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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Online Supplementary Design and Methods

Antibodies and flow cytometry

The following antibodies conjugated with FITC, PE or PC5 were used for flow cytometric analysis (EPICS XL-MCL, Coulter, Fullerton, CA, USA): HLA-G (clone MEM-G/9, Abcam, Cambridge, UK); anti-CD14, CD3, CD8 (Beckman Coulter, CA, USA); anti-CD4 (Becton-Dickinson, NJ, USA); anti-HLA-DR, CD80, CD86, CD33, CD11b (BD Biosciences, Pharmingen, CA, USA); FOXP3 (e-Bioscience, San Diego, USA); anti-ILT2/CD85j, (R&D, MN, USA). A goat anti-mouse Alexa Fluor 488 (Santa Cruz, Heidelberg, Germany) was used as secondary antibody for ILT4/CD85d (R&D). In control experiments, Ficoll isolated PBMCs were stained after pre-incubation for 30 min with 20% human serum or were stained with the Alexa Fluor 488-conjugated Fab-fragment of the MEM-G/9 antibody (Exbio, Praha, Czech Republic). No differences in the percentages of HLA-G positive cells were found and we therefore performed subsequent stainings with the intact MEM-G/9 antibody without serum pre-incubation (*Online Supplementary Figure S1*). Isotype controls were systematically used to subtract non-specific signals (FITC, PE or PC5- conjugated from BD Biosciences and Alexa Fluor 488-conjugated from Exbio). In control experiments, PE-conjugated IgG1 from BD Biosciences and "irrelevant" anti-CD20 (clone B-Ly1, IgG1) from Abcam generated identical low level background fluorescence on monocytes. Data analysis was performed with WinMDI 2.9 (free available software). The Specific Fluorescence Index (SFI) was calculated by using the formula: SFI= specific geometric mean/ geometric mean of unspecific isotype.

Cell isolation

Isolation of HLA-G^{pos} cells was performed by fluorescent-activated cell sorting (FACS-sorting) using the FACS Vantage SE (Becton Dickinson). PBMCs were stained for 30 min with HLA-G-PE, CD14 or CD3-PC5 and an isotype control. A gate was set on all non-aggregated cells followed by sorting of HLA-G positive and HLA-G negative cell populations. Cell purity was assessed by FACS and was always more than 98%. Isolation of peripheral blood monocytes was performed by FACS-sorting using the CD14-PC5 antibody or by negative selection after magnetic separation of T cells (Miltenyi Biotec, Bergisch Gladbach, Germany). T-cell enrichment from PBMCs (>90% CD3$^+$ population) for the *in vitro* immunoregulatory experiments was achieved by 24-h monocytic adherence, as previously described.[1] The non-adherent population was extensively washed and the CD3$^+$ cells were more than 90%.

In vitro *immunostimulatory and immunosuppression assays*

For alloproliferation studies, one-way mixed lymphocyte cultures (MLCs) were performed, as previously described in detail.[2] Briefly, unseperated PBMCs or enriched CD3$^+$ T cells (responders) were labeled with CFSE (CFSE Cell Proliferation Kit, Invitrogen, Paisley, UK) and were cultured with irradiated (25Gy) allogeneic PBMCs (stimulators) at a 1:1 ratio. Cultures were performed in U-bottom 96-well plates (Corning INC., Corning, NY, USA) in RPMI (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum (GIBCO) and 100 U/mL penicillin-streptomycin. Cell proliferation was measured according to CFSE intensity by flow cytometry at Day 6. In some MLCs, irradiated FACS-sorted CD14$^+$HLA-G^{pos} or CD14$^+$HLA-G^{neg} cells were used as stimulators. For suppression assays, irradiated FACS-sorted CD14$^+$HLA-G^{pos} or CD14$^+$HLA-G^{neg} cells were used as third-party cells in MLCs. MLCs without third-party cells were used as controls. Suppression assays were also conducted using transwell (0.4 μm) systems (Greiner bio-one, San Diego, CA, USA), in which the MLC was placed in the lower chamber and the third-party cells in the upper. For blocking experiments, anti-HLA-G (20 μg/mL, clone 87G, Exbio), anti-ILT2 and/or anti-ILT4 (10 μg/mL, R&D) or irrelevant IgG isotypes were added in suppression assays on Day 0 and Day 3.

T-cell pre-treatment procedure

Enriched T cells from peripheral blood were pre-treated for 18 h or four days with allogeneic irradiated FACS-sorted CD14$^+$HLA-G^{pos} or CD14$^+$HLA-G^{neg} cells. Pre-treatment was set up as MLC. At the end of the pre-treatment period, CD3$^+$ cells were isolated by FACS-sorting (purity >98%). Pre-treated CD3$^+$ cells were then either labeled with CFSE and used as responder cells in new MLCs, or were irradiated (25Gy) and used as third-party cells in suppression assays. Non-pre-treated paired CD3$^+$ cells following the same procedure of culture and FACS-sorting were used as controls. MLCs and suppression assays were set up as described above.

Immunohistochemistry

Four-micrometer sections of paraffin-embedded skin biopsies were incubated with the MEM-G/2 primary antibody which recognizes the free heavy chain of all HLA-G isoforms (Exbio, Praha) or an isotype antibody, after deparaffinization, antigen retrieval treatment, and blockage of endogenous peroxidase activity. To support our data, we also used the 4H84 anti-HLA-G antibody (Santa Cruz, Heidelberg, Germany). Positive (extravillous cytotrophoblast) and negative (villi) controls were included in each experiment. The binding sites of the primary antibody were then visualized using a Dako EnVison kit (Dako, Copenhagen, Denmark) after thorough washing in phosphate-buffered saline (PBS, GIBCO) solution. Finally, sections were faintly counterstained with hematoxylin. All sections were analyzed under light microscopy. Staining results were interpreted by two pathologists (EP, DP) who were blinded to the individual's diagnostic categories and were scored according to distribution (percentage of positive cells) and intensity as determined by comparison with positive control. Score for distribution was: (-) no positive cells, (+) less than 30% of positive cells, (++) 30-70% of positive cells and (+++) more than 70% positive cells. Score for staining intensity was: (-) no stain, (+) weakly, (++) moderately and (+++) strongly stained.

References

1. Le Rond S, Azema C, Krawice-Radanne I, Durrbach A, Guettier C, Carosella ED, et al. Evidence to support the role of HLA-G5 in allograft acceptance through induction of immunosuppressive/ regulatory T cells. J Immunol. 2006;176(5):3266-76.
2. Themeli M, Petrikkos L, Waterhouse M, Bertz H, Lagadinou E, Zoumbos N, et al. Alloreactive microenvironment after human hematopoietic cell transplantation induces genomic alterations in epithelium through an ROS-mediated mechanism: in vivo and in vitro study and implications to secondary neoplasia. Leukemia. 2010;24(3):536-43.

Online Supplementary Table S1. **Absolute numbers of HLA-G$^+$ peripheral blood cells in healthy individuals and in transplanted patients**

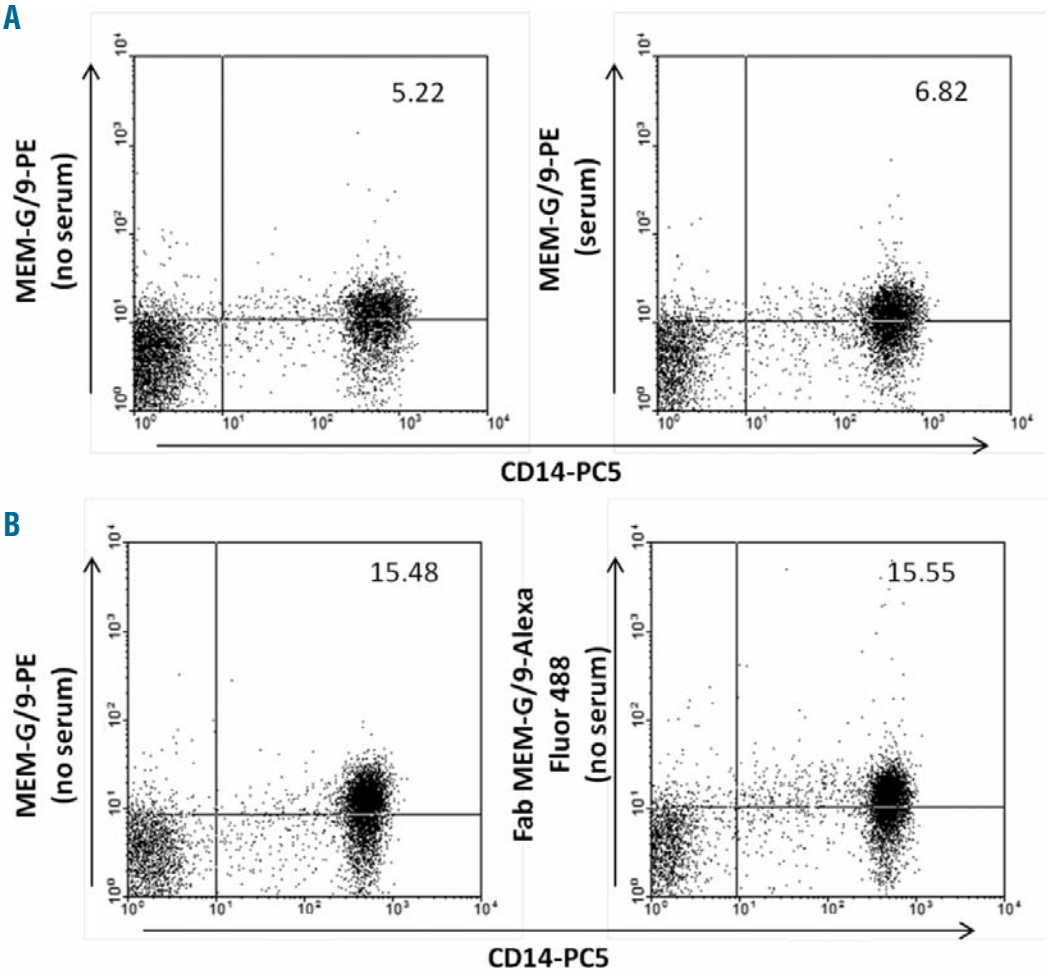
	Healthy subjects (N=8)[1]	Pre- HCT (N=4)	Post-HCT (N=27)	Healthy *vs.* post-HCT (P)	Pre-HCT *vs.* post-HCT (P)
HLA-G+ monocytes (x10^9/L)	0.07±0.02	0.06±0.02	0.15±0.05	0.0006	0.0014
HLA-G+ lymphocytes (x10^9/L)	0.024±0.006	0.018±0.005	0.14±0.012	< 0.0001	< 0.0001

[1]Transplanted patients are the same as in Figure 4 but healthy subjects are different. Absolute number (x10^9/L) of HLA-G$^+$ blood monocytes/ lymphocytes were calculated by multiplying the percentage of CD14$^+$HLA-G^{pos} /CD3$^+$HLA-G^{pos} cells by the absolute number of peripheral blood monocytes/ lymphocytes determined by an automated blood cell counter (CELL DYN 3700, ABBOTT, Illinois, USA). Mean ± standard deviation is shown; N: number of subjects analyzed; HCT: allogeneic hematopoietic cell transplantation.

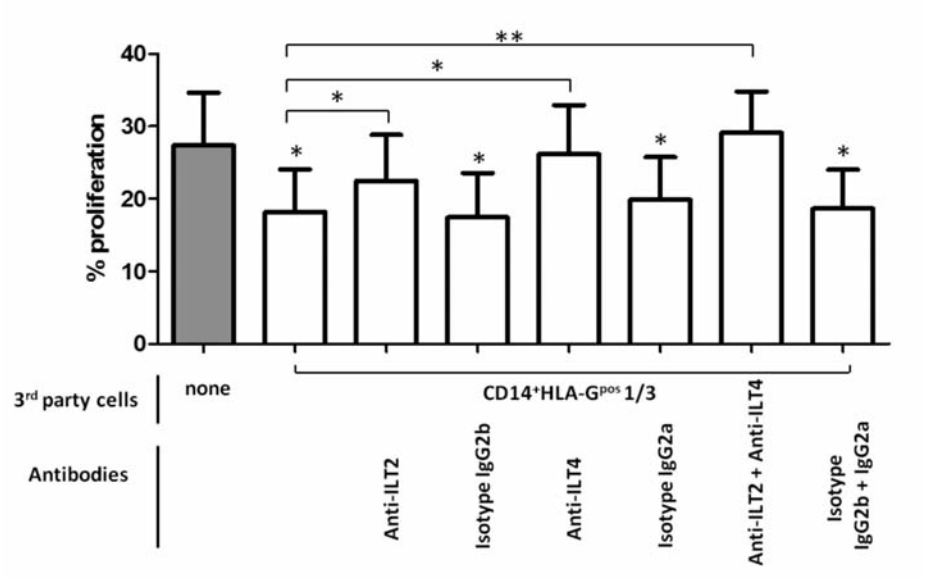
Online Supplementary Table S2. **HLA-G expression in skin from healthy individuals and transplanted patients.**

Subject	Day after HCT	Histological GvHD[1]	MEM-G/2 antibody[2]				4H84 antibody (basal)[4]
			Basal	Intermediate	External	Overall[3]	
Group 1: healthy subjects							
H1	NA	NA	-/-	-/-	-/-	negative	-/-
H2	NA	NA	+++/+	-/-	-/-	dim	-/-
H3	NA	NA	+++/++	-/-	-/-	dim	-/-
H4	NA	NA	++/+	++/+	-/-	dim	ND
Group 2: no GvHD							
P1	41	0	-/-	-/-	-/-	negative	-/-
P2	190	0	-/-	-/-	-/-	negative	-/-
P3	253	0	-/-	-/-	-/-	negative	-/-
Group 3: mild skin GvHD							
P4	162	I	+++/++	+++/++	++/+	positive	+++/+
P5	178	II	+++/++	+++/+	+++/++	positive	+++/+
P6	118	II	+++/+	++/+	++/+	positive	++/+
Group 3: moderate skin GvHD							
P7	323	I	+++/++	+++/+	+++/++	positive	+++/+
P8	279	II	+++/+++	+++/++	+++/+++	positive	+++/+
P9	251	II	+++/++	+++/++	+++/++	positive	+++/+
Severe skin GvHD							
P10	959	III	-/-	-/-	-/-	negative	ND

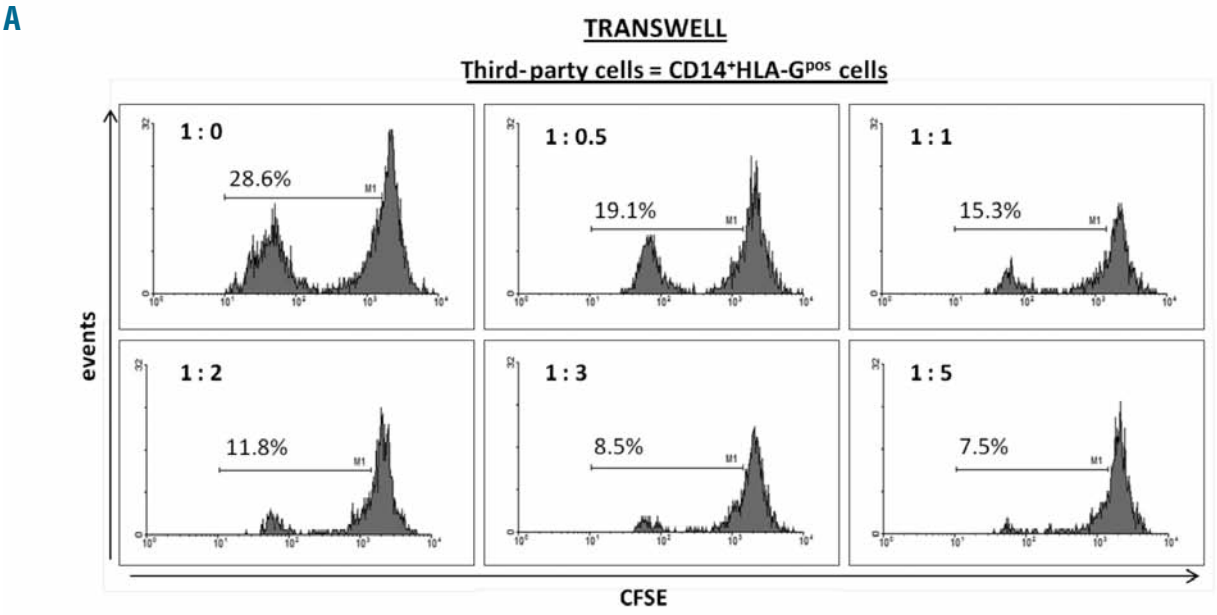
GvHD: graft-versus-host disease; H: healthy; HCT: allogeneic hematopoietic cell transplantation; P: transplanted patient; NA: not applicable; ND: not done. [1]Histological grading of GvHD was performed by pathologists who were blinded to the clinical grade of GvHD. [2]HLA-G expression in epidermis was evaluated after staining with the MEM-G/2 antibody and scored in the basal (internal), intermediate and external layer of the epidermis according to distribution / intensity as explained in detail in Design and Methods *section; [3]Overall estimation of MEM-G/2 staining in epidermis was assessed as follows: negative, lack of detection; dim, weak detection only in basal ± intermediate layer; positive: detection in all layers; [4]HLA-G expression in basal layer of epidermis after staining with the 4H84 antibody. Intermediate and external layers stained in all cases were negative. 4H84 stain evaluation was made by a pathologist who was blind to the MEM-G/2 results.*

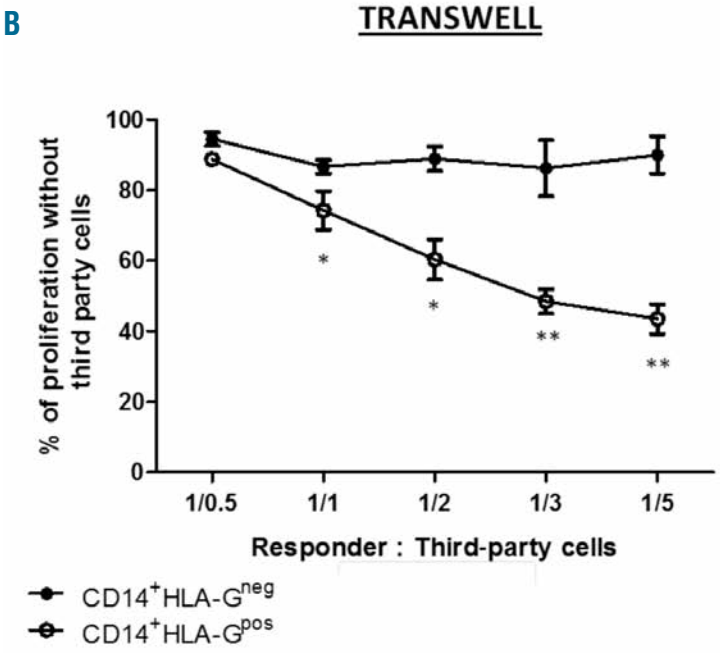


Online Supplementary Figure S1. Identification of CD14^{+}HLA-G^{pos} cells in peripheral blood of healthy individuals with the use of MEM-G/9 antibody. Images show examples of flow cytometric identification of CD14^{+}HLA-G^{pos} cells in peripheral blood of healthy individuals using different staining techniques. Text in parentheses denotes whether the cells were stained directly or after pre-incubation with 20% human serum for 30 min. Fab denotes staining with the commercially available Alexa Fluor 488-conjugated Fab-fragment of the MEM-G/9 antibody. Numbers indicate the percentage of CD14^{+}HLA-G^{pos} cells. (A) and (B) show representative examples of 5 and 8 experiments, respectively.

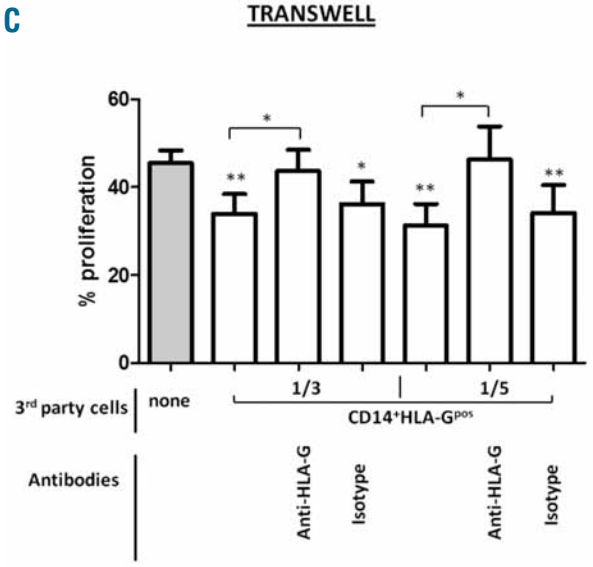


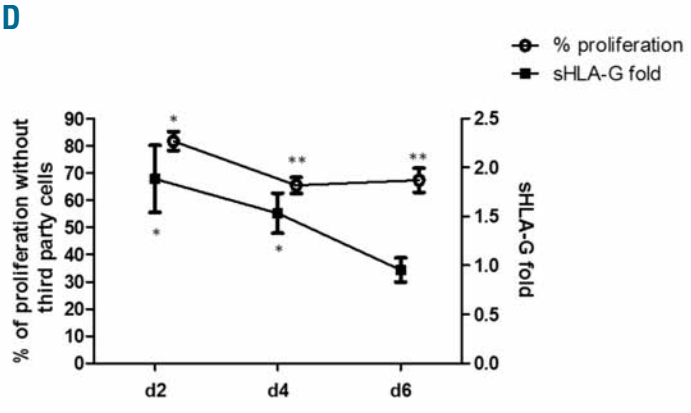
Online Supplementary Figure S2. Immunoglobulin-like transcript (ILT)-2 and ILT-4 receptors are involved in the *in vitro* suppressive function of CD14^{+}HLA-G^{pos} cells. The graph summarizes results of 4 independent suppression assays carried out after blocking the ILT-2 and/ or ILT-4 receptors. Anti-ILT2 or/and anti- ILT-4 antibodies or isotype-matched irrelevant antibodies were added on Days 0 and 3 in cultures containing third-party CD14^{+}HLA-G^{pos} cells. Cell proliferation was quantified at Day 6 by CFSE assay. Statistically significant proliferation between CD14^{+}HLA-G^{pos} containing cultures and control MLC without third-party cells is given by an asterisk directly above the column and between CD14^{+}HLA-G^{pos} containing cultures without *versus* with blocking or isotype antibody by an asterisk above the horizontal lines (*P<0.05, **P<0.005).

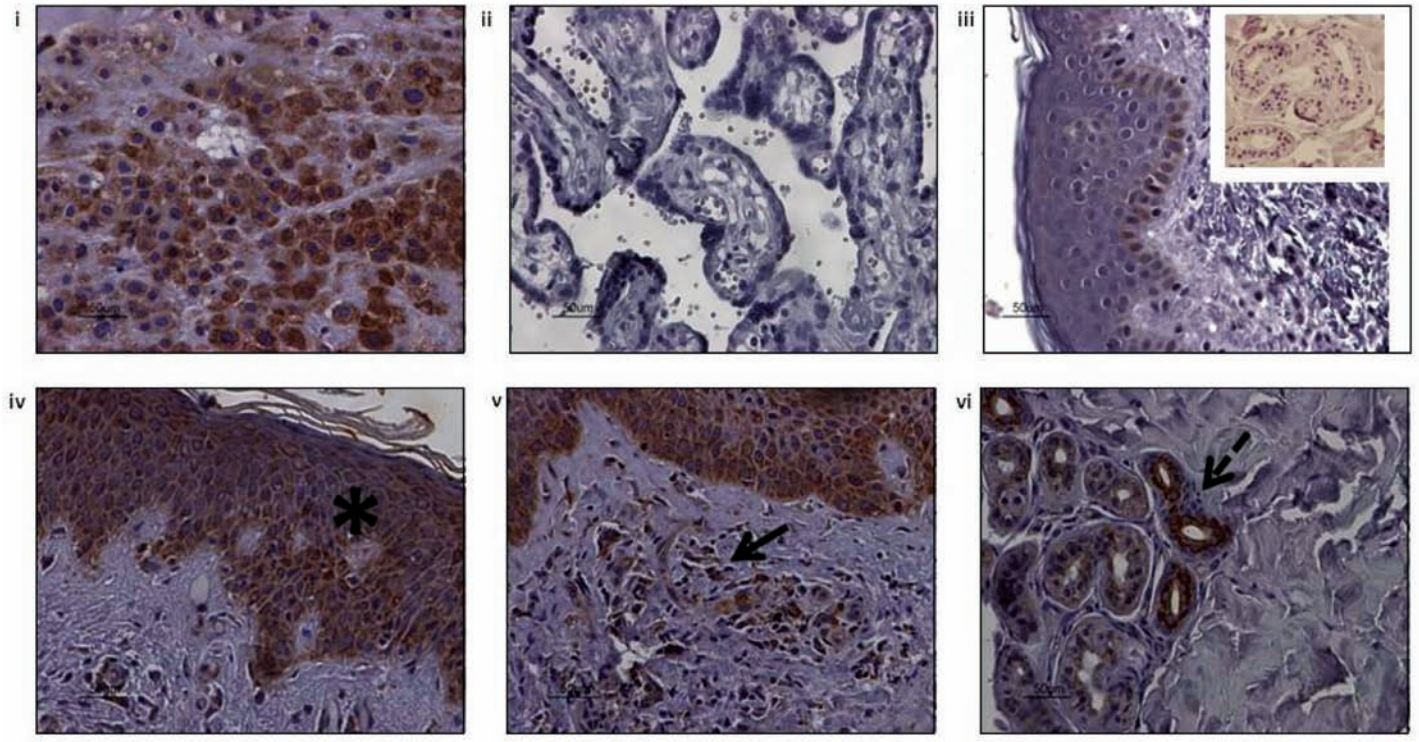




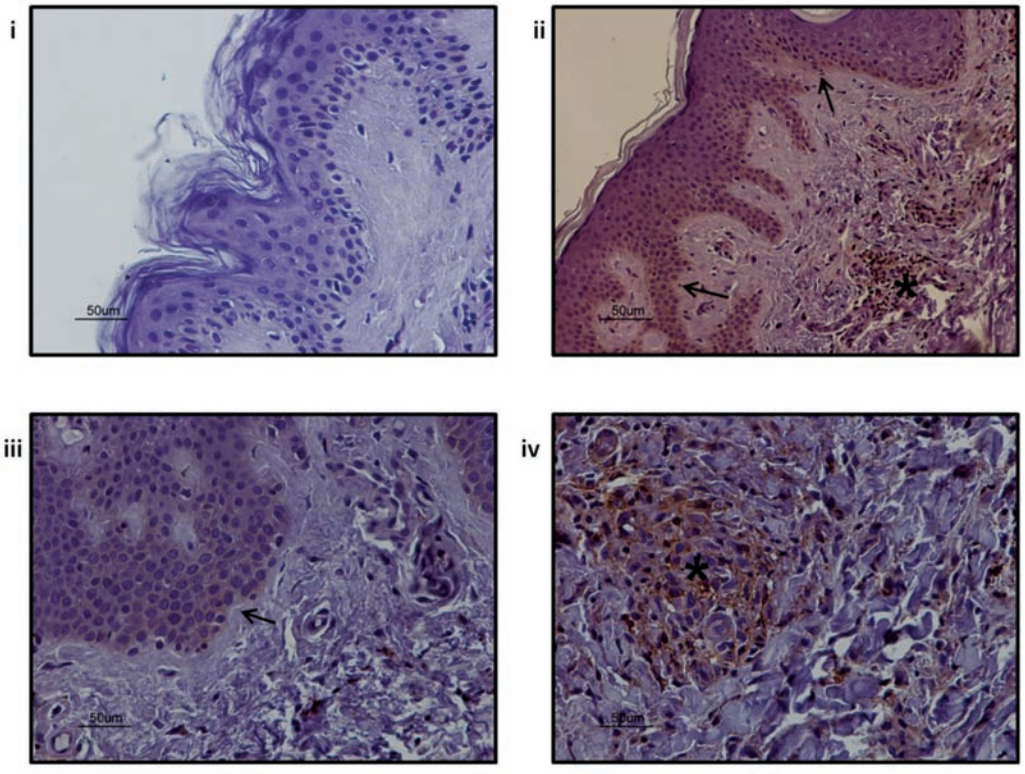
Online Supplementary Figure S3. CD14$^+$HLA-G^{pos} cells do not require cell contact to exert their suppressive functions. Suppression assays were performed by adding irradiated CD14$^+$HLA-G^{pos} or paired CD14$^+$HLA-G^{neg} cells at various ratios at the top of the chamber of a transwell culture system, while MLCs was at the bottom chamber. Analysis of proliferation was made by CFSE assay at Day 6. (A) A representative experiment is shown. The numbers in the upper corner indicate the ratio responder: third-party CD14$^+$HLA-G^{pos} cells and the numbers above the horizontal line indicate 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) The graph summarizes results of 3 independent anti-HLA-G blocking experiments performed at different responder : third-party ratios. Statistically significant proliferation between CD14$^+$HLA-G^{pos} containing cultures and control MLCs is given by an asterisk directly above the column and between CD14$^+$HLA-G^{pos} containing cultures without *versus* with neutralizing or isotype antibody by an asterisk above the horizontal lines (*P<0.05, **P<0.005). (D) Soluble HLA-G (sHLA-G) was measured by enzyme-linked immunosorbent assay (sHLA-G ELISA, Exbio, Praha, Czech Republic) in filtered (0.2 μm) supernatants of transwell cultures in which CD14$^+$HLA-G^{pos} cells were added to a responder : third-party ratio of 1:3 (n=6). sHLA-G were measured at Day (d) 2, 4 and 6 of culture. In 3 experiments, we also evaluated the alloproliferation by CFSE assay. The graph shows the percentage of proliferation (left y axis) and the fold-increase levels of sHLA-G (right y axis) as compared to control MLC without third-party cells (*P<0.05, **P<0.005).







Online Supplementary Figure S4. HLA-G expression in the skin of transplanted patients. Immunohistochemical staining of paraffin-embedded biopsies using the MEM-G/2 antibody. HLA-G was stained brown. (i) Extravillous trophoblast (positive control) and (ii) villous trophoblast (negative control). (iii) Skin specimen from a healthy donor showing HLA-G expression solely in the basal layer. In the upper corner the skin biopsy from another healthy donor is presented, focusing on the dermis and showing the negative stain of sweat eccrine glands. (iv-vi) Skin specimens from transplanted patients were stained positively for HLA-G within all layers of epidermis (asterisk), in inflammatory cells (solid arrow) and in sweat eccrine glands of the dermis (dash arrow). Original magnifications 40x.



Online Supplementary Figure S5. HLA-G expression in skin using the 4H84 anti-HLA-G antibody. Immunohistochemical staining of paraffin-embedded biopsies using the 4H84 anti-HLA-G antibody (Santa Cruz, Heidelberg, Germany). HLA-G was stained brown. Representative experiments are shown. (i) Skin specimen from a healthy donor showing no HLA-G expression. (ii-iv) Skin specimens from transplanted patients showing a weak expression of HLA-G in the basal level of epidermis (solid arrow) and a stronger expression in inflammatory cells (asterisk). Original magnification. 40x, 20x in (ii).