Induction of HLA-G-restricted human cytomegalovirus pp65 (UL83)-specific cytotoxic T lymphocytes in HLA-G transgenic mice

Françoise Lenfant, Nathalie Pizzato, Siyuan Liang, Christian Davrinche, Philippe Le Bouteiller and Anatolij Horuzsko

The non-classical major histocompatibility complex class I molecule HLA-G is expressed mainly by extravillous trophoblasts at the materno–foetal interface. HLA-G has been found to bind endogenously processed nonameric peptides but its function as a restriction element for a cytotoxic T cell response to viruses with tropism for trophoblastic cells has never been demonstrated. In this study, candidate viral peptides derived from human cytomegalovirus (HCMV) pp65 (UL83), which stabilized the HLA-G molecule on HLA-G-transfected T2 cells, were identified. The specific anti-pp65 cytotoxic T lymphocyte (CTL) response restricted by HLA-G in triple transgenic mice (HLA-G, human β2m, human CD8α) was then investigated by injection of dendritic cells loaded with synthetic pp65-derived peptides or by infection with canarypox virus expressing pp65. Results showed that CTLs from HLA-G mice have the capacity to kill target cells either infected with recombinant vaccinia viruses expressing pp65 or loaded with specific pp65-derived peptides using HLA-G as an antigen-presenting molecule. It was also demonstrated that these HLA-G-restricted pp65-specific T cells are able to kill the human astrocytoma cell line U373, which was transfected with HLA-G and infected with HCMV. Moreover, using HLA-G tetramers refolded with a synthetic pp65-derived peptide, peptide-specific CD8+ cells restricted by HLA-G have been detected in vivo. These findings provide the first evidence that HLA-G can select anti-HCMV-restricted CTLs in vivo, although the potency of this cytolytic response is limited (20–25%). The weak HLA-G-restricted anti-HCMV response is probably due to HLA-G-mediated inhibitory signals on the development of an antiviral CTL response.

INTRODUCTION

During pregnancy, the placenta is the first barrier that protects the foetus from pathogens. It is of relevance, therefore, that trophoblast cells in placental tissues lack major histocompatibility (MHC) class I expression. These highly polymorphic MHC molecules, HLA-A and HLA-B, play a central role in the detection and elimination of infected cells by CD8+ T cells and natural killer (NK) cells. However, despite the general lack of classical MHC molecules, extravillous trophoblasts, a subpopulation of trophoblasts that is in direct contact with maternal cells, express low levels of HLA-C and at a higher extent, the non-classical HLA-Ib molecules HLA-G and HLA-E (Kovats et al., 1990; Le Bouteiller et al., 1999; King et al., 2000), which are accompanied by abundant expression of transporter-associated proteins (TAP) (Clover et al., 1995). The non-classical HLA-Ib molecule HLA-E binds preferentially to leader peptides of many MHC class I molecules (Braud et al., 1998; Lee et al., 1998) and these complexes function as ligands for CD94/NKG2 receptors on NK cells (Braud & McMichael, 1999). HLA-G molecules have a peptide-binding groove that is homologous to those of classical MHC class I molecules. In addition, HLA-G was shown to present nonameric peptides derived from a variety of intracellular proteins (Diehl et al., 1996; Lee et al., 1995). It was proposed that HLA-G on trophoblast cells interacts with several inhibitory or triggering NK cell receptors to protect placental cells against the cytotoxic activity of NK cells (Lanier, 1999). Two reports using HLA-G transgenic mice have demonstrated that HLA-G molecules are recognized as a self-molecule capable of eliciting a cytotoxic T lymphocyte (CTL) response (Horuzsko et al., 1997; Schmidt et al., 1997). In humans, HLA-G expression has been detected also on thymic medullary epithelial cells (Criza et al., 1997; Mallet et al., 1999) and HLA-G molecules were found to bind CD8α/α with an affinity similar to that observed with classical HLA molecules (Sanders et al., 1991),
which may mediate positive and/or negative selection of HLA-G-restricted T cells in the thymus. Altogether, these observations raise the possibility that, in the absence of classical MHC molecules, HLA-G molecules are potentially capable of presenting viral peptides and may then function as a restriction element for a CTL response directed against the virus with tropism for trophoblast tissues.

Human cytomegalovirus (HCMV) infection remains the most common congenital virus infection and is the cause of neurological defects that affect from 0.4 to 2.3 % of live-born infants (Plotkin, 1994). Several reports on infection of placental cytotrophoblasts in vitro and in utero demonstrated that trophoblast cells are permisive to HCMV infection (Hemmings et al., 1998; Fisher et al., 2000). The rate of mother-to-child transmission, around 40 %, is consistent with the existence of placental mechanisms preventing virus transmission. During the clearance of HCMV-infected cells by the immune system, CD8 T lymphocytes are directed mainly against the matrix protein pp65 (UL83) (Kern et al., 1999; McLaughlin-Taylor et al., 1994). These CTLs play a prominent role in anti-HCMV defence in vivo, as the protein pp65 has been shown to be internalized immediately after virus input and then is rapidly available for presentation to specific CD8 T cells in the thymus. Altogether, these observations raise the possibility that, in the absence of CD8 T lymphocytes, CD8+ T cells capable of presenting viral peptides and may then function as a restriction element for a CTL response directed against HCMV infection.

In this study, we identified six pp65-derived peptides having the HLA-G consensus motif and analysed their binding capacity to HLA-G molecules. We then used the triple transgenic mice (HLA-G, human β2m, human CD8ε) and HLA-G tetrameric complexes refolded with a HCMV pp65 epitope to analyse the HLA-G-restricted CTL response against pp65-derived peptides or the pp65 protein. Our data showed a limited induction of an HLA-G-restricted CTL response in vivo directed against HCMV infection.

**METHODS**

**Cell lines.** The TAP1−/TAP2−-deficient T2 cell line (Salter & Cresswell, 1986) was electroporated with plasmid pcDNA3 (Invitrogen) containing a Clal–HindIII 4.0 kb DNA fragment of the structural gene HLA-G (Horuzsko et al., 1999). This HLA-G-transfected cell line was designated T2/G.

EL4 (H-2b), EL4/G [EL4 cells transfected with the HLA-G gene expressed under the control of H-2Kβ promoter and human β2-microglobulin (β2m)], EL4/G-pp65 (EL4/G cells transiently transfected with pcDNA3-pp65 plasmid) cell lines were maintained in IMDM medium (Life Technologies) supplemented with 10 % FCS, 2 mM glutamine, 100 U penicillin ml−1 and 100 μg streptomycin ml−1.

U373MG (U373) human astrocytoma cells (ATCC), U373/G [U373 cells stably transfected with pcDNA-G1 (Blasschitz et al., 1997)] and U373/G-IE1-pp65 [U373/G cells stably transfected with IE1-pp65 cDNA (Vaz-Santiago et al., 2001)] were used as HCMV-infected target cells.

**Peptide-binding assay.** Six pp65-derived peptides were designed based on the HLA-G-binding motif (Diehl et al., 1996; Lee et al., 1995). We also used peptides eluted from HLA-G molecules (Lee et al., 1995) and an HLA-B27-binding peptide (Solache et al., 1999) as positive and negative controls, respectively. Sequences of the peptides are described in Table 1. Peptides were synthesized at the Molecular Biology Core Facility of the Medical College of Georgia (Augusta, GA, USA) or at the Peptide Synthesis Core Facility of the IFR30 (Toulouse, France). The peptides were dissolved in PBS at a concentration of 2 mg ml−1 and stored at −20 °C before use.

Binding assays were performed with the T2/G cell line as follows: 2.5 × 10⁵ cells were incubated in the presence of 200 μM peptide for

<p>| Table 1. Synthetic HCMV-derived and control peptides used in this study |</p>
<table>
<thead>
<tr>
<th>Positive controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH9L</td>
</tr>
<tr>
<td>KG9L</td>
</tr>
<tr>
<td>R9L</td>
</tr>
<tr>
<td>Negative control</td>
</tr>
<tr>
<td>SR9R</td>
</tr>
<tr>
<td>pp65-derived peptides</td>
</tr>
<tr>
<td>LG9L</td>
</tr>
<tr>
<td>VI9L</td>
</tr>
<tr>
<td>HL9I</td>
</tr>
<tr>
<td>AL9I</td>
</tr>
<tr>
<td>LC9L</td>
</tr>
<tr>
<td>VF9L</td>
</tr>
</tbody>
</table>
16 h at 37 °C. Cell surface HLA-G molecules were detected using the HLA-G-specific antibody 87G [IgG2a; a gift of D. Geraghty, Seattle, WA, USA (Lee et al., 1995)] followed by incubation with F(ab')2 goat anti-mouse IgG antibody conjugated with FITC (Coulter Immunotech). Cells were analysed by flow cytometry using a FACSCalibur (Becton Dickinson).

**Generation of peptide-specific CTLs.** HLA-G transgenic mice (CBA/Ca, H-2d, H-2Kb/HLA-G; Igl2m, HCDba) (Horuzsko et al., 1997) at 6–8 weeks of age were used for experiments. Mice were bred and maintained in a specific-pathogen-free facility at the Medical College of Georgia, GA, USA.

Dendritic cells (DCs) from triple transgenic mice were generated from bone marrow, according to Inaba et al. (1992). In brief, bone marrow was flushed from femurs and tibias with air-buffered washed medium (IMDM) (Life Technologies) and centrifuged. Cell suspensions were adjusted to 5 × 10⁶ cells ml⁻¹ in culture medium containing 25 ng murine granulocyte/macrophage colony-stimulating factor (GM-CSF) ml⁻¹. IMDM medium supplemented with 10 % (v/v) foetal calf serum (FCS), 2 mM glucose, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ was used for cell culture. On day 3, non-adherent cells, which were mostly granulocytes, were gently removed and fresh medium containing GM-CSF was added. On day 10, non-adherent cells (DCs) were collected for immunizations. More than 95 % of cells produced in this way stained positively with the murine pan-DC marker, anti-CD11c monoclonal antibody (mAb) (PharMingen). DCs (10 × 10⁶) were washed twice in IMDM without FCS and incubated with 200 μg pp65-derived peptides. After 3 h of incubation at 37 °C, peptide-pulsed DCs were washed twice and 5 × 10⁶–10⁶ cells per mouse were injected subcutaneously (s.c.) into the hind footpads. To increase the maturation process of the DCs, DCs were subsequently incubated overnight with an anti-CD40 mAb (PharMingen) and then washed twice before injection. After 4 days, splenocytes were removed and restimulated in vitro once or twice with peptide-loaded DCs for 6 days in complete IMDM containing 5 U recombinant IL-2 ml⁻¹.

Alternatively, DCs (10 × 10⁶) were washed twice in IMDM without FCS and loaded with 10 μg IE1–pp65 fusion protein ml⁻¹. After 3 h, DCs were injected s.c. into the hind footpads. Splenocytes were removed 4 days later and restimulated in vitro with peptide-loaded DCs for 5 days. Cells were then used as effectors in T cell cytotoxicity assays. The production of the IE1–pp65 fusion protein in S9 cells infected with recombinant baculovirus and IE1–pp65 fusion protein purification were performed as described previously (Var-Santiago et al., 2001).

**Viruses and mice immunizations for generation of CTLs.** Recombinant canarypox virus, expressing the HCMV pp65 protein (ALVAC-pp65, vCP260), and vaccinia virus, either parental (vacwt, Lvar) or recombinant for pp65 (vac-pp65, vP1214), was a kind gift from J. Tartaglia (Virogenetics Corporation, NY, USA). Their identification of HLA-G-binding peptides derived from HCMV pp65 protein

HCMV Towne strain (ATCC) was propagated in MRC5 human fibroblasts (BioMérieux).

HLA-G transgenic mice were immunized through intraperitoneal (i.p.) injection with 2 × 10⁸ p.f.u. ALVAC-pp65 and were then transferred to the infectious quarters of the animal facility.

At 2 weeks after immunization, spleen cell cultures were removed and subpopulations of CD8⁺ cells were enriched using the Murine T cell CD8 Subset Column kit (R&D Systems). After purification, cells were grown in RPMI supplemented with 10 % FCS, 1 % sodium pyruvate, 1 % non-essential amino acids, 0·5–5 % l-glutamine, 0·5 % penicillin, 0·5 % streptomycin, 10 mM N-acetyl cysteine, pH adjusted to 7·2 (Sigma), 20 U human IL-2 ml⁻¹, 20 ng recombinant murine IL-12 ml⁻¹ and 20 ng human IL-7 ml⁻¹ (Fowler et al., 1996). Cells were restimulated in vitro in 24-well plates, once with autologous irradiated and UV-inactivated splenocytes infected previously with vac-pp65 at an m.o.i. of 0·5 for 5 days, and a second time with DCs loaded with pp65-derived peptides for 5 additional days. Alternatively, splenocytes were used directly for a 51Cr-release assay.

**Cytotoxicity assays.** Effector cells were tested for cytotoxicity in a standard 51Cr-release assay. Peptide-pulsed targets were prepared by incubating the 2T/G cell line or the EL4/G cell line for 3 h at 37 °C with peptides (200 μg ml⁻¹). EL4 and EL4/G target cells were uninfected or infected with vacwt or vac-pp65 vaccinia viruses at an m.o.i. of 2·5 for 3 h on a minimal volume. U373 and U373/G cells were infected with HCMV Towne strain at an m.o.i. of 3 for 2 h on a minimal volume of RPMI medium without FCS, and further incubated 4 h with complete RPMI medium. For 10⁶ cells, 100 μg (1 MBq) ⁵¹Cr was added for 1 h, with one drop of FCS. Labelled target cells were washed three times and mixed with effector cells at various effector: target (E:T) cell ratios in triplicate using 96-well U-bottomed microtitre plates. Cells were incubated for 6 h. The percentage of specific ⁵¹Cr release was calculated as follows: [(c.p.m. experimental release − c.p.m. spontaneous release) / c.p.m. maximal release − c.p.m. spontaneous release] × 100. Spontaneous ⁵¹Cr release (<20 %) and maximal release (100 %) were determined in the presence of either medium or 5 % Triton X-100, respectively. In blocking experiments, target cells were pre-incubated with 5 μg mAb 87G ml⁻¹ for 20 min and then washed before mixing with effector cells.

**HLA-G tetramers and labelling.** HLA-G tetramers were produced essentially as described previously (Allan et al., 1999), using synthetic self-peptide RIIPRHLQL (RI9L) derived from histone H2A, which is conserved in mice and humans and shown previously to bind HLA-G (Lee et al., 1995), and pp65-derived peptide VFPTKDVAL (VF9L). Tetramerization was performed by the addition of streptavidin–phycocerythrin (PE) (Molecular Probe). Tetramers were named HLA-G-control-tet/PE and HLA-G-pp65-tet/PE, respectively.

Mice were immunized s.c. with 5 × 10⁶–10⁷ VP9L-pulsed DCs and the lymph nodes were removed 6 days after immunization. Labelling of lymph nodes was performed at room temperature for 1 h using PE-labelled HLA-G tetramers, washed and incubated with the FITC-conjugated murine anti-CD8 (Ly 2 clone, PharMingen) for 30 min at 4 °C in staining buffer (PBS plus 0·1 % NaN₃ and 1 % BSA). Samples were analysed on a FACSCalibur using the CELL QUEST software (Becton Dickinson).

**RESULTS**

**Identification of HLA-G-binding peptides derived from HCMV pp65 protein**

The amino acid sequence of HCMV pp65 protein was screened for HLA-G-binding consensus anchor residues: proline at position 3 and leucine or isoleucine at position 9 (Diehl et al., 1996; Lee et al., 1995). Six peptides exhibiting the best position match were selected (Table 1) and then analysed for their capacity to stabilize HLA-G molecules (Lee et al., 1995), were used as positive controls, since they have high binding affinities for HLA-G. Fig. 1(A) shows that the density of HLA-G on the T2/G cell surface increased around twofold upon incubation with the positive control peptide RH9L. Incubation
V. F. Lenfant and others

with the other control peptide KG9L gave similar results (data not shown). In contrast, using an HLA-B27-binding peptide (SR9R), no significant binding to T2/G cells was observed, thus validating our assay to discriminate peptides with significant binding to the HLA-G molecule.

HCMV-pp65 peptide candidates displayed varying capacities of stabilizing HLA-G on T2/G cells (Fig. 1B). Five peptides were found to increase the expression of HLA-G molecules on the surface of T2/G cells but to different extents. Peptide VF9L bound with an efficiency as high as that observed with positive controls. In contrast, peptide AL9I had no stabilization effect, as it bound at level similar to that of the negative control peptide SR9R.

Thus, using the T2/G-binding assay, five HCMV-pp65-derived peptides that stabilize the HLA-G molecule have been identified and will be used for subsequent experiments.

**Induction of HLA-G-restricted CTLs directed against pp65 peptides**

To determine whether pp65-derived peptides that bind to HLA-G were immunogenic, we assessed their capacity to induce an HCMV-specific CTL response in vivo.

Injection of peptide-pulsed DCs in vivo has been shown to elicit virus-specific CTLs (Ludewig et al., 1998). Consequently, HLA-G transgenic mice were injected s.c. with DCs loaded either with synthetic pp65-derived peptides VF9L, VL9L or HL9I alone or with the three peptides together. To assess specific responses against the inoculated peptides, splenocytes were removed and after one in vitro restimulation, T2/G cells loaded with the same peptides were used as targets. Specific lysis against peptides-pulsed T2/G cells (15–20 % specific lysis) was obtained as opposed to unpulsed T2/G cells (5 % specific lysis), demonstrating a specific cytotoxicity against viral peptides (Fig. 2A). The CTL response against the peptide VF9L, which exhibits the...
strongest affinity for HLA-G, gave a CTL response identical to that obtained with the peptide VL9L. Immunization with DCs pulsed with the three peptides generated a similar range of lysis (around 20 % specific lysis) (Fig. 2B). Addition of the anti-HLA-G mAb 87G abolished cytotoxicity, thus demonstrating that the anti-pp65 cytotoxic activity was HLA-G restricted and specific (Fig. 2B).

**Restricted HLA-G peptide-specific CTLs recognize pp65 processed endogenously**

We investigated further the ability of CTLs to recognize viral peptides derived from pp65 processed intracellularly. HLA-G transgenic mice were immunized similarly with HCMV peptide-loaded DCs. Splenocytes were recovered after immunization and stimulated in vitro with peptide-loaded DCs. EL4 and EL4/G cells were infected with vaccinia virus (vacwt) or recombinant pp65-expressing vaccinia virus (vac-pp65) and used as targets in a standard $^{51}$Cr-release assay. In contrast to T2/G, the murine EL4 cell line ($H-2^d$) could present endogenously pp65-derived peptides. As shown in Fig. 3(A), significant lysis of EL4/G-vac-pp65 target cells (25 % specific lysis) was observed as compared to EL4/G target cells (6 % specific lysis) and to EL4/G-vacwt (15 % specific lysis), suggesting that these peptides were processed naturally (10 % anti-pp65 specific lysis). In addition, the absence of killing of the HLA-G-negative EL4 target cells loaded with peptides as well as the decrease of cytotoxicity observed when the specific anti-HLA-G mAb 87G was added to EL4/G-vac-pp65 cells demonstrated that the cytotoxic activity was HLA-G restricted.

To study whether the in vivo processing of pp65 was sufficient to induce a specific antiviral response, we also injected transgenic mice with DCs loaded with the recombinant fusion protein IE1–pp65, which has been produced and purified previously (Vaz-Santiago et al., 2001). Splenocytes were recovered after immunization and stimulated in vitro with peptide-loaded DCs. Cytotoxicity was tested on EL4 or EL4/G cells pulsed or not with peptides. As shown in Fig. 3(B), a pp65-specific CTL response was detected using either EL4/G-vac-pp65 target cells or EL4/G cells loaded with peptides (26 and 30 % specific lysis, respectively). Moreover, both the addition of the specific anti-HLA-G mAb 87G on peptide-pulsed EL4/G target cells, which decrease the specific lysis against the same target cells, and the insignificant lysis against the HLA-G-negative EL4 target cell loaded with peptides demonstrated that this specific anti-pp65 cytotoxicity was HLA-G restricted.

Altogether, these results provide evidence that HLA-G-restricted CTLs specific to pp65-derived peptides that are processed endogenously can be selected in HLA-G transgenic mice, although the specific CTL response was weak.

**Induction of HLA-G-restricted, pp65-specific CTLs using virus infection of transgenic mice**

Besides the use of professional antigen-presenting molecules (DCs), we also used viruses for immunizations in order to generate and increase the specific HLA-G-restricted CTL response. Since HCMV is not infectious in mice, we used recombinant canarypox virus expressing the pp65 protein (ALVAC-pp65), which has been used already for classical MHC class I molecules (Gonzol et al., 1995). HLA-G transgenic mice were inoculated first i.p. with a single dose of ALVAC-pp65. Splenocytes were removed, enriched in CD8 cells and then restimulated in vitro with autologous splenocytes infected with vac-pp65 for 5 days. In order to increase the specific CTL response against pp65, a second in vitro stimulation was also performed with DCs loaded with synthetic pp65-derived peptides. Under these conditions, a significant lysis of EL4/G target cells infected with vac-pp65 (50 % at 30 : 1) was obtained as compared to EL4/G target cells infected with vacwt (25 % at 30 : 1) (Fig. 4). The specificity of the anti-pp65 CTL response was also confirmed using the EL4/G-vac-pp65 transfectant cell line.
The reactivity of the HLA-G-restricted, pp65-specific CTLs was HLA-G restricted. HLA-G, was observed, demonstrating that the reactivity of pp65-specific CTLs was HLA-G restricted.

These data demonstrated that infection of mice with recombinant virus expressing pp65 induced an HLA-G-restricted CTL response against pp65.

HLA-G-restricted, pp65-specific CTLs are able to kill human cell lines infected with HCMV

The reactivity of the HLA-G-restricted, pp65-specific CTLs was tested finally on human cell lines infected with HCMV to assess their function in the control of HCMV infection. The HLA-G transgenic mice were immunized with the canarypox virus expressing pp65 and boosted twice with the same dose to increase CTL response (Gonzol et al., 1995). Mice were sacrificed 1 week after the third inoculation and the lysis efficiency of HLA-G-restricted CTLs specific for pp65 protein was then measured using the U373 cell line transfected with the HLA-G cDNA following infection with the HCMV Towne strain. The U373 astrocytoma cell line was used as the target cell line, as this human cell line is permissive to HCMV infection (Duclos et al., 1989). The pp65 structural protein is delivered rapidly to the cytosol by the infecting virion and is presented to CD8+ cells by MHC class I molecules.

Fig. 5 showed that only targets expressing HLA-G infected with HCMV or expressing the IE1–pp65 fusion protein were killed significantly. The anti-HCMV CTL response restricted by HLA-G increases by 16 % at an E : T ratio of 30 : 1, while no significant killing of HCMV-infected U373 target cells (negative for HLA-G expression) was observed (4 %). This result indicated that HLA-G functions as a restriction element to control HCMV infection. This specific lysis assessed in vivo CTL responses, since it was measured directly after immunization using bulk splenocytes without any restimulation procedures.

Consequently, these results demonstrated that the HLA-G-restricted, pp65-specific T cells are able to kill HCMV-infected astrocytoma cells.

HLA-G tetramers containing pp65-derived peptides bind to specific CD8+ T lymphocytes in transgenic mice

The in vivo selection of anti-pp65 HLA-G-restricted CTLs was also measured using HLA-G–peptide tetrameric complexes. The following HLA-G tetramers were constructed: one was refolded with the VF9L peptide derived from pp65 (HLA-G-pp65-tet/PE), while the second was refolded with a control RI9L peptide that had been eluted from HLA-G (HLA-G-control-tet/PE) (Lee et al., 1995). The percentage of peptide-specific CD8+ T cells was then determined in lymph node cells from mice injected with VP9L-loaded DCs. At 6 days after the injection, these cells were evaluated by flow cytometry using PE-labelled HLA-G tetramers. As shown in Fig. 6(A), tetramer staining revealed a significant frequency of HLA-G-restricted, CD8+ cell population specific for the pp65 peptide in the immunized mice (1 %). In contrast, the levels of tetramer positive CD8+ T cells in normal lymph nodes from non-immunized mice were below the limits of detection (0-2 % of CD8+ T cells). However, labelling with the control tetramer showed some binding capacity, although the intensity was lower (0-5 % for HLA-G-control-tet/PE versus 1 % for the specific HLA-G-pp65-tet/PE). The percentage of HLA-G-pp65-positive cells within the CD8+ cell population represents 4 versus 2 % for the control
HLA-G tetrameric complex. Repeat experiments showed a similar range of results and tetramer staining was always higher with HLA-G-pp65-tet/PE than with the control tetramer. We also observed that both HLA-G tetramers stained some CD8\(^+\) cells, especially on immunized mice. A similar observation was reported by others (Allan et al., 1999; Horuzsko et al., 2001): i.e. HLA-G tetramers are able to recognize both myelomonocytic cells and DCs. Consequently, this labelling of CD8\(^+\) T cells on immunized mice is likely to be associated to the DCs that we have injected.

More recently, a defect in spontaneous maturation of DCs has been observed in HLA-G mice (Horuzsko et al., 2001), although this effect can be rescued by addition of lipopolysaccharide to bone marrow-derived cultures. This observation may suggest that strong signals to DCs, such as signals during pathogen infection, deliver potent DC-maturation signals. Since the immunizations were performed with VF9L-loaded DCs, we then incubated the peptide-pulsed DCs with the mAb directed against CD40 to induce their maturation in vitro before mice immunization. This treatment resulted in a twofold increase in the

---

**Fig. 6.** (A) HLA-G tetramer staining of draining lymph node cells from mice immunized with pp65-pulsed DCs. Mice were immunized s.c. with VF9L-pulsed DCs and lymph nodes were removed 6 days after immunization. The percentage of pp65-specific CTLs was determined by flow cytometry using double labelling with anti-CD8-FITC, HLA-G-pp65-tet/PE (pp65-derived peptide, VF9L) and HLA-G-control-tet/PE (peptide R9L eluted from HLA-G). Non-immunized mice were used as a control. Acquisition and analysis were performed using the CELLQUEST software. The percentage of double positive (CD8 tetramer) cells is indicated. The plots at the right are gated on the CD8\(^+\) lymphocyte fraction. (B) Comparison of HLA-G tetramer staining in mice immunized with VF9L-loaded DCs (DC+VF9L) to mice immunized with mature VF9L-pulsed DCs that were obtained through incubation with the CD40 mAb (DC+VF9L+CD40 mAb). Results are representative of four independent experiments.
number of CD8+ T cells labelled by the specific tetramer. Unspecific labelling with the control tetramer always remained at a level below (Fig. 6B).

The use of HLA-G tetramers directly detected HLA-G-restricted, pp65-specific CD8+ T cells generated in vivo in the transgenic mice.

DISCUSSION

This work was directed at determining whether non-classical HLA-G molecules behave similarly to classical ones in their capability to elicit an antiviral-specific CD8+ T cell response in vivo. First, we selected five HCMV pp65-derived peptides that bound to the HLA-G molecule. One of these peptides, VP9L, was the most avid binder and was able to refold the HLA-G tetramer complex, a procedure that generally needs a high-affinity peptide. Immunization of HLA-G transgenic mice with peptide-pulsed DCs or with recombinant viruses expressing pp65 demonstrated that some pp65-derived peptides were processed endogenously and able to induce a specific HLA-G-restricted CTL response. The presentation of viral pp65-encoded peptides by the HLA-G molecules then appeared effective. This result is particularly important, since the overall diversity of HLA-G-derived peptides was estimated to be lower than HLA-A2-derived peptides (Lee et al., 1995) and could have limited the presentation of viral peptides. Moreover, recent data have demonstrated that the truncated cytoplasmic tail of HLA-G confers the ability of the endoplasmic machinery to discriminate between low- and high-affinity peptides (Park et al., 2001). HLA-G molecules loaded with low-affinity peptides were rerouted to the endoplasmic reticulum (ER) and only HLA-G molecules loaded with high-affinity peptide ligands were allowed to reach the plasma membranes (Park et al., 2001). Thus, our results demonstrate that some pp65 viral peptides are successful in reaching the control step of the post-ER compartments for HLA-G loading and presentation.

We have also used HLA-G tetrameric complexes to detect the HLA-G-restricted CTL response specific for pp65. Although labelling of lymph node cells from immunized mice with the HLA-G–peptide tetramer showed nonspecific staining with the control tetramer, a higher percentage of positive cells was always obtained with the HLA-G–pp65 tetramer. These data indicated selection of specific anti-pp65 CD8+ T cells restricted by HLA-G. Cross-reactions during tetramer labelling have been reported already for other MHC molecules, due to binding to nonspecific T cells by interacting with the co-receptor CD8 (Moris et al., 2001).

Immunizations of transgenic mice with recombinant canarypox virus expressing pp65 also demonstrated that an anti-pp65 CTL response restricted by HLA-G can be selected. Furthermore, the use of human astrocytoma HLA-G transfected U373 cells that could be infected by HCMV provide the first evidence that HLA-G is able to trigger a virus-specific CD8+ CTL response, in particular against HCMV, which is known to infect trophoblasts, suggesting that they might play a role in controlling HCMV infection. Indeed, trophoblasts are capable of presenting endogenously derived peptides, and a significant cytolysis of trophoblast cells has been observed (Gobin et al., 1997). Unfortunately, the use of HCMV-infected trophoblasts as target cells was complicated in our cytotoxicity assays, since the level of ex vivo infection remained low (10 %) (Hemmings et al., 1998).

Despite the use of different immunization schedules, one main concern was that the antigen-specific cytotoxic T cell response was consistent but relatively weak (20–25 %). The low level of HLA-G-restricted anti-HCMV CTLs in mice should be explained by poor immunogenicity of the pp65 epitopes presented by HLA-G or by the influence of HLA-G on the development of the CTL response. Using murine MHC molecules as antigen-presenting cells and the lymphocytic choriomeningitis virus (LCMV), comparison of the antiviral H-2Kd-restricted CTL response in HLA-G transgenic mice to wild-type CBA mice demonstrated that the anti-LCMV CTL response was also limited and less potent in the HLA-G mice than in wild-type mice (50 % specific lysis in wild-type mice as compared to 20 % in HLA-G mice at an E: T ratio of 30 : 1) (Horuzsko et al., 2001). This reduced immune responsiveness was attributed to a defect in DC functions in HLA-G mice (Horuzsko et al., 2001). Using the CD40 mAb to increase DC maturation, we showed an increase in the number of anti-pp65 CD8+ T cells using HLA-G–pp65 tetramer labelling. These data suggest that the reduction in HLA-G-specific CTLs is probably not specific for the pp65 response but is due to inhibitory triggering signals on the induction of antiviral CTL responses in transgenic mice. Further analysis showed that HLA-G modifies the function of murine DCs via interactions with the PIR-B inhibitory receptor, a homologue of the human inhibitory receptor ILT4 (Liang et al., 2002). This is consistent with results obtained in humans, where specific binding of HLA-G tetramers was associated with ILT4 on myelomonocytic cells (Allan et al., 1999).

In addition, the low CTL responses can be explained by specific functions of membrane-bound and soluble isoforms of HLA-G, expressed both in HLA-G mice and in target cells (Ishitani & Geraghty, 1992). In humans, transfection of membrane-bound HLA-G1 in target cells reduced the lytic activity of antigen-specific CTLs (Le Gal et al., 1999). Besides, soluble HLA-G1 has been found to induce apoptosis of activated CD8+ T cells through the engagement of CD8 (Fournel et al., 2000). While the production of this soluble HLA-G isoform was thought to contribute to the local elimination of CD8+ alloreactive maternal T cells, it could also participate in the elimination of antiviral-activated T cells in mice, where soluble HLA-G1 molecules have been detected in sera (data not shown). Although these different mechanisms might have turned off the immune...
system locally, generation of antiviral HLA-G-restricted CD8\(^+\) T cells still occurred, supporting the idea that activation of an antiviral response restricted by HLA-G molecules was initiated. The data obtained from the transgenic mice study probably reflect what might happen in humans, due to the similarity between the two organisms in the balance between activation of antiviral CTLs and HLA-G inhibitory triggering signals. However, the detection of such antiviral HLA-G-restricted CD8\(^+\) T cells still remains to be determined in humans during pregnancy.

In HLA-G transgenic mice, the HLA-G gene was expressed under the \(H2-K^b\) promoter. HLA-G was thus expressed in all tissues. We do not exclude the possibility that the absence of a wider distribution of HLA-G in humans prevents generation of the HLA-G-restricted CD8\(^+\) T cells. It remains to be determined what would occur if HLA-G expression was restricted to placental tissues and consequently, how HLA-G could regulate a peripheral cellular immune response. Several reports suggested that HLA-G has been found on antigen-presenting cells following infection. During HCMV infection, Fisher et al. (2000) demonstrated that virus is often transmitted from infected trophoblasts to fibroblasts, placental macrophages and endothelial cells in the villous cores. Infected placental macrophages – namely Hofbauer cells that express HLA-G – seem to be good candidates to enter the venous circulation of the placenta. Moreover, induction of HLA-G antigens was observed upon reactivation of latent peripheral blood mononuclear cells infected by HCMV (Onno et al., 2000) following treatment with recombinant HCMV IL-10 (Spencer et al., 2002) and on monocytes obtained from human immunodeficiency virus type 1 (HIV-1)-infected patients (Lozano et al., 2002), suggesting that HCMV or HIV-1 infection upregulates HLA-G expression on monocytes. Elucidation of these mechanisms could facilitate our understanding of induction of an antiviral peripheral immune response restricted by HLA-G during pregnancy.

Finally, our data agree with the fact that, in most uterine virus infections, maternal infection may spread to the placenta but fails to progress to the foetus, suggesting that trophoblast cells have established some mechanisms of antiviral defence (Lew & Fowler, 1998). The correlation between spontaneous pregnancy loss and HCMV infection during early pregnancy may also argue in favour of a strong maternal immune response against viruses (Kriel et al., 1970; Naib et al., 1970). A mixed villous infiltrate of lymphocytes has been reported in some virus infections and is associated commonly with tissue destruction (Fox, 1993).

The detection of antiviral HLA-G-restricted CD8\(^+\) T cells in humans will definitively prove this physiological function of HLA-G during pregnancy. Staining of human peripheral blood mononuclear cells (PBMCs) has been done already using HLA-G tetramers refolded with self-peptides (histone H2A) (Allan et al., 1999). They revealed an interaction of HLA-G tetramers with blood monocytes and failed to detect labelling of the CD8\(^+\) population. Moreover, these experiments have been done using PBMCs from healthy donors and HLA-G tetramers refolded with self-peptides, which would not be expected to interact with antiviral-specific CD8\(^+\) T cells. The constructed HLA-G tetramer containing the pp65 peptide may be a useful tool to examine the development of anti-HCMV-specific, HLA-G-restricted CTLs using PBMCs from an HCMV-infected pregnant woman.

In conclusion, these data provide the first evidence that HLA-G molecules have the capacity to present HCMV peptides to CD8\(^+\) T cells and function as restriction elements to recognize and kill HCMV-infected astrocytoma cells. Further studies are needed to evaluate how and whether such induction of the HLA-G-restricted, anti-HCMV response exists in humans.

**ACKNOWLEDGEMENTS**

We thank A. Tartaglia from Virogenetics, NY, USA, for providing us with the pp65 recombinant vaccinia virus and canarypox virus. We acknowledge Lei Huang for technical assistance in tetramer technology. We also thank Jean-Luc Davignon, Etienne Joly and Bent Rubin for helpful comments on the manuscript. This work was supported by grants from INSERM, Sidaction, ANRS, University Paul Sabatier (F. L.), from the Department of Medicine, Medical College of Georgia (A. H.), F. L. was supported by a short-term fellowship from the Human Frontier Science Program and UICC, and N. P. was supported by a fellowship from Ministère de la Recherche.

**REFERENCES**


