Alloreactive CD4+ and CD8+ T cells express the immunotolerant HLA-G molecule in mixed lymphocyte reactions: *in vivo* implications in transplanted patients

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HLA-G displays immunotolerogenic properties towards the main effector cells involved in graft rejection through inhibition of NK- and CTL-mediated cytolysis and CD4+ T cell alloproliferation. HLA-G expression is restricted in healthy tissues to trophoblast and thymus but is extended to various tissues under pathological conditions. HLA-G was detected in allograft biopsies and sera from transplanted patients who displayed a better graft acceptance. However, the cells involved in such *de novo* expression of HLA-G remain to be characterized. By flow cytometry and confocal microscopy, we demonstrated that, following allogeneic stimulation *in vitro,* both CD4+ and CD8+ T cell subsets can express membrane-bound HLA-G1 and/or soluble HLA-G5 molecules. Such HLA-G1/-G5 expression is regulated at the transcriptional level. Soluble HLA-G5 could be detected by using a novel monoclonal antibody, 5A6G7, specific for the intron 4-retaining sequence of HLA-G5. Finally, the biological relevance of these data was provided by analysis of transplanted patients in whom we identified both CD4+ and CD8+ T cells expressing HLA-G. The HLA-G-positive T cells we describe here may constitute a cellular source of HLA-G after allotransplantation and may be involved in the improved graft acceptance which is observed in HLA-G-positive transplanted patients.

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1 Introduction

In order to prevent graft rejection, immunosuppressors and antibody treatments have been developed, aimed at prolonging survival of the grafts in MHC-mismatched recipients [1, 2]. Nevertheless, these drugs have serious side effects and further investigations of alternative immunotolerant strategies are sought [3]. Recently, non-classical HLA class I molecule HLA-G, which is a well-known tolerogeneic molecule, was found to contribute to allograft acceptance in transplanted patients [4–8].

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Abbreviation: PI: Propidium iodide

Initially detected at the fetal-maternal interface, the expression of HLA-G protects the fetal semi-allograft from rejection by the mother's immune system [9–11]. In non-pathological conditions, HLA-G expression is restricted to immunoprivileged sites such as trophoblast and thymus. Contrary to the highly polymorphic classical HLA class I molecules, which constitute transplantation antigens leading to tissue-allograft rejection, *HLA-G* counts only fifteen alleles. HLA-G can be expressed under four membrane-bound (HLA-G1, -G2, -G3, and -G4) and three soluble (HLA-G5, -G6, and -G7) isoforms that are generated by alternative splicing of a unique primary transcript [12]. Among these seven isoforms, membrane-bound HLA-G1 and soluble HLA-G5 proteins have been studied the most. We focused our present work on both these isoforms.

The 39-kDa full-length HLA-G1 heavy chain is constituted by three extracellular domains (α1, α2 and α3) associated with the β2-microglobulin (β2m) and a nonapeptide. The 37-kDa soluble HLA-G5 heavy chain is encoded by an alternative transcript which retains part of intron 4 and lacks both transmembrane and cytoplasmic domains [13]. HLA-G5 also associates to β2m and peptide [13]. In terms of function, both HLA-G1 and HLA-G5 isoforms display immunotolerant properties [12]. In particular, the role of both isoforms with respect to their capability to reduce graft rejection may come from their ability to interact with inhibitory receptors, such as CD85j (ILT-2) [14, 15] and CD85d (ILT-4) [16, 17]. For instance, HLA-G1 protects targets from both NK cell- and CTL-mediated lysis, and only few HLA-G1-positive cells are required to exert significant inhibitory effect [15, 18]. Furthermore, HLA-G has been shown to impair the maturation of dendritic cells, resulting in prolonged allogeneic skin graft survival [19]. HLA-G leader peptide stabilizes the cell-surface expression of another non-classical HLA class I inhibitory molecule, HLA-E [20]. Finally, soluble HLA-G induces apoptosis of $CD8^+$ T and NK cells through ligation with CD8, and *via* a Fas/FasL-dependent mechanism [11, 21, 22].

In human allo-transplantation, ectopic HLA-G expression was demonstrated in graft biopsies and sera from heart- or kidney/liver-transplanted patients who exhibit a better graft acceptance [4, 5, 7]. However, the cells and mechanisms involved in such a *de novo* expression of HLA-G remain unknown. Attempts were made in the present study to investigate this point both *in vitro* in mixed lymphocyte reactions (MLR), and *in vivo* in transplanted patients. Using antibodies that recognize either all HLA-G isoforms or soluble HLA-G5 isoform specifically, we showed that HLA-G1 and/or -G5 proteins are expressed by $CD4^+$ and/or $CD8^+$ alloreactive T cells after MLR. Such HLA-G-positive T cells may act as inhibitory regulators of immune responses, thus limiting allo-graft rejection in human transplantation. Our observations are backed by two kidney/liver bi-transplanted patients from whom peripheral blood $CD4^+$ and $CD8^+$ T cells that also express HLA-G were identified *in vivo*.

2 Results

2.1 Up-regulation of HLA-G1 and/or HLA-G5 transcripts after mixed lymphocyte reactions

To analyze the induction of *HLA-G* transcription after 7 days of MLR, RT-PCR analysis was performed on 16 allogeneic and 9 autologous combinations. JEG-3 cells were used as HLA-G1 and HLA-G5 positive controls, and β-actin transcription was used as an internal control.

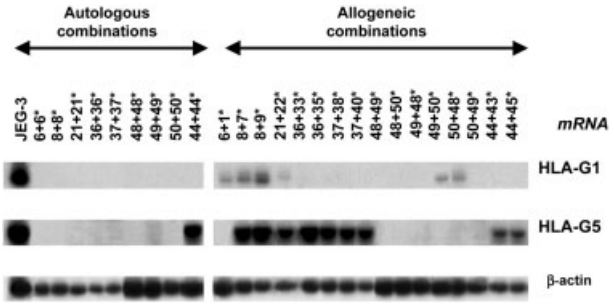

Fig. 1. Up-regulation of HLA-G1 and/or HLA-G5 transcription during allogeneic responses. Analysis of HLA-G1 and HLA-G5 transcription by RT-PCR was performed in 25 combinations after 7 days of MLR. For each combination, β-actin was used as an internal control and the JEG-3 cell line as HLA-G positive control. Numbers correspond to PBMC obtained from healthy adult donors, used as responder or irradiated stimulating cells (*) in MLR.

As shown in Fig. 1, only 1 of the 9 autologous combinations transcribed HLA-G5 mRNA. For allogeneic combinations, 25% (4/16) had no detectable level of HLA-G1 and HLA-G5 transcripts (group I), 19% (3/16) transcribed selectively the full-length HLA-G1 alternative transcript (group II), 37% (6/16) transcribed only HLA-G5 mRNA (group III), and 19% (3/16) had detectable levels of both HLA-G1 and HLA-G5 transcripts (group IV). HLA-G5 mRNA was the transcript expressed the most in allogeneic responses (56%). We then investigated whether this up-regulation of *HLA-G* transcription was associated with protein expression after allogeneic stimulation. Since no mAb was available for the specific detection of the HLA-G5 soluble protein, we produced such a specific mAb, namely 5A6G7.

2.2 Characterization of an mAb specific for the soluble HLA-G5 and -G6 intron 4-retaining isoforms

The 5A6G7 mAb was obtained after immunization of BALB/c mice with a peptide corresponding to the C-intron 4-encoded polypeptidic sequence of HLA-G5/-G6 proteins in order to produce HLA-G5/-G6 specific mAb. Characterization of this mAb was achieved by Western blot (Fig. 2A), immunocytochemistry (Fig. 2B) and confocal microscopy (Fig. 2C) using M8-pcDNA, M8-HLA-G1, M8-HLA-G5 and M8-HLA-G6 transfectants.

First, as shown in Fig. 2A, 5A6G7 recognizes 37- and 28-kDa proteins corresponding to HLA-G5 and HLA-G6, respectively. Secondly, 5A6G7 specifically stained M8-HLA-G5 cells but did not stain M8-pcDNA or M8-HLA-G1 (Fig. 2B and C). The expression of each HLA-G iso-

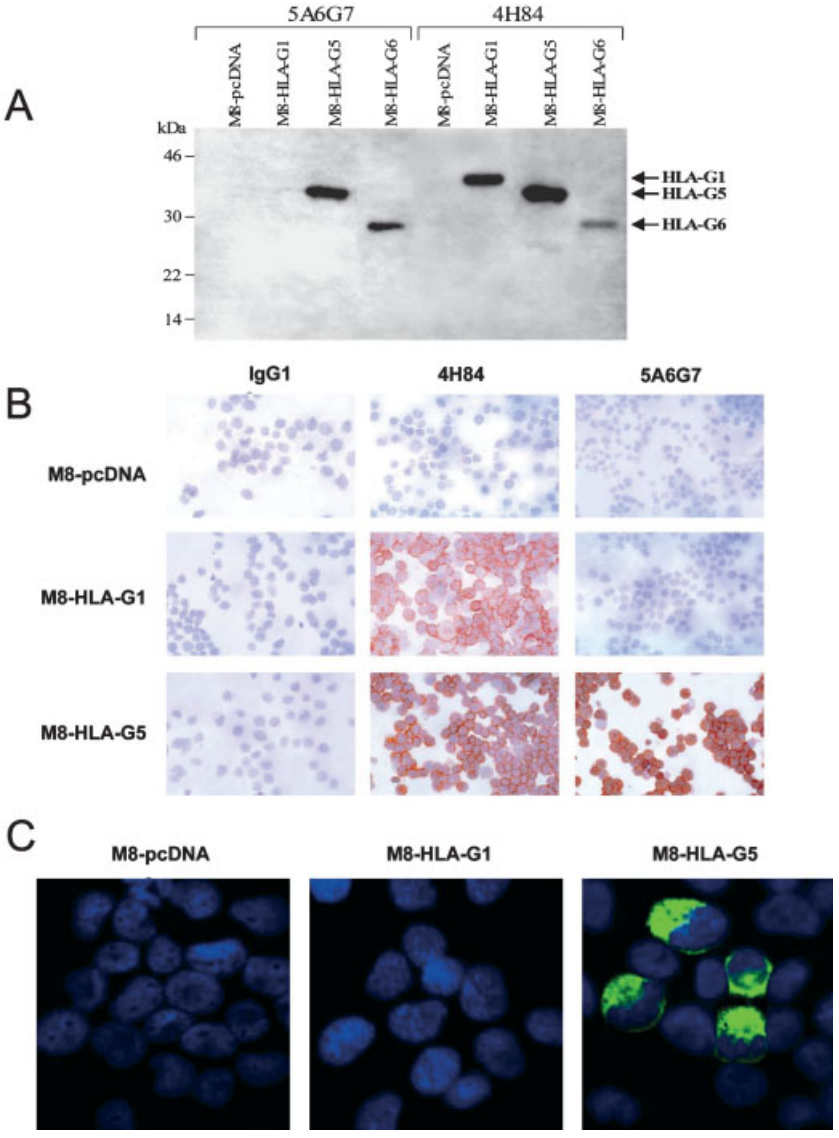

Fig. 2. The 37-kDa HLA-G5 and the 28-KDa HLA-G6 soluble proteins are revealed specifically by the 5A6G7 mAb. The antigen specificity of the 5A6G7 mAb using the M8 transfected cells was analyzed by (A) Western blot, (B) immunocytochemistry, and (C) confocal microscopy after cell permeabilization. The 4H84 mAb which recognizes all the HLA-G isoforms, was used as control.

form expressed by these M8-transfected cells was positively controlled using the 4H84 anti-HLA-G mAb (Fig. 2A and B). These results showed that the 5A6G7 mAb was specific for both HLA-G5 and HLA-G6 isoforms, and did not cross-react with the full-length HLA-G1 membrane-bound isoform or other HLA-class I molecules (A, B, C and E), expressed by the M8 melanoma cell line.

2.3 HLA-G1 and/or HLA-G5 expression is induced after mixed lymphocyte reactions and is regulated at the transcriptional level

The 16 allogeneic combinations and the 9 autologous combinations described in Sect. 2.1 were studied for HLA-G protein expression. Cells were collected on day 7 of the allogeneic response and analyzed by immunocytochemistry using the 4H84 mAb (specific for HLA-G α1 domain), the 5A6G7 mAb, and an isotype-matched control IgG1 mAb. Cells from autologous combinations were never stained by the HLA-G mAb, and the same was true for allogeneic combinations corresponding to group I. By

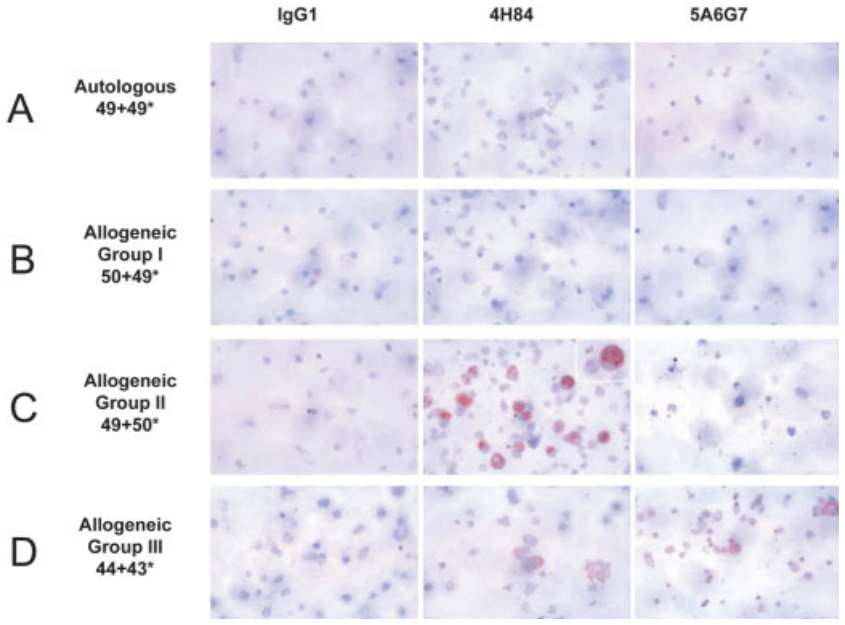

Fig. 3. Three profiles of HLA-G expression after 7 days of MLR. Immunocytochemistry images were obtained by using 5A6G7, 4H84 and IgG1-isotype-matched control Ab. Staining was visualized by a biotin-streptavidin-peroxidase detection system revealed by the 3, 3′-diamino benzidine substrate-chromogen solution, yielding a red-brown reaction. Representative results obtained for cells from autologous (A), and allogeneic Group I (B), Group II (C) and Group III (D) combinations are shown.

contrast, cells from allogeneic combinations were either stained only by 4H84 (group II), or stained by both anti-HLA-G mAb (4H84 and 5A6G7) (groups III and IV). These patterns of HLA-G expression are illustrated in Fig. 3, showing one autologous (A) and three allogeneic (B, C, and D) combinations at day 7 of the MLR. These results demonstrated that HLA-G was expressed in 75% of the allogeneic combinations (groups II, III and IV) and that soluble HLA-G5 and/or -G6 were the main HLA-G isoforms expressed during allogeneic responses. When evaluated by immunocytochemistry analysis, the proportion of HLA-G-positive cells in groups II, III and IV was variable and ranged from 5 to 80% of cells.

Taken together, these data showed that (i) for autologous combinations, absence of HLA-G1 and HLA-G5 mRNA correlated with no expression of HLA-G1 and -G5 proteins, excepted in one autologous combination for which HLA-G5 expression was detected at the mRNA level only, (ii) 25% of allogeneic combinations (4/16) did not transcribe *HLA-G*, nor did they express HLA-G protein (group I), (iii) 19% of allogeneic combinations (3/16) expresses HLA-G1 mRNA only and were stained only by 4H84 (group II), (iv) 37% of allogeneic combinations (6/16) expressed HLA-G5 mRNA only and were stained by both 4H84 and 5A6G7 (group III), and (v) 19% of allogeneic combinations (3/16) expressed both HLA-G1 and -G5 mRNA and were stained by both anti-HLA-G mAb (group IV). Thus, comparison of the results obtained by RT-PCR and immunocytochemistry demonstrated that expression of HLA-G which was induced under allogeneic stimuli is regulated at the transcriptional level (Table 1).

Since HLA-G inhibits T cell functions [23, 24], we analyzed the T cell alloproliferative response of the sixteen allogeneic combinations after 6 days of MLR. Strong proliferative responses were observed for all these allogeneic combinations. However, even though the number of combinations was too small for statistical significance, a lower alloproliferative response was observed for HLA-G-positive combinations than for HLA-G-negative ones (data not shown).

2.4 Both $CD4^+$ and $CD8^+$ alloreactive T cell subsets can express membrane-bound HLA-G1 and/or soluble HLA-G5 isoforms

To identify the cell population responsible for HLA-G expression during MLR, cells from HLA-G-positive allogeneic combinations were collected on day 7 and analyzed by confocal microscopy. These analyses showed that both responder $CD4^+$ and $CD8^+$ alloreactive T cell subsets could express HLA-G1 and/or HLA-G5. Four representative analyses are shown in Fig. 4A, B, C, and

Table 1. Summary of the results on both HLA-G gene transcription and protein expression in autologous and allogeneic combinations

Combinations		RT-PCR		Immunocytochemistry[a]		HLA-G isoform[b]	%[c]
		HLA-G1 mRNA	HLA-G5 mRNA	4H84	5A6G7		
Autologous	6+6*	-	-	-	-	none	100
	8+8*	-	-	-	-		
	21+21*	-	-	-	-		
	36+36*	-	-	-	-		
	37+37*	-	-	-	-		
	48+48*	-	-	-	-		
	49+49*	-	-	-	-		
	50+50*	-	-	-	-		
	44+44*	-	+	-	-		
Allogeneic	48+49*	-	-	-	-	none	25
	48+50*	-	-	-	-		
	49+48*	-	-	-	-		
	50+49*	-	-	-	-		
	6+1*	+	-	+	-	HLA-G1	19
	49+50*	+	-	+	-		
	50+48*	+	-	+	-		
	36+33*	-	+	+	+	HLA-G5	37
	36+35*	-	+	+	+		
	37+38*	-	+	+	+		
	37+40*	-	+	+	+		
	44+43*	-	+	+	+		
	44+45*	-	+	+	+		
	8+7*	+	+	+	+	HLA-G1 and HLA-G5	19
	8+9*	+	+	+	+		
	21+22*	+	+	+	+		

[a] HLA-G protein expression was analyzed by immunocytochemistry using the 4H84 and 5A6G7 mAb, which are specific for all HLA-G isoforms and only HLA-G5, respectively.

[b] The HLA-G isoform expressed by the responder cells of the corresponding allogeneic combination was deduced from both RT-PCR and immunocytochemical experiments.

[c] The numbers correspond to the percentages of combinations with the described pattern of HLA-G expression in all the autologous or allogeneic combinations tested.

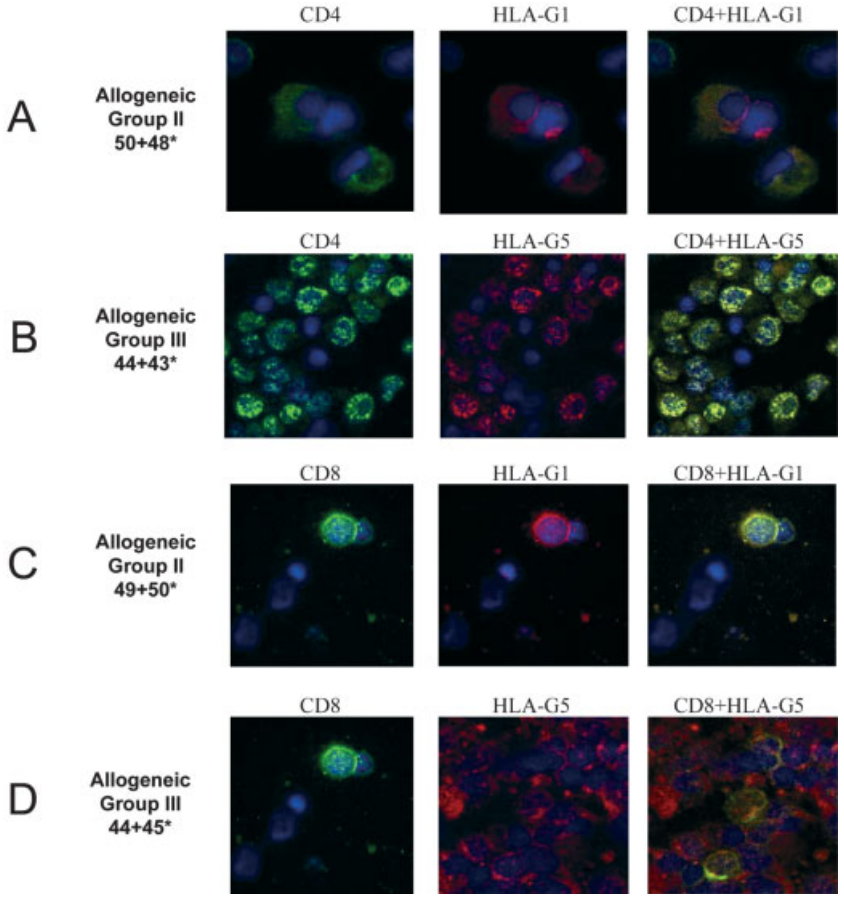

Fig. 4. Both $CD4^+$ and $CD8^+$ alloreactive T cells can express HLA-G1 and/or HLA-G5 molecules after 7 days of MLR. Confocal analysis images were obtained after double staining with the MEM-G/9 or the 5A6G7 mAb, conjugated to TR, in combination with FITC-conjugated anti-CD4 or with FITC-conjugated anti-CD8. Dapi was added for nuclear staining (blue). The firt column shows the staining with either anti-CD4 or anti-CD8 (green). The second column shows cells stained either by MEM-G/9 or 5A6G7 mAb (red). The third column is an overlay of the first and second columns and shows co-localization (yellow). The identification of the HLA-G isoform namely HLA-G1 or HLA-G5 expressed by the corresponding allogeneic combination was deduced from both transcription and protein expression studies (see Table 1). Four representative HLA-G-positive allogeneic combinations from groups II and III (A, B, C and D) are shown.

D. It has to be noted that not all $CD4^+$ or $CD8^+$ T cells were positive for HLA-G1 or HLA-G5. Next, we investigated whether HLA-G-positive T cells expressed a memory phenotype. As shown in Fig. 5 for one representative allogeneic combination, both memory ($CD45\ RO^+$, Fig. 5A) and naive ($CD45\text{-}RA^+$, Fig. 5B) T cell subsets expressed HLA-G.

Since soluble HLA-G5 was also detected *de novo* in $CD8^+$ and/or $CD4^+$ responder T cells, we measured by enzyme-linked immunosorbent assay (ELISA) the level of soluble HLA-G proteins in supernatants from groups III and IV allogeneic combinations on day 7 of the MLR. Soluble HLA-G was consistently detected in all these allogeneic combinations (mean =15±3 ng/ml) with a range of 7 to 24 ng/ml.

Subsequently, we focused on the allogeneic combinations in which the full-length HLA-G1 transcript and protein expression were found (n=6: group II and IV), in order to determine whether HLA-G1 was present at the cell surface of $CD4^+$ and/or $CD8^+$ responder T cell populations. HLA-G1 cell-surface expression could not be detected on $CD8^+$ T cells, but HLA-G1-positive $CD4^+$ T cells were found as shown in Fig. 6 for one allogeneic combination (61% of the total $CD4^+$ T cells). However, cell-surface expression of HLA-G1 by T cells remained a rare event.

Of note, during primary MLR, HLA-G1 and/or HLA-G5 could be detected after day 3 in T lymphocytes and MLR supernatants. Restimulation with allogeneic cells in secondary MLR enhanced HLA-G expression by T lymphocytes, as illustrated by (i) an average of a threefold increase of soluble HLA-G in MLR supernatants and (ii) an average of a twofold increase of HLA-G-positive cells,

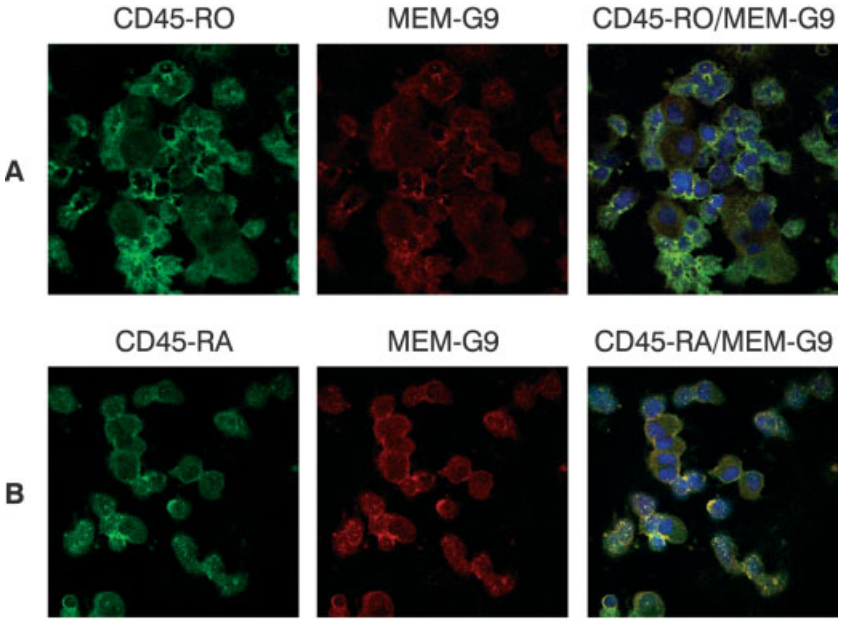

Fig. 5. Both CD45-RA and CD45-RO T cells can express HLA-G1 and/or HLA-G5 molecules after 7 days of MLR. Confocal analysis images were obtained after double staining with the MEM-G/9 mAb in combination with FITC-conjugated anti-CD45-RO (A) or anti-CD45-RA (B), as described in Fig. 4. One out of ten HLA-G-positive allogeneic combinations is shown as a representative example.

at day 18 of secondary MLR compared to day 7 of the primary one.

2.5 *In vivo* identification of CD4+ and CD8+ T cell subsets expressing HLA-G in liver-kidney bi-transplanted patients

To investigate the biological relevance of our *in vitro* results, we analyzed by flow cytometry HLA-G expression on CD4+ and CD8+ T cells from PBMC of two kidney-liver bi-transplanted patients. As shown in Fig. 7, both CD8+ and CD4+ T cell subsets from both patients expressed cell-surface HLA-G1. For these patients, HLA-G1-positive CD4+ T cells represented 12 and 9% of total CD4+ T cells and HLA-G1-positive CD8+ T cells represented 20 and 9.5% of total CD8+ T cells. None of these patients suffered from allograft rejection at the time of the experiment. Finally, a high level of soluble HLA-G was detected by ELISA in the sera of both patients and was titrated at 83 and 47 ng/ml, respectively, whereas in healthy donors, HLA-G5 was consistently titrated at 18±9 ng/ml (n=30).

3 Discussion

T cells play a central role in both allograft rejection and tolerance [25]. Indeed, allograft rejection is mainly mediated by recipient T cells either upon direct stimulation by donor antigen-presenting cells (APC), or upon indirect stimulation by recipient APC which present processed peptides from allo-HLA molecules [26]. Furthermore, allograft tolerance can be transferred between recipients by T cells through active suppression mediated by regulatory T cells. Peripheral tolerance is maintained either by passive mechanisms such as deletion or functional non-responsiveness (anergy) of alloreactive T cells, or by active suppression mediated by regulatory T cells [27, 28]. Recently, evidence has been provided that all these mechanisms are crucial to prevent rejection of transplanted allogeneic tissues [25].

In the present study, we described the expression of the tolerogeneic HLA-G molecule by responder T cells in primary MLR. We focused our study on the two main HLA-G isoforms, namely the membrane-bound HLA-G1 and the soluble HLA-G5 proteins. In physiological conditions, both isoforms are expressed at the fetal-maternal interface in trophoblast where they act as specific immunosuppressors and prevent the rejection of the semi-allogeneic fetus by the maternal immune system [9–11]. In the context of human allotransplantation, these HLA-G molecules have been detected in graft biopsies and sera from patients who had undergone heart or kidney/liver transplantation [4–7]. In these cases, HLA-G was associated with allograft acceptance, suggesting that titration of seric soluble HLA-G levels may be useful for

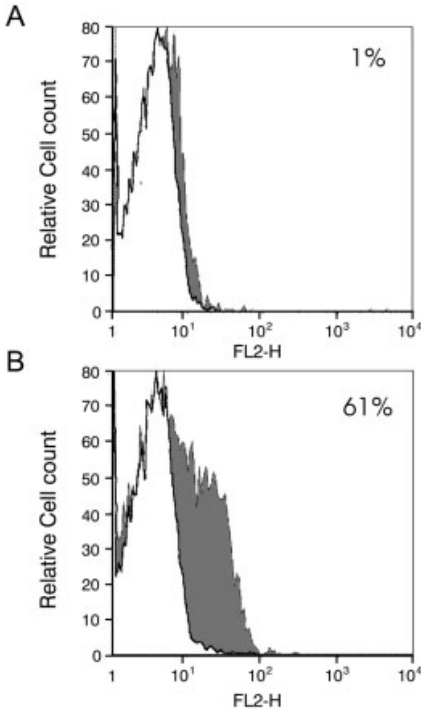

Fig. 6. CD4$^+$ alloreactive T cells can express HLA-G1 cell surface molecules. One allogeneic (B) and its corresponding autologous combinations (A) were labeled by the following Ab: MEM-G/9 stained with a PE-conjugated $(Fab')_2$ goat anti-mouse IgG (H+L) antibody, followed by FITC-conjugated anti-CD4 and PI staining. FITC-conjugated IgG1 and PE-conjugated $(Fab')_2$ goat anti-mouse IgG (H+L) antibody were used as controls (dotted line). A gate on CD4$^+$ T cells was performed enabling the visualization of HLA-G1 expression on day 7 of MLR. The percentages in A and B indicate the HLA-G-positive cells among the CD4$^+$ cell population in the autologous and allogeneic combinations, respectively.

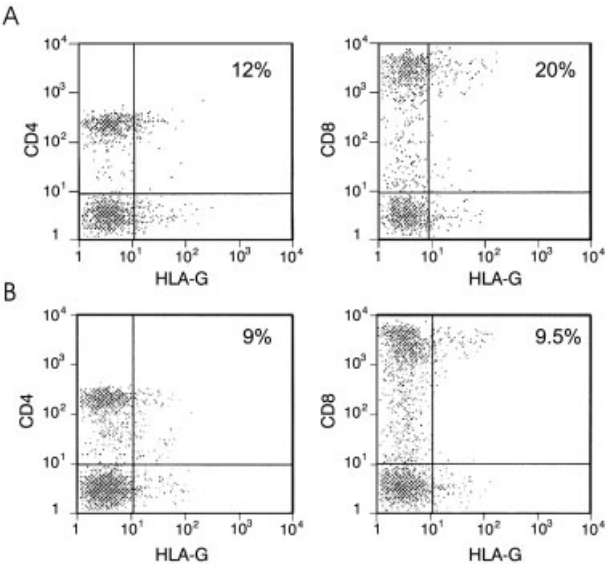

Fig. 7. Both CD4$^+$ and CD8$^+$ T cell subsets from two kidney/liver bi-transplanted patients express HLA-G1 cell surface molecules. PBMC from two distinct kidney-liver bi-transplanted patients (A) and (B) were labeled by the following mAb: MEM-G/13 stained by PE-conjugated $(Fab')_2$ goat anti-mouse IgG (H+L) antibody, followed by anti-CD4 conjugated to ECD and anti-CD8 conjugated to PC5. PI staining was used to gate out dead cells. Numbers on the right of each quadrant correspond to the percentage of double-labeled CD4$^+$ or CD8$^+$ HLA-G$^+$ T cells among the CD4$^+$ or CD8$^+$ T cell population.

monitoring the clinical course of allotransplantation [8]. By inhibiting both NK cell- and CD8$^+$ T cell-mediated cytolysis, and by suppressing CD4$^+$ T cell alloproliferative response, HLA-G molecules affect the main effector cells involved in graft rejection [12]. In particular, soluble HLA-G induces apoptosis of CD8$^+$ T and NK cells and may participate in allograft tolerance through peripheral deletion of alloreactive T cells after allotransplantation *in vivo* [21, 22]. Previous analysis of the functional role of both HLA-G1 and HLA-G5 in mixed lymphocyte reaction showed that both suppress the alloproliferative response of T cells [23, 24, 29]. Such inhibition could be reversed by the use of anti-HLA-G mAb [24].

In the present work, we first demonstrated by immunocytochemistry that alloreactive cells from particular MLR combinations expressed membrane-bound HLA-G1 and/or soluble HLA-G5 after 7 days of allostimulation, whereas no HLA-G expression was detected in the corresponding autologous combinations. HLA-G expression was detected in 75% of allogeneic combinations, with a predominance of the soluble HLA-G5 isoform (56% of cases). Given that the 5A6G7 mAb recognizes both HLA-G5 and -G6 soluble isoforms, we cannot exclude that, in addition to HLA-G5, HLA-G6 may also be expressed in our experiments. It is known that the production of HLA-G depends on various factors such as cytokines (IL-10, IFN-α, β, γ) [30, 31], stress [32], or chemical agents (corticoids, demethylating agents) [33, 34]. However, it also has to be noted that the induction of HLA-G1 and/or HLA-G5 expression by responder T cells from a given donor is influenced by the allogeneic stimulator cell population. For instance, responder T cells from donor 49 expressed HLA-G1 when stimulated by donor 50 but not when stimulated by donor 48. We can also note that it is always the same pattern of alternative HLA-G transcripts that is switched on in a given responder T cell population. For instance, responder T cells from donors 8, 36, 37, or 44, when transcribing *HLA-G* after allostimulation, give always rise to HLA-G1 and/or HLA-G5 mRNA.

Confocal microscopy analysis demonstrated that both CD4+ and CD8+ T cell subsets from the responder cell populations can express HLA-G1 and HLA-G5. Notably, both naive and memory T lymphocytes were able to express HLA-G, suggesting that such expression may be influenced by microenvironmental factors such as cytokines provided upon the allogeneic response, and not only by the allosensitization itself. However, by flow cytometry, and despite extensive analysis, HLA-G1 cell-surface expression on the responder CD4+ T cell population remained a rare event *in vitro*. A possible explanation is that membrane-bound HLA-G1 molecules have been released by shedding. The use of an HLA-G-specific ELISA allowed the detection of soluble HLA-G in HLA-G-positive allogeneic MLR combinations supernatants, even though the levels of soluble HLA-G varied between combinations.

These *in vitro* results were confirmed *in vivo* by showing that circulating CD4+ and CD8+ T cells from two kidney-liver bi-transplanted patients expressed HLA-G1 at their surface. Moreover, these patients had high seric levels of soluble HLA-G. We chose these patients on the basis of their kidney and liver allografts acceptance. The aim of our present *in vivo* investigation was not to carry out a statistical analysis of HLA-G expression in transplanted patients, but rather to determine whether HLA-G positive T cells after *in vitro* allogeneic stimulation had any biological relevance. Whether the presence of such T cells is frequent in patients who have undergone allotransplantation remains to be defined.

A minor subpopulation of regulatory CD4+CD25+ T cells (~10%), produced by the normal thymus as a functionally distinct subpopulation of T cells has the ability to protect the integrity of tissues and organs *in vivo,* since they are naturally anergic and suppressive [35]. They play critical roles, not only in promoting transplantation tolerance, but also in tumor immunity and autoimmunity [36]. In our study, whether the allogeneic CD4+ T cells expressing HLA-G molecules are CD4+ CD25+ regulatory T cells remains an open question. Similarly, it is unknown whether the HLA-G-positive CD8+ T cells are suppressor T cells [37]. Of note, T cells could express soluble HLA-G upon mitogenic and antigenic stimuli such as anti-CD3 mAb and tetanus toxoid (data not shown), and not selectively upon alloantigenic stimulation. These data strongly suggest that the immune/biological role of HLA-G, as a negative regulatory molecule, may be broader than previously assumed.

In conclusion, our description of circulating HLA-G-expressing T cells constitutes the first *in vivo* identification of a cellular source of HLA-G in transplanted patients. This observation is of particular interest since T cells have previously been described in the literature as potential cellular sources of HLA-G *in vivo* under three other pathological situations, *i.e.* inflammatory skin lesions [38], cancer [39], and HIV infection [40]. Based on the well-described tolerogeneic properties of HLA-G, the present results lead us to hypothesize that HLA-G expression by T cells after allostimulation may constitute a way by which T cells regulate immune responses, thereby preventing allograft rejection.

4 Materials and methods

4.1 Cells and patients

The following transfectants from the M8 melanoma cell line: M8-pcDNA (mock transfected), M8-HLA-G1, M8-HLA-G5, and M8-HLA-G6 (transfected with a vector containing the cDNA of HLA-G1, HLA-G5, or HLA-G6, respectively) were obtained and cultured as described [41]. The HLA-G-positive human choriocarcinoma, JEG-3, was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured as recommended. Peripheral blood mononuclear cells (PBMC) and serum were isolated from two kidney-liver bi-transplanted patients at 1 or 5 years post-transplantation, respectively. Neither of these patients suffered from allograft rejection.

4.2 Monoclonal antibodies

The following antibodies were used: 4H84, IgG1 anti-HLA-G α1-domain recognizing all HLA-G isoforms, kindly provided by S. Fisher and M. McMaster (University of California, San Francisco); 5A6G7, IgG1 anti-HLA-G5 and -G6 produced by our laboratory and described in Sect. 2; MEM-G/9 and MEM-G/13, IgG1 reacting exclusively with native HLA-G1 and -G5 [41] (Exbio, Prague, Czech Republic) and kindly provided by V. Horejsi (Academy of Sciences of the Czech Republic, Videnska, Czech Republic); anti-human CD4 or anti-human CD8 directly conjugated to either FITC, or phycoerythrin-Texas Red (ECD), or phycoerythrin-cyanin 5.1 (PC5) (Immunotech, Marseille, France or Becton Dickinson, Le Pont-de-Claix, France); anti-human CD45-RA or CD45-RO directly conjugated to FITC (Immunotech); IgG1 isotype-matched control antibody alone or conjugated to either FITC, ECD, or PC5 (Immunotech); $(Fab')_2$ goat anti-mouse IgG (H+L) secondary antibody conjugated to phycoerythrin (PE) (Immunotech); IgG1 anti-tubulin (Sigma).

4.3 Mixed lymphocyte reactions and T cell proliferation assay

PBMC were isolated from healthy donors (Hôpital Saint-Louis, Paris, France) as follows: mononuclear cells were separated by Ficoll-Histopaque gradient 1077 (Sigma). In total, 10^5 allogeneic responder PBMC from healthy donors

were stimulated with 10^5 irradiated PBMC (25 Gy) and T cell proliferation was measured as described [23]. Concomitantly, MLR were performed in flasks, and both cells and supernatants were collected after 7 days. Restimulation was carried out under similar conditions on day 10 and the resulting secondary response was analyzed on day 18.

4.4 Isolation of RNA, reverse transcription, PCR amplification and Southern blot

Total RNA was isolated from up to 5×10^6 PBMC before their use and after 7 days of MLR with the RNeasy® Mini total ARN kit (Qiagen S.A., Courtaboeuf, France) according to the manufacturer's recommendations. cDNA were prepared from 1–5 μg of total RNA using Ready To Go ™ kit and oligo $(dT)_{12-18}$, according to the manufacturer's recommendations. For HLA-G1 and HLA-G5 transcript detection, PCR amplification and hybridization of the PCR products were performed as described [34]. The JEG-3 cell line was used as an *HLA-G* transcription control.

4.5 Immunocytochemical staining

Cells were harvested from the culture flasks after 7 days of primary MLR and after 18 days of secondary MLR and cytospins were made using superfrost/plus® slides (Merck, Strasbourg, France), and a Cytospin 3® (Shandon). For staining, cytospins were incubated for 1 h with mAb 4H84, 5A6G7 or IgG1 isotype-matched control and treated as previously described. Briefly, endogenous peroxidase was blocked with 3% H_2O_2 for 5 min and FcR were blocked by human AB serum.

4.6 Immunofluorescence double staining and confocal microscopy analysis

Cytospins were performed as described above. Fc receptors were blocked by 50% human AB serum in PBS. The MEM-G/9, 5A6G7, or control IgG1 antibody was incubated for 30 min, followed by a TR-conjugated $(Fab')_2$ goat anti-mouse IgG secondary Ab. Then, FITC-conjugated anti-CD4, anti-CD8, anti-CD45-RA, or anti-CD45RO mAb was applied. Slides were mounted in Dapi-Vectashield to stain nuclei and analyzed using a fluorescence microscope (Bio-Rad, MRC1024). Cell permeabilization was controlled by using anti-tubulin Ab.

4.7 Flow cytometry analysis

PBMC (0.5×10^6) after 7 days of MLR, or PBMC obtained from two kidney-liver bi-transplanted patients were analyzed for HLA-G1 cell-surface expression as described [41]. Cells were analyzed on a flow cytometer EPICS XL (FACS Vantage Expo-32; Becton Dickinson, Le-Pont-de-Claix, France) and gated to exclude propidium iodide (PI)-positive dead cells. The mAb used were: MEM-G/9 or MEM-G/13 followed by a PE-conjugated $(Fab)_2$ goat anti-mouse IgG (H+L), followed by either FITC- or ECD-conjugated anti-CD4, or FITC- or ECD-conjugated anti-CD8, and finally with PI. IgG1 isotype-matched control antibody conjugated to FITC, ECD, PC5 or PE-$(Fab)_2$ goat anti-mouse IgG (H+L) was used in the corresponding experiments.

4.8 ELISA

Soluble HLA-G concentrations were measured in MLR supernatants at day 7 of the primary MLR and at day 18 of the secondary MLR or in sera from transplanted patients. Briefly, microtiters plates (Corning Costar, France) were coated in PBS, pH 7.4 with the MEM-G/9 mAb (10 μg/ml). Plates were saturated with 250 μl of PBS containing 2% BSA for 30 min at room temperature. MLR supernatants or serum were added to each well (100 μl) in triplicate and incubated for 1 h. Anti-β2m-HRP (Dako, France) was then added and plates were incubated for 1 h. The chromogenic substrate (OPD, orthophenylenediamine dihydrochloride, Dako, France) was added for 30 min in the dark. Finally, the reaction was stopped by adding H_2SO_4 (1 N), and optical densities were measured at 490 nm. Standard curves were performed using serial dilutions of purified soluble HLA-G from HLA-G5-transfected cells. Detection limit of the ELISA was 5 ng/ml.

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