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Evidence to Support the Role of HLA-G5 in Allograft Acceptance through Induction of Immunosuppressive/Regulatory T Cells

Solène Le Rond,* Christine Azéma,* Irène Krawice-Radanne,* Antoine Durrbach,† Catherine Guettier,‡ Edgardo D. Carosella,* and Nathalie Rouas-Freiss2*

The soluble HLA-G5 isoform encoded by intron-4 retaining spliced transcript has been previously detected in vivo in sera and grafts from transplanted patients who had significantly better graft acceptance. These findings led us to investigate the role of HLA-G5 in tolerance induction in vitro and its biological relevance in allograft acceptance in vivo. We demonstrated that engagement of Ig-like transcript-2 and Ig-like transcript-4 receptors by HLA-G5 is involved in inhibition of T cell alloproliferative responses. Naïve T cells sensitized in vitro with HLA-G5, for as little as 18 h, 1) lost their ability to respond to subsequent allogeneic stimulus, and 2) acquired regulatory properties because they inhibited the reactivity of other T cells. These HLA-G5-induced T cells act in an Ag-nonspecific fashion and through soluble factors. Biological relevance was provided by ex vivo analyzes of samples from liver-kidney cotransplanted patients who had high HLA-G5 serum levels and no graft rejection. We showed that addition of HLA-G5-containing sera from these patients inhibited T cell alloresponses and that serum HLA-G5 was responsible for this inhibition. Notably, PBMC from transplanted patients exposed to high levels of circulating HLA-G5 did not respond to allostimulation and inhibited alloreactivity of other T cells. These results demonstrate that HLA-G5-mediated tolerance involves the induction of immunosuppressive T cells. These findings provide evidence supporting the tolerogenic properties of HLA-G and emphasize its potential application as a relevant therapeutic candidate capable of limiting allograft rejection. The Journal of Immunology, 2006, 176: 3266–3276.

HLA-G differs from other HLA-class I molecules by a restricted distribution in nonpathological cells and tissues, such as trophoblast, thymus, cornea, and erythroid lineage and by an alternative splicing of its primary transcript, resulting in at least seven HLA-G isoforms, of which four are membrane-bound (HLA-G1–HLA-G4) and three are soluble proteins (HLA-G5–HLA-G7) (see review in Ref. 1). However, HLA-G expression can be extended to various tissues under pathological conditions such as transplantation (2, 3). In this context, the role of HLA-G in reducing graft rejection may be due to its direct interaction with inhibitory receptors, such as Ig-like transcript (ILT)2/IL1RBL1/CD85j, expressed by human monocytes, T cells, B cells, NK cells, and dendritic cells (DC) (4) and myeloid-specific ILT4/IL1RBL2/CD85d (5). ILT2 and ILT4 are both characterized by a broad specificity for HLA-class I molecules, but have the highest affinity for HLA-G (6). All cell subsets involved in graft rejection bear at least one receptor for HLA-G. Therefore, HLA-G may be tolerogenic by exerting its inhibitory functions on all main effector cells responsible for graft rejection. Indeed, in vitro data indicate that HLA-G inhibits both NK cell- and CD8+-T cell-mediated cytolysis (7), suppresses CD4+ T cell allograft responses (8), and induces apoptosis of activated CD8+ T cells through ligation with CD8 and via a Fas/Fas ligand-dependent mechanism (9, 10). Furthermore, HLA-G mediates up-regulation of its own receptors (ILT2, ILT4, and KIR2DL4) on APC, NK cells, and T cells, thus boosting its own inhibitory effects (11). Moreover, HLA-G1-expressing APC can induce immunosuppressive CD4+ T cells (12), showing that HLA-G acts not only on effector inhibition, but also upstream on T cell clonal amplification and effector maturation.

Peripheral tolerance mechanisms are crucial to prevent rejection of transplanted allogeneic tissues and are maintained either by passive mechanisms such as deletion, ignorance, or anergy of alloreactive T cells, or by active suppression mediated by regulatory T cells. T cells play a central role in both allograft rejection and tolerance. Indeed, allograft rejection is mainly mediated by recipient T cells either upon direct stimulation by donor APC, or upon indirect stimulation by recipient APC, which present processed peptides from allo-HLA molecule (13). Both pathways can be inhibited by particular subsets of T cells with suppressor functions. Attention has focused in particular on a minor subset of regulatory CD4+ T cells that express CD25 (IL-2Rα-chain) (14, 15).

The nonclassical HLA class I molecule HLA-G contributes to allograft acceptance (16). Initially detected at the fetal-maternal interface, HLA-G protects the semiallogeneic fetus from rejection.

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3 Abbreviations used in this paper: ILT, Ig-like transcript; DC, dendritic cell; LKT, liver-kidney transplantation; KT, kidney transplantation; β2-m, β2 microglobulin; SN, supernatant.

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by the mother’s immune system (17, 18). In human allotransplantation, HLA-G was found in endomyocardial biopsies of 18% of heart-transplanted patients (2, 19). The soluble HLA-G5 or HLA-G6 isoform was systematically detected in the serum of these HLA-G-positive patients. By contrast, when HLA-G was not detected in the heart allograft, it was not detected in the serum either. Interestingly, HLA-G-positive patients had significantly fewer episodes of acute rejection than HLA-G-negative patients and no chronic rejection. HLA-G expression was also studied in combined liver-kidney transplantation (LKT), because in this case, the liver allograft is known to increase the survival of the cotransplanted kidney (20). In LKT patients, HLA-G expression was found in 1) liver biliary and renal tubular epithelial cells, which are the prime targets of acute rejection, 2) graft-infiltrating mononuclear cells (3, 21), and 3) peripheral T lymphocytes (22). Furthermore, increased concentrations of soluble HLA-G were found in the serum of these patients but not in those of kidney single transplanted (KT) patients or healthy controls. Similarly to heart-transplanted patients, LKT patients in whom HLA-G expression was observed accepted their double graft better and had no acute and no chronic graft rejection (21).

A recent report highlighted the role of HLA-G-induced suppressive T cells during transplantation. Indeed, HLA-G tetrameric complexes inhibited the maturation of murine DC in vitro through interaction with paired Ig-like inhibitory receptor-B, the murine homologue of human ILT4 (23). Injection of HLA-G tetramer-coated beads to recipient mice before allogeneic skin grafting induced suppressive T cells and resulted in an increase of skin allograft survival (24, 25). Thus, we hypothesized that HLA-G5 does not have to be present at the time of the immune reaction to exert its immunosuppressive function but rather acts by tolerizing immune cells such as T cells or APC. Because of these observations, we conducted experiments in which we presensitized T cells with HLA-G5 in vitro and then investigated whether these T cells had immunomodulatory functions. We have previously reported that following allogeneic stimulation in MLR, both CD4+ and CD8+ T cell subsets could express soluble HLA-G5 molecules (22), which in return inhibited the proliferation of alloreactive CD4+ T cells (8). In this study, we investigated the mechanisms by which HLA-G5 controls allogeneic responses in vitro and in vivo after transplantation. We found that HLA-G5 induces a regulatory/suppressive T cell population both in vitro and in vivo that explains, in part, why transplanted patients with high levels of serum HLA-G5 had a better graft acceptance.

To define the time necessary for HLA-G to be expressed and to exert immunosuppressive effects, we analyzed three newly LKT patients for which serum and PBMC were collected during the first-month posttransplantation. These three newly LKT patients were as follows: two males and one female of an average of 52 ± 11 years. They had not had a previous transplantation.

In parallel, previously described HLA-G5-negative sera from KT patients (n = 3) (21) were used as HLA-G5-negative controls. These KT patients have stable graft function and did not develop acute graft rejection. Thirteen sera and 20 PBMC were obtained from healthy donors (Hôpital Saint-Louis, Paris, France). Some PBMC from healthy donors were depleted of monocytes/macrophages by adherence and were depleted of B lymphocytes using anti-CD19-coated Dynabeads (Dynal Biotech). The resulting T cell-enriched population was >90% pure and contained no monocytes/macrophages. Approval for this study was obtained from the local ethics committee.

M8-pcDNA cells (mock-transfected) and M8-HLA-G5 cells (transfected with a vector-containing HLA-G5 cDNA) were obtained and cultured as described previously (7). M8-pcDNA and M8-HLA-G5 transfectants were controlled for HLA-G expression by Western blot and ELISA, as described previously (22).

mAbs and flow cytometry analysis

The following Abs were used: 4H84, IgG1 anti-HLA-G-α1 domain specific for all HLA-G isoforms (provided by Dr. M. McMaster, University of California, San Francisco, CA) (26, 27); W6/32, IgG2a specific for all HLA class I molecules associated to β2 microglobulin (β2m) (Sigma-Aldrich); 5A6G7, IgG1 anti-HLA-G5 and -G6 (22); MEM-G9, IgG1 specific for native HLA-G1 and -G5 (Exbio) (28); TP25.99, IgG1 anti-HLA-A,-B,-C, and -E but not anti-HLA-G (provided by S. Ferrone, Roswell Park Cancer Institute, Buffalo, NY) (27, 29); GH175, IgG2 anti-ILT2 and finally, 2D1, anti-ILT4 (provided by Dr. M. Colonna, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO).

CD4 and CD25 cell surface expressions were analyzed on PBMC (2 × 10^6) after 6 days of MLR, and on PBMC obtained from LKT patients. For this purpose, we used PE-conjugated anti-CD4 or PC5-conjugated anti-CD25 (BD, mouse; and as IgG1 isotype-matched control Ab conjugated to PE and PC5. Furthermore, ECD-conjugated anti-CD4, or anti-CD8, or anti-CD14 (Inmunotech) and PE-conjugated anti-CD18, or anti-CD152, or anti-CD154 (Inmunotech), or anti-CD137 (BD Biosciences), and FITC-conjugated anti-CD62L (Inmunotech) or anti-PD1 (BD Biosciences) were analyzed on the cell surface of PBMC (2 × 10^6) after 18-h sensitization with HLA-G5 and at days 3 and 6 of MLR. Cells were analyzed on a flow cytometer EPICS XL using Expo-32 software (Beckman Coulter) as described previously (22).

Production of recombinant HLA-G5 protein adsorbed onto microbeads

Recombinant HLA-G5 protein was 1) produced in SF9 insect cells, cultured in 5% SFV TNMH medium (Invitrogen Life Technologies) and infected with HLA-G5-baculovirus alone (HLA-G5) or together with human β2m-baculovirus (HLA-G5/β2m) (Quantum Appligene) and 2) cultured for 5 days at 27°C, 5% CO2. Aplyrogén HLA-G5 protein was purified from supernatant (SN) of infected SF9 cells by immunofinity chromatography with W6/32 mAb (HLA-G Technologies). Purified HLA-G5 and HLA-G5/β2m were adsorbed onto 4.8-μm diameter latex microbeads (In-dicia Biotechnology).

Capture of naturally produced HLA-G5 or HLA-A,-B,-C, and -E molecules from LKT patients’ serum

HLA-G5 molecules present in serum from LKT patients or in SN from M6-HLA-G5 transfec tant cells were captured using magnetic beads coated with goat anti-mouse IgG Ab (Ademtech). Beads were incubated overnight at 4°C with anti-HLA-G5, 5A6G7 mAb, or anti HLA-A,-B,-C and -E, TP25.99 mAb. After three washing steps, 5A6G7 or TP25.99-coated beads were incubated with serum from LKT patients, KT patients, or healthy controls (14.10^6 beads for 300 μl of serum) and with SN from M8-HLA-G5 transfec tants. After extensive washes, beads were collected by magnetic separation and then used in functional assays.

ELISA

Soluble HLA-G concentrations were measured in sera from 19 LKT patients, three KT patients, and 13 healthy donors, as well as in SNs from M8 transfec tants. Two distinct ELISA were conducted, as described previously (21, 30). On one hand, 96-well plates (Corning Costar) were coated in PBS...
(pH 7.4) with MEM-G/9 at 10 µg/ml. Plates were saturated with 250 µl of PBS 2% BSA for 2 h at 37°C. Samples were added to each well (100 µl) in triplicate. After incubation overnight at 4°C, anti-β2-m-HRP (DakoCytomation) was added as detection Ab for 1 h at 37°C. The chromogenic substrate (tetramethylbenzidine; Sigma-Aldrich) was added for 30 min in the dark. Finally, the reaction was stopped by adding HCl (1N). In contrast, a similar approach was performed using 5A6G7 mAb at 5 µg/ml as capture Ab, and W6/32-biotin (Interchim) plus streptavidin-HRP as detection Ab (Amersham). This 5A6G7/W6/32 combination can only detect HLA-G5 and not HLA-G6 due to the inability of W6/32 to bind HLA-G6. Optical densities were measured at 450 nm. Standard curves were calculated using serial dilutions of purified soluble recombinant HLA-G5 protein. Detection limit of both ELISA was 5 ng/ml.

**MLR**

Total PBMC or T cell-enriched population was used as responder cells and plated into 96-well plates at 50,000 cells/well together with 50,000 gamma irradiated (25 Gy) stimulator PBMC. In some assays, third-party cells were gamma irradiated (25 Gy) and added to the MLR. T cell proliferation was measured at day 6 of MLR as described previously (8). For large-scale allostimulation experiments, MLR were performed in flasks at a responder:stimulator ratio of 1:1.

**Sensitization procedure**

Total PBMC or T cell-enriched preparations were sensitized for 18 h or 4 days with adherent gamma-irradiated (125 Gy) M8-pcDNA or M8-HLA-G5 cells, or their corresponding SNs. At the end of this sensitization period, PBMC or T cell-enriched preparations were extensively washed and used in MLR, either as responder cells, or as gamma-irradiated (25 Gy) third-party cells, as indicated. When indicated, suppression assays were conducted using Transwell (0.4 µm) culture system (Greiner Bio-One).

**Immunoprecipitation and Western blot analysis**

Soluble HLA-G immunoprecipitation was performed by incubating 48-h SNs from M8-pcDNA and M8-HLA-G5 cultures with MEM-G/9 mAb overnight at 4°C. Immune complexes were captured with protein A-Sepharose beads for 1 h at 4°C. After extensive washing, HLA-G5 associated or not with β2-m and coated or not on microbeads were boiled in Laemmli buffer. Aliquots of total protein were separated on 12% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond; Amersham Pharmacia). The membranes were immunolabeled with 4H84 mAb and the revelation was done, as described previously (7).

**Cell apoptosis**

PBMC were incubated for 18 h together with SNs from M8-pcDNA or M8-HLA-G5 cells, and their viability was evaluated before and after allogeneic stimulation (2 and 4 days of MLR). Trypan blue exclusion and flow cytometry analysis of annexin-V FITC staining (apoptosis) vs propidium iodide uptake (necrosis) (Annexin V-FITC kit; Immunotech) were performed on nonpermeabilized cells. Cells treated with 3% formaldehyde were used as positive controls for apoptosis.

**Statistical analysis**

All data are representative of experiments performed at least three times. Significance was assessed by Mann-Whitney U test assuming p < 0.05 as significant.

**Results**

*T cell allogeneic response is inhibited by HLA-G5-positive cells and recombinant HLA-G5 protein*

Evidence has been previously provided that HLA-G5 expression by heart and liver-kidney cotransplanted patients is associated with better allograft acceptance (3, 19). Thus, we hypothesized that, in addition to immunosuppressive therapy, HLA-G5 may be involved in the tolerance status of transplanted patients, by modulating the function of immunocompetent cells.

First, to confirm that HLA-G5 inhibits allogeneic responses, we conducted MLRs between healthy donors in HLA-G5-containing medium. HLA-G5 was provided either by HLA-G5-secreting irradiated third-party cells (i.e., M8-HLA-G5), or as SNs of HLA-G5-secreting cells, or as recombinant HLA-G5 protein. Results showed that HLA-G5-positive cells added as third-party cells at the first day of MLR induced a significant inhibition of T cell alloproliferation, whereas HLA-G5-negative control cells (i.e., M8-pcDNA) did not. Three representative allogeneic combinations are shown for which the mean percentage of T cell alloproliferation inhibition ≥ SD was of 70.7 ± 5.0 (p < 0.001) compared with M8-pcDNA (Fig. 1A), 47.8 ± 6.1 (p < 0.005) (Fig. 1B), and 75.1 ± 5.7 (p < 0.0001), respectively (Fig. 1C). Inhibition was obtained with 1) very low amounts of HLA-G5-positive cells (i.e., 20-fold less HLA-G5-positive inhibitory cells than responder cells) (Fig. 1, B and C) and with 2) HLA-G5-containing medium (SN-M8-HLA-G5) (Table I and II) in a dose-dependent manner (data not shown).

The role of HLA-G5 in inhibiting alloproliferative response was definitively attested by using recombinant HLA-G5 protein. Four distinct forms were analyzed: 1) HLA-G5-free H chain (HLA-G5); 2) HLA-G5 H chain associated with β2-m (HLA-G5/β2-m); 3) HLA-G5-free H chain coated on microbeads (HLA-G5 beads); and 4) HLA-G5 H chain associated with β2-m and coated onto microbeads (HLA-G5/β2-m beads). Characterization of these proteins by Western blot analysis showed a 37-kDa band corresponding to HLA-G5 (Fig. 2). Functional experiments showed that neither monomeric HLA-G5 nor HLA-G5/β2-m forms did inhibit T cell alloproliferative response (Table I). By contrast, once coated onto microbeads, they both did (50–70% inhibition). These data show that protein aggregation is a critical factor for the biological activity of HLA-G5, whereas β2-m association is not.

**FIGURE 1.** Inhibition of T cell alloproliferative response by HLA-G5-positive cells. Various concentrations of gamma-irradiated (125 Gy) M8-HLA-G5 cells or M8-pcDNA cells were added as third-party cells at the first day of MLR between stimulator and responder PBMC from two histoincompatible healthy donors. Tritiated thymidine incorporation after 6 days of MLR was measured. Three representative allogeneic combinations (A–C) of seven are shown. Results are expressed as the mean of thymidine incorporation (cpm) in triplicate wells, corrected for background values (Δcpm). R:S:T ratio corresponds to the responder:stimulator:third-party cells ratio. Stimulator cells are indicated by *.
Having shown that HLA-G5 inhibits alloproliferative responses in vitro, we analyzed the in vivo biological relevance in transplanted patients. For this purpose, we studied nine liver-kidney co-transplant recipients who had been transplanted for >1 year. A previous study on these patients showed an association between HLA-G expression and allograft acceptance (3, 21). We measured soluble HLA-G levels in the serum of these patients by two distinct ELISA, detecting either shed HLA-G1 plus secreted HLA-G5 or HLA-G5 (HLA-G5-free H chain) or HLA-G5/β2m (HLA-G5 H chain associated with β2m) using the 4H84 mAb. SNs were collected from M8-pcDNA cells (SN M8-pcDNA) and from M8-HLA-G5 cells (SN M8-HLA-G5) cultured at a concentration of 6000 cells/ml. Supernatants were centrifuged at 300 g to remove cells before being used for ELISA. The mean of HLA-G5 serum levels was initially and 2 years later over a longitudinal study was conducted on seven of these 19 LKT patients to analyze the stability of HLA-G serum levels with time. The mean of HLA-G5 serum levels was initially and 2 years later as follows: 386 ± 15 and 464 ± 31 ng/ml, respectively. This result reveals that high HLA-G serum level is maintained in time. Notably, no acute liver and kidney graft rejection and only one case of chronic kidney graft rejection was observed in these 19 HLA-G-positive LKT patients. These data are in agreement with previous clinical reports that associated HLA-G expression by transplanted patients with better acceptance of their allograft.

As described above for recombinant HLA-G5 protein, addition of HLA-G5-containing serum from these LKT patients (LKT1–LKT9) had high serum levels of soluble HLA-G, compared with healthy individuals (1–13). The mean of soluble HLA-G1 plus HLA-G5 serum levels ± SD in LKT patients vs healthy controls was 459 ± 179 vs 16 ± 23 ng/ml (p < 0.0001), and the mean of soluble HLA-G5 serum levels was 216 ± 70 vs 7 ± 13 ng/ml (p < 0.0001). Similar levels of serum HLA-G were obtained for 10 other LKT patients (data not shown). Moreover, a longitudinal study was conducted on seven of these 19 LKT patients to analyze the stability of HLA-G serum levels with time.

Table I. Effect of β2m association and protein aggregation on biological activity of HLA-G5a

<table>
<thead>
<tr>
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<th>59 + 59*</th>
<th>59 + 57*</th>
<th>Percentage of Inhibition</th>
<th>59 + 67*</th>
<th>Percentage of Inhibition</th>
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<td></td>
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<td>Medium</td>
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<td>14,300 ± 373</td>
<td>50</td>
<td>9,700 ± 3142</td>
<td>70</td>
</tr>
<tr>
<td>HLA-G5/β2m beadsd</td>
<td>520 ± 46</td>
<td>10,800 ± 442</td>
<td>59</td>
<td>9,700 ± 3142</td>
<td>70</td>
</tr>
</tbody>
</table>

a Raw proliferations are presented as the mean of thymidine incorporation (cpm) in triplicate wells of MLR carried out in presence of several forms of HLA-G5. Percentage of alloproliferation inhibition (percentage of inhibition) in treated MLR was calculated according to the alloproliferation observed in medium and was corrected from background values obtained with SN-M8-pcDNA or beads alone. Two of five representative allogeneic combinations (59 + 57* and 59 + 67*) using PBMC from healthy individuals as responder and stimulator cells are shown. Corresponding autologous combination is shown as control (59 + 59*). Stimulator cells are indicated by *.

b SNs from M8-HLA-G5 (SN M8-HLA-G5) were added as negative control or beads coated with SN from healthy donor (13).

c HLA-G5 concentrations were inferior to 5 ng/ml for SN M8-pcDNA and of 67 ng/ml for SN M8-HLA-G5.

d HLA-G5 (HLA-G5-free H chain) or HLA-G5/β2m (HLA-G5 H chain associated with β2m) was added at the first day of MLR at the concentration of 1 μg/ml.

e HLA-G5 beads (HLA-G5-free H chain coated on microbeads) or HLA-G5/β2m beads (HLA-G5 H chain associated with β2m and coated on microbeads) were added at the first day of MLR at the ratio of 2 beads:cell corresponding to 0.1 ng of HLA-G/BEad.

FIGURE 2. Characterization of HLA-G5 proteins. The 37 kDa purified HLA-G5 protein was produced in SF9 insect cells infected with HLA-G5 cDNA baculovirus alone (HLA-G5) or together with human β2m baculovirus (HLA-G5/β2m). These proteins were coated on microbeads (HLA-G5 beads and HLA-G5/β2m beads) and analyzed by Western blot using the 4H84 mAb. SNs were collected from M8-pcDNA cells (SN M8-pcDNA) and from M8-HLA-G5 cells (SN M8-HLA-G5) cultured at a similar level of confluence, and HLA-G5 expression was analyzed by carrying out immunoprecipitation with the MEM-G/9 mAb followed by Western blot analysis using the 4H84 mAb.
LKT9) suppressed the T cell alloproliferative response from 70 to 100% (Fig. 4A). By contrast, the T cell alloresponse was not inhibited by addition of serum from individuals that were devoid of HLA-G (i.e., inferior to 39 ng/ml), such as those of six healthy donors (donors 1–6) and three KT patients (KT1–KT3) (Fig. 4A). The addition of LKT serum to the MLR inhibited the alloresponse by 85.5 ± 9.9% (p < 0.002), whereas KT serum and serum from healthy donors did not significantly inhibit the alloresponse.

**FIGURE 3.** Increased levels of soluble HLA-G in serum from liver-kidney cotransplanted patients. Soluble HLA-G levels were measured in sera from nine LKT patients (LKT1–LKT9) by ELISA, using as capture Ab either MEM-G9 (A) or 5A6G7 (B) and as detection Ab either anti-β2m (A) or W6/32 (B). HLA-G levels were also measured in sera from 13 healthy donors (donors 1–13).

**FIGURE 4.** Inhibition of T cell alloproliferative response by HLA-G5-positive sera from liver-kidney cotransplanted patients. A. Sera (50 μl) from six healthy controls (1–6), from three KT patients (KT1–KT3), and from nine LKT patients (LKT1–LKT9) were added at the first day of MLR (97 ± 98*). SN from M8-HLA-G5 cell cultures (SN M8-HLA-G5) was used as HLA-G5-positive control. B. HLA-G5-containing serum dilutions (50 μl) from one LKT patient (LKT9) were added at the first day of MLR (97 + 98*). Number indicated at the top of each histogram corresponds to the HLA-G concentration in the corresponding diluted serum. Tritiated thymidine incorporation after 6 days of MLR was measured. Results are expressed as the mean of thymidine incorporation (cpm) in triplicate wells, corrected for background values (Δcpm). Some bars are not visible because of the very small SD.
Inhibition mediated by HLA-G5-positive serum was dose-dependent as illustrated with the serum from one patient (LKT9), which contained 720 ng/ml soluble HLA-G (Figs. 3A and 4B). More than 90% inhibition of the alloprolific response was achieved when soluble HLA-G5 concentration exceeded 50 ng/ml (Fig. 4B).

Finally, the involvement of HLA-G5 in inhibiting the proliferation of alloreactive T cells was definitively attested by immuno-purifying HLA-G5 from LKT serum. For this purpose, microbeads bearing anti-HLA-G5 mAb were used to capture naturally produced HLA-G5. Such HLA-G5 beads were added to MLR and inhibited alloproliferation of T cells >60% (p < 0.05) (Table III). By contrast, beads alone, or beads treated with serum from patients with no increased HLA-G serum levels, had no inhibitory effect. To determine the role of the other HLA class I molecules, similar experiments were performed in which the soluble HLA-A, -B, -C, and -E molecules circulating in the serum of the LKT patients were captured by using magnetic beads coated with the TP25.99 mAb (recognizing all HLA class I molecules except HLA-G). Results showed that HLA-A, -B, -C, -E coated beads did not inhibit alloproliferation of T cells, whereas HLA-G5 extracted from the same sera and under an aggregated form inhibited alloproliferation (Table III). These data demonstrate the biological relevance of HLA-G5 that is present in biological fluids as a molecule that sufficiently contributes to tolerance induction by limiting T cell alloresponse.

**Inhibitory receptors ILT2 and ILT4 are involved in HLA-G5-mediated inhibition of T cell alloproliferative response**

To further understand how HLA-G5 inhibits the proliferative response of alloreactive T cells, we investigated the involvement of HLA-G inhibitory receptors, namely ILT2 and ILT4 (5), which both bind HLA-G with high affinity (6). First, expression of both receptors was analyzed on PBMC before and after allogeneic stimulation. Flow cytometry analysis showed that both receptors are expressed at a high level on CD14+ monocytes before (Fig. 5) and at day 3 (data not shown) of the MLR, whereas these receptors were not present at the cell surface of CD4+ and CD8+ T cells (data not shown). Then, functional experiments were performed using SNs from M8-pcDNA cells or M8-HLA-G5 cells that were added at the first day of MLR together with anti-ILT2 or anti-ILT4. Results showed that blocking these receptors reversed significantly (p < 0.05 compared with control Ab) HLA-G5-mediated inhibition of MLR, whereas control Ab did not (Fig. 5). These data demonstrate the involvement of both ILT2 and ILT4 expressed by myeloid APC in HLA-G5-mediated inhibition of alloproliferation.

**HLA-G5 renders naive T cells unable to respond to subsequent allogeneic stimulus**

To further investigate the mechanisms by which HLA-G5 induces tolerance, naive PBMC from healthy individuals were pretreated with HLA-G5-positive cells (sensitization step), and their ability to proliferate under allogestimulation was measured after HLA-G5 was removed. For this purpose, HLA-G5-pretreated T cells were used as responder cells toward histoincompatible stimulator PBMC (Fig. 6A). The sensitization step was also performed using SN from M8-HLA-G5 cells, which gave similar inhibition of T cell alloresponses than irradiated M8-HLA-G5 cells (data not shown). As shown in Fig. 6A, after pretreatment with HLA-G5-positive cells, naive T cells from two healthy donors (77 and 78) lost their ability to proliferate under allogeneic stimulation in comparison with a pretreatment with HLA-G5-negative cells (mean percentage inhibition ± SD = 97.6 ± 1.6; p < 0.0001).

**FIGURE 5.** Both ILT2 and ILT4 receptors expressed by CD14+ cells are involved in HLA-G5-mediated inhibition of T cell alloresponse. Before the MLR, PBMC were double labeled with anti-CD14 mAb together with either anti-ILT2 or anti-ILT4 Ab and then analyzed by flow cytometry (insert at right top). SN from M8-HLA-G5 (HLA-G concentration of 86 ng/ml) or medium alone was added at the first day of MLR concomitantly to the addition of anti-ILT2 or anti-ILT4 Ab at the first and third days. Control Ab corresponds to isotype-matched irrelevant Ab. Tritiated thymidine incorporation was measured after 6 days of MLR. One representative allogeneic combination (92 + 94*) of three is shown. Results are expressed as the mean of thymidine incorporation (cpm) in triplicate wells, corrected for background values (Δcpm). Some bars are not visible because of the very small SD.
In line with these experiments, we used PBMC from transplanted patients to investigate the allogeneic capabilities of these PBMC, which had been exposed to high levels of circulating HLA-G5 in vivo. Results showed that PBMC from all HLA-G5-positive LKT patients (LKT1–LKT9) were unresponsive to allogeneic stimulation in vitro (mean percentage inhibition of T cell positive LKT patients (LKT1–LKT9) were unresponsive to alloresponse ± SD = 98.3 ± 0.3%; p < 0.02), compared with responder PBMC from one healthy donor (Fig. 6B).

**HLA-G5 induces immunosuppressive T cells**

Tolerance can be achieved by apoptosis-induced deletion of T cells and/or by inducing immunosuppressive cells that will regulate the function of other immunocompetent cells. We first determined whether HLA-G5-mediated inhibition of T cell allogeneic proliferation was due to apoptosis. For this purpose, we sensitized PBMC from a healthy donor for 18 h with SN from M8-pcDNA or M8-HLA-G5 cells, allostimulated them, and evaluated apoptosis levels. No difference was observed between PBMC pretreated with SN from M8-pcDNA or M8-HLA-G5 cells, nor did we observe differences in apoptosis levels before or after allogeneic stimulation (Table IV). These results show that apoptosis is not involved in HLA-G5-mediated inhibition of allogeneic T cells.

We then analyzed whether regulatory T cells could be induced by HLA-G5. For this purpose, PBMC from healthy donors (77, 78, and 79), or the T cell-enriched population from one healthy donor (64) were pretreated with HLA-G5 (sensitization step) and then used as irradiated third-party cells in MLR (Fig. 7A). Third-party cells that had not been pretreated with HLA-G5 did not affect the extent of the alloresponse and hence had no immunosuppressive function. On the contrary, third-party cells (PBMC or the T cell-enriched population) that had been pretreated with HLA-G5 inhibited the alloresponse of naive T cells by >80% (mean percentage inhibition ± SD = 87.4 ± 6.1; p < 0.001). This was true when the responder cells were autologous or allogeneic to the HLA-G5-pretreated third-party cells (64 responder + 60* stimulator + 64** third-party cell, and 60 responder + 56* stimulator + 64** third-party cell, respectively) (Fig. 7A). Similar results were obtained by using HLA-G5-containing medium instead of irradiated M8-HLA-G5 cells for the sensitization step (data not shown). These data demonstrate that HLA-G5 can induce immunosuppressive T cells in vitro.

Using a similar approach, PBMC from the nine LKT patients (LKT1–LKT9) were used as irradiated third-party cells in MLR. T cell allogeneic proliferative response was significantly inhibited by the addition of PBMC from LKT patients (mean percentage inhibition ± SD = 70.1 ± 23.0% (p < 0.03), compared with no third-party cells added), but not by that of PBMC from healthy individuals (donors 1–5) (mean percentage inhibition ± SD = 5.9 ± 10.4%; p > 0.1). The percentages of inhibition given by patient’s PBMC varied between patients and were superior to 50% for LKT1, LKT2, LKT4, LKT7, and LKT9 patients (Fig. 7B). These

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**Table IV. Percentage of apoptosis of HLA-G5-pretreated responder PBMC before and after allogeneic stimulation**

<table>
<thead>
<tr>
<th></th>
<th>Medium&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SN M8-pcDNA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SN M8-HLA-G5&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PBMC 77&lt;sup&gt;e&lt;/sup&gt;</strong></td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>Day of MLR&lt;sup&gt;f&lt;/sup&gt;</strong></td>
<td><strong>Medium</strong>&lt;sup&gt;g&lt;/sup&gt;</td>
<td><strong>SN M8-pcDNA</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td><strong>SN M8-HLA-G5</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>22</td>
<td>21</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are from flow cytometry experiments and are expressed as the percentage of cells positively stained by Annexin-V-FITC and propidium iodide to determine apoptotic cells.

<sup>b</sup> PBMC from one healthy donor (77) was untreated (medium) or pretreated with SN from either M8-pcDNA (SN M8-pcDNA) or M8-HLA-G5 (SN M8-HLA-G5) for 18 h.

<sup>c</sup> These pretreated PBMC were then extensively washed and used as responder cells in MLR towards stimulator histoincompatible PBMC (78*).

<sup>d</sup> Percentage of apoptosis of responder PBMC 77 was analyzed on days 2 and 4 of MLR.
results show that immunosuppressive cells are present among PBMC from LKT patients.

To define the critical time necessary for HLA-G to be expressed and that for HLA-G to exert its immunosuppressive effects after transplantation, we analyzed three newly LKT patients whose serum and PBMC were collected during the first month posttransplantation. High HLA-G5 serum levels were detected (mean ± SEM 683 ± 13 ng/ml) showing that HLA-G5 is present at high levels early after transplantation. This result suggests that HLA-G5 may tolerize cells precociously after liver-kidney allotransplantation.

To answer this point, we analyzed HLA-G5-containing serum from these three recently transplanted patients. Results showed that such HLA-G5 was biologically active because it inhibited T cell alloresponses from 90 to 100% \( (p < 0.005, \text{compared with control without HLA-G5}) \). Tritiated thymidine incorporation was measured after 6 days of MLR. Results are expressed as the mean of thymidine incorporation (cpm) in triplicate wells, corrected for background values (Δcpm). Some bars are not visible because of the very small SD.

**Table V.** Percentage of CD4⁺ CD25⁺ regulatory T cells in responder PBMC from healthy donors sensitized with or without HLA-G5

<table>
<thead>
<tr>
<th>Hours of Sensitization</th>
<th>Medium⁺</th>
<th>SN M8-pcDNA⁺</th>
<th>SN M8-HLA-G5⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC 77</td>
<td>18</td>
<td>0.2⁺</td>
<td>0.8</td>
</tr>
<tr>
<td>PBMC 81</td>
<td>48</td>
<td>6.4</td>
<td>6.0</td>
</tr>
<tr>
<td>PBMC 82</td>
<td>120</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>PBMC 83</td>
<td>96</td>
<td>6.8</td>
<td>5.4</td>
</tr>
<tr>
<td>PBMC 83</td>
<td>72</td>
<td>7.3</td>
<td>6.0</td>
</tr>
</tbody>
</table>

⁺ Data are from flow cytometry experiments after double staining using anti-CD4-PE and anti-CD25-PC5 on naive PBMC from healthy donors (77, 81, 82, and 83) that were presensitized and then used as responder cells in MLR. Results are expressed as the percentage of CD4⁺ CD25⁺ among the CD4⁺ T cell population at day 6 of MLR.

HLA-G5 induces immunosuppressive T cells that are not CD4⁺ CD25⁺ regulatory cells

Having shown that inhibition of the allogeneic response after HLA-G5 sensitization was due to suppressive T cells, we then investigated whether these were CD4⁺ CD25⁺ regulatory T cells. CD4⁺ CD25⁺ regulatory T cells can down-regulate the immune response by affecting T cell function, Ab production, cytokine secretion, and APC function. This CD4⁺ CD25⁺ regulatory T cell-mediated inhibition is not Ag specific (31, 32). We hypothesized that sensitization of naive PBMC with HLA-G5 triggers the expansion of CD4⁺ CD25⁺ regulatory T cells and that therefore, this subset should be expanded in HLA-G5-positive LKT patients.

No differences were observed in the percentages of CD4⁺ CD25⁺ regulatory T cells in PBMC from healthy individuals after sensitization with or without HLA-G5 (Table V; SN-M8-pcDNA or SN-M8-HLA-G5 vs medium; \( p > 0.1 \)) or 2) in PBMC from HLA-G5-positive LKT patients (4–18%) (Fig. 8) vs PBMC from healthy donors after sensitization with or without HLA-G5 (Table V; SN-M8-pcDNA or SN-M8-HLA-G5 vs medium; \( p > 0.1 \)).
PBMC from healthy individuals (data not shown). The percentage of CD4^+CD25^+ T cells varied between LKT patients and was related neither to HLA-G serum levels nor to the ability of PBMC to inhibit the reactivity of other T cells. For instance, PBMC from LKT3 that contained 16% of CD4^+CD25^+ T cells inhibited alloproliferative responses 27%, whereas PBMC from LKT4 that contained 4% of CD4^+CD25^+ T cells suppressed alloresponses completely (>83%) (Figs. 7 and 8).

To determine the phenotype of the HLA-G5-induced T cells, several T cell markers (CD4, CD18, CD62L, CD137, CD152, CD154, and PD1) were analyzed on HLA-G5-sensitized PBMC, by flow cytometry, after 18-h sensitization and at days 3 and 6 of MLR. At day 6 of MLR, comparison of the mean fluorescence intensity, between PBMC presensitized with control medium vs HLA-G5-containing medium, gave the following significant differences: mean 26 vs mean 7 for CD4 (p < 0.01); and mean 9 vs mean 3 for CD62L (p < 0.01). Moreover, the percentage of CD4^+CD25^+ was decreased when PBMC were presensitized with HLA-G5 (3%), compared with control (16%) (p < 0.01). This lower expression of CD4 and CD62L appeared since 18-h sensitization (data not shown) and became significant at day 6 of MLR. On the contrary, the expression of the other markers did not significantly vary (data not shown). Taken together, these data obtained from four separate experiments showed that the HLA-G5-sensitized CD4^+ T cells exhibited a significant decrease of the costimulatory CD4 molecule and the L-selectin CD62L.

**HLA-G5-induced regulatory T cells do not require cell-contact to inhibit T cell alloresponses**

Having shown that inhibition of the allogeneic response after HLA-G5 sensitization was due to suppressive T cells, we then investigated whether these T cells require cell-contact to exert their function. For this purpose, PBMC from one healthy donor (8) were pretreated with HLA-G5 (sensitization step) and then used as irradiated third-party cells in MLR. These sensitized T cells were either added directly to the MLR or at the top chamber of a Transwell culture system, which allows only soluble factors secreted in the culture medium to diffuse into the bottom chamber where the MLR was conducted. Results showed that HLA-G5-induced suppressive T cells inhibited T cell alloproliferative responses, whatever they were, or not, in close contact with alloreactive T cells from the MLR (Fig. 9). Thus, HLA-G5-induced regulatory T cells can, at least, exert their immunosuppressive properties through a soluble factor.

**Discussion**

HLA-G expression has been proposed to contribute, in addition to immunosuppressive therapy, to human allograft acceptance. Indeed, HLA-G expression found in heart-transplant recipients, both in serum and in grafted endomyocardial cells, was associated with a decreased number of acute rejection episodes and an absence of chronic rejection, compared with patients who did not express HLA-G (2, 19). Furthermore, following liver-kidney cotransplantation, ectopic expression of HLA-G in grafted liver and in serum was associated with an absence of acute and chronic rejection of both liver and kidney transplants (3). By contrast, serum levels of total HLA class I molecules was not associated with graft outcome in these LKT patients (21). The aim of the present study was to investigate the role of HLA-G in tolerance induction. We focused our investigations on the role of the soluble HLA-G5 protein in modulating the functions of T cells that are the main effectors involved in graft rejection, in vitro and in vivo.

In vitro experiments showed that HLA-G5 required aggregation, rather than association with β_2m, to have biological inhibitory activity. This is consistent with a previous study that reported that cell surface HLA-G1 oligomers bind ILT2 with increased avidity, which might enhance HLA-G inhibitory function (33). Thus, it is possible that in our in vitro system, HLA-G5 requires oligomerization-dependent high-affinity binding to inhibitory receptors to have its inhibitory functions. Thus, it is possible that HLA-G5 is already in an aggregated form in the sera from LKT patients.

In this study, both ILT2 and ILT4 inhibitory receptors were involved in HLA-G5-mediated inhibition. We previously showed that HLA-G can bind to NK cells and CTL cells via ILT2, which lead to their functional inhibition (7, 34, 35). In our system, both ILT2 and ILT-4 are exclusively expressed by myeloid APCs, such as CD14^+ monocytes, which are thus clearly involved in HLA-G5-mediated inhibition of T cell alloproliferation. This is consistent with other studies, which showed in the murine system that HLA-G tetramer interacts with the murine homologue of ILT4, paired Ig-like inhibitory receptor-B, which impairs DC maturation and alloresponses of T cells (23). These same authors also showed that immunization of HLA-G-tetramers 1 day before skin grafting induces anergic and CD4^+CD25^+ regulatory T cells (23–25).

In this study, we show that HLA-G5 induce immunosuppressive T cells in vitro. Indeed, naive T cells from healthy individuals sensitized 18 h with HLA-G5-positive cells or with HLA-G5-containing medium lost their ability to respond to allostimulation and
acquired suppressive functions. These data indicate that HLA-G5 does not need to be present at the time of the allogeneic reaction to exert its inhibitory functions. Indeed, HLA-G sensitized T cells were still immunosuppressive even when HLA-G5 is no longer present. Moreover, HLA-G5 can act in a completely Ag nonspecific fashion, because its effects are shown when no Ag stimulation is given. Thus, based on these in vitro observations, a microenvironment of high HLA-G5 content would be efficiently immunoinhibitory in suppressing function of recipient alloreactive T cells after organ transplantation.

To analyze the biological relevance of these observations, we studied liver-kidney cotransplanted patients. In agreement with previous reports (21), increased levels of soluble HLA-G were detected in the 19 LKT patients analyzed. By the use of the 5A6G7 mAb, which distinguishes between shed HLA-G1 from the membrane and HLA-G5 soluble form, we could clearly measured very high concentrations of HLA-G5 in serum from all patients. This high HLA-G5 serum level was maintained in time. Notably, no acute hepatic or renal graft rejection and only one case of chronic kidney graft rejection were observed in these 19 HLA-G5-positive patients, supporting the association between HLA-G expression and better graft acceptance. Such association could either mean that HLA-G expression is directly responsible for tolerance or that HLA-G expression is a consequence of tolerance. To address this question, we analyzed the suppressive function of HLA-G5 from LKT patients. In accordance with our in vitro studies, we observed that immunopurified HLA-G5 from LKT serum suppressed T cell alloresponses. This effect was not observed with serum from healthy donors and KT patients who had baseline levels of HLA-G. It is of note that we previously showed that serum concentrations of soluble HLA-class I molecules were similar between LKT patients, KT patients, liver transplanted patients, and healthy individuals, demonstrating that soluble HLA-class I molecules may not be responsible for the inhibition presently observed (21). This hypothesis was confirmed by the immunopurification of soluble HLA-A, -B, -C, and -E molecules (circulating in the serum from LKT patients) that did not exert inhibitory effects on the T cell alloresponse. Moreover, the functions of T cell from HLA-G5-positive LKT patients were altered. These T cells did not respond to allogeneic stimulation and had regulatory/immunosuppressive functions, in opposition to T cells from HLA-G-negative healthy donors. Thus, soluble HLA-G5 induces a regulatory/immunosuppressive T cell population that may, in part, explain why transplanted patients with increased serum levels of HLA-G5 accept their allograft better than transplanted patients with baseline levels of HLA-G5. Time course analysis of HLA-G expression and function after LKT showed that HLA-G5 is present at high levels in serum since the first week’s posttransplantation, allowing the HLA-G5-mediated immunoregulatory process to take place rapidly. Regarding the influence of immunosuppression levels on HLA-G5 expression by LKT compared with KT patients, it is of note that the dosage of calcineurin inhibitors was reduced for LKT patients followed in Paul Brousse hospital (tacrolimus levels between 5 and 7 ng/ml), compared with KT patients (tacrolimus levels between 10 and 15 ng/ml). Such decrease of immunosuppressive treatment in LKT patients was motivated by their better immunological status, and may be responsible for HLA-G5 induction and its associated immunoregulatory state. Such hypothesis would deserve particular attention in next clinical investigations on a larger number of patients.

In opposition to what was observed in the murine system (23, 24), in vitro sensitization of naive T cells with HLA-G5 did not induce an expansion of CD4+CD25+ T cells, and no difference was observed in the percentage of peripheral CD4+CD25+ T cells between LKT patients and healthy controls. However, extensive phenotypic analysis led us to conclude that HLA-G5-induced T cells exhibit a CD4dimCD62Llow phenotype. Down-modulation of the coreceptor CD4 on HLA-G5-sensitized CD3+ T cells may limit the TCR-mediated activation of such T cell and may be responsible for their inability to respond to allogeneic stimulus. Moreover, the reduced expression of the lymph node homing receptor L-selectin (CD62L) on HLA-G5 sensitized CD4+ T cells may have important in vivo implications by altering T cell migration and homing into lymphoid tissue (36). Regarding their immunosuppressive/regulatory properties, we have demonstrated that HLA-G5-induced T cells act in an Ag-nonspecific fashion and through soluble factor(s). It is known that regulatory T cells down-modulate immune responses through secretion of immunosuppressive cytokines IL-10 and TGF-β (37, 38). The involvement of these cytokines in HLA-G5-mediated tolerance now requires further attention.

Unexpectedly, we could not detect HLA-G expression in the liver graft biopsies of the patients analyzed in this study, whose transplantation was performed more than 1 year before our analysis (data not shown). This contrasts with our previous study in which HLA-G was found in grafted biliary epithelial cells from LKT patients more recently transplanted (3). Moreover, a retrospective analysis on 14 LKT patients, whose biopsies were analyzed monthly during the first year posttransplantation showed that HLA-G is expressed in the liver graft as of the second month after transplantation and that its expression is maintained through the first year (data not shown). One can hypothesize that after a certain time posttransplantation, HLA-G-positive cells are preferentially localized in immunoreactive sites such as draining lymph nodes, from which soluble HLA-G is delivered to the peripheral blood.

In conclusion, our results can be extended to other solid organ transplants such as heart, which also express HLA-G. The in vitro demonstration that soluble HLA-G5 suppresses T cell functions and induces regulatory T cells, together with the in vivo results showing inhibitory properties of serum HLA-G5 from patients who had an absence of acute hepatic or renal episode rejections, led us to propose that HLA-G5 contributes to tolerance in transplanted patients. These data have the following important clinical implications: 1) measurement of HLA-G5 serum level may serve to monitor transplanted patients expected to better accept their allograft; 2) in such HLA-G-positive patients, the amount of immunosuppressive drugs may thus be decreased thereby reducing deleterious side effects; and 3) treatment with HLA-G5 or derivatives may constitute in the future a novel efficient antigraft rejection therapy.

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Disclosures
The authors have no financial conflict of interest.

References
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