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# Modulation of HLA-G Antigens Expression by Human Cytomegalovirus: Specific Induction in Activated Macrophages Harboring Human Cytomegalovirus Infection<sup>1</sup>

Myriam Onno,<sup>2\*</sup> Céline Pangault,\* Gaëlle Le Friec,\* Valérie Guilloux,\* Patrice André,<sup>†</sup> and Renée Fauchet\*

After infection, human CMV (HCMV) establishes a latent and persistent infection in immature myeloid progenitors and peripheral blood monocytes. Completion of the HCMV life cycle is possible upon maturation of monocytes to tissue macrophages and under permissive circumstances, e.g., immunosuppression. We investigated the hypothesis that HLA-G molecules could be induced during HCMV reactivation in activated macrophages to favor virus dissemination. In this study, we provide evidence that HLA-G Ags are produced during viral reactivation in macrophages generated after allogeneic stimulation of HCMV latently infected monocytes. While HLA-G surface expression is up-regulated, classical MHC-I molecules are partially down-regulated by HCMV. In vivo, bronchoalveolar macrophages collected from patients suffering from acute HCMV pneumonitis also express HLA-G molecules. The direct correlation between HLA-G Ag induction and HCMV infection was confirmed in U-373 MG astrocytoma cells. Soluble HLA-G expression is stimulated upon HCMV infection, and this modulation depends on the cooperative action of the two immediate-early-1 pp72 and immediate-early-2 pp86 products. Because HLA-G transcription is active in macrophages and U-373 MG astrocytoma cells, it is likely that the modulation of HLA-G protein expression during HCMV replication occurs at a post-transcriptional level. Our data suggest that induction of HLA-G molecules could be an additional mechanism that helps HCMV to subvert host defenses. *The Journal of Immunology*, 2000, 164: 6426–6434.

Human CMV (HCMV)<sup>3</sup> belongs to the  $\beta$  herpesvirinae subfamily and, as observed with other herpesviruses, primary HCMV infection is followed by viral persistence with >50% of healthy adults being latently infected. Reactivation is defined as intermittent virus replication resulting in sub-clinical or clinical infections mainly in immunocompromised patients. HCMV primary or recurrent infections are also the major cause of viral congenital diseases in the Western world (1).

Despite the important roles latency and reactivation play in the pathogenesis of HCMV disease, knowledge of the underlying mechanisms controlling these processes remains limited. Herpesviruses have selected different strategies to ensure their persistence in latently infected cells and to evade host immunity during their active replication (2). During the infectious cycle, the assembly and trafficking of classical class I (MHC-I) molecules have been

targeted by HCMV to protect infected cells from attack by MHC-I-restricted cytotoxic T cells (3). pp65 inhibits the generation of HCMV-specific T cell epitope. US3 retains stable MHC-I heterodimers in the endoplasmic reticulum, whereas US2 and US11 mediate the cytosolic degradation of heavy chains by the proteasome. US6 interacts with TAP, thereby inhibiting TAP-mediated peptide loading of MHC-I molecules. HCMV-infected cells should be more vulnerable to attack by NK cells, because MHC-I molecules mediate inhibitory messages through various receptors. The HCMV MHC-I homologue UL18 may contribute to evasion from NK lysis as it binds to the leukocyte Ig-like receptor 1 (LIR-1) (4). Surface expression of HLA-E is enhanced by binding the leader sequence of the HCMV glycoprotein UL40 and inhibits NK cell-mediated lysis by interacting with CD94/NKG2a receptors (5).

The function of the classical MHC-I molecules, HLA-A, -B, and -C in immune recognition is well understood in both functional and structural terms. These highly polymorphic molecules constitute transplantation Ags that may be recognized by alloreactive T cells. These molecules also play an important role in the induction of a specific immune response by presenting tumoral or viral peptide Ags to T cells. In contrast, nonclassical MHC-I HLA-G molecules have been described as inhibitors of the cellular immune response. The HLA-G gene is characterized by a limited polymorphism and the alternative transcription of spliced mRNAs that encode at least six different isoforms, namely the membrane-bound HLA-G1, -G2, -G3, and -G4 and soluble HLA-G5 and HLA-G6 proteins (6). Both cell surface and soluble HLA-G molecules can bind an identical set of peptides derived from a variety of intracellular proteins. HLA-G Ags are primarily expressed in fetal trophoblast cells that invade the maternal decidua. These invading trophoblast cells fail to express MHC-I HLA-A, -B, and MHC-II molecules (6). It was proposed that HLA-G expression on trophoblast cells affects NK cell cytotoxicity through binding to killing

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<sup>3</sup> Abbreviations used in this paper: HCMV, human CMV; BAL, bronchoalveolar lavages; IE, immediate-early; gB, CMV glycoprotein B; HFF, human foreskin fibroblasts; LIR-1, leukocyte Ig-like receptor; MHC-I, class I genes of the MHC; MFI, mean fluorescent intensity.

inhibitory receptors present on NK cells. Although controversial results were reported by different groups, three NK or other cell inhibitory receptors that bind HLA-G have been now recognized: LIR-1, LIR-2, and p49 (7–11). LIR-1 is expressed on NK and T subsets, B lymphocytes, and myelomonocytic cells (12). LIR-2 is selectively expressed in monocytes, macrophages, and dendritic cells (13). Recognition of LIR-1 and LIR-2 inhibitory receptors by membrane-bound HLA-G molecules may modulate Ag-presenting functions or inflammatory responses mediated by myelomonocytic cells. Cytotoxic activity is also down-regulated by HLA-G surface expression, probably by engaging an inhibitory receptor. Thus, the inhibition of an HLA-A2-restricted CTL response specific for an influenza viral peptide has been recently demonstrated (14). Furthermore, HLA-G is capable of suppressing the allogeneic proliferative response of T lymphocytes in classical mixed lymphocyte reactions *in vitro* (15).

Soluble HLA-G molecules may have synergistic or complementary tolerance effects with membrane-bound HLA-G proteins. In cytotoxicity assays using the K562 cell line as a NK target, the soluble GST-HLA-G fusion proteins produced in *Escherichia coli* impair peripheral blood NK lytic activity (16). The supernatants of CHO cells transfected by soluble HLA-G cloned cDNAs exhibit NK inhibitory properties and show strong MLR suppression (17). These different *in vitro* functional studies strongly suggest that cell surface and soluble HLA-G Ags may act as strong immunosuppressive molecules *in vivo*.

In previous works, we showed that HLA-G translation is tightly regulated in myelomonocytic cells. The HLA-G gene is preferentially translated in macrophages and dendritic cells infiltrating lung carcinoma (18). Furthermore, IFN- $\gamma$ , in synergy with IL-2 and GM-CSF, induces HLA-G protein expression at the cell surface of the U937 monohistiocytic leukemia cell line (19). Interestingly, in peripheral blood, CD14<sup>+</sup> cells are the predominant site of latent HCMV viral genome (20, 21), and virus reactivation occurs during inflammatory responses when macrophages or dendritic cells differentiate (22, 23). Macrophages are the major HCMV-infected cells infiltrating tissues that could serve as cellular vectors spreading virus to different organs (24).

A virus would increase its likelihood of survival and transmission by inducing synthesis of molecules that down-modulate both the innate and adaptive immunities. We investigated the hypothesis that HCMV may modulate HLA-G expression in virally infected macrophages. Our study demonstrates the induction of HLA-G molecules during reactivation of HCMV in allogeneic-stimulated primary macrophage cultures and in alveolar macrophages isolated from patients with acute HCMV pneumopathy. The direct correlation between HLA-G Ag production and HCMV replication was confirmed in U-373 MG astrocytoma cells.

## Materials and Methods

### Cell lines

Human foreskin fibroblasts (HFF) and U-373 MG human astrocytoma cells were grown in DMEM (Life Technologies, Cergy-Pontoise, France). The JEG 3 human choriocarcinoma cell line expressing HLA-G Ags was maintained in Eagle's MEM (Life Technologies). This choriocarcinoma cell line was used as a positive control in the different HLA-G expression experiments. Media were supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were consistently free of mycoplasma infection.

Transfected U-373 MG human astrocytoma cells expressing IE-pp72 (A1b) or IE-pp86 and IE-pp72 plus IE-pp86 (A45) (a gift from S. Michelson, Paris, France) were maintained in DMEM containing 400  $\mu$ g/ml of the neomycin analogue, G418 (Life Technologies). The establishment of the A1b and A45 cell lines has been described previously (25, 26). In transfected cells expressing IE-pp86 alone, the viral gene was inserted in

the pMam-neo plasmid under the control of the dexamethasone-inducible Moloney murine tumor virus-long terminal repeat promoter. This vector was cotransfected with the pCT-TK-GR3-795 plasmid expressing a glucocorticoid receptor (27). U-373 MG astrocytoma cells transfected with empty vector expressing the G418 resistance gene were used as negative controls.

### Isolation and culture of monocyte-derived macrophages

Blood samples were collected from a pool of donors at the Etablissement Français du Sang Bretagne. PBMCs were isolated by Ficoll density gradient centrifugation on Lymphoprep (density: 1.077) (Nycomed Pharma, Oslo, Norway), washed twice with sterile HBSS and resuspended at  $1 \times 10^7$  cells/ml in Iscove's medium (Life Technologies) containing penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml), L-glutamine (2 mM), and 10% human AB group serum. Cells ( $2 \times 10^7$ /well) were plated in six-well tissue culture Falcon plates (Becton Dickinson, Meylan, France) at 37°C with 5% CO<sub>2</sub>. For allogeneic stimulation experiments, equal numbers of cells from two unrelated blood donors at a cell concentration of  $1 \times 10^7$ /ml were mixed in complete Iscove's medium before plating in six-well plates. After 48 h of culture at 37°C in 5% CO<sub>2</sub>, nonadherent cells were removed. Cultures were washed and maintained in complete 60/30 medium (60% AIMV medium (Life Technologies) and 30% Iscove's medium) composed of 50% spent medium and 50% fresh medium, which was replenished every 3–4 days for up to 45 days poststimulation (23). For analysis, macrophages were collected at different time points by trypsinizing and gentle scraping.

### Bronchoalveolar lavages (BAL) collected from patients

Forty bronchoalveolar samples were obtained from the Laboratoires de Bactériologie-Virologie et de Parasitologie at the Centre Hospitalier de Pontchaillou in Rennes after being analyzed for bacterial, fungal, and viral infections. In particular, the presence of infectious HCMV was verified by the shell vial centrifugation culture assay (28). Cells were washed twice in PBS (pH 7.5), cytocentrifuged onto glass slides, and stored wrapped in aluminum foil at –20°C until used.

### Viral strain and infection

HCMV strain Toledo (a gift from J. Nelson, Oregon Health Sciences University, Portland, OR) was harvested from the culture medium of fibroblasts, clarified by centrifugation at  $1500 \times g$  for 20 min, and stored at –80°C. U-373 MG monolayers were grown to confluence and infected at a ratio of 5 PFU/cell. After adsorption of virus during 1 h at 37°C, the inoculum was removed, the cells were washed in PBS, and complete medium was added. At various times postinfection, the cells were analyzed for the expression of HLA-G and viral Ags.

### Antibodies

The 87G and 16G1 mAbs were provided by D. Geraghty (Fred Hutchinson Cancer Research, Seattle, WA). 87G is a murine IgG2a mAb recognizing the membrane-bound HLA-G1 and the soluble isoform HLA-G5. 87G shows no cross-reactivity with other MHC-I molecules when tested on diverse MHC-I type transfected cells (29). 16G1 is a conformation-independent murine IgG1 mAb showing complete specificity for HLA-G soluble molecules (29). The W6/32 mAb is a murine IgG2a mAb that binds to MHC-I heavy chains associated with  $\beta_2m$ . Differentiated macrophages were phenotyped with an anti-CD68 murine IgG1 mAb (KP1 clone; Dako, Trappes, France). Three Abs against HCMV genes products were used: two polyclonal rabbit sera against the IE-pp86 and IE-pp72 Ags (gifts from J. Nelson) and a mAb against glycoprotein B (gB; clone HCMV37, Chemicon, Temecula, CA).

### RT and amplification of HLA-G transcripts

Poly(A)<sup>+</sup> mRNAs from peripheral blood monocytes were purified using Dynabeads oligo(dT)<sub>25</sub> (Dyna, Great Neck, NY). cDNAs were prepared by random hexamer priming of 100 ng of poly(A)<sup>+</sup> mRNA. First-strand cDNA reactions were performed using Superscript II RNase H<sup>-</sup> reverse transcriptase (Life Technologies). Hot start PCR amplifications were conducted with half the cDNA reaction mixtures in a total volume of 50  $\mu$ l containing 50 pmol of specific HLA-G primers. The pan-HLA-G primers G257 (exon 2), 5'-GGA AGA GGA GAC ACG GAA CA-3'; G1225 (3' untranslated region), 5'-TGA GAC AGA GAC GGA GAC AT-3' detect all alternative spliced transcripts (30). PCR amplifications were run at 94°C for 1 min, at 61°C for 1 min, and at 72°C for 1 min 30 for 35 cycles, with a final 10-min extension at 72°C. In the same way, amplification of  $\beta$ -actin cDNA was performed to check RNA quality. Ten microliters of each PCR



reaction was analyzed following electrophoresis on a 1.2% agarose gel and staining with ethidium bromide.

#### Detection of viral HCMV DNA in monocytes

Because not all HCMV-seropositive individuals carry HCMV in their monocytes, and a substantial proportion of seronegative individuals are indeed HCMV carriers (31), latent infection was tested by detection of HCMV DNA. DNA was prepared from adherent PBMCs using the DNA Qiagen Blood Kit (Qiagen, Courtaboeuf, France). HCMV-specific primers from exons 1 and 2 of the major immediate-early UL123 gene were used in nested PCR reactions (31). PCR reaction mixture consisted of 100 ng of DNA, PCR buffer (50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 10 mM Tris-HCl, pH 9.0), 200 μM dNTP, 1 μM of amplification primers, and 0.5 U of AmpliTaq DNA polymerase (Pharmacia Biotech, Orsay, France). Amplifications were first conducted with primers MIE I 5' (5'-GAG TCC TCT GCC AAG AGA AA-3') and MIE I 3' (5'-GAG TTC TGC CAG GAC ATC TTT-3') using 30 cycles at 94°C for 20 s, 63°C for 50 s, and 72°C for 20 s. Two microliters of the PCR reaction were added to the nested PCR reaction mixture. Nested PCR was conducted with primers MIE II 5' (5'-GAG AAA GAT GGA CCC TGA TAA T-3') and MIE II 3' (5'-CTC GGG GTT CTC GTT GCA AT-3') using 30 cycles at 94°C for 20 s and at 60°C for 1 min. DNAs prepared from uninfected and infected HFF were used as negative and positive controls, respectively. Final amplification products (332 bp) were run on a 1.7% agarose gel containing ethidium bromide and were visualized with UV light.

#### Flow cytometry

Flow cytometry was performed on 3 × 10<sup>5</sup> macrophages. Fc receptors were blocked by preincubating cells for 60 min in 5% human AB group serum. Cells were incubated with 87G or W6/32 mAbs at a final concentration of 1 μg/ml for 45 min at 4°C. In each experiment an equivalent concentration of a normal mouse IgG2a mAb (clone DAK-GO5; Dako) was substituted for the specific Ab. After washing, cells were incubated for 45 min with an anti-mouse goat IgG F(ab')<sub>2</sub> fraction conjugated with PE (Immunotech, Marseille, France). Fluorescence analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson).

#### Immunocytochemistry

Cells were cytocentrifuged on glass slides and fixed for 10 min in acetone at 4°C. Samples were stored wrapped in aluminum foil at -20°C until used. Staining was conducted at room temperature using the LSAB 2 kit peroxidase (Dako). Cells were incubated for 20 min in 3% BSA-40% human AB group serum in Tris-buffered saline (TBS) (pH 7.5) to eliminate nonspecific binding. Samples were then incubated with the following primary Abs: anti-HLA class I mAb, W6/32 (2 μg/ml); anti-HLA-G mAbs, 87G (5 μg/ml) and 16G1 (4 μg/ml); anti-CD68 mAb, KP1 (4 μg/ml); anti-HCMV IE pp86 (1:100); anti-HCMV IE pp72 (1:100); and anti-HCMV gB (10 μg/ml). They were then washed twice in Tris-buffered saline and incubated for 10 min with biotinylated anti-mouse or anti-rabbit Abs. After washing, endogenous peroxidase activity was blocked in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in TBS, samples were incubated for 10 min with peroxidase-labeled streptavidin, and staining was completed with a freshly prepared substrate chromogen, 3% 3-amino-9-ethylcarbazole in *N,N*-dimethylformamide. Finally, specimens were counterstained with Harris' hematoxylin and mounted in aqueous Aquatex mounting medium (Merck, Darmstadt, Germany). To test the specificity of immunocytochemical techniques, primary Abs were replaced by isotype-matched control Abs. No labeling was detected using negative control Abs at the same concentration, thereby indicating that immunostaining was specific, and endogenous activities were indeed suppressed.

#### Double-label immunofluorescence

Cells were cytocentrifuged onto glass slides and fixed for 10 min in acetone at 4°C. Samples were then rehydrated in PBS, and endogenous fluorescence was quenched through incubation of fixed cells in 50 mM NH<sub>4</sub>Cl for 10 min. Cells were incubated for 20 min in 3% BSA-40% human AB group serum in PBS so as to eliminate nonspecific binding. The 87G (5 μg/ml) or 16G1 (4 μg/ml) mAbs were applied for 30 min to slides that were then washed twice in PBS. Bound 87G or 16G1 was detected by sequential incubations with biotinylated goat anti-mouse mAb and FITC-labeled streptavidin. To search for HCMV expression in HLA-G-positive cells, the same preparations were saturated once again with 3% BSA-40% human AB group serum in PBS. Samples were then incubated for 30 min with the anti-HCMV IE polyclonal serum or the anti-gB mAb. After washing, goat anti-mouse or anti-rabbit tetramethylrhodamine isothiocyanate (TRITC)-labeled Abs were applied for 30 min. Samples were then mounted in an

anti-fade medium and viewed with a Leica confocal laser scanning microscope (Rockleigh, NJ).

#### SDS-PAGE and Western blotting

Proteins were extracted using a lysis buffer containing 1% Nonidet P-40 and 0.5% sodium deoxycholate and were sonicated for 30 s to shear off chromosomal DNA. Solubilized proteins were loaded on a 12% mini-SDS-PAGE (75 μg/lane) and transferred to a polyvinylidene difluoride membrane using an electrophoretic transfer apparatus. The quality of protein samples was checked by incubating the membrane in the Ponceau S reagent. Membranes were saturated with 5% nonfat dry milk in PBS and incubated with the 16G1 mAb (4 μg/ml). Alkaline phosphatase-linked anti-mouse Ab was added, and the mixture was reacted with CDP-Star Chemiluminescent substrate (New England Biolabs, Schwalbach, Germany).

#### HCMV detection by virus recovery on human foreskin fibroblasts

Sonicated macrophages were plated onto HFF at subconfluence in six-well plates. After 1 h of inoculation, HFF were washed, and fresh medium was added. Cultures were incubated for 5–10 wk and regularly controlled for the apparition of a cytopathic effect. Cells were fixed with acetone and stained for the presence of HCMV IE Ags using the polyclonal serum against the IE-pp86 Ag.

## Results

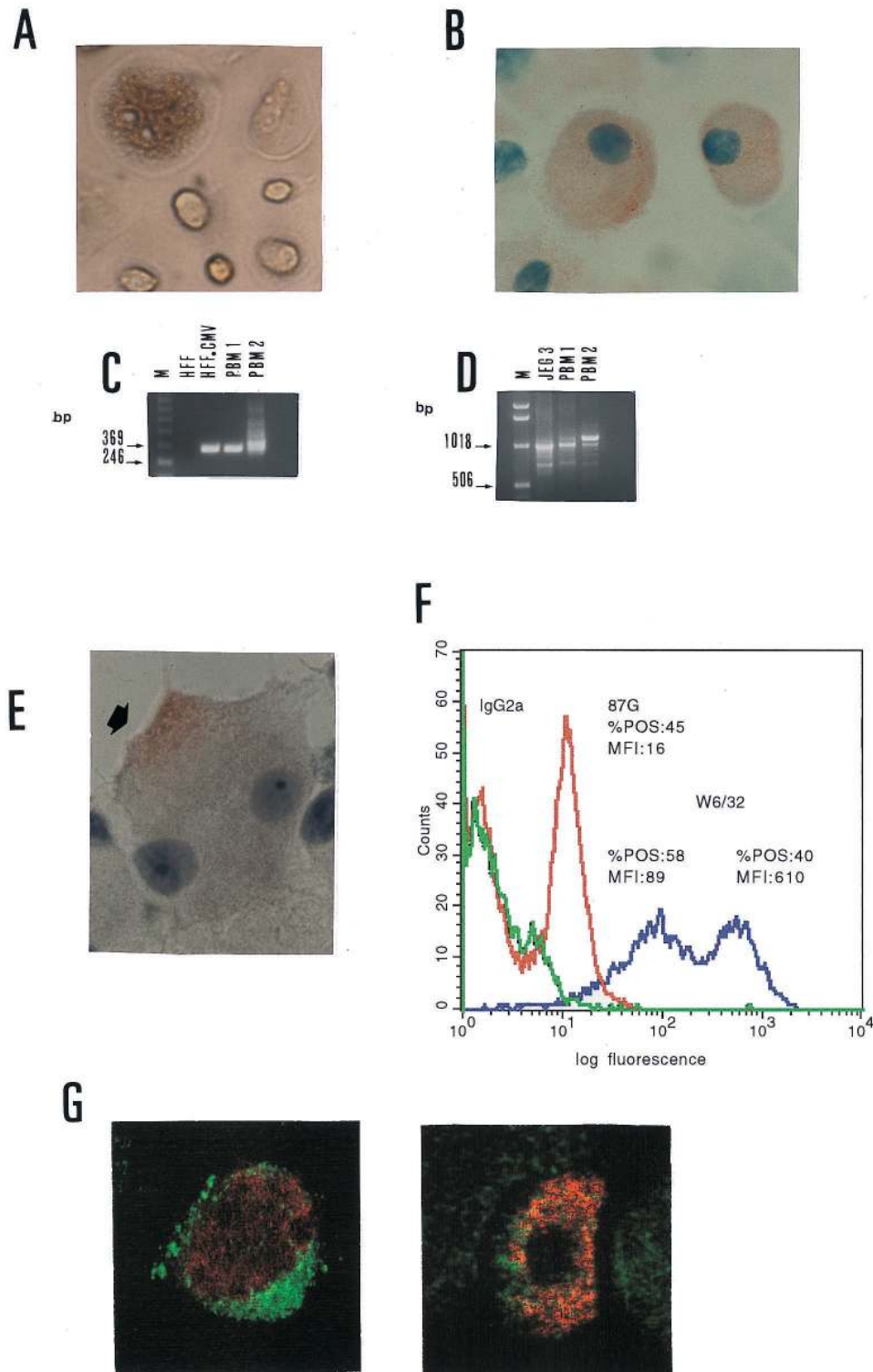
#### Detection of HLA-G Ags in macrophages generated following allogeneic stimulation of latently HCMV peripheral blood monocytes

We tested the potential up-regulation of HLA-G gene activity upon HCMV reactivation in allogeneically stimulated monocyte-derived macrophages. Primary macrophage cultures were established by mixing PBMCs from unrelated blood donor pairs. After 48 h of allogeneic stimulation, nonadherent cells were removed from the culture, and macrophages were maintained for 5–6 wk. Adherent-stimulated monocytes differentiated into morphologically different phenotypes of macrophages, including multinucleated giant cells (Fig. 1A). At 10 days poststimulation, monocyte-macrophage differentiation was demonstrated by uniform intracellular expression of CD68 Ag (Fig. 1B).

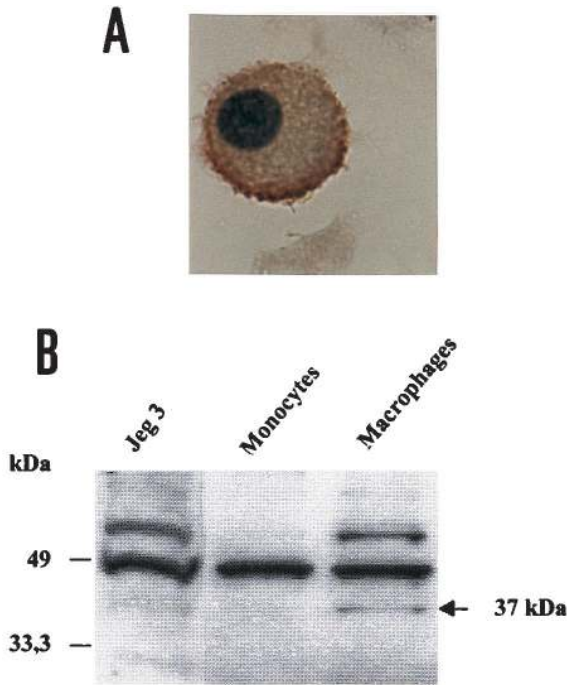
We studied 10 primary macrophage cultures established from PBMCs collected from healthy donors; six cultures were established by mixing PBMCs from six unrelated HCMV DNA-positive donor pairs (Fig. 1C), and four others by mixing PBMCs from four unrelated HCMV DNA-negative donor pairs. HLA-G expression was monitored by immunocytochemistry and flow cytometry every 5–6 days. Several alternatively spliced HLA-G mRNAs were expressed in monocytes before differentiation (Fig. 1D), but cell surface and soluble HLA-G proteins were never detectable.

Using the 87G mAb, HLA-G-expressing macrophages were first detected by immunocytochemistry in five primary cultures established from latently infected monocytes at 2 wk poststimulation. At that time, intracellular HLA-G-positive staining was found in 0.05–2.5% of differentiated macrophages (Fig. 1E). On day 20 poststimulation, analyses by flow cytometry of two primary cultures demonstrated HLA-G cell surface expression in 30 and 45% of macrophages, respectively (Fig. 1F). At this stage, while HLA-G Ags were up-regulated, expression of classical MHC-I molecules was substantially decreased. However, two-color staining analyses using 87G and W6/32 mAbs showed that induction of HLA-G Ags always occurred in macrophages that still expressed classical MHC-I molecules (data not shown). In both cultures no HLA-G cell surface expression was detectable after 5 wk poststimulation.

Using the 16G1 mAb, soluble HLA-G molecules were also identified by immunocytochemistry in three primary cultures generated from latently infected monocytes ~2 wk poststimulation



**FIGURE 1.** HLA-G expression in one allogene-stimulated primary macrophage culture generated from two HCMV latently infected donors. *A*, Macrophage differentiation was conducted by allogeneic stimulation of PBMCs from two unrelated healthy blood donors. *B*, At ~10 days poststimulation, macrophages expressed the CD68 macrophage marker (red-brown staining). *C*, The presence of latent HCMV genome is demonstrated by the amplification of a 332-bp IE product. Human foreskin fibroblasts (HFF) and HCMV-infected fibroblasts (HFF-CMV) were used as negative and positive controls, respectively. *D*, HLA-G is transcribed in undifferentiated peripheral blood monocytes collected from the two donors (PBM1 and PBM2). JEG 3, HLA-G-expressing choriocarcinoma cells (positive control). *E*, Expression of HLA-G Ags was analyzed by immunocytochemistry using the 87G mAb. In these macrophages, HLA-G-positive staining was first obtained at 20 days poststimulation. *F*, Flow cytometric analysis demonstrated cell surface expression of HLA-G molecules in 45% of macrophages (red curve). The profile also shows that one population of macrophages displayed a decrease in MHC-I cell surface expression. MFI, mean fluorescent intensity; % POS, percentage of positive cells. *G*, Double-label immunofluorescence was performed with the anti-HCMV IE-pp86 rabbit serum (*left*) or the anti-HCMV gB mAb (*right*) (rhodamine) and the anti-HLA-G mAb 87G (fluorescein). Fluorescence staining is visualized with a confocal microscope. *Left*, Cellular colocalization of IE-86 (red nucleus) and HLA-G (green cytoplasm) was observed in few macrophages at 20 days poststimulation. *Right*, The photograph illustrates the colocalization of HCMV glycoprotein and HLA-G Ags in the cytoplasm of macrophages displaying HCMV replication (orange fluorescent staining).



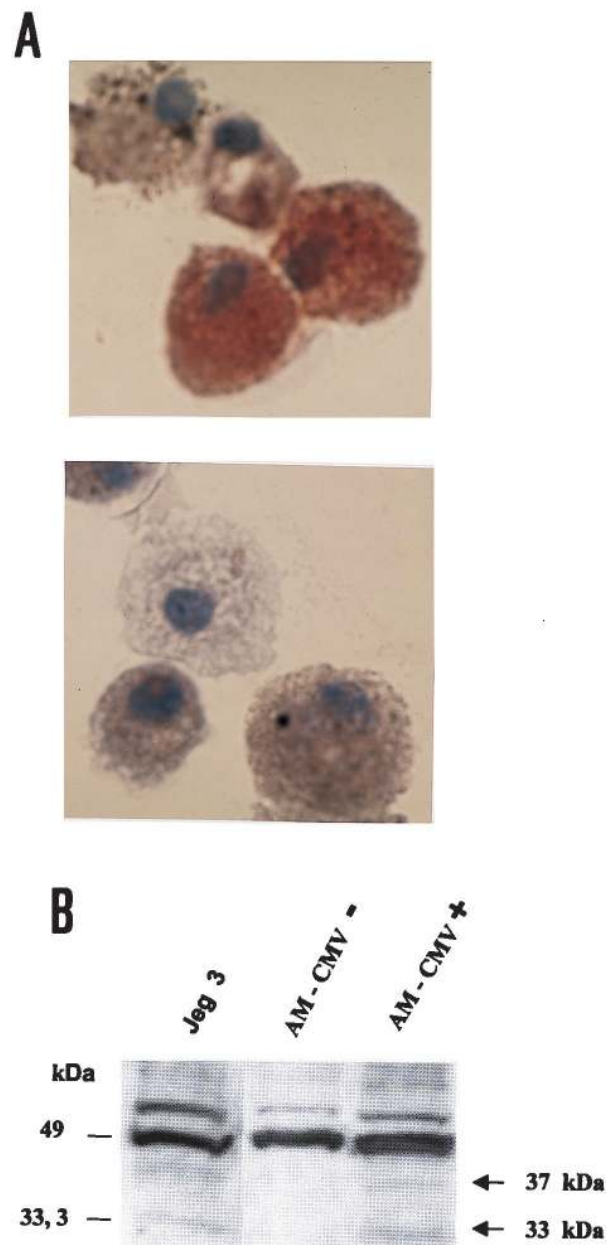
**FIGURE 2.** Identification of HLA-G-soluble molecules in one macrophage culture generated from two HCMV latently infected donors. *A*, Immunocytochemical staining, using the 16G1 mAb, demonstrated a strong expression of HLA-G-soluble molecules in macrophages (red-brown staining). *B*, The Western blot was incubated with the 16G1 mAb. A 37-kDa HLA-G-soluble protein was found in macrophages at 19 days postinfection. No HLA-G-soluble Ags were detected in lysates prepared from peripheral blood monocytes before starting the primary culture.

(Fig. 2A). On Western blot, these soluble molecules migrated as a 37-kDa soluble HLA-G5 isoform (Fig. 2B). HLA-G Ags were never detected in the primary cultures produced by mixing uninfected PBMCs. During the culture, these uninfected macrophages always displayed an usually high level of classical MHC-I Ags at their cell surface (data not shown).

We simultaneously verified whether virus was reactivated from its latent state during the macrophage differentiation process. Macrophage samples were collected every 5–6 days for 50 days and evaluated for HCMV gene expression by immunocytochemistry and for virus production by coculture of sonicated macrophage samples with human fibroblasts. IE-pp72 and IE-pp86 Ags were detected in both allogene-stimulated cultures expressing HLA-G proteins between 16 and 20 days poststimulation. Double-label immunofluorescence with the anti-HCMV IE-pp86 and 87G Abs demonstrated the presence of HCMV IE-pp86 product in the nucleus of some HLA-G expressing macrophages (Fig. 1G). Coexpression of HCMV gB late Ag and HLA-G molecules was also observed a few days later in macrophages of five cultures (Fig. 1G). Consistent with this HCMV late Ag expression, a cytopathic effect, indicating infectious virus production, was demonstrated in human fibroblasts inoculated with macrophage sonicates after 5–10 wk of coculture. No cytopathic effect was observed when fibroblast cultures were inoculated with macrophages generated from HCMV-negative donor pairs.

#### *HLA-G Ags are expressed in alveolar macrophages during acute HCMV pneumonia*

Because alveolar macrophages harbor replicative virus in lungs during acute HCMV pneumonia, we searched for HLA-G protein expression in alveolar cells collected by BAL. Immunocytochem-



**FIGURE 3.** Expression of HLA-G molecules in alveolar macrophages collected from patients with HCMV pulmonary infection. *A*, Immunocytochemical staining was monitored using the 87G mAb. Alveolar macrophages displayed a strong cytoplasmic HLA-G expression (red-brown staining). No staining was observed by using the isotypic IgG2a control. *B*, On Western blot, like in JEG 3 choriocarcinoma cells, the 16G1 mAb detected two different soluble isoforms (37 and 33 kDa) in alveolar macrophages harboring HCMV replication (AM-CMV<sup>+</sup>). Alveolar macrophages collected from a patient with interstitial pneumopathy without HCMV infection did not express HLA-G-soluble molecules (AM-CMV<sup>-</sup>).

ical staining with the anti-CD68 mAb, KP1, revealed the presence of at least 85% alveolar macrophages in each cytoцентрифуг preparation. Of 40 BAL performed on patients suffering from pneumonitis, five yielded a positive HCMV culture using the shell vial centrifugation assay. They were collected from three bone marrow-transplanted patients, and two HIV-infected patients. HLA-G-expressing macrophages were observed by immunocytochemical staining in four of these five HCMV-positive samples (Fig. 3A). Between 5 and 25% of alveolar macrophages demon-



strated expression of HLA-G Ags. Furthermore, 33- and 37-kDa HLA-G soluble isoforms were identified in the protein lysate of one sample tested by Western blot (Fig. 3B). No HLA-G expression was found in alveolar macrophages of lungs infected by bacterial or other viral pathogens ( $p < 0.0001$ ). Interestingly, herpes simplex viruses were isolated in two HCMV-negative viral cultures, and these did not display HLA-G expression.

#### *Modulation of HLA-G Ags expression in HCMV-infected U-373 MG astrocytoma cells*

To confirm the direct correlation between HCMV replication and HLA-G Ag production, U-373 MG astrocytoma cells were infected with the HCMV Toledo strain. U-373 MG astrocytoma cells are known to be permissive for HCMV replication and to express HLA-G transcripts without detectable translation products (32). HLA-G Ag expression was analyzed from the end of the virus adsorption period up to 4 days postinfection by immunocytochemistry. Between 5 and 10% of the cells produced HLA-G Ags at ~24 h postinfection in different experiments (Fig. 4A). At that time, HCMV IE-pp72 and IE-pp86 were highly expressed in 90 and 80% of inoculated astrocytoma cells, respectively, and only 1% of the cells contained the late viral protein gB (data not shown). Double-label immunofluorescence with the anti-HCMV IE-pp86 and 87G Abs showed the presence of IE-pp86 Ags in the nucleus of HLA-G expressing infected U-373 MG astrocytoma cells (Fig. 4B). Simultaneous monitoring of HLA-G cell surface expression by flow cytometry using mAb 87G showed an absence of cell surface expression. On the other hand, immunocytochemistry and Western blot analyses with 16G1 mAb demonstrated that induced HLA-G Ags are soluble isoforms. The amount of induced soluble HLA-G Ags increased at 48 h postinfection and peaked at 72 h (Fig. 4C). HLA-G production decreased at 96 h postinfection and was down-regulated after this time. At 96 h postinfection, 10 and 90% of astrocytoma cells contained nuclear IE-pp72 and IE-pp86 Ags, respectively. At that time, the late viral protein gB was detectable in 90% of the cells (data not shown).

To further explore the mechanism involved in the induction of HLA-G Ags, we analyzed stably transfected U-373 MG astrocytoma cells expressing HCMV IE products. Double-label immunofluorescent staining was performed using the anti-HLA-G and anti-HCMV IE Abs. There was no HLA-G-specific fluorescence in control U-373 MG cells and transfected U-373 MG cells expressing IE-pp72 or IE-pp86 alone. In contrast, soluble HLA-G Ags were observed in the cytoplasm of U-373 MG cells coexpressing the two IE proteins (Fig. 4D). Around 20% of these transfected U-373 MG astrocytoma cells displayed soluble HLA-G production. Interestingly, by flow cytometry, no membrane staining was detected on any of the transfected astrocytoma cells expressing HCMV IE products.

## Discussion

In this study, we demonstrated that HCMV induces the expression of nonclassical MHC-I HLA-G molecules. In allogeneic-stimulated macrophage cultures, HLA-G Ags were expressed upon differentiation of latently HCMV-infected monocytes during HCMV reactivation. Although HLA-G transcription persists, there was no translation of these transcripts in uninfected macrophages up to 50 days poststimulation. Thus, cellular factors, which dictate macrophage differentiation, are not sufficient for HLA-G up-regulation. *Ex vivo*, alveolar macrophages collected from patients undergoing HCMV pneumonitis also displayed HLA-G molecules. Although subjects from whom HCMV was not recovered had immunologi-

cal abnormalities and other pathogens due to their underlying disease, no HLA-G Ags were detected.

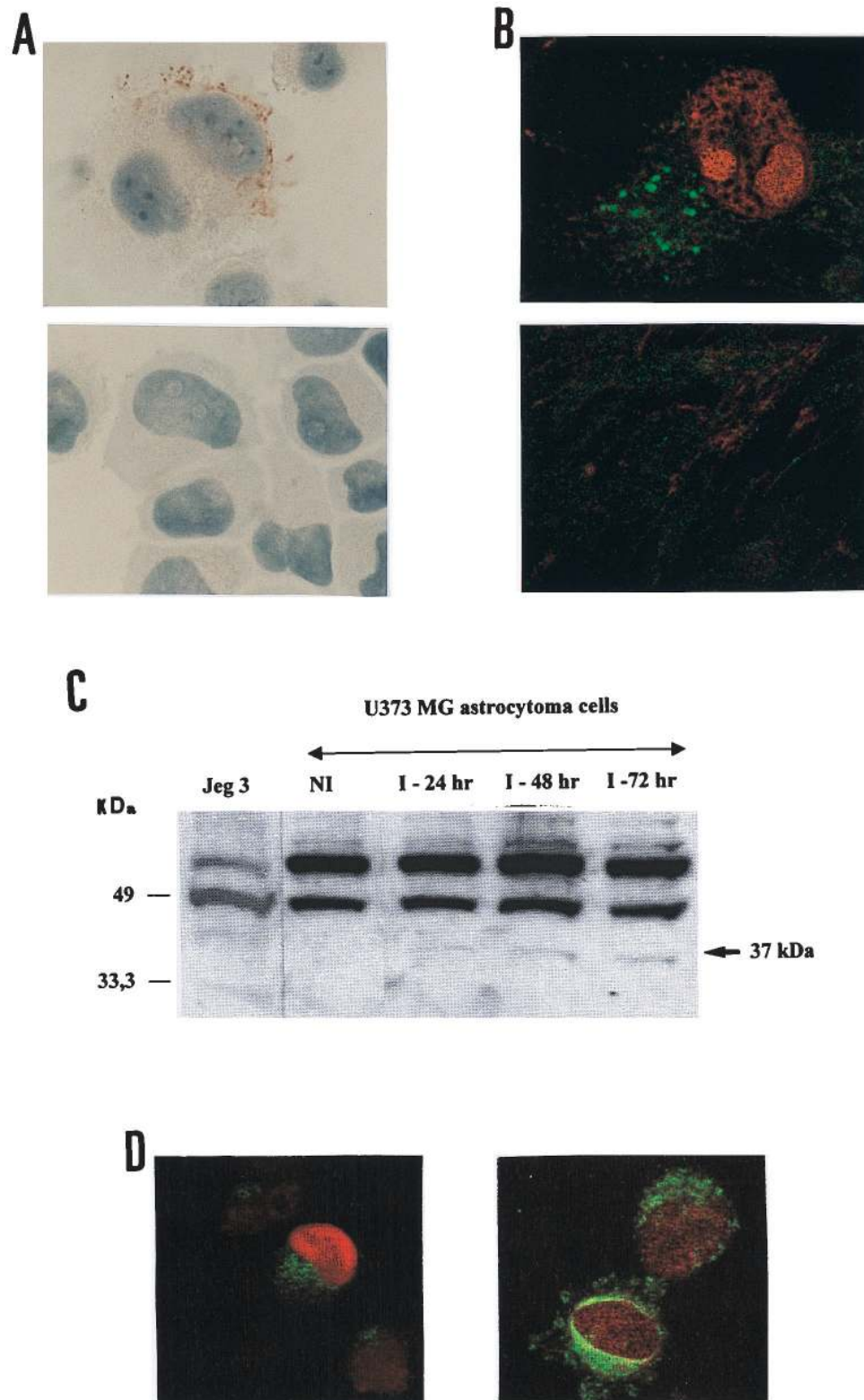
To demonstrate the modulation of HLA-G translation by HCMV products, we assayed direct infection of primary culture of monocyte-derived macrophages. Our investigations were shown to be difficult for different reasons: variability in the growth kinetic of HCMV in monocyte-derived macrophages, difficulty of obtaining fully permissive macrophages, lack of good isolates of HCMV, and small number of infected macrophages. We therefore tested the HLA-G-HCMV interactions in permissive U-373 MG astrocytoma cells, which, like monocytes and macrophages, express transcripts but not translated products (32). Inoculation of U-373 MG astrocytoma cells with HCMV resulted in the production of soluble HLA-G Ags; this specific modulation was mediated by the cooperation of HCMV IE1-pp72 and IE2-pp86 products.

Unlike astrocytoma cells, membrane-bound and soluble HLA-G molecules were detectable in macrophages displaying HCMV replication. However, variations in the modulation of HLA-G isoforms were also observed in macrophages. The failure to detect HLA-G cell surface expression in infected astrocytoma cells suggests that the virus has settled different molecular mechanisms to induce HLA-G expression in these two cell types. As EBV (33), HCMV may have developed multiple strategies to ensure its long term persistence and its replication in different infected cells; these may include the establishment of a cell phenotype-specific program of viral gene expression and the differential stimulation of HLA-G isoforms expression. The genotype of HCMV strains and/or the MHC-I phenotype of infected donors might be also important. For example, the shedding of soluble classical MHC-I molecules is known to be phenotype dependant, suggesting that the secretion is under genetic control. The soluble HLA-G level in the plasma of HLA-A11-positive donors was shown to be significantly lower than that in HLA-A11-negative donors (34).

Different results acquired to date strongly support that HLA-G expression is tightly regulated by post-transcriptional events such as increased mRNA stability or protein translation and control on the secretory pathway (35, 36). We favor the hypothesis that HCMV is likely to affect these post-transcriptional molecular regulatory mechanisms. Viral products could modulate HLA-G expression, either directly by regulating HLA-G gene activity or indirectly by *trans*-activating the promoter of cellular regulatory factors. The IE proteins are regulatory proteins known to be promiscuous *trans*-activators, particularly when they act synergistically (for review, see Ref. 37).

A recent report suggests that HLA-G may possess characteristics of structure or trafficking that allow escape from HCMV-associated MHC-I degradation pathway. HCMV-infected cells synthesize four genes products, US2, US3, US6, and US11, which play diverse roles in blocking classical MHC-I membrane expression (3). This reduction of MHC-I molecules abrogates the presentation of viral Ags by HCMV-infected cells. Unlike their classical MHC-I counterparts, HLA-G molecules stably expressed in JEG 3 trophoblastic cells were indeed shown to be resistant to rapid degradation imposed by the HCMV gene products US2 and US11 (38).

Recently, surface expression of the class Ib HLA-E was shown to be enhanced on infected human foreskin fibroblasts. This up-regulation depends on binding of the leader sequence derived from the HCMV glycoprotein UL40 (5). Loading a viral peptide may also stimulate HLA-G cell surface expression in infected macrophages. Nevertheless, Fish et al. (39) observed HCMV disruption of the microtubule network in monocyte-derived macrophages,



**FIGURE 4.** Expression of HLA-G molecules in U-373 MG astrocytoma cells infected with HCMV and in transfected U-373 MG astrocytoma cells expressing HCMV IE products. *A, Top*, Immunocytochemical staining, using the 87G mAb, demonstrated intracellular expression of HLA-G Ags in HCMV-inoculated cells at 24 h postinfection. *Bottom*, No staining was observed by using the isotypic IgG2a control. *B, Top*, Double-label immunofluorescence was performed with the anti-HCMV IE-pp86 rabbit serum (rhodamine) and the anti-HLA-G mAb 87G (fluorescein). By visualizing with a confocal microscope, cellular colocalization of IE-pp86 (red nucleus) and HLA-G (green cytoplasm) was observed in infected cells. *Bottom*, No fluorescence was detectable using a normal rabbit serum or an isotypic IgG2a control. *C*, On Western blot, using the 16G1 mAb, a 37-kDa HLA-G-soluble isoform was detected in infected cells at 24 h (I-24 h), 48 h (I-48 h), and 72 h (I-72 h) postinfection. NI, noninfected; JEG3, choriocarcinoma cells expressing HLA-G-soluble isoforms (positive control). *D*, HLA-G-soluble isoforms (fluorescein) are expressed in stably transfected U-373 MG cells coexpressing HCMV IE-pp72 (*left*) and IE-pp86 products (*right*) (rhodamine).



suggesting that the trafficking of proteins involved in Ag-presenting pathways to the cell surface may be indirectly altered. As induction of HLA-G molecules required the cooperation of the two IE proteins in astrocytoma cells, it is likely that another mechanism contributes to the up-regulation of HLA-G molecules.

Because classical MHC-I molecules are partially down-regulated in infected macrophages and deliver inhibitory messages to NK cells, similar inhibitory signals mediated by HLA-G molecules seem redundant. Thus, HLA-G molecules may provide additional signals to other immune effector cells. For example, HLA-G molecules could inhibit an anti-HCMV CTL response induced by viral peptides loaded on classical MHC-I complexes. In transplant recipients, the development of disease, e.g., pneumonitis, is indeed clearly correlated with absent or diminished HCMV-specific CD8<sup>+</sup> CTL responses (40, 41). A marked consequence of HCMV infection of monocyte-macrophages is the suppressive effect of these virally infected cells on lymphoproliferative responses (42, 43). In addition, it is conceivable that HLA-G molecules affect the functions of myelomonocytic cells, such as Ag presentation, cytokine production (12), and down-regulate the anti-viral adaptive immune response.

In conclusion, our observations provide evidence that HCMV infection stimulates HLA-G molecule production, with regulatory controls operating at a post-transcriptional level. In vivo, this modulation may be intimately linked to the differentiation of latently infected monocytes. The transient appearance of HLA-G molecules suggests that modulation is tightly controlled during the progression of viral infection, perhaps before the establishment of other evasive mechanisms such as blockade of classical MHC-I molecules. Elucidation of the mechanisms by which viral IE proteins govern the stimulation of HLA-G Ags will facilitate the understanding of HLA-G gene regulation.

Circulating blood monocytes immigrate into extravascular tissue sites and differentiate as macrophages at sites of infection, injury, allograft, or tumor rejection. Initiation of an immune response at these different sites of inflammation may result in reactivation of latent virus. Then, immunosuppression provided by HLA-G molecules would favor virus dissemination and exacerbate the severity of HCMV diseases. The immunological or clinical consequences of HLA-G expression in natural infection remain to be unraveled.

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## References

- de Jong, M. D., G. J. Galasso, B. Gazzard, P. D. Griffiths, D. A. Jabs, E. R. Kern, and S. A. Spector. 1998. Summary of the II International Symposium on Cytomegalovirus. *Antiviral Res.* 39:141.
- Ploegh, H. L. 1998. Viral strategies of immune evasion. *Science* 280:248.
- Ahn, K., A. Angulo, P. Ghazal, P. A. Peterson, Y. Yang, and K. Fruh. 1996. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc. Natl. Acad. Sci. USA* 93:10990.
- Chapman, T. L., A. P. Heikeman, and P. J. Bjorkman. 1999. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity* 11:603.
- Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael, and G. W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287:1031.
- Le Bouteiller, P. 1997. HLA-G: on the track of immunological functions. *Eur. J. Immunogenet.* 24:397.
- Cosman, D., N. Fanger, L. Borges, M. Kubin, W. Chin, L. Peterson, and M. L. Hsu. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* 7:273.
- Colonna, M., J. Samaridis, M. Cella, L. Angman, R. L. Allen, C. A. O'Callaghan, R. Dunbar, G. S. Ogg, V. Cerundolo, and A. Rolink. 1998. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.* 160:3096.
- Cantoni, C., S. Verdiani, M. Falco, A. Pessino, M. Cilli, R. Conte, D. Pende, M. Ponte, M. S. Mikaelsson, L. Moretta, et al. 1998. p49, a putative HLA class I-specific inhibitory NK receptor belonging to the immunoglobulin superfamily. *Eur. J. Immunol.* 28:1980.
- Rajagopalan, S., and E. O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.* 189:1093.
- Allan, D. S., M. Colonna, L. L. Lanier, T. D. Churakova, J. S. Abrams, S. A. Ellis, A. J. McMichael, and V. M. Braud. 1999. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *J. Exp. Med.* 189:1149.
- Samaridis, J., and M. Colonna. 1997. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur. J. Immunol.* 27:660.
- Colonna, M., F. Navarro, T. Bellon, M. Llano, P. Garcia, J. Samaridis, L. Angman, M. Cella, and M. Lopez-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.* 186:1809.
- Le Gal, F. A., B. Riteau, C. Sedlik, I. Khalil-Daher, C. Menier, J. Dausset, J. G. Guillet, E. D. Carosella, and N. Rouas-Freiss. 1999. HLA-G-mediated inhibition of antigen-specific cytotoxic T lymphocytes. *Int. Immunol.* 11:1351.
- Rouas-Freiss, N., I. Khalil-Daher, B. Riteau, C. Menier, P. Paul, J. Dausset, and E. D. Carosella. 1999. The immunotolerance role of HLA-G. *Semin. Cancer Biol.* 9:3.
- Carosella, E. D., N. Rouas-Freiss, P. Paul, and J. Dausset. 1999. HLA-G, a tolerance molecule from the major histocompatibility complex. *Immunol. Today* 20:60.
- Mitsuishi, Y., K. Miyazawa, A. Sonoda, and P. I. Terasaki. 1997. Cellular function of soluble HLA-G. In *Proceedings of the Twelfth International Histocompatibility Workshop and Conference*, Vol. 2. D. Charron, ed. EDK, Paris, France, p. 251.
- Pangault, C., L. Amiot, S. Caulet-Maugendre, F. Brasseur, F. Burtin, V. Guilloux, B. Drenou, R. Fauchet, and M. Onno. 1999. HLA-G protein expression is not induced during malignant transformation. *Tissue Antigens* 53:335.
- Amiot, L., M. Onno, B. Drenou, C. Monvoisin, and R. Fauchet. 1998. HLA-G class I gene expression in normal and malignant hematopoietic cells. *Hum. Immunol.* 59:524.
- Taylor-Wiedeman, J., J. G. Sissons, L. K. Borysiewicz, and J. H. Sinclair. 1991. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* 72:2059.
- Bolovan-Fritts, C. A., E. S. Mocarski, and J. A. Wiedeman. 1999. Peripheral blood CD14(+) cells from healthy subjects carry a circular conformation of latent cytomegalovirus genome. *Blood* 93:394.
- Taylor-Wiedeman, J., P. Sissons, and J. Sinclair. 1994. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J. Virol.* 68:1597.
- Soderberg-Naucler, C., K. N. Fish, and J. A. Nelson. 1997. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* 91:119.
- Sinzger, C., B. Plachter, A. Grefte, T. H. The, and G. Jahn. 1996. Tissue macrophages are infected by human cytomegalovirus in vivo. *J. Infect. Dis.* 173:240.
- Lafemina, R. L., M. C. Pizzorno, J. D. Mosca, and G. S. Hayward. 1989. Expression of the acidic nuclear immediate-early protein (IE1) of human cytomegalovirus in stable cell lines and its preferential association with metaphase chromosomes. *Virology* 172:584.
- Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward. 1988. Trans-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. *J. Virol.* 62:1167.
- Wieland, S., U. Dobbeling, and S. Rusconi. 1991. Interference and synergism of glucocorticoid receptor and octamer factors. *EMBO J.* 10:2513.
- Gerna, G., M. G. Revello, E. Percivalle, M. Zavattoni, M. Parea, and M. Battaglia. 1990. Quantification of human cytomegalovirus viremia by using monoclonal antibodies to different viral proteins. *J. Clin. Microbiol.* 28:2681.
- Lee, N., A. R. Malacko, A. Ishitani, M. C. Chen, J. Bajorath, H. Marquardt, and D. E. Geraghty. 1995. The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous peptides but differ with respect to TAP association. *Immunity* 3:591.
- Kirszenbaum, M., P. Moreau, E. Gluckman, J. Dausset, and E. Carosella. 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.
- Larsson, S., C. Soderberg-Naucler, F. Z. Wang, and E. Moller. 1998. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. *Transfusion* 38:271.
- Maier, S., D. E. Geraghty, and E. H. Weiss. 1999. Expression and regulation of HLA-G in human glioma cell lines. *Transplant. Proc.* 31:1849.

33. Masucci, M. G., and I. Ernberg. 1994. Epstein-Barr virus: adaptation to a life within the immune system. *Trends Microbiol.* 2:125.
34. Rebmann, V., K. Pfeiffer, M. Passler, S. Ferrone, S. Maier, E. Weiss, and H. Grosse-Wilde. 1999. Detection of soluble HLA-G molecules in plasma and amniotic fluid. *Tissue Antigens* 53:14.
35. Guillaudeux, T., A. M. Rodriguez, M. Girr, V. Mallet, S. A. Ellis, L. L. Sargent, R. Fauchet, E. Alsat, and P. LeBouteiller. 1995. Methylation status and transcriptional expression of the MHC class I loci in human trophoblast cells from term placenta. *J. Immunol.* 154:3283.
36. Chu, W., J. Gao, W. J. Murphy, and J. S. Hunt. 1999. A candidate interferon- $\gamma$  activated site (GAS element) in the HLA-G promoter does not bind nuclear proteins. *Hum. Immunol.* 60:1113.
37. Mocarski, E. S. 1996. Cytomegalovirus and their replication. In *Fields Virology*, Vol. 2. B. N. Fields, D. M. Knipe, and P. M. Howley, eds. Lippincott-Raven, Philadelphia, p. 2447.
38. Schust, D. J., D. Tortorella, J. Seebach, C. Phan, and H. L. Ploegh. 1998. Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. *J. Exp. Med.* 188:497.
39. Fish, K. N., W. Britt, and J. A. Nelson. 1996. A novel mechanism for persistence of human cytomegalovirus in macrophages. *J. Virol.* 70:1855.
40. Krause, H., H. Hebart, G. Jahn, C. A. Muller, and H. Einsele. 1997. Screening for CMV-specific T cell proliferation to identify patients at risk of developing late onset CMV disease. *Bone Marrow Transplant.* 19:1111.
41. Ljungman, P., J. Aschan, J. N. Azinge, L. Brandt, A. Ehrnst, V. Hammarstrom, S. Klaesson, A. Linde, B. Lonnqvist, O. Ringden, et al. 1993. Cytomegalovirus viraemia and specific T-helper cell responses as predictors of disease after allogeneic marrow transplantation. *Br. J. Haematol.* 83:118.
42. Carney, W. P., and M. S. Hirsch. 1981. Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. *J. Infect. Dis.* 144:47.
43. Rinaldo, C. R., Jr., P. H. Black, and M. S. Hirsch. 1977. Interaction of cytomegalovirus with leukocytes from patients with mononucleosis due to cytomegalovirus. *J. Infect. Dis.* 136:667.