Identification of a novel HLA-G$^+$ regulatory population in blood: expansion after allogeneic transplantation and *de novo* HLA-G expression at graft-*versus*-host disease sites

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ABSTRACT

Background
The human leukocyte antigen-G (HLA-G) has been considered to be an important tolerogeneic molecule playing an essential role in maternal-fetal tolerance, which constitutes the perfect example of successful physiological immunotolerance of semi-allografts. In this context, we investigated the putative role of this molecule in the allogeneic hematopoietic cell transplantation setting.

Design and Methods
The percentage of HLA-G$^+$ cells in peripheral blood of healthy donors and allo-transplanted patients was evaluated by flow cytometry. Their immunoregulatory and tolerogeneic properties were investigated in *in vitro* immunostimulatory and immunosuppression assays. Immunohistochemical analysis for HLA-G expression was performed in skin biopsies from allo-transplanted patients and correlated with the occurrence of graft-*versus*-host disease.

Results
We identified a CD14$^+$HLA-G^{pos} population with an HLA-DR^{low} phenotype and decreased *in vitro* immunostimulatory capacity circulating in peripheral blood of healthy individuals. Naturally occurring CD14$^+$HLA-G^{pos} cells suppressed T-cell responses and exerted an immunotolerogenic action on T cells by rendering them hyporesponsive and immunosuppressive *in vitro*. After allogeneic hematopoietic cell transplantation, HLA-G^{pos} cells increase in blood. Interestingly, besides an increase in CD14$^+$HLA-G^{pos} cells, there was also a pronounced expansion of CD3$^+$HLA-G^{pos} cells. Of note, CD3$^+$HLA-G^{pos} and CD14$^+$HLA-G^{pos} cells from transplanted patients were suppressive in *in vitro* lymphoproliferation assays. Furthermore, we found an upregulation of HLA-G expression in skin specimens from transplanted patients that correlated with graft-*versus*-host disease. Inflammatory cells infiltrating the dermis of transplanted patients were also HLA-G^{pos}.

Conclusions
We report the presence of naturally occurring HLA-G^{pos} monocytic cells with *in vitro* suppressive properties. HLA-G expressing regulatory blood cells were found in increased numbers after allogeneic transplantation. Epithelial cells in skin affected by graft-*versus*-host disease revealed elevated HLA-G expression.

Key words: HLA-G, GvHD, myeloid suppressor cells.

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Introduction

Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule, initially found to be expressed during embryogenesis playing a role in mediating maternal tolerance of the fetal 'semi-allogeneic' graft.[1,2] Specifically, HLA-G was found to be expressed at the fetal–maternal interface of human placenta where it inhibits maternal natural killer cells protecting the fetus from immune attack and rejection.[3] Contrary to classical HLA class I molecules, HLA-G is not ubiquitously expressed after prenatal life. In healthy adults, HLA-G translation is epigenetically repressed and constitutive HLA-G expression was found only in immune-privileged tissues, such as thymus and cornea.[2] Besides this highly tissue-restricted expression, some authors have observed HLA-G cell surface expression in some peripheral blood (PB) monocytes from healthy individuals[4-7] while Feger *et al.*[8] have recently found that thymus generates a discrete subset of naturally occurring HLA-G^{pos} T cells with regulatory properties. Beyond physiological conditions, ectopic HLA-G expression has been found in tumor cells,[9] virus infected cells,[7] transplanted organs or immune cells infiltrating grafts,[3] and has been suggested to constitute an immune escape mechanism. After solid organ transplantation, HLA-G expression by the transplant organ[10-12] and high soluble HLA-G plasma levels[13,14] have been associated with better graft acceptance. After allogeneic bone marrow transplantation, soluble HLA-G plasma levels have been suggested to correlate with the occurrence of acute graft-*versus*-host disease (GvHD), suggesting an involvement of HLA-G in GvHD prevention.[15]

Unlike the highly polymorphic classic HLA class I molecules, HLA-G is expressed as only seven protein isoforms (HLA-G1 to -G4 membrane-bound and soluble HLA-G5 to -G7) that are generated by alternative splicing.[3] *In vitro* functional studies with membrane-bound HLA-G[16] or soluble HLA-G5[17] expressing cell lines have recognized the HLA-G molecule as an important mediator of immune tolerance, as it may: i) inhibit natural killer and T-cell cytotoxicity; ii) induce apoptosis in activated CD8$^+$ T cells; iii) suppress alloreactive CD4$^+$ T-cell proliferation; iv) generate suppressor T cells; and v) impair antigen-presenting cell function. HLA-G exerts the above immunoregulatory functions by engaging inhibitory receptors (immunoglobulin-like transcript (ILT)-2, ILT-4 and the killer immunoglobulin-like(KIR)2DL4) on immune effector cells without any need for peptide presentation, resulting in a transient block in their functions.[2] Furthermore, it has been shown that HLA-G molecules can be intercellularly transferred from HLA-G$^+$ cells to activated T cells and NK cells by trogocytosis, making them from effector to immunoregulatory cells.[18,19] The immunosuppressive functions of HLA-G are strengthened by *in vivo* studies showing that injection of HLA-G tetramer-coated beads into mice[20] or use of HLA-G/human β2-microglobulin transgenic mice[21] promoted skin allograft survival and that HLA-G expression in tumor cells favored their evasion in xenotumor mouse models.[22]

Allogeneic hematopoietic cell transplantation (allo-HCT) is an established curative treatment for several hematologic malignancies, but its success is hampered by GvHD in which immunocompetent donor cells attack the antigenically foreign tissues of the transplant recipient.[23] GvHD is still a major cause of post-transplant morbidity and mortality, indicating the need to develop novel preventive and therapeutic strategies. On the basis of the concept that the HLA-G molecule plays an essential role in pregnancy (the clearest demonstration of successful physiological immunotolerance of semi-allografts) and that HLA-G can be up- or down-regulated during the course of various pathologies, we aimed to investigate the putative role of this molecule in the allo-HCT transplant setting. We report the presence of HLA-G expressing cells with regulatory properties in human peripheral blood under physiological conditions and we monitored their evolution after allo-HCT. Furthermore, we analyzed skin biopsies after transplantation and we found an ectopic expression of HLA-G in susceptible target cells of GvHD.

Design and Methods

Subjects and sample collection

Peripheral blood mononuclear cells (PBMCs) were obtained from 22 healthy volunteers and 27 unselected patients who underwent allo-HCT at the Bone Marrow Transplantation Unit of Patras University, after obtaining informed consent. PBMCs were isolated from heparinized whole blood by density-gradient centrifugation over Ficoll-Histopaque PLUS (GE Healthcare, Bio-Sciences, Uppsala, Sweden). Transplanted patients were treated as previously described in detail.[24] Acute and chronic GvHD was staged and graded according to consensus criteria. Severity of chronic GvHD was assessed according to functional impairment and response to first-line therapy as mild, moderate or severe, as previously described in detail.[24] Skin punch biopsies were taken either for verification of clinically suspected skin GvHD or as routine follow up. Histological grading of GvHD was performed according to Lerner *et al.*[25] Briefly, grade I showed only vacuolar degeneration of epidermal cells, grade II showed in addition spongiosis and dyskeratotic (apoptotic) keratinocytes, grade III revealed epidermiolysis and cleft formation, and grade IV showed total loss of epidermis. Normal skin specimens from adults were resected at the border of the wound after surgical removal of melanocytic nevus. The study protocol was approved by the institutional ethics committee (n. 297/ 15.03.11).

Statistical analysis

Student's t-test, Mann-Whitney or Fisher's exact test were carried out according to the nature of the data, using GraphPad Prism 5.00 software. Results are described as mean + standard error of the mean (SEM). $P<0.05$ was considered significant.

Information concerning antibodies and flow cytometry, cell isolation, *in vitro* immunostimulatory and immunosuppression assays, T-cell pre-treatment procedure and immunohistochemistry is available in the *Online Supplementary Appendix*.

Results

Naturally occurring HLA-G^{pos} monocytic cells in peripheral blood of healthy individuals

Freshly isolated PBMCs from healthy donors (n=14) were assessed for HLA-G membrane expression by flow cytometry using the monoclonal antibody MEM-G/9. A mean of 3.2% HLA-G^{pos} cells (range 0.4-7.8%) was found. HLA-G^{pos} cells were mainly presented in the CD14$^+$ monocytic compartment. Indeed, 17.8% (+ 4.4) of the CD14$^+$

cells were HLA-G^{pos} as compared to only 1.6% (±0.4) HLA-G^{pos} cells found in the CD3$^+$ subpopulation (Figure 1A). To obtain further proof of this, we isolated the monocytic population either by FACS-sorting (n=12) or by CD3-negative MACS selection (n=7). It was found that, independently of the purification method used, 18.7% (±0.3) of the CD14$^+$ cells were HLA-G^{pos}. On the contrary, when we isolated the HLA-G^{pos} cells by FACS-sorting we found that these were exclusively CD14$^+$.

Further phenotypic analysis of the CD14$^+$HLA-G^{pos} PB cells (n=8) revealed that they were CD33$^+$, CD11b$^+$, ILT-2$^+$, ILT-4$^+$, CD80$^-$, CD86$^{-/+}$, and HLA-DR$^+$ (Figure 1B). We analyzed in more detail the expression level of HLA-DR, an HLA class II molecule known to be crucial for eliciting an immune response. CD14$^+$HLA-G^{pos} monocytes were found to have significantly lower HLA-DR expression than CD14$^+$HLA-G^{neg} cells (n=13, mean SFI 62.4 ± 34.2 *vs.* 189.6 ± 66.3, P<0.0001) (Figure 1C). We also analyzed the immunostimulatory properties of the CD14$^+$HLA-G^{pos} cells in *in vitro* alloproliferation assays. Irradiated PBMCs or their paired FACS-sorted CD14$^+$HLA-G^{pos} or CD14$^+$HLA-G^{neg} fractions were used as stimulators of allogeneic enriched CD3$^+$ lymphocytes (n=5) or whole PBMCs (n=2). In all cases, CD14$^+$HLA-G^{pos} cells induced a significantly reduced lymphoproliferative response when compared to their negative counterparts (CD14$^+$HLA-G^{neg}) or whole PBMCs, and that was true for all ratios of stimulatory/responder cells that were used (Figure 1D). Taken together, a distinct population of CD14$^+$HLA-G^{pos} cells with low HLA-DR^{low} expression and decreased *in vitro* immunostim-

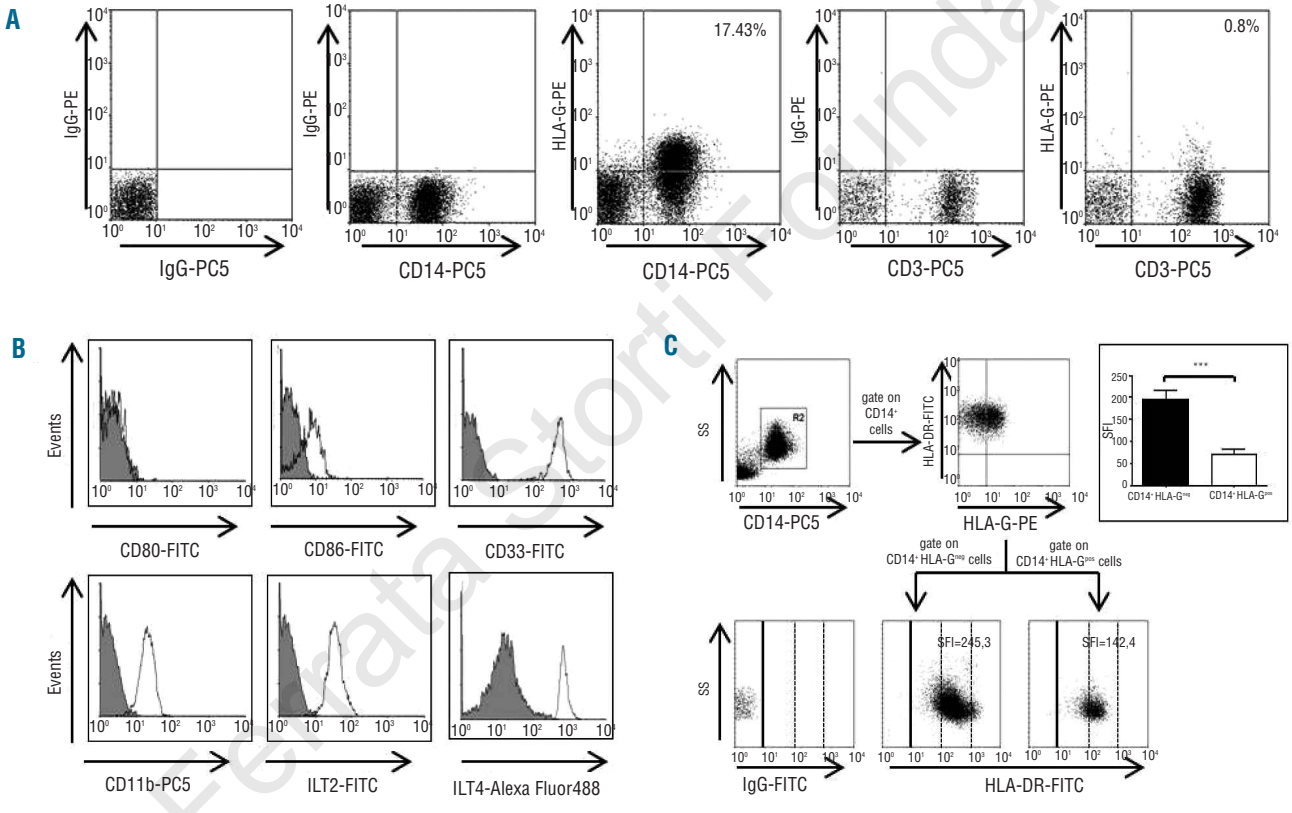

Figure 1. Naturally occurring HLA-G^{pos} cells with reduced immunostimulatory capacity in peripheral blood of healthy individuals. (A) PBMCs from healthy donors were analyzed by flow cytometry for the presence of HLA-Gpos monocytes (CD14$^+$) and lymphocytes (CD3$^+$). Numbers indicate the percentage of double positive cells. A representative example is shown. (B) Phenotype of CD14$^+$HLA-G^{pos} cells. Filled histograms represent isotypic controls. One representative experiment is shown. (C) Expression level of HLA-DR on CD14$^+$HLA-G^{pos} cells as compared to CD14$^+$HLA-G^{neg} cells. The numbers represent the specific fluorescence index (SFI). Raw data from one representative experiment is shown. The graph summarizes the results of 13 independent experiments (mean ± SEM, ***P<0.0005). (D) PBMCs (gray columns) or their paired FACS-sorted CD14$^+$HLA-G^{pos} (white columns) or CD14$^+$HLA-G^{neg} cells (black columns) were used as stimulators (Stim) and allogeneic enriched CD3$^+$ lymphocytes as responders (Resp) in MLCs, in various ratios as indicated. Proliferation was measured at Day 6 by using the CFSE assay and shown as mean ± SEM of 5 experiments. Statistically significant proliferation between CD14$^+$HLA-G^{pos} cultures *versus* CD14$^+$HLA-G^{neg} and unseparated PBMCs is shown by an asterisk, ***P<0.0005.

ulatory capacity could be detected in the peripheral blood of healthy individuals.

Naturally occurring CD14^{+}HLA-G^{pos} cells are immunosuppressive in vitro

Given the known immunoregulatory function of the HLA-G molecule, we investigated the potent *in vitro* suppressive properties of the naturally occurring CD14^{+}HLA-G^{pos} cells. For this purpose, irradiated CD14^{+}HLA-G^{pos} cells or their HLA-G negative counterparts were used as third-party cells in allogeneic MLCs where PBMCs (n=17) or enriched CD3^{+} cells (n=5) were used as responders. Addition of CD14^{+}HLA-G^{neg} cells in MLCs did not significantly affect proliferation as compared to control MLCs (n=5), at any responder to third-party ratio. In contrast, CD14^{+}HLA-G^{pos} cells induced an inhibition of alloproliferation as compared to both control MLCs (n=17) and to MLCs containing the paired CD14^{+}HLA-G^{neg} cells (n=5) (Figure 2A and B). The suppression was dependant on the responder to suppressor ratio and was statistically significant over the ratio of 1:1. To address whether the immunosuppressive effect of CD14^{+}HLA-G^{pos} cells is indeed attributed to the HLA-G molecule, we performed blocking experiments (n=5) by adding the anti-HLA-G antibody 87G to MLCs. This antibody neutralizes HLA-G1 as well as soluble HLA-G5. The suppressive capacity of the CD14^{+}HLA-G^{pos} cells was significantly reversed (up to 100%) by HLA-G neutralization, suggesting a specific role of HLA-G of this regulatory population (Figure 2C and D). Furthermore, masking of the ILT-2 and/ or ILT-4 receptors (n=4) with specific antibodies in cultures containing CD14^{+}HLA-G^{pos} cells restored the proliferation to similar levels to those of control MLC (*Online Supplementary Figure S2*).

When suppression assays were performed in a transwell system (n=5) in which the CD14^{+}HLA-G^{pos} cells were separated from the responder cells with a 0.4 μm membrane, we found similar inhibition of alloproliferation by CD14^{+}HLA-G^{pos} cells as in non-transwell cultures and abrogation of this effect after HLA-G neutralization with 87G blocking antibody (*Online Supplementary Figure S3A-C*). Serial measurements of soluble forms of HLA-G (sHLA-G) in supernatants from transwell experiments revealed significantly increased levels in Day 2 and Day 4 cultures including third-party CD14^{+}HLA-G^{pos} cells as compared to control MLCs without third-party cells. At Day 6, sHLA-G levels were not increased (*Online Supplementary Figure S3D*).

Naturally occurring CD14^{+}HLA-G^{pos} cells act immunotolerogenic on T cells

HLA-G-transfected antigen-presenting cell lines have been shown to exert their regulatory functions either by inducing T-cell hyporesponsiveness or by promoting the differentiation of T cells into suppressor CD3^{+}CD4^{low} and CD3^{+}CD8^{low} cells. We wondered whether these naturally occurring CD14^{+}HLA-G^{pos} cells mediate their suppressive effects through the same mechanisms. For this purpose, enriched T cells were pre-treated with allogeneic CD14^{+}HLA-G^{pos} or CD14^{+}HLA-G^{neg} cells. CD3^{+} cells were then removed by FACS-sorting and used in MLCs either as responder cells (in order to analyze their ability to respond to subsequent allogeneic stimulus) or as irradiated third-party cells (to analyze whether they became suppressive). In the first case, CD3^{+} cells pre-treated for four days with CD14^{+}HLA-G^{pos} cells revealed a decrease in alloproliferation in MLCs as compared to untreated-CD3^{+} cells (n=4, P=0.002) and to CD14^{+}HLA-G^{neg}-pre-treated CD3^{+} cells (n=4, P=0.029) (Figure 3A). In the second case, when 4-day CD14^{+}HLA-G^{pos}-pre-treated-CD3^{+} cells were added as third-party cells in MLCs, they significantly suppressed the alloproliferative response by an average of 39% (range 32.8-51%) as compared to: a) control MLCs without third-party cells (n=4, P=0.003); b) untreated-CD3^{+} third-party cells (n=4, P=0.03); or c) CD14^{+}HLA-G^{neg}-pre-treated-CD3^{+} cells (n=4, P=0.02) (Figure 3B). Though less pronounced, a statistically significant induction of hyporesponsiveness and suppressive function in CD3^{+} cells was also seen after 18 h pre-treatement with CD14^{+}HLA-G^{pos} (n=3, *data not shown*).

Having shown that pre-treatment of CD3^{+} cells with CD14^{+}HLA-G^{pos} cells renders them immunosuppressive, we then carried out phenotypical analysis; 4-day CD14^{+}HLA-G^{pos}-pre-treated-CD3^{+} cells were Foxp3 negative but revealed reduced surface expression of CD4 and CD8. Indeed, the mean CD4 and CD8 SFI of CD14^{+}HLA-G^{pos}-pre-treated-CD3^{+} cells was 1.8-fold and 1.9-fold decreased, respectively, as compared to untreated-CD3^{+} cells (mean CD4 SFI of CD14^{+}HLA-G^{pos}-primed-T cells 79.0 ± 6.9 *vs.* 139.1 ± 2.2 for unprimed, P=0.005, mean CD8 SFI of CD14^{+}HLA-G^{pos} primed-T cells 76.75 ± 15.3 *vs.* 142±34.9 for unprimed, P=0.04), (Figure 3C). Taken together, naturally occurring CD14^{+}HLA-G^{pos} cells exert an immunotolerogenic action on CD3^{+} cells by rendering them hyporesponsive and differentiating them to CD3^{+}CD4^{low} and CD3^{+}CD8^{low} suppressor cells.

Expansion of HLA-G^{pos} cells with immunosuppressive properties in peripheral blood of allo-HCT patients

We asked whether there was a potent involvement and clinical significance of HLA-G expression in the allogeneic hematopoietic cell transplantation (allo-HCT) setting. Therefore, we analyzed the numbers of HLA-G^{pos} cells in the peripheral blood of allo-HCT patients (n=27). The total percentage of HLA-G^{pos} cells in peripheral blood post-transplantation was significantly increased (8.1±0.8), as compared to healthy donors (mean fold increase 2.5, P<0.0001) or to respective percentages before transplantation (mean fold increase 2.8, P=0.01) (Figure 4A, B and D). Interestingly, this increase in HLA-G^{pos} cells post-transplantation was not only due to a higher number of CD14^{+}HLA-G^{pos} cells (39±3.4, P=0.0005), but also to an expansion of HLA-G^{pos} CD3^{+} lymphocytes (8.7±0.8) as compared to both healthy subjects (1.6±0.4, P<0.0001) or pre-transplantation values (1.1±0.2, P=0.005) (Figure 4A and B). Similar results were found when the absolute numbers of HLA-G^{+} cells were determined (*Online Supplementary Table S1*). Further characterization of these CD3^{+}HLA-G^{pos} cells revealed that they were restricted to the CD8^{+} cell subpopulation (Figure 4A). In allogeneic MLC assays, FACS-sorted CD3^{+}HLA-G^{pos} (n=3) and CD14^{+}HLA-G^{pos} cells (n=3) from transplanted patients were able to suppress T-cell responses (P=0.002 and P=0.0005, respectively). HLA-G^{pos} cells, both CD3^{+} and CD14^{+}, were detected early after transplantation (d<100) and continued to be high at later time periods (100<d<365, d>365) (Figure 4C). By serially sampling 7 patients at different time points after transplantation, we confirmed that the numbers of HLA-Gpos cells remained increased over time (Figure 4D). No correlation was

observed between the percentages or absolute numbers of CD14^{+}HLA-G^{pos} or CD3^{+}HLA-G^{pos} cells in patients with or without active GvHD or history of GvHD and in patients receiving or not receiving immunosuppressive drugs.

Upregulation of HLA-G expression in the skin of transplanted patients

To investigate whether HLA-G expression is up-regulated in tissues which are targets of GvHD, we performed HLA-G immunohistochemistry in skin biopsies.

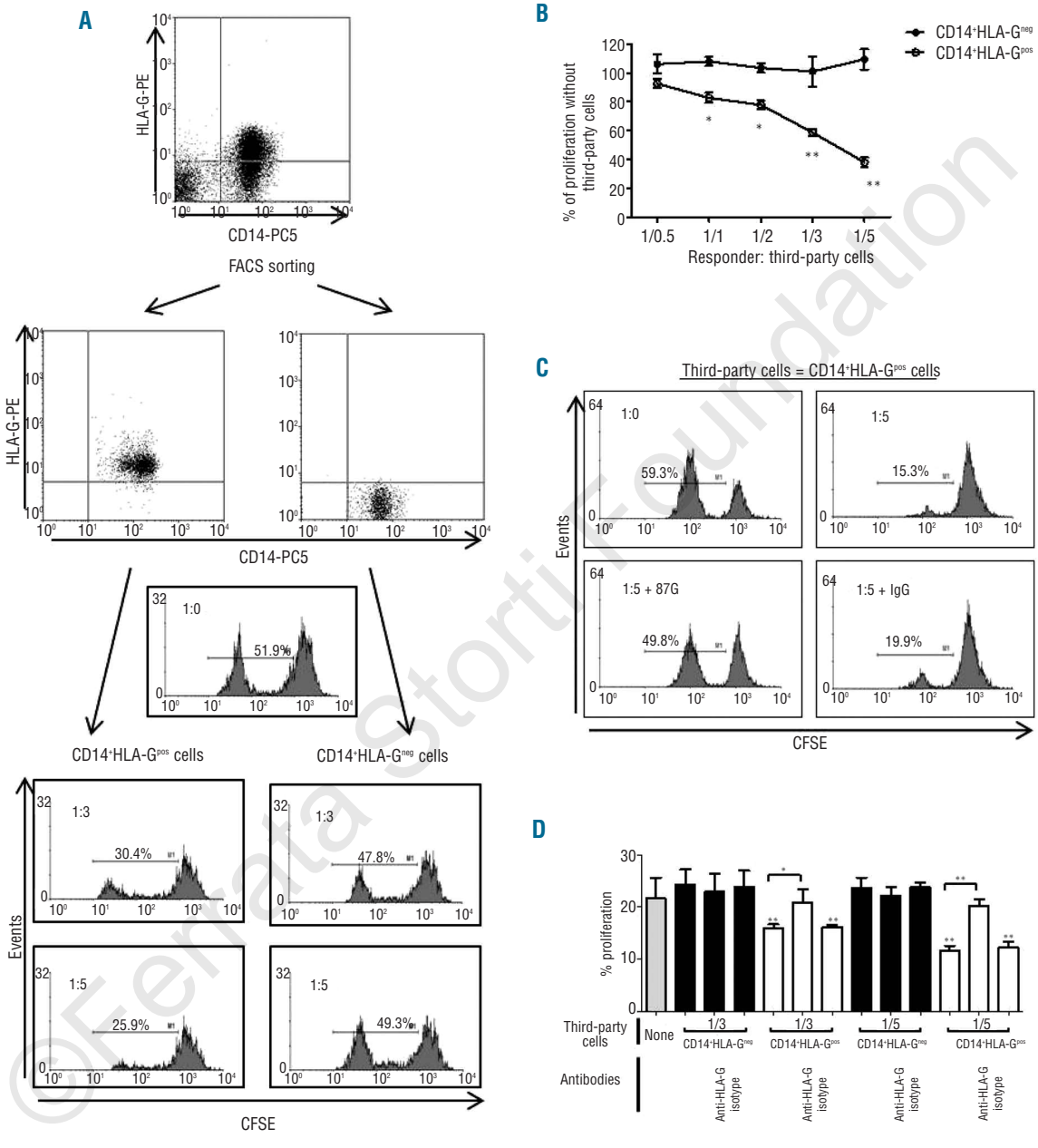

Figure 2. Naturally occurring CD14^{+}HLA-G^{pos} cells have immunosuppressive functions which are HLA-G specific. Figures show suppression assays, in which CD14^{+}HLA-G^{pos} or paired CD14^{+}HLA-G^{neg} cells were added as third-party cells in MLCs conducted between irradiated PBMCs (stimulators) and CFSE-labeled PBMCs or enriched T cells (responders). (A) A representative experiment is shown. From top to bottom, PBMCs were stained for CD14 and HLA-G and then the CD14^{+}HLA-G^{pos} (left panels) and CD14^{+}HLA-G^{neg} (right panels) fractions were isolated by FACS sorting and used as third-party cells in MLCs in increasing ratio of responder: third-party cells, as indicated in the upper corner of the CFSE histograms. MLC without third-party cells is shown as 1:0. Cell proliferation was quantified at Day 6 by CFSE flow cytometric analysis. The numbers above the horizontal line represent the percentage of proliferating responder cells. (B) The graph summarizes the results of 5 independent experiments. Results are shown as mean ± SEM of proliferation as compared to MLC without third-party cells. Asterisks indicate statistically significant inhibition of proliferation as compared to control MLC or to MLC containing CD14^{+}HLA-G^{neg} cells *P<0.05, **P<0.005 (C) and (D) show results of blocking experiments, in which neutralizing HLA-G antibody (87G) or isotype control was added on Days 0 and 3 in cultures containing third-party CD14^{+}HLA-G^{pos}. In (C), a representative example of anti-HLA-G blocking experiment at a responder : third-party cell ratio of 1:5 is shown. (D) Results of 5 independent blocking experiments performed at different responder : third-party ratios. Statistically significant proliferation between CD14^{+}HLA-G^{pos} and corresponding CD14^{+}HLA-G^{neg} containing cultures is given by asterisk direct above the column and between CD14^{+}HLA-G^{pos} containing cultures without *versus* with neutralizing or isotype antibody by asterisk above the horizontal lines (*P<0.05, **P<0.005)

Interestingly, by using the MEM-G/2 antibody, we found a weak HLA-G expression in the basal layer of the epidermis in 3 out of 4 healthy skin specimens. In transplanted patients, HLA-G was found to be expressed not only in the basal layer but also in the intermediate and external layers of the epidermis in 6 out of 10 skin specimens, as well in the eccrine sweat glands of the dermis in 5 out of 6 specimens (*Online Supplementary Figure S4A*). Skin biopsies revealing ectopic HLA-G expression in the intermediate and external layer were considered to be positive (HLA-G^{pos} patients). A positive correlation was found between HLA-G^{pos} patients and histological signs of GvHD (no *vs*. any grade, *P*=0.033) (*Online Supplementary Table S2*) and history of GvHD (no *vs*. history of acute or chronic, any grade, *P*=0.033). In addition, in cases in which inflammatory cells (macrophages and lymphocytes) were infiltrating the dermis, they stained strongly positive for HLA-G, except one patient (P10 in *Online Supplementary Table S2*) with lethal 'acute-like' chronic skin GvHD, in whom no HLA-G expression was found either in the inflammatory cells or in the epidermal cells.

We confirmed these results on transplanted patients by using another anti-HLA-G antibody. By using the 4H84 anti-HLA-G antibody, no HLA-G expression was detected in healthy skin specimens (subjects H1-3 in *Online Supplementary Table S2*) and in specimens from the 3 HLA-G (MEM-G/2)- negative patients (patients 1-3 in *Online Supplementary Table S2)*, whereas transplanted patients

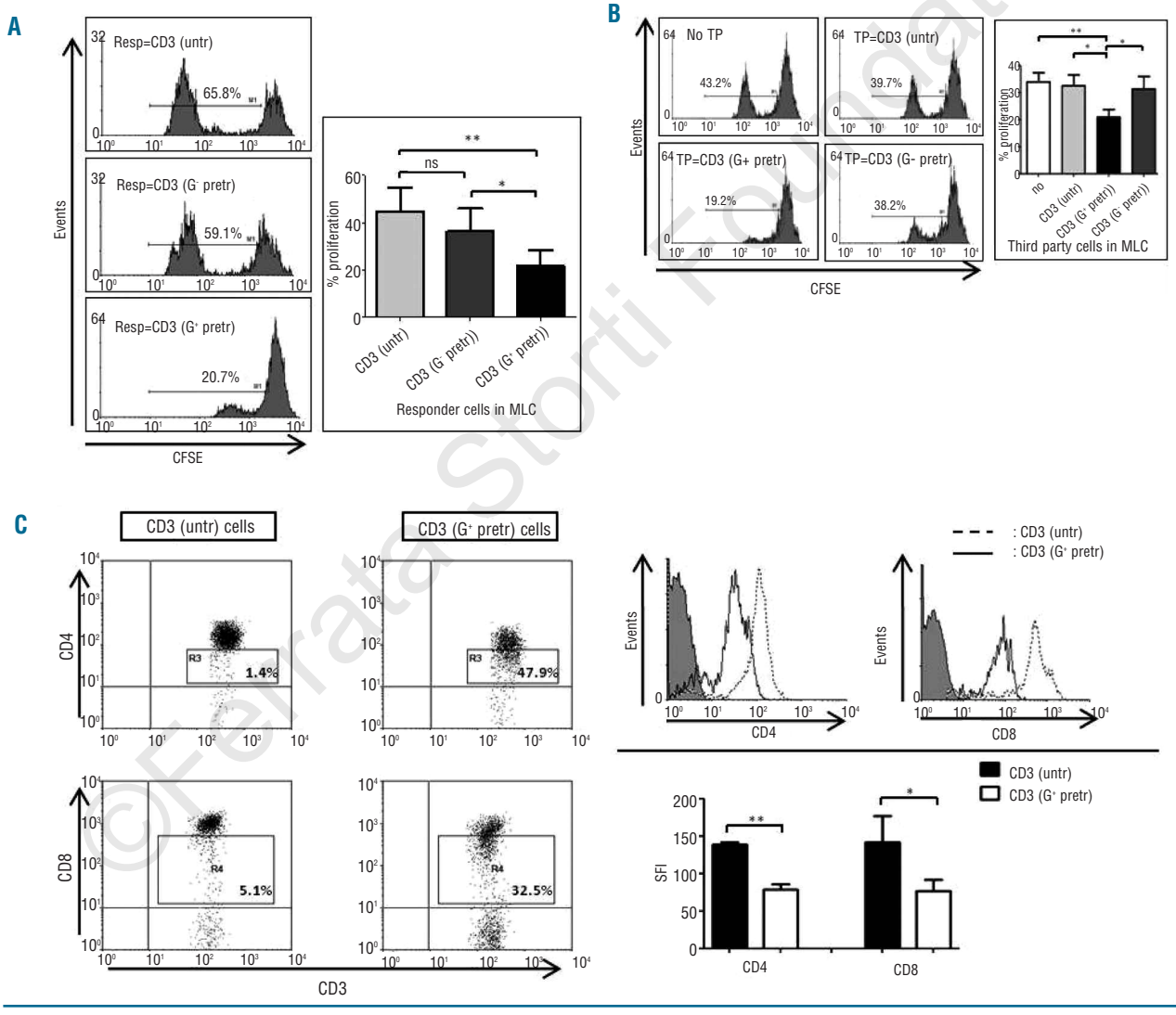

Figure 3. Tolerogenic effects of naturally occurring CD14^{+}HLA-G^{pos} cells. Enriched T lymphocytes from healthy donors were pre-treated for four days with CD14^{+}HLA-G^{pos} [CD3 (G^{+} pretr)] cells. After this pre-treatment period, CD3^{+} T cells were isolated by FACS sorting and used in secondary MLCs either as (A) responder cells (resp) at a ratio 1:1 responders: stimulators or (B) as third-party cells (TP) at a ratio 1:1 responders: third-party cells. Untreated CD3^{+} lymphocytes [CD3 (untr)] or CD3^{+} cells pre-treated with CD14^{+}HLA-G^{neg} [CD3(G- pretr)] cells were used as controls. The numbers above the horizontal line represent the percentage of proliferating responder cells. Panels show representative experiments and graphs show mean ± SEM of 4 and 4 experiments, respectively. (C) CD14^{+}HLA-G^{pos}-pre-treated-CD3^{+} cells show reduced CD4 and CD8 surface expression, as compared to untreated-CD3^{+} cells. Two representative examples are shown either as dot plots gated on CD3^{+} cells (left panel) or histograms (upper right panel). The boxes (R3 and R4 gates) indicate the CD4low and CD8low populations and the shaded histogram the isotype control. Lower right panel: mean CD4 and CD8 SFI ± SEM of 6 independent experiments. *P<0.05 **P<0.005, ns: not significant

who revealed extended HLA-G/(MEM-G/2) expression showed also a weak HLA-G/(4H84) expression in the basal level of epidermis and a stronger expression in inflammatory cells in the dermis (*Online Supplementary Table S2* and *Online Supplementary Figure S5*).

Discussion

In this study, we detected a small subset of HLA-G^{pos} T cells (median 1.6%) and a sizable population of HLA-G^{pos} monocytic cells (median 17.8%) circulating in the peripheral blood of healthy individuals. Feger *et al.*[8] found also a similar low number of naturally occurring HLA-G^{pos} lymphocytes in blood of healthy adults which were able to suppress immune responses *in vitro* and were increased at sites of inflammation in patients with acute neuroinflammatory disorders and idiopathic myositis. The existence of HLA-G^{pos} monocytes in steady state blood has been previously reported by different authors.[4-7] However, other groups could not verify this by using the same antibody (87G).[26] Differences in the purification method used could in part explain the reported discrepancy. In our study, we analyzed HLA-G expression in freshly isolated cells without any pre-cultivation period. Furthermore, when we analyzed the expression of HLA-G in monocytes that were isolated either by FACS sorting or magnetic beads, we found similar results independently of the purification method used. By using the same antibody as we did (MEM-G/9), Lozano *et al.*[7] demonstrated an average of 14% CD14+HLA-Gpos cells in healthy donors, similar to our results.

HLA-G has been considered to be a key mediator in immune tolerance. Experiments with HLA-G transfected antigen presenting cells revealed that HLA-G inhibits the cytolytic function of both NK and cytotoxic T cells, the alloproliferative response of CD4$^+$ cells and the maturation and function of dendritic cells.[2,3] Though whether CD14$^+$HLA-G^{pos} monocytes presented under physiological

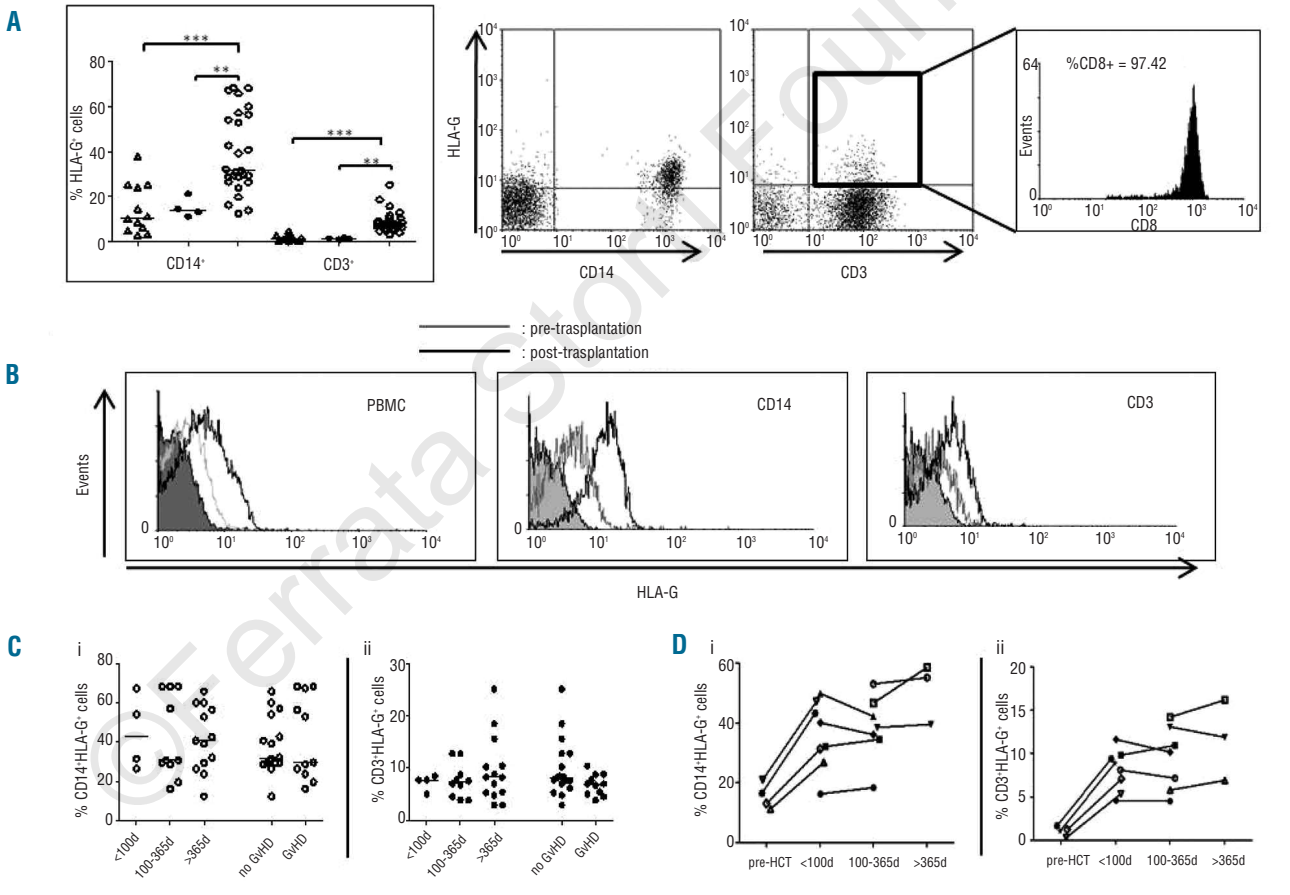

Figure 4. CD14$^+$HLA-G^{pos} and CD3$^+$HLA-G^{pos} cells with immunosuppressive properties in peripheral blood of allo-transplanted patients. (**A**) The graph represents the percentage of peripheral blood HLA-G^{pos} monocytes (CD14$^+$) and HLA-Gpos lymphocytes (CD3$^+$) from healthy subjects (triangles) or from different patients pre-transplantation (filled circles) and post-transplantation (open circles). The short solid lines represent median values. ***P*<0.005, ****P*<0.0005. FACS images show a representative example of increased HLA-G^{pos} monocytes and lymphocytes 384 days after transplantation. Gated CD3$^+$HLA-G^{pos} cells were exclusively CD8$^+$ (boxed area). (**B**) A representative example of the expansion of circulating HLA-G^{pos} cells post-transplant is shown. The gray line represents the HLA-G^{pos} cells before transplantation and the black line after transplantation. Shaded histogram is the isotype control. (**C**) CD14$^+$HLA-G^{pos} (i) and CD3$^+$HLA-G^{pos} (ii) cell frequency in blood of transplanted patients, according to the day (d) after transplantation and the history of acute or chronic GvHD. (**D**) Frequency of CD14$^+$HLA-G^{pos} (i) and CD3$^+$HLA-G^{pos} (ii) in patients who were sampled at two different time points. Each line represents different patients and each dot different time points post-transplant. Two patients shown in (**D**), who in contrast to others reveal a slight decrease in HLA-G^{pos} cells over time, were the only ones in this group who were started on imatinib mesylate (tyrosine kinase inhibitor) between the two measurements.

conditions in blood exert similar immunoregulatory functions remains to be clarified. In our study, we found that naturally occurring CD14^{+}HLA-G^{pos} cells from healthy individuals have reduced *in vitro* immunostimulatory function and inhibit T-cell alloproliferation when added as third-party cells in MLCs. The impaired immune stimulatory capacity could be attributed to their low surface expression of the HLA-DR stimulatory molecule. Naturally occurring CD14^{+}HLA-G^{pos} cells may belong to the heterogenous population of myeloid-derived suppressor cells. Similar to the HLA-Gpos population found here, human myeloid-derived suppressor cells are CD33^{+} and CD11b^{+} and reveal decreased expression of HLA-DR.[27] Interestingly, CD14^{+}HLA-DR^{low} monocytes with reduced *in vitro* immunostimulatory capacity and immunosuppressive function have been found in non-malignant conditions such as sepsis, pancreatitis, and liver failure,[28,29] as well as in cancer patients,[30-32] and their numbers have been associated with poor clinical outcome. Whether the immunosuppressive HLA-DR^{low} population found in such conditions expresses HLA-G has yet to be evaluated. The suppressive function of the naturally occurring CD14^{+}HLA-G^{pos} cells could be antagonized by blocking HLA-G, demonstrating that HLA-G expressed by monocytes plays a key role in their immunomodulatory properties. Similar to studies with HLA-G transfected cell lines, transwell experiments indicated that the immunosuppressive effect of naturally occurring CD14^{+}HLA-G^{pos} cells may be also mediated through soluble HLA-G. This soluble HLA-G can be produced either by the alternative splicing of the primary HLA-G transcript or by shedding the membrane-bound forms after proteolytic cleavage. Mitsdoerffer *et al.*[6] suggested also an immunosuppressive function of peripheral blood HLA-G^{pos} monocytic cells from healthy individuals by showing that monocyte-derived soluble HLA-G was able to inhibit cytokine (IFN-γ, IL-2) production by activated CD4^{+} T cells.

Besides their direct suppressive function on immune cells, HLA-G transfected cell lines have been shown to exert additional immunoregulation by turning naïve T cells into anergic and suppressor CD3^{+}CD4^{low} and CD3^{+}CD8^{low} cells.[33] Consistent with this, in our experiments, we also noticed that after pre-treatment with naturally occurring CD14^{+}HLA-G^{pos} cells, T cells become hyporesponsive, acquire *in vitro* immunosuppressive functions, and decrease CD4 and CD8 cell surface antigen expression. Downmodulation of the CD4 antigen on CD14^{+}HLA-G^{pos} pre-treated T cells could limit their TCR-mediated activation and subsequently explain their inability to respond to secondary allogeneic stimulus.[17] The CD8 downmodulation may be due to the direct interaction between HLA-G and CD8 co-receptor.[33] An association between HLA-G and T-cell tolerance was suggested by Naji *et al.*[33] who found a correlation between soluble HLA-G plasma levels, percentages of CD3^{+}CD4^{low} and CD3^{+}CD8^{low} suppressor T cells, and graft acceptance in liver/kidney transplanted patients. In addition, in a very recent study, Giuliani *et al.*[34] reported an *in vitro* expansion of CD3^{+}CD4^{low} and CD3^{+}CD8^{low} T cells by mesenchymal stem cells in an HLA-G mediated fashion.

Increased HLA-G expression in peripheral blood cells has been described in HIV patients[7] and in patients after liver/kidney transplantation.[35,36] Here, we described for the first time a significantly higher frequency of HLA-G^{pos} blood cells after allo-HCT as compared to pre-transplant values and healthy subjects. Interestingly, there was a more pronounced expansion of CD3^{+}HLA-G^{pos} cells (5.4-fold increase) compared to the increase in CD14^{+}HLA-G^{pos} cells (2.2-fold increase). Similarly to the liver/kidney transplanted patients, the HLA-Gpos T cells after allo-HCT were mainly CD8^{+}.[36] What regulates this expansion of HLA-G^{pos} cells in the bone marrow transplanted patients is not known. The fact that increased HLA-G^{pos} cells appeared early post-transplantation and remained high also at later time points after transplantation makes it very likely that the alloreactive environment in the transplanted patient played a causative role in the long-term expansion of the peripheral blood HLA-G^{pos} cells. Indeed, there are data suggesting that an alloreaction itself might up-regulate the HLA-G expression.[36] In the only study analyzing the role of HLA-G in the allo-HCT setting, Le Maux *et al.*[15] found increased soluble HLA-G plasma levels in patients without acute GvHD as compared to those patients with, and suggested a preventative role of this molecule in the occurrence of acute GvHD. In our study, we did not find any correlation between the frequency of the HLA-G^{pos} cells and active GvHD, history of acute or chronic GvHD, severity of chronic GvHD or treatment with immunosuppressive drugs. However, this could be attributed to the fact that only one patient in our cohort experienced grade II or higher acute GvHD. Nevertheless, isolated CD14^{+}HLA-G^{pos} and CD3^{+}HLA-G^{pos} blood cells from allo-transplanted patients were found to be immunosuppressive *in vitro* and inflammatory cells in skin biopsies of patients with skin GvHD stained positive for HLA-G.

Ectopic HLA-G expression has been found in graft biopsies after solid organ transplantation and has been correlated with a decrease in the number of rejection episodes, suggesting a role for HLA-G in allograft tolerance.[3] It must be noted that, in these cases, expression of HLA-G was detected *in situ* in cells that are the primary targets of the immune system, e.g endomyocardial cells in heart grafts,[11,37] biliary epithelial cells in liver transplants[12] and tubular epithelial cells after kidney transplantation.[35] Despite the large amount of information concerning HLA-G expression in solid organ transplantation, there have been no similar studies in the allogeneic hematopoietic cell transplantation (allo-HCT) setting. Here, we investigated for the first time HLA-G expression in target organs of graft-*versus*-host-disease (GvHD) after allo-HCT and we found a neo-expression of HLA-G in epidermis and in the glands of the dermis. More importantly, a significant correlation between HLA-G protein expression and histological grading of GvHD or history of clinical GvHD was found, suggesting a link between GvHD and HLA-G upregulation. The exact mechanism by which GvHD might induce HLA-G expression in skin epithelial cells, which are the primary targets of the allogeneic immune system, remains to be clarified. It has been shown that microenvironmental factors, such as TNFa or interferons which are also identified at GvHD sites,[23] may up-regulate the HLA-G expression.[4,6] However, whether the ectopic HLA-G expression in the skin after transplantation is a consequence of GvHD or is part of an internal regulatory system to control GvHD remains to be defined in prospective, longitudinal studies with a larger study cohort, and with the additional application of sensitive methodologies, such as quantitative PCR determination of HLA-G transcripts. Interestingly, one patient with very severe skin GvHD who died from GvHD-related causes showed com-

pletely negative expression of HLA-G, both in epidermis as well as in inflammatory cells of the dermis. One could speculate that this absence of HLA-G adaptation, for example due to presence of HLA-G allelic variants associated with low HLA-G mRNA levels,[38] resulted in failure to control immune aggression in this particular patient. Indeed, La Nasa *et al.*[39] have observed an association between the HLA-G 14-basepair deletion allele and an increased risk of acute GvHD.

A striking finding from our study is that HLA-G protein expression was detected in the basal layer of the epidermis of healthy adults. At first glance, this finding disagrees with the concept that HLA-G expression is highly restricted in adult tissues and with previous studies showing no HLA-G expression in healthy skin. Such a discrepancy could be attributed to the different antibodies used to detect HLA-G expression. In our experiments, and in contrast to previous studies of HLA-G expression in skin, we used the new commercially available MEM-G/2 antibody which was proposed for immunohistochemical analyses during the International Workshop for HLA-G.[40] Like us, Lodererova *et al.*[41] using the MEM-G/1 antibody detected for the first time HLA-G expression on skeletal muscle biopsies from healthy subjects. Our observation contributes to the growing opinion that HLA-G expression in adults might be more widespread than initially thought. Nevertheless, we clearly showed that there is an upregulation of HLA-G expression in the supra-basal layers and in the eccrine sweat glands of the skin after allogeneic bone marrow transplantation.

In summary, we report here for the first time a population of immunosuppressive and immunotolerogenic monocytes in the systemic circulation of healthy adults characterized by the cell surface expression of HLA-G. These cells probably belong to the normal repertoire of the immune system which, depending on physio-pathological status, may putatively be expanded in order to maintain immune tolerance. After allo-HCT, the number of HLA-G^{pos} cells increase in blood and are detected at sites of GvHD. Furthermore, we showed that in primary targets of GvHD, such as skin, HLA-G expression is up-regulated after transplantation. Overall, we believe that this adaption of HLA-G expression after transplantation acts as a shield against immune aggression. Since HLA-G positive cells are easy to isolate from blood, naturally occurring HLA-G^{pos} monocytes may in the future constitute a novel strategy for adoptive cell immunosuppressive therapy against GvHD. To date, no orthologs of HLA-G have been identified in other species, meaning that this concept cannot be proved in *in vivo* experiments. Of note, mesenchymal stem cells which are used for prevention and treatment of GvHD in humans have been shown to exert an immunosuppressive action through HLA-G.[42] The mechanisms of post-transplant HLA-G upregulation in immune cells and in epithelium remain unclear. In this context, various drugs such as hypomethylating agents have been shown to induce HLA-G expression *in vitro*.[43] Selective induction of HLA-G expression in susceptible target cells of GvHD may represent an innovative strategy to protect against GvHD.

Authorship and Disclosures

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