Capacity of myeloid and plasmacytoid dendritic cells especially at mature stage to express and secrete HLA-G molecules

Gaëlle Le Friec,[1] Frédéric Gros,[1] Yasmine Sebti, Valérie Guilloux, Céline Pangault, Renée Fauchet, and Laurence Amiot[2]

Laboratoire Universitaire d'Hématologie et de la Biologie des Cellules Sanguines, UPRES EA 22-33, Faculté de Médecine, Université de Rennes 1, France

Abstract: Human leukocyte antigen (HLA-G), a class Ib major histocompatibility complex molecule, is potentially relevant in the immune response through its various immune cell functions. Its expression noticed in some malignancies has also been shown on macrophages and dendritic cells (DC) in tumoral and inflammatory diseases. As DC constitute a key component in the immune response, this work aimed at assessing the expression of HLA-G at transcriptional and proteic levels during differentiation and maturation of the different DC subsets. We show that HLA-G transcription was induced during CD34+-derived DC differentiation and is associated with a cell-surface expression in half of cases and with a substantial secretion of soluble HLA-G in all cases. Results were very similar for monocyte-derived DC, but there was still a weak HLA-G cell-surface expression and a lower level of secretion. On the contrary, HLA-G transcription was weak in plasmacytoid DC without any HLA-G cell-surface expression and with a basal level of secretion. The mechanisms involved in HLA-G expression appear transcriptional and post-transcriptional. However, the amount of HLA-G transcripts and the expression of the protein are not related. HLA-G expression or secretion by DC may have negative consequences on the function of effective immune cells and also on DC themselves via the interaction with inhibitory receptors expressed by these cells. The capacity of DC to express or secrete HLA-G should be studied in the context of cellular therapy using DC in addition to its suppressive action in immune response. *J. Leukoc. Biol.* 76: 1125–1133; 2004.

Key Words: *CD34+-derived DC · monocyte-derived DC · soluble HLA-G · real-time PCR · ELISA*

INTRODUCTION

The new insights into the role of the nonclassic major histocompatibility complex class I antigen human leukocyte antigen (HLA-G) show that this molecule is likely to modulate immune responses by favoring the escape of innate [natural killer (NK) cells] and acquired immunity [T cells and dendritic cells (DC)] from the effectors. Initially, the trophoblast was the first tissue where HLA-G has been shown to be expressed in contrast to classic class I antigens, which suggests its implication in fetal maternal tolerance [1]. Further studies have described its expression in pathological cases such as tumoral processes [2], inflammatory diseases [3–5], and post-transplantations [6–9]. In addition to a restrictive distribution, HLA-G molecules exhibit specific features such as a low polymorphism and an expression through several isoforms, generated by alternative splicing from primary transcript HLA-G1. Four membrane-bound isoforms (HLA-G1, HLA-G2, HLA-G3, HLA-G4) [10, 11] and three soluble isoforms (HLA-G5, HLA-G6, HLA-G7) [12, 13] have been described. The HLA-G1 and -G5 appear as the major isoforms, sharing immunosuppressive functions through interaction with inhibitory receptors present on immunocompetent cells [14]. To date, four receptors recognizing HLA-G have been identified: immunoglobulin (Ig)-like transcript 2 (ILT2; LIR1, CD85j), ILT4 (LIR2, CD85d), Ig-like receptor (KIR)2DL4 (CD158d), and By55. These receptors are expressed on NK cells (ILT2, KIR2DL4, By55), on T and B cells (ILT2), and on monocytes/macrophages and DC (ILT4). DC or macrophages may express HLA-G molecules under particular conditions, such as tumoral diseases (lung carcinomas, breast carcinomas, cutaneous lymphomas) [15–18], inflammatory pulmonary diseases [16], and transplanted tissues [7].

DC are considerable antigen-presenting cells (APC) with a unique capacity to induce primary immune responses [19]. These properties explain their key role in the initiation and also during the progress of the immune response. DC constitute a heterogeneous population of hematopoietic cells with two different types: myeloid DC (mDC) [20] and plasmacytoid DC (pDC) [21]. These two subtypes are derived from CD34+ stem cells depending on two pathways of differentiation. mDC

[1] These authors contributed equally to this work.

[2] Correspondence: Laboratoire Universitaire d'Hématologie et de la Biologie des Cellules Sanguines, UPRES EA 22-33, 2 Avenue du Professeur Léon Bernard, CS34317, 35043 Rennes Cedex, France. E-mail: laurence.amiot@chu-rennes.fr

Received January 12, 2004; revised July 16, 2004; accepted August 2, 2004; doi: 10.1189/jlb.0104015.

(DC1), native to myeloid bone marrow precursors, differentiate from the monocyte/macrophage lineage and require the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) for surviving [22]. Immature DC1 (iDC1), identified as CD11c+/DR+ cells [23], circulate in human peripheral blood and then infiltrate the tissues. Heterogeneity of this subtype is reflected by different anatomical areas such as skin epidermal DC—Langherans cells—dermal DC, or interstitial DC. After antigen capture, they migrate to lymphoid organs. Their maturation is linked with their migration and results in the acquisition of costimulation molecules (CD40, CD80, CD86) and maturation marker (CD83) and functionally, by the secretion of interleukin (IL)-12 [24], driving polarization of T cells into T helper type 1 (Th1) cells [25]. Moreover, mDC can favor Th2 orientation when stimulated with prostaglandin E_2.

Lymphoid DC precursor cells, pre-DC2 or pDC, are present in human peripheral blood, in the lymphoid tissues, and correspond to lineage (Lin)–/CD11c–/DR+/CD4+ low/CD45RA+/IL-3 receptor α+ cells [26]. They are also positive for the recently described blood DC antigen 2 (BDCA-2) and BDCA-4 antigens [27]. Their survival and differentiation depend on IL-3 [26]. The lymphoid origin of this subtype, although controversial, could explain the high levels of pre-T cell receptor α [28] and Ig λ-like transcripts detected in these cells. After the appropriate activation, they can cause T cell differentiation into Th2 cells [29]. Conversely, pre-DC2 constitute the major cell type, which can produce a high level of type I interferons [(IFN)-α, -β] upon viral stimulation [30]. However, the mouse fetal liver tyrosine kinase 3 (Flt3) pDC induce IL-12 production and Th1 cell development when they are stimulated with CpG. Subsequently, the plasticity skews the T cell orientation more than DC subset.

Put together, these data underline the interest in searching for the link between DC playing a role in the innate and acquired immunity and immunomodulating HLA-G molecules. To examine such a hypothesis, we have studied the transcriptional and proteic expressions of these HLA-G molecules during lymphoid and mDC differentiation and maturation. We demonstrated the potential capacity of DC to express or secrete HLA-G, especially for the CD34+ myeloid-derived DC. Secretion/expression may have negative consequences for the immune response, in particular, via an autocrinic action by DC expressing a potential, inhibitory HLA-G receptor, ILT4, or in the murine model, its homologue, paired Ig-like receptor (PIR)-B [31, 32].

MATERIALS AND METHODS

Cytokines, reagents, and cell lines

Human GM-CSF (sp act 1.2×10^8 U/mg) was provided by Shering Plough (Lyon, France), whereas IL-4 (sp act 10^8 U/ml) was provided by Promocell (Heidelberg, Germany). Transforming growth factor-β (TGF-β; sp act 2.5×10^7 U/mg), Flt3 (sp act 2×10^5 U/mg), tumor necrosis factor α (TNF-α; sp act 2×10^7 U/mg), stem cell factor (SCF; sp act 5×10^5 U/mg), and IL-3 (sp act 1×10^7 U/mg), IL-2 (sp act 10^4 U/μg), IFN-γ (sp act 1×10^7 U/mg), and IL-10 (sp act $>5\times10^5$ U/mg) were supplied by Tebu (Le Perray-en-Yvelines, France). ^{3}H-Thymidine (sp act 5 Ci/mmol) was purchased from Amersham Pharmacia Biosciences (Uppsala, Sweeden). CD40 ligand (CD40-L) (0.1 mg/ml) and enhancer CD40-L (1 mg/ml) were supplied by Alexis (Illkirch, France).

MEM-G/9 monoclonal antibody (mAb), detecting HLA-G1 and -G5 molecules; MEM-G/1, recognizing the free heavy chain of all the HLA-G isoforms; as well as MEM-E/02, reacting with denaturated heavy chain of human HLA-E were purchased from Exbio (Prague, Czech Rep.). 16G1, recognizing HLA-G5 and HLA-G6 soluble isoforms, was kindly provided by Dan Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA).

Fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated mouse mAb against CD1a, CD14, CD34, or HLA-DR were purchased from Immunotech/Beckman (Marseille, France), whereas CD40, CD83, and CD86 mAb were provided by PharMingen (Le Pont de Claix, France).

Lin-FITC, CD123-PE, and HLA-DR-PCy5 mAb, supplied by Becton Dickinson (San Jose, CA), were used in a triple staining to characterize pDC.

The B lymphoblastoid cell line, LCL.721.221-G5, kindly provided by D. Geraghty (Fred Hutchinson Cancer Research Center) was obtained by transfection of the intron 4 containing cDNA HLA-G as described previously [12]. JEG3, supplied by the American Type Culture Collection (ATCC; Manassas, VA), is a choriocarcinoma cell line expressing HLA-G. The acute myeloblastic leukemia cell line KG1 (ATCC) an HLA class I-positive and HLA-G-negative cell lines was used as negative control in the different experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

In accordance with the manufacturer's recommendations, total RNA was isolated from 1–3 × 10^6 cells using TRIzol reagent (Invitrogen, Cergy Pontoise, France). cDNA synthesis was performed on 5 μg RNA by random hexamers priming using Superscript II RT (Invitrogen) for 50 min at 42°C. After denaturation of enzyme at 70°C for 15 min, RNA template was digested by adding RNase H for 20 min at 37°C. Hot-start PCR was carried out in a volume of 50 μl containing 10 μl RT reaction, 50 pmol each primers, 10 μl 10× PCR buffer, and 2.5 U Taq polymerase (Amersham Pharmacia Biosciences). As described previously [33], the primer sets, which were used, are the following: G257 (exon 2, 5′-GGAAGAGGAGACACGGAACA)/G1004 (exon 5 and exon 6 junction, 5′-CCTTTTCAATCTGAGCTCTCTTT). PCR cycle conditions were for 1 min at 94°C, 1 min 30 s at 61°C, and for 1 min 30 s at 72°C for 35 cycles with a final increase at 72°C for 10 min. β-Actin cDNA was amplified under the same conditions to ensure RNA quality of each sample. PCR products were separated in an ethidium bromide stained 1.7% agarose gel. Alkaline DNA transfer was performed under vacuum on Hybond-N+ membrane. Southern blot was prehybridized for 1 h at 68°C and then hybridized for 3 h at 57°C in buffer containing 5′ digoxigenin-labeled GR probe (exon 2-specific, 5′-GGTCTGCAGGTTCATTCTGTC), defined as pan HLA-G probe. Revelation was performed using an antidigoxigenin system.

Real-time PCR analysis

Duplex PCR was carried out for 40 cycles in the presence of Taqman universal PCR master mix on an ABI prism 7000 (Applied Biosystems, Foster City, CA) using HLA-G-specific primers located in conserved exon 5 (G1046) and exon 6 (G1099) and a specific probe located in exon 5 [6-carboxy-fluorescein reporter and 6-carboxy-N,N,N′,N′-tetramethylrhodamine (TAMRA) quencher] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control (VIC reporter and TAMRA quencher, Applied Biosystems) as described previously [34]. The location of primers and probe enables amplification of all alternative mRNA isoforms. Quantifications relative to JEG3, a HLA-G-expressing choriocarcinoma cell line, were carried out in duplicate, using the calculation $\Delta_{Ct} = C_{T\ HLA\text{-}G} - C_{T\ GAPDH}$ and $\Delta\Delta Ct = \Delta_{CT\ sample} - \Delta_{CTJEG3}$. The quantity of the HLA-G level is defined as $2^{-\Delta\Delta CT}$.

Specific sHLA-G enzyme-linked immunosorbent assay (ELISA)

HLA-G5 concentrations were measured using a specific sandwich ELISA. Microtiter plates (Corning Costar, Issy-les-Moulineaux, France) were coated with MEM-G/9 (10 μg/ml) in 0.01 M phosphate-buffered saline (PBS), pH 7.4. After three washes in PBS containing 0.05% Tween 20, plates were saturated with 250 μl PBS containing 2% bovine serum albumin for 30 min at room temperature. Cell culture supernatants (100 μl) were added to each well and were tested three times. After a 1-h incubation at room temperature, plates

were washed three times in PBS with 0.05% Tween 20. Anti-β2-microglobulin–horseradish peroxidase (β2m–HRP; Dako, Trappes, France; 100 μl) was added to each well, and plates were incubated for 1 h at room temperature. Plates were washed three times and then incubated for 30 min with the substrate (ortho-phenylenediamine dihydrochloride, Dako). After addition of H_2SO_4 (1N), optical densities (ODs) were measured at 490 nm. Standard curves were performed using serial dilutions of calibrated supernatant of LCL-G5, kindly provided by Vera Rebmann (Institute for Immunology, Essen, Germany). Thus, the concentrations of HLA-G5 were determined by the value of OD according to the standard curves.

Western blotting

Proteins from LCL721.221-G5 and LCL721.221-wild-type (WT) cell lines and DC were extracted from 1–3 × 10^6 cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's recommendations. Solubilized proteins were loaded in a 12% mini-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, thanks to an electrophoretic transfer apparatus. Membranes were saturated with 5% nonfat dry milk in PBS and incubated with the 16G1 mAb (4 μg/ml) or MEM-G/1 (4 μg/ml) or MEM-E/02 (1 μg/ml). A peroxidase-conjugated anti-IgG mouse was thereafter used as secondary antibody. After washing, blots were developed by chemiluminescence using a 100-mM Tris-HCl solution (pH 8.5) containing 0.9% (w/w) H_2O_2, 225 μM coumaric acid, and 1.25 mM luminol.

DC generation

mDC

Monocyte-derived DC. DC were obtained from peripheral blood monocytes (n=6) as described previously [35]. Briefly, mononuclear cells were obtained by Ficoll gradient (d=1.077 g/ml; Amersham Pharmacia Biosciences) centrifugation of blood buffy coat from healthy volunteers provided by the EFS-Bretagne. Twenty millions of cells per well were seeded into six-well culture plates in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics, and 10% fetal calf serum (FCS). After a 37°C incubation for 2 h, nonadherent cells were removed, and adherent cells were further cultured during 7 days with 800 U/ml GM-CSF and 500 U/ml IL-4. Half of the medium was replaced at days 3, 5, and 7 by fresh medium. Mature DC were generated by further addition of TNF-α (10 ng/ml) for 2 days as previously reported [36].

CD34+-derived DC. CD34+ cells were purified from apheresis products (n=6) using magnetic beads (CD34 progenitor cell isolation kit, Miltenyi Biotec, Paris, France) with a 95% mean purity. Isolated CD34+ cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen), 10% FCS, supplemented with 200 U/ml GM-CSF, 20 ng/ml SCF, 50 ng/ml Flt3-L, 20 ng/ml TNF-α, and 0.5 ng/ml TGF-β. Cultures were fed by replacing half of the culture medium by fresh medium at days 5, 9, and 14. At day 9, the medium was only supplemented with TNF-α, GM-CSF used at the previous concentrations, and 500 U/ml IL-4. Maturation of DC cells was obtained by addition of 1 μg/ml lipopolysaccharide (LPS) at day 14.

pDC

Isolation of pDC was performed from apheresis products (n=3) by two magnetic separations. First, blood DC were pre-enriched of peripheral blood mononuclear cells by immunomagnetic depletion of CD3+ T cells, CD11b+ monocytic cells, and CD16+ NK cells [blood DC isolation kit, magnetic cell sorter (MACS), Miltenyi Biotec]. Secondly, $BDCA^{2+}$ cells were positively selected on a magnetic column (BDCA-2 cell isolation kit, MACS, Miltenyi Biotec). The purity of pDC was evaluated by flow cytometry using a FACSCalibur cytometer (Becton Dickinson) using triple staining with a cocktail of Lin-mAb, Lin-FITC, CD123-PE, and HLA-DR-PCy5 (Becton Dickinson).

Purified pDC were matured into DC2 during 48 h under culture of 5 × 10^5 cells/ml in the presence of 10 ng/ml IL-3 with 0.5 μg/ml CD40-L and 1 μg/ml enhancer CD40-L.

Flow cytometry DC studies

In vitro-generated DC

Phenotypic analysis of CD34+ cell-derived DC, monocyte-derived DC, and pDC was performed using flow cytometric direct immunofluorescence as described previously [35, 37]. Cells were first incubated for 30 min in human AB serum at 4°C to avoid nonspecific binding. Then, cells were labeled using direct immunofluorescence and were thereafter analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson). imDC correspond to CD1a+/CD14–/CD34– cells, and maturation results in the appearance of CD40, CD80, CD83, and CD86 and the increase in HLA-DR expression. pDC correspond to the Lin–/HLA-DR+/CD123+ population.

HLA-G expression on DC was studied with the anti-HLA-G MEM-G/9 (10 μg/ml) recognizing HLA-G1 and -G5 for 30 min at 4°C. Murine isotype-matched, control IgG1 was used as negative control. After washing, cells were incubated for 30 min at 4°C with a goat anti-mouse IgG (Fab'_2) fraction conjugated with PE. Fluorescence was detected by a FACSCalibur flow cytometer (Becton Dickinson).

Ex vivo blood mDC analysis

Mononuclear cells from apheresis products were obtained using Ficoll-Hypaque density gradient centrifugation (Novamed, Jerusalem, Israel). Cells were labeled by four-color staining using the following mAb: MEM-G/9 conjugated with FITC (Exbio), CD11c conjugated with PE (Becton Dickinson), HLA-DR conjugated with energy coupled dye (ECD) (Beckman Coulter), and lineage cocktail mAb, including CD3, CD14, CD16, CD19, CD20, and CD56 conjugated with PE-Cyanine 5.5 (Lin, PE-Cy5; Beckman-Coulter). Briefly, 2 × 10^6 cells were stained for 30 min at 4°C with saturating amounts of mAb. Then, after erythrocytes lysis and washing, acquisition was performed using an ARIA flow cytometer (Becton Dickinson) operating with argon laser and DIVA software. A cytogram characterizing HLA-G and CD11c expression was gated by Lin–, HLA-DR– cells. A total of 1 × 10^6 cells was stored in list mode and analyzed.

Cytokinic stimulations of monocyte-derived DC

After maturation, monocyte-derived DC were cultured for 48 h in the presence of different cytokines as the following: IL-2 (220 U/ml), IFN-γ

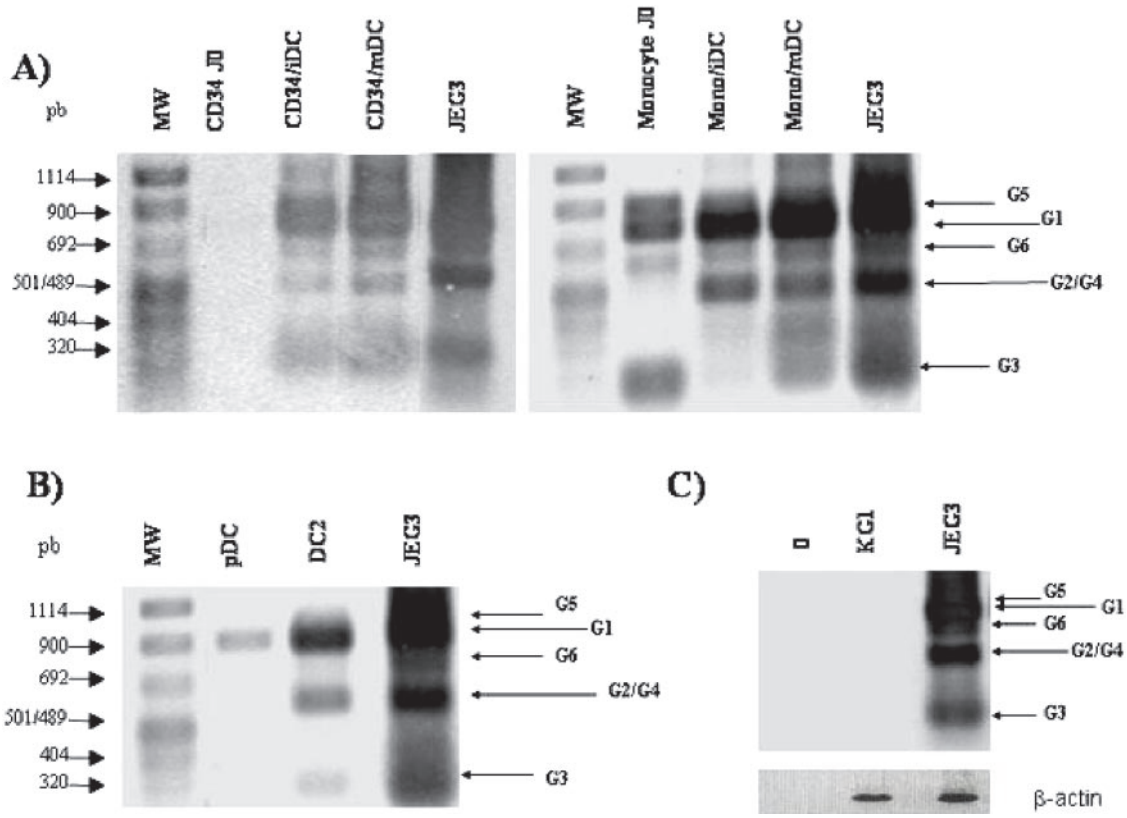

Fig. 1. RT-PCR/Southern blot analysis of HLA-G mRNA expression during differentiation and maturation of DC. Representative cases of CD34+-derived DC, monocyte-derived DC (A); pDC (B); negative (KG1) and positive (JEG3) controls (C) are shown. Detection of HLA-G transcripts was carried out using pan HLA-G primers set (G257/G1004) followed by hybridization with exon 2-specific GR probe recognizing all isoforms. The molecular weight markers (MW) are indicated on the left. Arrows indicate the bands corresponding to G5, G1, G2/G4, G6, and G3 mRNA isoforms. Several HLA-G transcriptional isoforms such as HLA-G5, -G1, -G6, -G2/G4, and -G3 were detected in iDC and mature mDC, whatever their subtype, and in mature DC2, whereas no transcript and only the G1 isoform were, respectively, found in CD34+ progenitor cells and in pDC. As expected, several bands corresponding to G5, -G1, -G6, -G2/G4, and -G3 isoforms are found in JEG3, and no band is observed for KG1 in spite of positive β-actin control. pb, base pairs; JO, day 0.

assigned to a value of 1 (**Fig. 2**). The amount of HLA-G transcripts in CD34+ cells is very weak and could be considered as negative. It is interesting that this quantity increases by a 1.7-fold factor in immature, CD34+-derived DC and more significantly, by a tenfold factor in mature, CD34+-derived DC, but it remains low compared with JEG3 (respectively, 0.28% and 1.6%; Fig. 2). The quantity of HLA-G transcripts in monocytes seems more important, as it represents ~9% of JEG3. This transcription level, respectively, increases by a 1.8- and ninefold factor in immature and mature monocyte-derived DC, corresponding to 17% and 79% of JEG3. Real-time PCR analysis shows various amounts of HLA-G transcripts depending on the samples with an important standard deviation (Fig. 2). The HLA-G transcription level appears close to being undetectable in ipDC. A weak level is detectable in mature pDC (DC2) representing 0.04% of JEG3 (Fig. 2).

HLA-G cell-surface expression during DC differentiation

HLA-G cell-surface expression in CD34+-derived DC is detected using flow cytometry with MEM-G/9 in half of the cases. In the positive cases (n=3), MEM-G/9 staining could be detected on iDC as well as on mature cells. Expression on iDC from a particular donor is not systemically correlated with expression at a mature stage and reciprocally.

The expression level also appears variable depending on the samples, and it ranges from 7.2% to 52% of positive cells (**Fig. 3B**). In contrast, HLA-G cell-surface expression was only observed in one out of six cases of monocyte-derived DC at a mature stage (Fig. 3C).

HLA-G cell-surface expression on blood DC

No HLA-G expression is found at the cell surface of mDC, defined as Lin–, CD11c+, HLA-DR+ (**Fig. 4**), as well as on pDC, whatever its maturation stage (data not shown), according to flow cytometry analysis.

sHLA-G expression during dendritic differentiation

To precise HLA-G protein expression, Western blotting experiments using 16G1 were performed in 14 cases (six CD34+-derived DC, six monocyte-derived DC, and two pDC) in parallel to negative (LCL721.221-WT) and positive (LCL721.221-G5) controls. A single band of 45 kDa is obtained in lymphoid and mDC generated from monocytes or CD34+ cells, whatever their maturation status in all cases (**Fig. 5**). A similar band of 45 kDa is also found in the specific HLA-G5-transfected cell line LCL721.221-G5, which also displays the expected 37-kDa band [1], whereas LCL721.221-WT cell line exhibits no band.

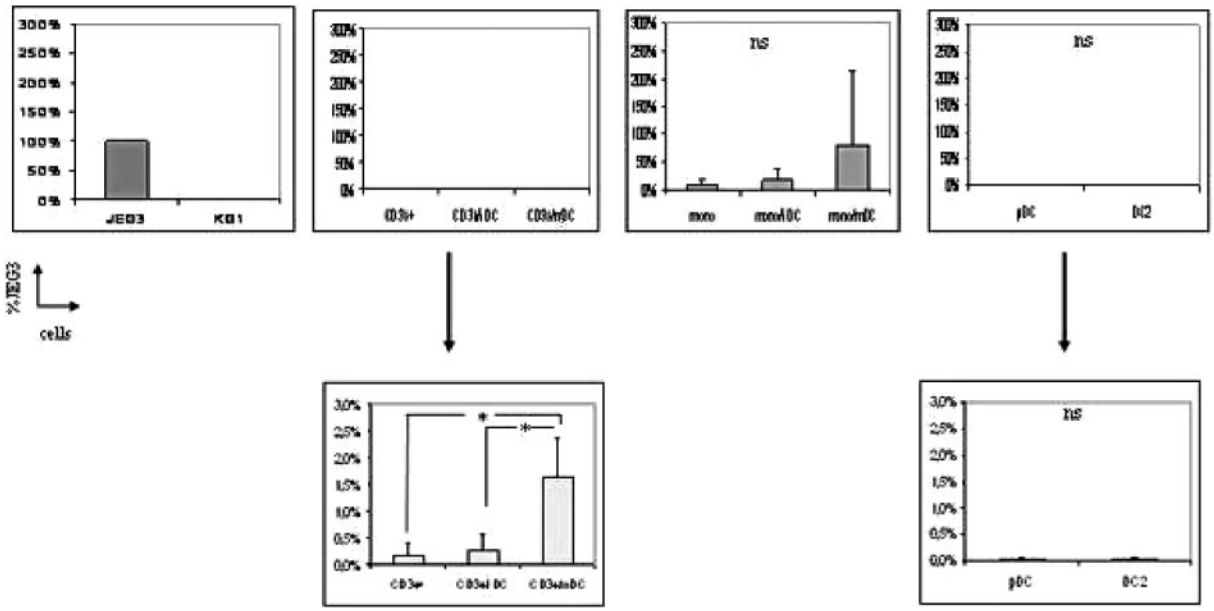

Fig. 2. Quantitative analysis of HLA-G transcripts during DC differentiation and maturation by real-time RT-PCR. Variable amounts of HLA-G transcripts were observed according to the subtype of DC. The upper panels are represented under the same scale (0–300%) including the negative (KG1) and positive (JEG3) controls. The low amounts of mRNA in CD34+-derived DC and in pDC are shown under a smaller scale (0–3%) in the lower panels. Results are expressed in percentage of the amount of HLA-G mRNA in JEG3: Weak levels (0–0.04%) for pDC and DC2, intermediate levels for CD34+-derived DC (0.28–1.6%), and higher levels correspond to monocyte-derived DC (17–79%). Only CD34+-derived DC showed a significant increase during maturation (*, $P<0.05$; ns, nonsignificant).

To check the presence of protein in these cell lines, a control was performed with MEM-E/02, demonstrating a band corresponding to HLA-E protein. To confirm these results, MEM-G/1, used as blotting mAb, was tested for Western blot. A similar pattern with the 45-kDa band appears in the nine tested cases comprising four CD34+-derived DC, three monocyte-derived DC, and two pDC, confirming the specificity of that band, which probably corresponds to additional glycosylation.

To search a potential HLA-G secretion by DC, the level of sHLA-G was determined in the supernatants of DC complete culture medium at immature and mature stages using a specific ELISA with MEM-G/9. The culture medium including FCS was used to determine the negative threshold. The results are represented in **Figure 6**. The level of sHLA-G in immature CD34+-derived DC was 17.34 ± 1.37 ng/ml (n=3) and increased slightly but not significantly at 24.68 ± 8.85 at mature stage. This secretion in iDC and mature DC appears significant compared with control ($P<0.001$). In monocyte-derived DC, the amount of sHLA-G is lower: 5.72 ± 1.41 ng/ml at immature stage and 8.76 ± 0.56 at mature stage ($P<0.01$). Mature DC2 exhibit a similar secretion level with 6.85 ± 0.7 ng/ml ($P<0.01$).

sHLA-G secretion after in vitro stimulation of monocyte-derived DC with cytokines

Mature monocyte-derived DC (n=3) were cultured for 48 h with different cytokines. sHLA-G secretion was determined in culture supernatants. The results are illustrated in **Figure 7**. The sHLA-G level does not significantly increase in the presence of cytokines compared with controls (15.72±9.08 ng/ml) with values comprised between 17.34 ± 8.2 and 27.5 ± 15.8 ng/ml. The highest levels of sHLA-G are observed at the mature stage (22.9±12.6) as well as with LPS (27.5±15.8 ng/ml), also known as maturation agent.

DISCUSSION

DC represent a major component of the immune response besides effector cells such as NK cells and T8 cytotoxic cells. Expression of HLA-G molecules may interfere with the function of these cells, contributing to a down-modulated immune response. Subsequently, the potential expression of HLA-G by DC could constitute an additional factor, which reinforces its negative action on T cells in particular. In this study, we demonstrate the capacity of DC, which are generated in vitro to express or secrete HLA-G molecules, whatever their subtype.

The expression of HLA-G on APC, such as monocytes/macrophages and DC, has been found ex vivo in several types of tumors including lung carcinoma [16, 17], breast carcinoma [15], and cutaneous lymphoma [18]. This expression on APC does not seem restricted to the tumoral process, as we also demonstrated an expression in inflammatory pulmonary diseases [16]. These data are consistent with previous results showing an unexpected expression in psoriasis [3], atopic dermatitis [4], and inflammatory myopathies [5], suggesting a protector role of HLA-G in case of inflammatory aggressions [38]. Moreover, sHLA-G was mainly secreted in physiological situations by monocytes [39].

In this study, we examine HLA-G expression at different levels during differentiation of mDC and pDC. As planned, HLA-G transcription was absent in CD34+ progenitor cells

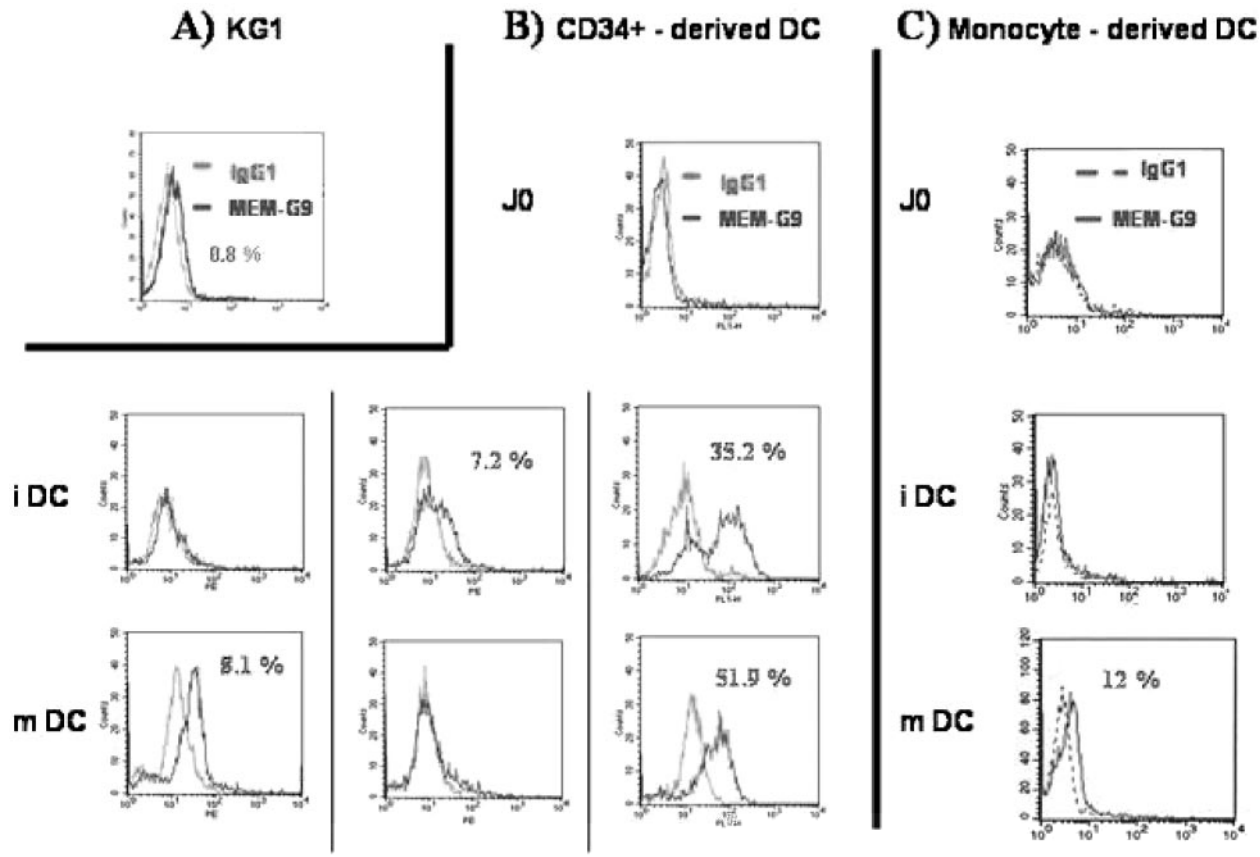

Fig. 3. Analysis of HLA-G cell-surface expression using flow cytometry in KG1 (A) and CD34+ (B)- and monocyte-derived DC (C) during mDC differentiation and maturation. MEM-G/9 was represented in red (A–C), whereas IgG1 mAb used as isotypic control was green (A and B) and in a dotted line (C). (A) No significant MEM-G/9 staining is found on KG1. (B) The three cases of CD34+-derived DC expressing membrane-bound HLA-G are shown at both stages of differentiation with variable intensity of expression according to the case. (C) MEM-G/9 staining was only observed in one out of six cases of monocyte-derived DC at the mature stage.

and displayed by monocytes [40]. HLA-G transcripts are detected in CD34+-derived DC at an immature stage, demonstrating a transcriptional regulation of HLA-G expression in this model. In half of the cases, this transcription is associated with a frequent cell-surface expression and a relative high capacity of secretion. In monocyte-derived DC, despite the higher quantity of HLA-G transcripts, cell-surface expression is rarely found, and the level of secretion is lower than the one of CD34+-derived DC. Moreover, addition of inflammatory cytokines (IL-2, IFN-γ, GM-CSF) or cytokines linked to tumoral environment (IL-10, TGF-β) or TLR ligand (LPS) has not acquired any effect on the level of sHLA-G secretion by mature monocyte-derived DC. These data suggest that the secretion is mainly linked to maturation and could be caused by other factors of the microenvironment [41, 42]. However, the results show a similar pattern of expression in both mDC but with a different level of expression. In contrast, pDC display a weak HLA-G transcription without any cell-surface expression and a low level of secretion. The amount of HLA-G transcripts does not seem to be related to protein expression, as intracellular proteins are detected in all cases using Western blot, such as in pDC. These results confirm that HLA-G expression is subject to two independent regulation mechanisms, transcriptional and post-transcriptional.

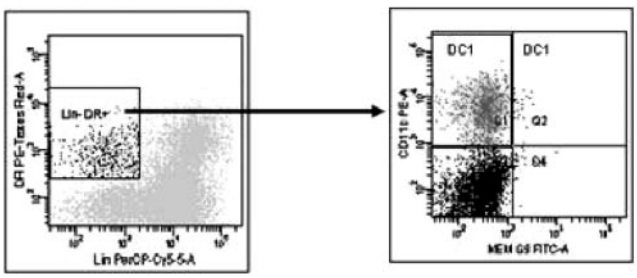

Fig. 4. Absence of HLA-G cell-surface expression on blood mDC (DC1). A representative case of HLA-G cell surface on mDC (n=3) is illustrated. Using cytometric analysis, cells were analyzed after four-color labeling (MEM-G/9-FITC, CD11c-PE, HLA-DR-ECD, CD3/CD14/CD16/CD19/CD20/CD56-Pc5). The first graph allowed to define a region Lin– (horiz.) and HLA-DR+ (vert.). The second graph represented HLA-G expression (horiz.) on CD11c+ cells (vert.) in the predefined region. No significant MEM-G/9 staining was observed on Lin– and HLA-DR+CD11c+ cells or DC1.

The consequences of HLA-G expression or secretion by DC concern different types of DC partner cells implicated in immunity. Membrane and sHLA-G molecules are likely to inhibit the cytolytic function of NK cells and T cells [43–45]. Moreover, sHLA-G may induce apoptosis of NK and CD8+ cells via ligation with CD8 involving the Fas/Fas-L pathway

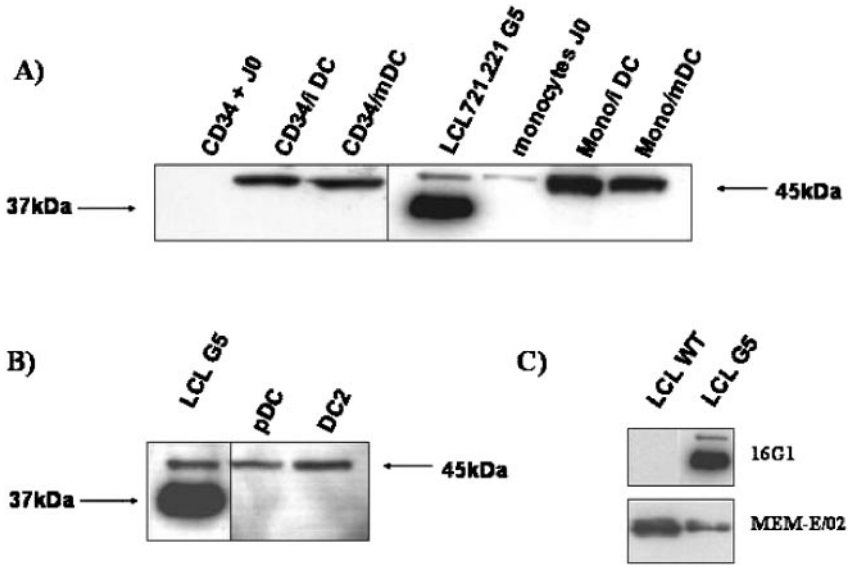

Fig. 5. Western blot analysis of sHLA-G protein expression during mDC and pDC differentiation. Representative examples of Western blot analysis obtained for (A) mDC differentiation (CD34+ and monocytes), (B) lymphoid differentiation, and (C) negative (LCL WT) and positive (LCL G5) controls were shown. Using 16G1 mAb recognizing specifically sHLA-G isoforms, a 45-kDa band was observed in cell lysates providing for all subtypes of DC and monocytes but not for the CD34+-purified cell lysate. This band corresponded to the one exhibited by the LCL721.221-G5 cell line, used as a positive control, displaying also the expected 37-kDa band. No band was observed in LCL-WT using 16G1, although a sufficient amount of protein was present in this sample as shown by MEM-E/02 staining.

[46–49]. Both forms present the capacity to inhibit T cell alloproliferation in a mixed lymphocyte reaction [2, 44, 45, 50]. In the same way, we have shown previously that sHLA-G molecules can reduce allogeneic T cell proliferation during interaction between DC and T lymphocytes [51]. It was proven that CD4+ T cells stimulated by HLA-G transfected cells considered as APC were inhibited for their capacity of proliferation in response to new antigenic stimulations [52]. These T4+ cells stimulated by these cell lines were differentiated into suppressor cells, which exerted, in return, suppressive effects on other T cells [53]. HLA-G-expressing APC would be able to modulate the transcription of inhibitory receptors, such as ILT2 decrease or KIR2DL4 increase [52–54] in T lymphocytes.

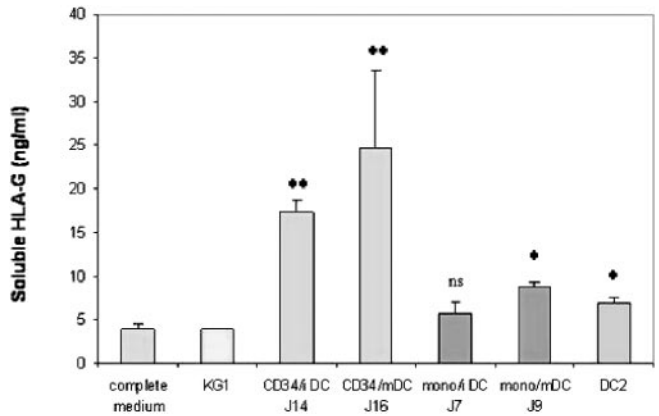

Fig. 6. Comparative level of sHLA-G during mDC and pDC differentiation. sHLA-G level (ng/ml) was detected in culture supernatant during mDC and pDC differentiation using a specific sandwich ELISA (MEM-G/9/anti-β2m–HRP). The results are represented through histograms corresponding to the means of three independent experiments in iDC and mature (mDC) CD34+ and monocyte-derived DC and in DC2. The complete culture medium and the culture supernatants of KG1 were used as negative controls. The detection of sHLA-G highlights the capacity of DC to secrete sHLA-G whatever their origin, but CD34-derived DC represent the mainly DC-secreting subtype (ns, Nonsignificant; *, $P<0.01$; **, $P<0.001$).

More generally, the consequences of HLA-G expression or secretion by the different DC subsets have to be examined considering their specific function. imDC could be tolerogeneic in view of their capacity to capture apoptotic cells and then present self-antigens to T cells [55], whereas mature DC are said to improve the immunity in polarizing, naive Th cells into effector/memory cells. As for pDC, their role is rather to induce immune tolerance. Previous data about HLA-G suggest that the expression of this molecule would be induced in tolerogeneic situations. Contrary to what was expected, our results show that HLA-G is more expressed or secreted in mDC than in pDC and a level of expression/secretion more substantial at the mature stage. These findings allow us to speculate that HLA-G expression/secretion enables regulation of mature DC function, constituting a way to control the intensity of the immune response. These hypotheses are partially confirmed by recent data demonstrating that HLA-G+ APC cell lines are immunoinhibitory cells that inhibit proliferation of CD4+ T cells and induce CD4+ T cell anergy or long-term unresponsiveness [52]. Con-

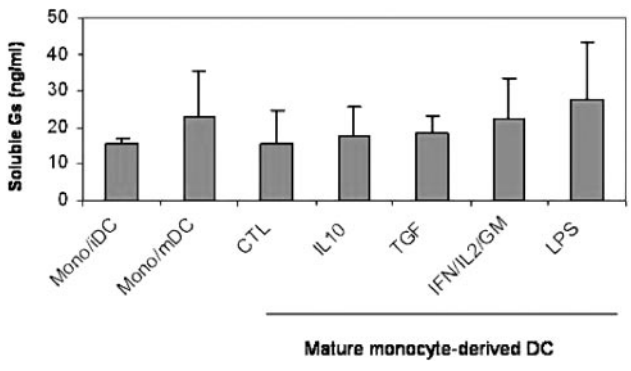

Fig. 7. Influence of cytokines on HLA-G secretion by mature monocyte-derived DC. Results are illustrated through histograms expressing the means of sHLA-G level (ng/ml) in culture supernatants of three independent experiments. No significant variation of sHLA-G secretion was observed after 48 h of stimulation with any of these conditions: IL-10, TGF-β, IFN-γ/IL-2/GM-CSF, and LPS compared with the control (CTL) situation without stimulation.

versely, the expression or secretion of HLA-G by DC may have an autocrinic action on DC themselves, which express ILT4, an inhibitory receptor likely to interact with HLA-G. Some contradictory results concern the role of HLA-G on differentiation and maturation of DC. Using HLA-G tetrameric constructions, Liang and co-workers [31, 32] demonstrated that HLA-G molecules could interact with PIR-B or ILT4 and that ligation led to generation of an inhibitory signal in blocking the nuclear factor-κB activation pathway stimulated by CD40-L. This mechanism would alter the maturation process and subsequently, the allostimulatory capacities of DC in murine skin allograft [56]. This blockage of maturation may represent a negative feedback on DC differentiation. Conversely, no effect on differentiation or maturation of human DC was found with purified HLA-G5 [51]. This discrepancy between these results can be explained by the difference in protein conformation. Tetrameric complexes may favor the recruitment of an inhibitory receptor. A special organization of HLA-G protein through complexes on the cell surface has been described, subsequently increasing the avidity for ILT2 [57, 58]. Moreover, the apoptosis of DC was not induced by sHLA-G [51].

DC are key actors of antitumoral reaction-promoting, tumor-specific, cytotoxic T lymphocytes and have been used as tools for some protocols of cellular therapy. HLA-G expression or secretion by these cells may counteract the efficiency of the immune response. In this field, tumor-loaded, DC-derived exosomes have been proposed to constitute a new way for cancer vaccination [59]. As it was recently demonstrated that HLA-G could be present in exosomes of a HLA-G-positive melanoma cell line [60], our data suggest that exosomes bearing HLA-G could be released from DC expressing HLA-G. The effects of HLA-G-positive exosomes on NK and T cells remain to be investigated but would constitute an additional way to escape immune surveillance in tumoral malignancies.

In conclusion, our data indicate that DC can potentially express and secrete HLA-G. This might have consequences regarding the function of NK cells, T cells, and DC themselves on innate and acquired immunity in malignant, inflammatory, and post-transplantation cases.

ACKNOWLEDGMENTS

This work was supported by grants from the Ligue Nationale Contre le Cancer (Comité Ille et Vilaine) 2001 and 2002. G. L. F., F. G., and Y. S. are recipients of fellowships of the Ligue Nationale Contre le Cancer (Comité des Côtes d'Armor). G. L. F. and F. G. contributed equally to this work. We thank D. Geraghty for providing the 16G1 mAb and LCL.721.221-G5 cell line and H. Grosse-Wilde and V. Rebmann for the calibrated standard sHLA-G.

REFERENCES

1. Kovats, S., Main, E. K., Librach, C., Stubblebine, M., Fisher, S. J., DeMars, R. (1990) A class I antigen, HLA-G, expressed in human trophoblasts. *Science* **248,** 220–223.
2. Rouas-Freiss, N., Moreau, P., Menier, C., Carosella, E. D. (2003) HLA-G in cancer: a way to turn off the immune system. *Semin. Cancer Biol.* **13,** 325–336.
3. Aractingi, S., Briand, N., Le Danff, C., Viguier, M., Bachelez, H., Michel, L., Dubertret, L., Carosella, E. D. (2001) HLA-G and NK receptor are expressed in psoriatic skin: a possible pathway for regulating infiltrating T cells? *Am. J. Pathol.* **159,** 71–77.
4. Khosrotehrani, K., Le Danff, C., Reynaud-Mendel, B., Dubertret, L., Carosella, E. D., Aractingi, S. (2001) HLA-G expression in atopic dermatitis. *J. Invest. Dermatol.* **117,** 750–752.
5. Wiendl, H., Behrens, L., Maier, S., Johnson, M. A., Weiss, E. H., Hohlfeld, R. (2000) Muscle fibers in inflammatory myopathies and cultured myoblasts express the nonclassical major histocompatibility antigen HLA-G. *Ann. Neurol.* **48,** 679–684.
6. Creput, C., Le Friec, G., Bahri, R., Amiot, L., Charpentier, B., Carosella, E., Rouas-Freiss, N., Durrbach, A. (2003) Detection of HLA-G in serum and graft biopsy associated with fewer acute rejections following combined liver-kidney transplantation: possible implications for monitoring patients. *Hum. Immunol.* **64,** 1033–1038.
7. Creput, C., Durrbach, A., Menier, C., Guettier, C., Samuel, D., Dausset, J., Charpentier, B., Carosella, E. D., Rouas-Freiss, N. (2003) Human leukocyte antigen-G (HLA-G) expression in biliary epithelial cells is associated with allograft acceptance in liver-kidney transplantation. *J. Hepatol.* **39,** 587–594.
8. Lila, N., Carpentier, A., Amrein, C., Khalil-Daher, I., Dausset, J., Carosella, E. D. (2000) Implication of HLA-G molecule in heart-graft acceptance. *Lancet* **355,** 2138.
9. Lila, N., Amrein, C., Guillemain, R., Chevalier, P., Latremouille, C., Fabiani, J. N., Dausset, J., Carosella, E. D., Carpentier, A. (2002) Human leukocyte antigen-G expression after heart transplantation is associated with a reduced incidence of rejection. *Circulation* **105,** 1949–1954.
10. Ishitani, A., Geraghty, D. E. (1992) Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc. Natl. Acad. Sci. USA* **89,** 3947–3951.
11. Kirszenbaum, M., Moreau, P., Gluckman, E., Dausset, J., Carosella, E. (1994) An alternatively spliced form of HLA-G mRNA in human trophoblasts and evidence for the presence of HLA-G transcript in adult lymphocytes. *Proc. Natl. Acad. Sci. USA* **91,** 4209–4213.
12. Fujii, T., Ishitani, A., Geraghty, D. E. (1994) A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. *J. Immunol.* **153,** 5516–5524.
13. Paul, P., Adrian Cabestre, F., Ibrahim, E. C., Lefebvre, S., Khalil-Daher, I., Vazeux, G., Moya Quiles, R. M., Bermond, F., Dausset, J., Carosella, E. D. (2000) Identification of HLA-G7 as a new splice variant of the HLA-G mRNA and expression of soluble HLA-G5, -G6, and -G7 transcripts in human transfected cells. *Hum. Immunol.* **61,** 1138–1149.
14. Hofmeister, V., Weiss, E. H. (2003) HLA-G modulates immune responses by diverse receptor interactions. *Semin. Cancer Biol.* **13,** 317–323.
15. Lefebvre, S., Antoine, M., Uzan, S., McMaster, M., Dausset, J., Carosella, E. D., Paul, P. (2002) Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. *J. Pathol.* **196,** 266–274.
16. Pangault, C., Le Friec, G., Caulet-Maugendre, S., Lena, H., Amiot, L., Guilloux, V., Onno, M., Fauchet, R. (2002) Lung macrophages and dendritic cells express HLA-G molecules in pulmonary diseases. *Hum. Immunol.* **63,** 83–90.
17. Urosevic, M., Kurrer, M. O., Kamarashev, J., Mueller, B., Weder, W., Burg, G., Stahel, R. A., Dummer, R., Trojan, A. (2001) Human leukocyte antigen G up-regulation in lung cancer associates with high-grade histology, human leukocyte antigen class I loss and interleukin-10 production. *Am. J. Pathol.* **159,** 817–824.
18. Urosevic, M., Willers, J., Mueller, B., Kempf, W., Burg, G., Dummer, R. (2002) HLA-G protein up-regulation in primary cutaneous lymphomas is associated with interleukin-10 expression in large cell T-cell lymphomas and indolent B-cell lymphomas. *Blood* **99,** 609–617.
19. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., Palucka, K. (2000) Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18,** 767–811.
20. Caux, C., Vanbervliet, B., Massacrier, C., Dezutter-Dambuyant, C., de Saint-Vis, B., Jacquet, C., Yoneda, K., Imamura, S., Schmitt, D., Banchereau, J. (1996) CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF α. *J. Exp. Med.* **184,** 695–706.
21. Blom, B., Ho, S., Antonenko, S., Liu, Y. J. (2000) Generation of interferon α-producing predendritic cell (Pre-DC)2 from human CD34(+) hematopoietic stem cells. *J. Exp. Med.* **192,** 1785–1796.

22. Palucka, K. A., Taquet, N., Sanchez-Chapuis, F., Gluckman, J. C. (1998) Dendritic cells as the terminal stage of monocyte differentiation. *J. Immunol.* **160,** 4587–4595.
23. Ito, T., Inaba, M., Inaba, K., Toki, J., Sogo, S., Iguchi, T., Adachi, Y., Yamaguchi, K., Amakawa, R., Valladeau, J., Saeland, S., Fukuhara, S., Ikehara, S. (1999) A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. *J. Immunol.* **163,** 1409–1419.
24. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184,** 747–752.
25. Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R., Liu, Y. J. (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283,** 1183–1186.
26. Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J., Liu, Y. J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. Exp. Med.* **185,** 1101–1111.
27. Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W., Schmitz, J. (2000) BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* **165,** 6037–6046.
28. Res, P. C., Couwenberg, F., Vyth-Dreese, F. A., Spits, H. (1999) Expression of pTα mRNA in a committed dendritic cell precursor in the human thymus. *Blood* **94,** 2647–2657.
29. Arpinati, M., Green, C. L., Heimfeld, S., Heuser, J. E., Anasetti, C. (2000) Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. *Blood* **95,** 2484–2490.
30. Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., Colonna, M. (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med.* **5,** 919–923.
31. Liang, S., Baibakov, B., Horuzsko, A. (2002) HLA-G inhibits the functions of murine dendritic cells via the PIR-B immune inhibitory receptor. *Eur. J. Immunol.* **32,** 2418–2426.
32. Liang, S., Horuzsko, A. (2003) Mobilizing dendritic cells for tolerance by engagement of immune inhibitory receptors for HLA-G. *Hum. Immunol.* **64,** 1025–1032.
33. Paul, P., Rouas-Freiss, N., Moreau, P., Cabestre, F. A., Menier, C., Khalil-Daher, I., Pangault, C., Onno, M., Fauchet, R., Martinez-Laso, J., Morales, P., Villena, A. A., Giacomini, P., Natali, P. G., Frumento, G., Ferrara, G. B., McMaster, M., Fisher, S., Schust, D., Ferrone, S., Dausset, J., Geraghty, D., Carosella, E. D. (2000) HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression. *Hum. Immunol.* **61,** 1177–1195.
34. Moreau, P., Mouillot, G., Rousseau, P., Marcou, C., Dausset, J., Carosella, E. D. (2003) HLA-G gene repression is reversed by demethylation. *Proc. Natl. Acad. Sci. USA* **100,** 1191–1196.
35. Chapuis, F., Rosenzwajg, M., Yagello, M., Ekman, M., Biberfeld, P., Gluckman, J. C. (1997) Differentiation of human dendritic cells from monocytes in vitro. *Eur. J. Immunol.* **27,** 431–441.
36. Zhou, L. J., Tedder, T. F. (1996) CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc. Natl. Acad. Sci. USA* **93,** 2588–2592.
37. Laupeze, B., Fardel, O., Onno, M., Bertho, N., Drenou, B., Fauchet, R., Amiot, L. (1999) Differential expression of major histocompatibility complex class Ia, Ib, and II molecules on monocyte-derived dendritic and macrophagic cells. *Hum. Immunol.* **60,** 591–597.
38. Carosella, E. D., Moreau, P., Aractingi, S., Rouas-Freiss, N. (2001) HLA-G: a shield against inflammatory aggression. *Trends Immunol.* **22,** 553–555.
39. Rebmann, V., Busemann, A., Lindemann, M., Grosse-Wilde, H. (2003) Detection of HLA-G5-secreting cells. *Hum. Immunol.* **64,** 1017–1024.
40. Kirszenbaum, M., Moreau, P., Teyssier, M., Lafon, C., Gluckman, E., Dausset, J., Carosella, E. (1995) Evidence for the presence of the alternatively spliced HLA-G mRNA forms in human mononuclear cells from peripheral blood and umbilical cord blood. *Hum. Immunol.* **43,** 237–241.
41. Kilburn, B. A., Wang, J., Duniec-Dmuchowski, Z. M., Leach, R. E., Romero, R., Armant, D. R. (2000) Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. *Biol. Reprod.* **62,** 739–747.
42. Chang, C. C., Murphy, S. P., Ferrone, S. (2003) Differential in vivo and in vitro HLA-G expression in melanoma cells: potential mechanisms. *Hum. Immunol.* **64,** 1057–1063.
43. Marchal-Bras-Goncalves, R., Rouas-Freiss, N., Connan, F., Choppin, J., Dausset, J., Carosella, E. D., Kirszenbaum, M., Guillet, J. (2001) A soluble HLA-G protein that inhibits natural killer cell-mediated cytotoxicity. *Transplant. Proc.* **33,** 2355–2359.
44. Wiendl, H., Mitsdoerffer, M., Hofmeister, V., Wischhusen, J., Bornemann, A., Meyermann, R., Weiss, E. H., Melms, A., Weller, M. (2002) A functional role of HLA-G expression in human gliomas: an alternative strategy of immune escape. *J. Immunol.* **168,** 4772–4780.
45. Wiendl, H., Mitsdoerffer, M., Hofmeister, V., Wischhusen, J., Weiss, E. H., Dichgans, J., Lochmuller, H., Hohlfeld, R., Melms, A., Weller, M. (2003) The non-classical MHC molecule HLA-G protects human muscle cells from immune-mediated lysis: implications for myoblast transplantation and gene therapy. *Brain* **126,** 176–185.
46. Fournel, S., Aguerre-Girr, M., Campan, A., Salauze, L., Berrebi, A., Lone, Y. C., Lenfant, F., Bouteiller, P. L. (1999) Soluble HLA-G: purification from eukaryotic transfected cells and detection by a specific ELISA. *Am. J. Reprod. Immunol.* **42,** 22–29.
47. Contini, P., Ghio, M., Poggi, A., Filaci, G., Indiveri, F., Ferrone, S., Puppo, F. (2003) Soluble HLA-A,-B,-C and -G molecules induce apoptosis in T and NK CD8+ cells and inhibit cytotoxic T cell activity through CD8 ligation. *Eur. J. Immunol.* **33,** 125–134.
48. Puppo, F., Contini, P., Ghio, M., Indiveri, F. (2002) Soluble HLA class I molecules/CD8 ligation trigger apoptosis of CD8+ cells by Fas/Fas-ligand interaction. *ScientificWorldJournal* **2,** 421–423.
49. Spaggiari, G. M., Contini, P., Carosio, R., Arvigo, M., Ghio, M., Oddone, D., Dondero, A., Zocchi, M. R., Puppo, F., Indiveri, F., Poggi, A. (2002) Soluble HLA class I molecules induce natural killer cell apoptosis through the engagement of CD8: evidence for a negative regulation exerted by members of the inhibitory receptor superfamily. *Blood* **99,** 1706–1714.
50. Lila, N., Rouas-Freiss, N., Dausset, J., Carpentier, A., Carosella, E. D. (2001) Soluble HLA-G protein secreted by allo-specific CD4+ T cells suppresses the allo-proliferative response: a CD4+ T cell regulatory mechanism. *Proc. Natl. Acad. Sci. USA* **98,** 12150–12155.
51. Le Friec, G., Laupeze, B., Fardel, O., Sebti, Y., Pangault, C., Guilloux, V., Beauplet, A., Fauchet, R., Amiot, L. (2003) Soluble HLA-G inhibits human dendritic cell-triggered allogeneic T-cell proliferation without altering dendritic differentiation and maturation processes. *Hum. Immunol.* **64,** 752–761.
52. LeMaoult, J., Krawice-Radanne, I., Dausset, J., Carosella, E. D. (2004) HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4+ T cells. *Proc. Natl. Acad. Sci. USA* **101,** 7064–7069.
53. Suciu-Foca, N., Manavalan, J. S., Cortesini, R. (2003) Generation and function of antigen-specific suppressor and regulatory T cells. *Transpl. Immunol.* **11,** 235–244.
54. LeMaoult, J., Le Discorde, M., Rouas-Freiss, N., Moreau, P., Menier, C., McCluskey, J., Carosella, E. D. (2003) Biology and functions of human leukocyte antigen-G in health and sickness. *Tissue Antigens* **62,** 273–284.
55. Steinman, R. M., Turley, S., Mellman, I., Inaba, K. (2000) The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med.* **191,** 411–416.
56. Horuzsko, A., Lenfant, F., Munn, D. H., Mellor, A. L. (2001) Maturation of antigen-presenting cells is compromised in HLA-G transgenic mice. *Int. Immunol.* **13,** 385–394.
57. Gonen-Gross, T., Gazit, R., Achdout, H., Hanna, J., Mizrahi, S., Markel, G., Horejsi, V., Mandelboim, O. (2003) Special organization of the HLA-G protein on the cell surface. *Hum. Immunol.* **64,** 1011–1016.
58. Gonen-Gross, T., Achdout, H., Gazit, R., Hanna, J., Mizrahi, S., Markel, G., Goldman-Wohl, D., Yagel, S., Horejsi, V., Levy, O., Baniyash, M., Mandelboim, O. (2003) Complexes of HLA-G protein on the cell surface are important for leukocyte Ig-like receptor-1 function. *J. Immunol.* **171,** 1343–1351.
59. Andre, F., Schartz, N. E., Chaput, N., Flament, C., Raposo, G., Amigorena, S., Angevin, E., Zitvogel, L. (2002) Tumor-derived exosomes: a new source of tumor rejection antigens. *Vaccine* **20** (Suppl. 4), A28–A31.
60. Riteau, B., Faure, F., Menier, C., Viel, S., Carosella, E. D., Amigorena, S., Rouas-Freiss, N. (2003) Exosomes bearing HLA-G are released by melanoma cells. *Hum. Immunol.* **64,** 1064–1072.