression of all HLA-G isoforms in monocytes-HLA-G1$^-$ and monocytes-HLA-G1$^+$ by immunohistochemistry using anti-pan-HLA-G mAb 4H84 (red staining). Original magnification ×10.

INDO Activity Assay

INDO activity was assessed using the method described by Yoshida et al. [52]. Briefly, frozen cell pellets were diluted in antiprotease buffer (Roche Molecular Diagnostics, Mannheim, Germany). Extracts were centrifuged at 13 000 × *g* for 10 min at 4°C and added to the assay reagent (100 mM potassium phosphate buffer pH 6.5, 40 mM ascorbate, 20 pM methylene blue, 200 μg/ml catalase, and 800 μM L-tryptophan). After 30 min of incubation at 37°C, the reaction was stopped by adding 200 μl of 30% (w/v) trichloroacetic acid. The solution was boiled for an additional 10 min and centrifuged at 2500 × *g* for 10 min. Kynurenine in the supernatant was measured in a high-performance liquid chromatography system with a Waters C18 column (4.5 mm × 15 cm). The mobile phase was 15 mM acetic acid-sodium acetate (pH 4.0) containing 27 ml/L acetonitrile, and kynurenine was detected by absorbance at 360 nm. One unit of the enzyme activity was defined as the amount that produced 1 nmol of kynurenine per hour. Protein was determined using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada) with BSA as the standard.

Western Blotting

Frozen cell pellets were diluted in antiprotease buffer (Roche). Extracts were centrifuged at 13 000 × *g* for 10 min at 4°C, and 10 μg of the total protein was run on 12% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany) by a Mini Trans-Blot Cell (Bio-Rad). Blots were incubated with either anti-INDO mAb (USBiological, Euromedex, Souffelweyersheim, France), or 4H84 antibody, which recognizes all isoforms of HLA-G, or 5A6G7B12, which recognizes secreted HLA-G5 and HLA-G6, or anti-beta-actin mAb (Abcam, Cambridge, United Kingdom). HRP-conjugated secondary antibody (Amersham Biosciences) was used, and the bands were visualized using an ECL+ Plus Western blotting detection system (Amersham Biosciences).

Statistical Analyses

To determine the significance of HLA-G expression differences before and after treatment, percentages of HLA-G-positive cells were compared before and after treatment with a positivity cutoff set using isotypic controls. Experiments performed more than six times were analyzed, and significance was assessed using the Fisher exact test. Differences were considered significant when $P < 0.05$.

RESULTS

Experimental Model: Unmodified HLA-G-Positive and HLA-G-Negative U937 and THP-1 APC Line; HLA-G-Positive and HLA-G-Negative Monocytes

To study the relationship between INDO and HLA-G, we searched for a cellular model in which HLA-G and INDO could be naturally expressed. Screening for HLA-G expression in APC lines revealed that monocytic U937 and THP-1 lines could express cell surface HLA-G in culture, in the absence of any treatment. Of note, THP-1 and U937 cell lines constitutively express INDO, but with a low basal activity (less than 12 nmol kynurenine per milligram of protein per hour; data not shown) and have been shown to express a low basal transcription level of HLA-G that can be up-regulated upon cytokine treatment [53]. U937 and THP-1 cultures were therefore divided into cell surface HLA-G1-negative U937 and THP-1 lines (U937-HLA-G1$^-$ and THP-1-HLA-G1$^-$), and cell surface HLA-G1-positive U937 and THP-1 lines (U937-HLA-G1$^+$ and THP-1-HLA-G1$^+$). Similarly, when screening for cell surface HLA-G-positive monocytes, we could identify a donor in which, exceptionally, all monocytes expressed cell surface HLA-G1 (thereafter named monocytes-HLA-G1$^+$). U937-HLA-G1$^-$ and U937-HLA-G1$^+$, THP-1-HLA-G1$^-$ and THP-1-HLA-G1$^+$, and monocytes-HLA-G1$^-$ and monocytes-HLA-G1$^+$ constituted our working experimental model. Expression of HLA-G1 for THP-1 cells and monocytes is illustrated in Figure 1.

1MT (INDO Functional Inhibitor) Induces Cell-Surface Expression by HLA-G-Negative APC Lines and Monocytes

To investigate the role of INDO in the expression regulation of HLA-G, we treated U937-HLA-G1$^-$ and THP-1-HLA-G1$^-$ lines, macrophage-differentiated THP-1-HLA-G1$^-$ (MΦ-THP-1-HLA-G1$^-$), and monocytes with tryptophan, 1MT, IFNG plus LPS, IFNG plus LPS plus tryptophan, or IFNG plus LPS plus 1MT. The effectiveness of the different treatments with respect to INDO function was assessed by kynurenine titration in culture supernatants and expression of cell-surface HLA-G1 by the treated cells (24 h for cell lines, and 48 h for monocytes) was investigated by flow cytometry. Cell surface expression of HLA-G and HLA-A,-B,-C,-E was measured using MEM-G/09, and TP25.99 mAbs, respectively, in monocytes, THP-1 cells, and macrophage-differentiated THP-1 cells (MΦ-THP-1) after no treatment (N.T.), or treatment with 1MT, IFNG plus LPS (IFNG+LPS), or IFNG plus LPS plus 1MT

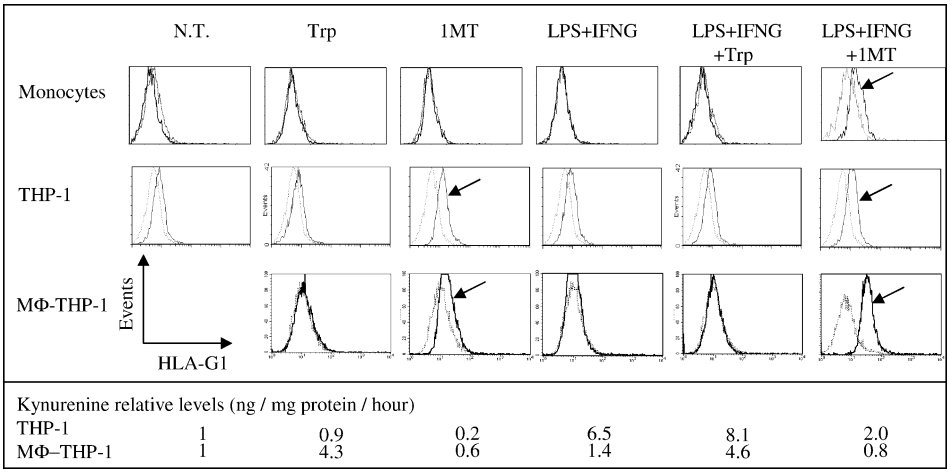

FIG. 2. 1MT induces cell-surface HLA-G1 up-regulation in APC lines and monocytes. Cell surface HLA-G1-negative monocytes, THP-1 cells, and macrophage-differentiated THP-1 cells (MΦ-THP-1) were untreated (N.T.), or treated as detailed in *Materials and Methods* with tryptophan (Trp), 1MT, IFNG+LPS, IFNG+LPS+Trp, or IFNG+LPS+1MT, as indicated. Histograms: HLA-G1 cell surface expression was evaluated by flow cytometry after 24 h for THP-1 cells and monocyte-differentiated THP-1 cells, and after 48 h for monocytes. Dark lines, MEM-G/09 staining; dotted lines, isotypic control. Arrows point to increased cell surface expression of HLA-G1.

(IFNG+LPS+1MT). Results obtained for one representative experiment out of seven for THP-1-HLA-G1$^-$, out of five for MΦ-THP-1-HLA-G1$^-$ cells, and out of six for monocytes-HLA-G1$^-$ are shown Figure 2. Results obtained for six experiments with U937-HLA-G1$^-$ cells were identical to those obtained for THP-1-HLA-G1$^-$ and are not shown. THP-1-HLA-G1$^-$ cells and U937-HLA-G1$^-$ cells significantly up-regulated HLA-G1 cell surface expression after treatment with 1MT alone or in combination with IFNG+LPS, whereas treatment with tryptophan alone, or IFNG+LPS, or the two combined, had no effect. Similarly, HLA-G1 cell surface expression was significantly up-regulated in monocytes after treatment with IFNG+LPS+1MT, but not after any other treatment. Thus functional inhibition of INDO with 1MT induced HLA-G1 cell surface expression in APC lines and monocytes. This effect seemed to be specific for HLA-G, because none of the treatments used on any of the cells described had any effect on other HLA class I cell-surface expression, as shown by concomitant staining with TP25.99, an antibody specific for HLA-A,-B,-C,-E, but not for HLA-G (Table 1).

HLA-G Cell-Surface Expression Up-Regulation on APC by 1MT Is Transitory

It has been reported that HLA-G cell surface up-regulation on U937 cells following IFNG treatment was transcriptional and transitory, and that such an induced cell-surface expression lasted less than 48 h [54]. Even though in our system IFNG alone or used in combination with LPS had no effect on HLA-G cell surface expression after 24 h, we investigated whether INDO inhibition-dependent HLA-G cell surface up-regulation was permanent or transitory. To address this question, kinetic studies were set up using cell surface HLA-G-negative monocytes, and U937-HLA-G1$^-$ and THP-1-HLA-G1$^-$ cells. These cells were treated 1) once with IFNG+LPS or IFNG+LPS+1MT, for 12, 24, 48, and 72 h; or 2) every 24 h with IFNG+LPS or IFNG+LPS+1MT for 72 h. In this latter case, LPS, IFNG, and 1MT were added to the existing culture medium. At each time point, HLA-G1 cell surface expression was measured by flow cytometry, and release of shed HLA-G1 or secreted HLA-G5 (or both) in the culture medium was analyzed by ELISA. The obtained results from four experiments show that treatment of either cell type with IFNG+LPS+1MT induced HLA-G cell surface expression as early as 12 h for THP-1-HLA-G1$^-$ cells, and 24 h for U937-HLA-G1$^-$ cells. This cell surface up-regulation was indeed transitory, and 72 h after treatment, all cells had lost HLA-G1 cell surface expression. However, cell surface HLA-G1 could still be detected at 72 h after renewed IFNG+LPS+1MT treatment every 24 h, even if it was at lower levels than those detected after 12 or 24 h of treatment (Fig. 3). The presence of HLA-G1/HLA-G5 was not detected at either time point after one treatment, probably due to assay sensitivity. However, renewed treatment with IFNG+LPS+1MT but not with IFNG+LPS allowed the detection of shed HLA-G1 (35 ng/ml), but not of HLA-G5 in the culture supernatants, ruling out the eventuality of flow cytometry artifacts (data not shown).

HLA-G Cell-Surface Expression Up-Regulation on APCs by 1MT Is Posttranslational

To gain insight into the mechanism involved in cell-surface HLA-G up-regulation, we investigated whether it was a transcriptional, posttranscriptional, translational, or post-translational mechanism. Cell surface HLA-G1-negative THP-1 cells were stimulated once with IFNG+LPS or IFNG+LPS+1MT for 12 h, corresponding to maximal cell

TABLE 1. Up-regulation of cell-surface expression of HLA-G, but not other HLA Class I molecules after treatment with 1-Methyl-Tryptophan.[a]

		Treatment			
Cells[b]	Antigen	No treatment	1MT	IFNG+LPS	IFNG+LPS+1MT
Monocytes	HLA-G	3.50 ± 1.50	5.5 ± 2.1	3.40 ± 1.30	6.00 ± 2.10
(N = 6)	HLA-A-B-C-E	89.00 ± 5.00	68.0 ± 2.00	81.00 ± 18.00	67.00 ± 14.00
THP-1	HLA-G	0.83 ± 0.10	2.75 ± 0.90	1.10 ± 0.17	4.10 ± 2.20
(N = 7)	HLA-A-B-C-E	4.50 ± 3.20	4.30 ± 1.00	4.55 ± 3.60	5.20 ± 2.10
MΦ-THP-1	HLA-G	1.75 ± 0.20	2.2 ± 0.40	2.58 ± 0.67	13.00 ± 8.10
(N = 5)	HLA-A-B-C-E	25.30 ± 4.00	26.00 ± 2.00	26.6 ± 4.00	25.5 ± 2.00

[a] Results are expressed as the mean fluorescence intensity ± SEM of the total cell population.
[b] N = number of independent experiments analyzed.

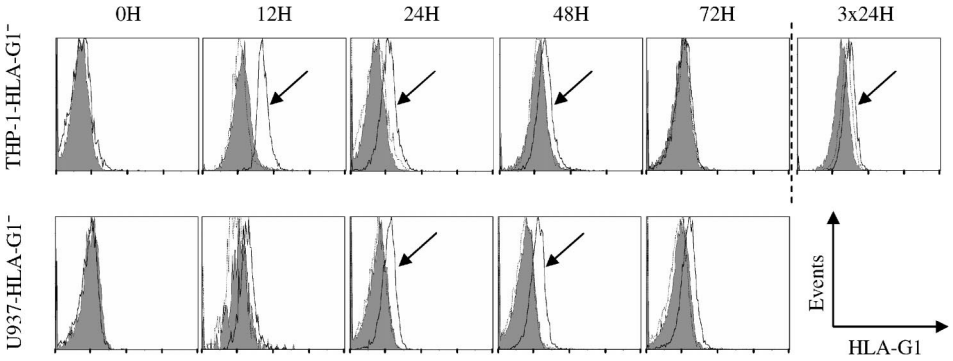

FIG. 3. HLA-G1 cell surface up-regulation by 1MT is transitory. HLA-G1 cell surface expression in THP-1-HLA-G1⁻ and U937-HLA-G1⁻ cells after treatment with IFNG+LPS (dotted lines) or with IFNG+LPS+1MT (dark lines) were investigated after 12, 24, 48, or 72 h, or after three 24-h treatments. The isotypic control is the shaded histogram. Arrows point to increased HLA-G1 cell surface expression.

surface HLA-G1 up-regulation. At this time point, analyses were carried as follows: 1) cell surface expression of HLA-G1 and total HLA-G1 contents were investigated by flow cytometry and intracellular flow cytometry, respectively, and 2) total HLA-G cellular contents were evaluated by Western blotting and immunohistochemistry.

Intracellular flow cytometry, immunohistochemistry, and Western blot analyses first revealed that even though HLA-G could not be detected at the cell surface, THP-1-HLA-G1⁻ cells, even untreated, expressed a basal level of HLA-G1 protein. Furthermore, total HLA-G cellular contents were identical following either treatment: No differences were observed between cells that were untreated, treated with IFNG+LPS, and treated with IFNG+LPS+1MT. Western blot analyses also revealed that HLA-G1 was the sole HLA-G isoform detected (see Fig. 4, which is representative of three such experiments).

Thus, these experiments showed that treatment of HLA-G1-expressing, but cell surface HLA-G1-negative APC lines with IFNG+LPS+1MT induced a fast but transitory HLA-G1 cell surface expression that occurred without HLA-G cellular content increase, hence, via a posttranslational mechanism. Renewed treatment with IFNG+LPS+1MT was the only treatment that allowed HLA-G1 cell surface expression to last.

Tryptophan Induces Loss of HLA-G1 Cell Surface Expression in HLA-G1-Positive APC Lines and Monocytes

1MT is a competitive inhibitor of tryptophan degradation by INDO. Because 1MT induced up-regulation of cell surface HLA-G1 expression in HLA-G-negative APC lines and monocytes, we investigated whether the reverse was true (i.e., whether increased concentrations of tryptophan, mimicking increased INDO function, induced down-regulation of HLA-G1 cell surface expression in HLA-G1-positive APC lines and monocytes).

As can be seen in Figure 5, a 24-h treatment of U937-HLA-G1⁺ (n = 3) and THP-1-HLA-G1⁺ (n = 3) lines and cell surface HLA-G1-positive monocytes-HLA-G1⁺ (n = 1) with IFNG+LPS+tryptophan induced a complete loss of HLA-G1 cell surface expression, whereas IFNG and LPS treatment alone or IFNG+LPS+1MT had no effect (Fig. 5). Concomitant staining with HLA-A,-B,-C,-E, but not with HLA-G-specific antibody showed that the treatments used had no effects on the expression levels of HLA class I molecules other than HLA-G1 (data not shown).

HLA-G1 Cell Surface Up-Regulation by 1MT Is Not a Mechanism Restricted to APC and Is Active in Tumor Lines

We wanted to determine whether up-regulation of cell-surface HLA-G1 expression was a mechanism that was restricted to APCs. Because INDO in mice and HLA-G in humans were shown to be involved in escaping antitumor immunity [41, 55], we treated the HLA-G1-expressing human melanoma line FON with 1MT for 72 h. This treatment induced a cell surface HLA-G1 up-regulation in the FON line, as had been observed for APC lines and monocytes (Fig. 6).

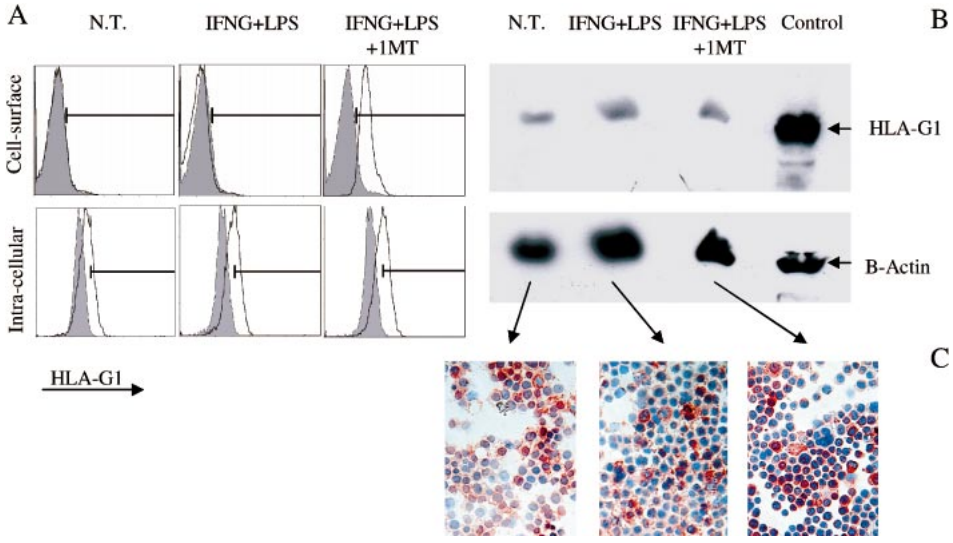

FIG. 4. HLA-G protein expression in THP-1 cells after treatment with 1MT using three different anti-HLA-G antibodies. HLA-G expression was analyzed in THP-1 cells after 12 h of no treatment (N.T.), IFNG+LPS treatment, or IFNG+LPS+1MT treatment. **A**) HLA-G1 cell surface expression and intracellular content using MEM-G/09 mAb. Shaded histograms are the isotypic control. **B**) HLA-G total cellular content (all HLA-G isoforms) with MEM-G/01 mAb (JEG-3 was used as a positive HLA-G-expressing control and beta-actin was used as the internal control). **C**) Total HLA-G expression by immunohistochemistry using 4H84 mAb (red staining). Original magnification ×10.

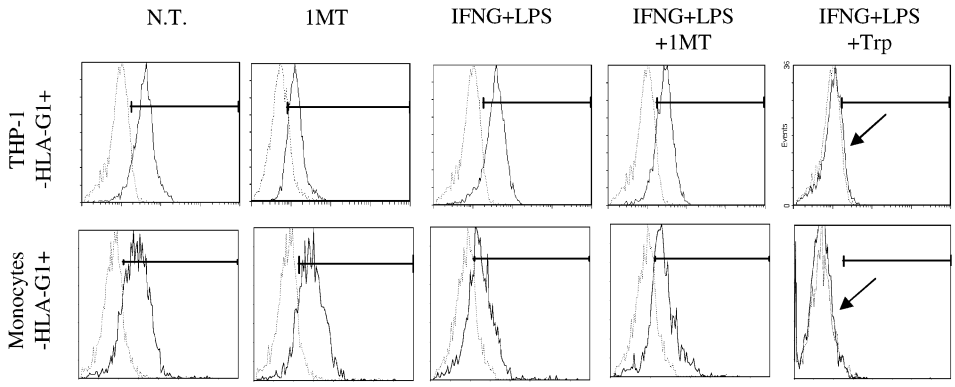

FIG. 5. Tryptophan induces loss of HLA-G1 cell surface expression in HLA-G1-positive APC lines and monocytes. THP-1-HLA-G1$^+$ cells and monocytes-HLA-G1$^+$ were untreated (N.T.) or treated as detailed in *Materials and Methods* with tryptophan (Trp), 1MT, IFNG+LPS, IFNG+LPS+Trp, or IFNG+LPS+1MT, as indicated. Dotted lines represent the isotypic control, plain lines are HLA-G1 cell surface expression levels, and arrows point to down-regulation of cell surface HLA-G1 expression.

DISCUSSION

INDO and nonclassical HLA class I HLA-G are two molecules involved in immune suppression, and both are involved in the protection of fetal tissues against destruction by the mother's immune system [23, 33, 56]. The most widely accepted hypothesis for INDO immunosuppressive function is that it acts by depleting the microenvironment that surrounds the cells of the essential amino acid tryptophan, thus inhibiting proliferation of immune cells, among others. HLA-G is the main HLA class I molecule expressed at the surface of extravillous cytotrophoblast at the fetal-maternal interface. HLA-G inhibits the function of immune cells (NK cells, T cells, and APCs) by high-affinity interaction with inhibitory receptors LILRB1 (CD85j/ILT2), LILRB2 (CD85d, ILT4), and KIR2DL4 (CD158d) [23]. We investigated whether these immunosuppressive mechanisms were linked, and we focused our efforts on the effects of INDO function on HLA-G expression.

Working with cell lines and monocytes that did or did not express cell surface HLA-G, we found that INDO function and HLA-G expression were indeed linked. Our results show that INDO function seems to prevent HLA-G cell surface expression. Indeed, cell surface HLA-G-negative APC lines and monocytes expressed cell surface HLA-G1 when treated with 1MT, a competitive inhibitor of INDO function. In the reverse experiments, cell surface HLA-G1-positive APC lines and monocytes lost HLA-G1 cell surface expression after treatment with tryptophan. Thus, HLA-G cell surface expression was up-regulated by INDO functional inhibition, and down-regulated by INDO functional boost. HLA-G cell surface expression up-regulation by INDO inhibition is fast and transient: HLA-G cell-surface expression was maximal 12–24 h after 1MT treatment, and unless this treatment is renewed, cell surface HLA-G is no longer detectable after 72 h. This might indicate that 1) 1MT is degraded during this period and no longer promotes HLA-G cell surface expression, or that 2) it is not INDO functional levels per se, but rather sudden stress-like variations in INDO functional levels that trigger HLA-G cell surface expression changes. Of note, cell surface HLA-G levels after three 24-h treatments were much lower than those detected 12–24 h after the first treatment. This would argue in favor of the latter hypothesis of INDO-related stress-inducing HLA-G cell surface expression regulation. Cell lines and monocytes responded to treatment with different kinetics: Maximal HLA-G1 cell surface up-regulation occurred at 12 h after 1MT treatment for THP-1-HLA-G1$^-$ cells, at 24 h for U937-HLA-G1$^-$ cells, and between 24 and 48 h for monocytes. The reasons for these differences are not known, but the fast kinetics of THP-1-HLA-G1$^-$ response led us to reason that HLA-G1 cell surface up-regulation might have little to do with transcription up-regulation, and more with posttranscriptional mechanisms. Thus, HLA-G protein production after treatment with 1MT was investigated. As had been reasoned, cell surface HLA-G up-regulation did not relate to HLA-G cellular content that was unaffected by any treatment, and was stable throughout the kinetics study. These experiments showed that inhibition of INDO induces a cell surface expression of HLA-G that is a posttranslational mechanism.

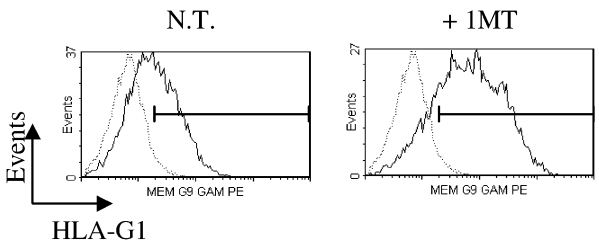

FIG. 6. 1MT induces HLA-G1 cell surface up-regulation in a tumor line. HLA-G1 cell surface expression in the HLA-G1-positive FON melanoma line after 72 h of no treatment (N.T.) or treatment with 1 mM 1MT (+1MT). Dotted lines are the isotypic control, plain lines are HLA-G1 cell surface expression.

The reverse experiments were performed and it was found that increased tryptophan concentrations induced a down-regulation of cell surface HLA-G1 levels in cell surface HLA-G1-positive APC lines and monocytes. In the experiments with 1MT described above, kinetic studies showed that HLA-G cell surface down-regulation by INDO functional boost was fast but temporary, complete after 12–24 h of treatment, and finished by 72 h. Protein expression studies could not be carried out, but given the fast and transitory kinetics of HLA-G modulation by INDO functional boost, it is likely that the mechanism involved is once again posttranslational.

The precise nature of the mechanisms involved in INDO-dependent HLA-G cell surface expression regulation is unknown. However, it is clear that the mechanisms described here do not fit the tryptophan-depletion immunosuppressive theory, and that tryptophan degradation or its lack thereof is not involved. The tryptophan-depletion theory proposes that tryptophan concentrations affect the proliferation rate of cells and pathogens. Accordingly, increased INDO function would deplete the surrounding microenvironment of tryptophan and have immunosuppressive effects. In our systems, treatment of APCs with IFNG plus LPS induced an increased INDO function, and therefore tryptophan depletion. However, this had no effect on

HLA-G1 cell surface expression modulation in HLA-G1-positive or HLA-G1-negative APCs. Furthermore, addition of tryptophan or addition of 1MT both led to a nondepletion of tryptophan in the medium. Yet these two treatments had opposite effects, both in terms of INDO activity and HLA-G1 cell surface expression regulation. Thus, it is INDO activity that seems to be responsible for HLA-G1 cell surface expression regulation, not tryptophan concentrations. Tryptophan could also affect HLA-G expression, not because of its availability/unavailability, but possibly because of some tryptophan metabolite, either present or not present, in the INDO degradation pathway. Because it was established that INDO function rather than tryptophan concentrations were involved in cell surface HLA-G1 expression regulation, we first thought that some product of tryptophan degradation would be responsible for the observed effect. In the tryptophan degradation pathway, it is INDO that is limiting. Thus, supplying kynurenine is a more efficient way of generating tryptophan degradation products than adding tryptophan at an equivalent concentration. We treated APC lines with kynurenine to generate tryptophan degradation products and to investigate whether one of these would be responsible for HLA-G1 cell surface expression modulation. This treatment had no affect, and no modification of HLA-G1 cell-surface expression was observed. This ruled out involvement of the tryptophan degradation pathway in INDO-driven HLA-G cell surface expression regulation.

INDO is an enzyme that breaks the indoleamine ring of tryptophan, leading to kynurenine production. This is an oxidative reaction that consumes oxygen free radicals [57]. Because INDO uses superoxide anion as its substrate and cofactor, it is considered that INDO functions as an antioxidant [58]. Because HLA-G cell surface expression regulation is related to INDO function, but not to tryptophan degradation metabolism, one can hypothesize that it is the INDO antioxidative function that is involved in HLA-G1 cell surface expression modulation. As a matter of fact, oxygen free radicals have been shown to play a role in the regulation of HLA expression and immune reactions [59].

Based on in vivo expression studies and in vitro functional studies, INDO in mice [41] and HLA-G in humans [44, 55], respectively, are suspected to play a role in the protection of tumors against antitumoral immunity. Treatment of HLA-G-expressing FON melanoma lines with 1MT leads to HLA-G cell surface up-regulation. This indicates that this mechanism is not restricted to APCs, but that HLA-G and INDO expression might co-contribute to tumor immune escape mechanisms.

It is our belief that HLA-G expression regulation by INDO function in APCs might be a novel mechanism of immune regulation. It not clear whether this mechanism plays a role in HLA-G expression regulation during pregnancy. However, one can hypothesize that HLA-G and INDO, which have been associated with fetal-maternal tolerance in humans and in mice, respectively, act cooperatively. Indeed, it is interesting to consider that although INDO functional inhibition in mice leads to abortion, an INDO knockout mouse was generated [35], indicating that INDO is not the key to murine fetal-maternal tolerance by itself. Cell surface expression modulation of HLA-G in humans or of its functional homologue in mice might compensate INDO functional fluctuations, the reverse being still unknown. Because HLA-G is crucial to early human pregnancy [12, 24], INDO is expressed primarily after the 14th week of pregnancy in humans [37], and INDO seems to be involved in later pregnancy in mice [33], it is conceivable that HLA-G and INDO could have two complementary, linked, but not redundant roles in maintenance of the maternal tolerance toward the fetus.

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