SUPPLEMENTARY APPENDIX

HLA-G expression levels influence the tolerogenic activity of human DC-10

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Supplementary material

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Running heads: HLA-G-mediated tolerance *via* DC-10

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Methods

Cells preparation

Human peripheral blood was obtained from healthy donors (HDs) upon informed consent in accordance with local ethical committee approval (Protocol TIGET03) and with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation over Lymphoprep (Nycomed Amersham).

T-cell purification

$CD4^+$ T cells were purified from PBMC by negative selection using the untouched $CD4^+$ T cell Isolation kit (Miltenyi Biotech, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were then depleted of $CD45RO^+$ cells using anti-CD45RO-coupled magnetic beads and LD negative selection columns (Miltenyi Biotech, Germany). In the purified cells the proportion of $CD4^+CD45RO^-CD45RA^+$ was consistently greater than 90%.

Flow cytometric analysis

DC were initially incubated for 15' with FcR blocking reagent (Miltenyi Biotech, Germany) and stained for additional 30' with anti-CD14 (MϕP9, BD Bioscience, CA, USA), anti-CD11c (B-ly6, BD Bioscience, CA, USA), anti-CD1a (HI149, BD Bioscience, CA, USA), anti-HLA-DR (TU36, BD Bioscience, CA, USA), anti-CD86 (BU63, Invitrogen, Camarillo CA, USA), anti-ILT4 (42D1, Beckman Coulter, Marseille, France) and, anti-HLA-G (MEM-G9, Exbio, Praha, Czech Republic) mAbs. T cells were stained with anti-CD45RA (HI100, Biolegend, San Diego, CA, USA), anti-CD4 (SK3, BD Bioscience, California, USA), anti-CD49b (AK-7, Biolegend, San Diego, CA, USA), anti-LAG-3 (FAB2319, R&D System), mAbs. The staining for CD49b and LAG-3 was performed at 37°C for 15 minutes. Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, CA, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). Quadrant markers were set accordingly to unstained controls.

Intracellular IL-10, IL-4, IFN-γ, and IL-2 were detected by flow cytometry. Briefly, T cells (1×10^6/ml) were stimulated with leukocyte activation kit according to manufacturers' instructions (BD Bioscience, California, USA) in complete medium. 6 hours after activation, T cells were collected, washed in PBS, and incubated with anti-hCD4 (SK3, BD Bioscience, California, USA). Subsequently, T cells were permeabilized with Cytofix/Cytoperm™ kit according to manufacturers' instructions (BD Bioscience, California, USA) and incubated with anti-hIL-2 (MQ1-17H12, BD Pharmingen), anti-hIL-4 (MP4-25D2, BD Bioscience, California, USA), anti-hIL-10 (JES3-9D7, BD Bioscience, California, USA), and anti-hIFNγ (B27, BD Bioscience, California, USA) mAbs. Samples were acquired using a FACS Canto II flow cytometer (Becton Dickinson, CA, USA), and data were analyzed with FCS express (De Novo Software, CA, USA). Quadrant markers were set accordingly to unstained controls.

T-cell differentiation

$1x10^5$ DC were cultured with $1x10^6$ allogeneic $CD4^+CD45RO^-$ T cells in 1ml of X-vivo 15 medium (Lonza, Italy), supplemented with 5% pooled AB human serum (Lonza, Italy), and 100 U/ml penicillin/streptomycin (Lonza, Italy). After 7 days, rhIL-2 (20U/ml) (Chiron) was added, and cells were expanded for additional 7 days. Fourteen days after initiation of the culture, T cells were collected, washed, and analyzed for their functions. T cells stimulated with DC-10 are referred as T(DC-10), and T cells stimulated with mDC as T(mDC). Cultures with DC-10 typically resulted in 8-10-fold reduction in T-cell expansion compared to cultures stimulated with mDC.

Proliferation and suppression of T cells

$CD4^+CD45RO^-$ T cells were stimulated with allogeneic DC (10:1, T:DC ratio) in a final volume of 200 μl of X-vivo 15 medium (Lonza, Italy), supplemented with 5% pooled AB human serum (Lonza, Italy), and 100 U/ml penicillin/streptomycin (Lonza, Italy) for 4 days and then pulsed for 16h with 1 μCi/well ^{3}H-thymidine.

To analyze the proliferative capacity of T(DC-10) and T(mDC) cell lines in response to allogeneic stimulation, T cells were stimulated with allogeneic mDC (10:1, T:DC ratio) in a final volume of 200 μl of medium for two day and then pulsed for 16h with 1 μCi/well ^{3}H-thymidine.

To evaluate the suppressive activity of T(DC-10), or T(mDC) cells, autologous $CD4^+$ T cells (Responders) were thawed and stained with Dye eFluor® 670 (eBioscience, California, USA) and activated with allogeneic mDC (10:1, T:DC ratio). Suppressor cells were added at a ratio of 1:1. The percentage of divided responder T cells was calculated by gating on $CD4^+$ cells, as described elsewhere [1].

Cytokine determination: ELISA

To measure IL-10, IL-12, IL-6, and TNFα, DC were left un-stimulated or activated with 50 ng/ml of rhIFNγ (R&D Systems, Minneapolis MN, USA) and 200 ng/ml of LPS (Sigma, CA, USA) for 2 days. Cytokine levels were determined in culture supernatants by ELISA according to the manufacturer's instructions (BD Bioscience, California, USA). The limits of detection were as follows: IL-6: 15 pg/ml, IL-10: 5 pg/ml, IL-12: 30 pg/ml, and TNFα: 5 pg/ml. To measure IFNγ production by T cells, culture supernatants were harvested after 48, 72, and 96 hours of culture and levels of IFNγ were determined by ELISA according to the manufacturer's instructions (BD Biosciences). The limit of detection was 30 pg/ml,

Detection of soluble HLA-Gs

Levels of shed HLA-G1 and soluble HLA-G5 were determined by enzyme-linked immunosorbent assay (ELISA), as previously described [2,3]. To detect sHLA-G (shed HLA-G1 and HLA-G5) plates (Nunc-Immuno Plate PolySorp, Thermoscientific, Denmark) were coated with the mAb G233 (Exbio, Czech Republic), whereas to detect HLA-G5 plates were coated with the mAb 5A6G7 (Exbio, Czech Republic). sHLA-G or HLA-G5 were

detected with biotinylated β_2-microglobulin and W6/32 mAbs (Exbio, Czech Republic), respectively. Supernatants from HLA-G transfected LCL721.221 cells [4], and HeLa HLA-G5-transfected cells (kindly provided by Dr. Rizzo, Università di Ferrara) purified by affinity chromatography by using the W6/32 mAb were used for the generation of standard calibration curves for shed HLA-G and HLA-G5, respectively. The limit of sensitivity was 1 ng/ml.

Amplification and sequencing of 3'UTR of the HLA-G gene

Genomic DNA was extracted from PBMCs using a commercial kit (QIAamp, QIAGEN, Italy) according to the manufacturer' s instructions. Briefly, 100 ng of genomic DNA were amplified in a 25 μl reaction containing 1X polymerase chain reaction (PCR) buffer (Roche, USA), 0.2 mM dNTP mix (Roche, USA), 1.5 mM $MgCl_2$ (Roche, USA), 0.8 U *Taq* Polymerase (Roche, USA), and 1 μM of each primer (For: 5' TCACCCCTCACTGTGACTGA 3'; Rev: 5' TTCTCATGTCTTCCATTTATTTTGTC 3'). The initial denaturation step was carried out at 95° C for 3 min, followed by 30 cycles at 93° C for 60 s, 58° C for 60 s, 72° C for 60 s, and by a final extension step at 72° C for 10 min. The amplification product was evaluated using a 2.5% agarose gel, purified using a commercial kit (Wizard SV Gel and PCR Clean-Up System, Promega, WI, USA) according to the manufacturer' s instructions, and subjected to direct sequencing on both strands. All polymorphic sites observed at the 3'UTR were individually annotated and named according to previous reports [5].

miRNA extraction and quantification

Cells were thawed as dry pellet, small RNA were extracted using mirVana Isolation Kit following manufacturer's instructions (Ambion, Austin, TX). Real-time RT-PCR was used to quantify the amounts of miR-152-3p, miR-2110 and, miR-93* (Applied Biosystems, Foster City, CA) transcripts in DC-10 compared to those of $CD14^+$ precursor cells. Briefly, PCR was carried out for 40 amplification rounds in the presence of Taqman® microRNA Assay system (Applied Biosystems, Foster City, CA), using miR-let-7a (Applied Biosystems, Foster City, CA) as endogenous control. Reactions were carried out in duplicate or triplicate in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) and SDS 2.2.1 software was used to analyze the data. The same threshold was applied to calculate the Ct values for each PCR reaction. Quantification relative to $CD14^+$ precursor cells was carried out using the comparative CT method: $\Delta CT = CT_{miR\text{-}X} - CT_{miR\text{-}let\text{-}7a}$; $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{CD14+}$; relative miR-X expression = $2^{-\Delta\Delta CT}$.

Real-time quantitative PCR analyses

Total RNA was extracted with the RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized with the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time analysis were performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) and SDS 2.2.1 software to analyze the data. Real-

time RT-PCR was used to quantify the amounts of HLA-G transcripts compared with those of JEG-3 as previously described [6]. Briefly, duplex PCR was carried out for 40 amplification rounds in the presence of TaqMan Universal PCR Master Mix, using the pre-developed TaqMan assay reagent GAPDH as endogenous control [probe with VIC reporter and 6-carboxytetramethylrhodamine (TAMRA) quencher (Applied Biosystems, Foster City, CA)], HLA-G-specific probe located in exon 5 [200 nM; (Applied Biosystems, Foster City, CA): 5'-CACTGGAGCTGCGGTCGCTGCT; 6-carboxyfluorescein (FAM) reporter and TAMRA quencher] and HLA-G-specific primers [300 nM (Applied Biosystems, Foster City, CA): forward 5'-CTGGTTGTCCTTGCAGCTGTAG; reverse 5'-CCTTTTCAATCTGAGCTCTTCTTTCT]. Quantification relative to JEG-3 was carried out in duplicate, using the comparative CT method: $\Delta CT = CT_{HLA\text{-}G} - CT_{GAPDH}$; $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{JEG\text{-}3}$; relative HLA-G expression = $2^{-\Delta\Delta CT}$.

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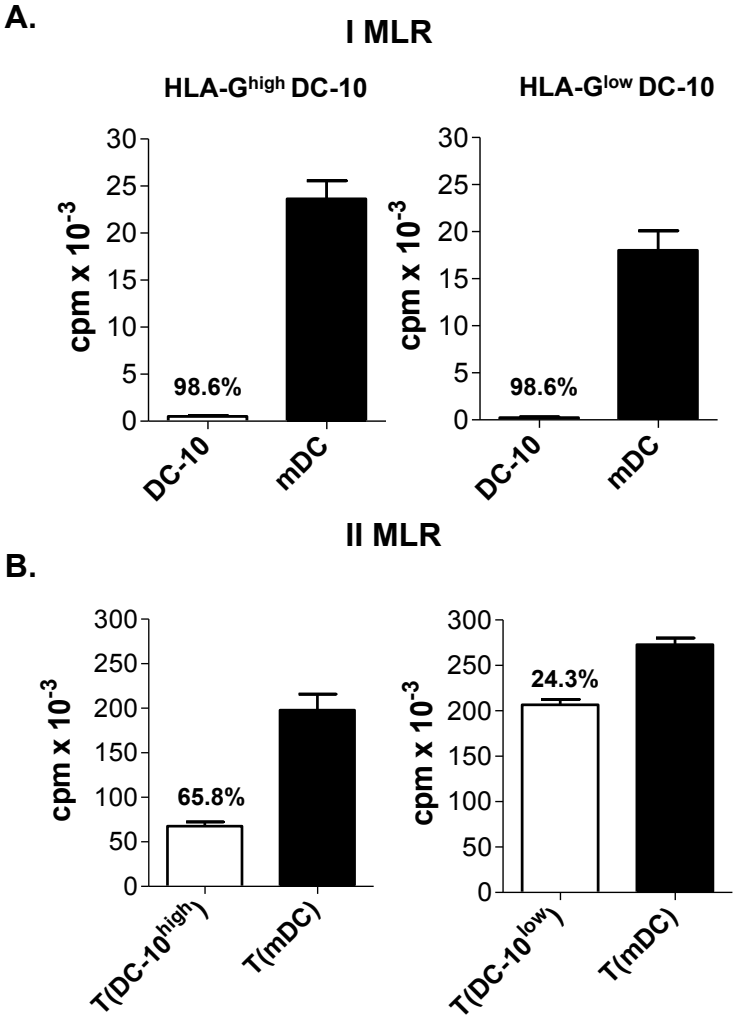
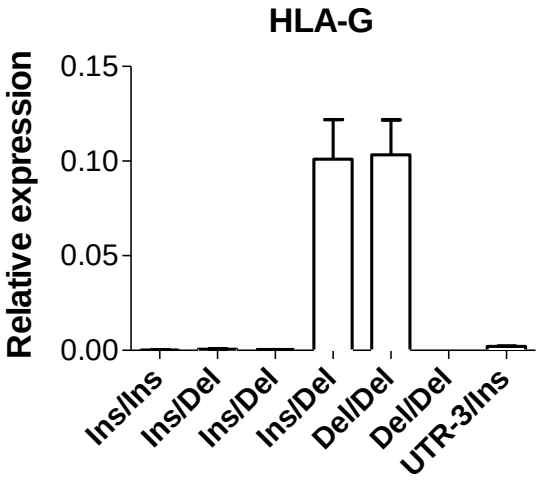
Supplementary Figure legend

Figure S1. DC-10 with high levels of membrane-bound HLA-G promote T-cell anergy. (A) Naive $CD4^+$ T cells were cultured with allogeneic *in vitro* differentiated HLA-G^{high} DC-10, HLA-G^{low} DC-10, and mDC (ratio 10:1). Proliferative responses were evaluated 4 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours. One representative donor out of 6 for HLA-G^{high} DC-10 and one out of 8 for HLA-G^{low} DC-10 independent donors tested are presented. Numbers represent the percentages of reduction in proliferation of naïve $CD4^+$ T cells stimulated with DC-10 compared to those activated with mDC. **(B)** Naive $CD4^+$ T cells were stimulated with allogeneic HLA-G^{high} DC-10 [T(DC-10^{high})], HLA-G^{low} DC-10 [T(DC-10^{low})], or mDC [T(mDC)] for 14 days. After culture, T cells were tested for their ability to proliferate in response to mDC from the same allogeneic donor used in priming. Proliferative responses were evaluated 2 days after culture by [^{3}H]-thymidine incorporation for additional 16 hours. One representative donor out of 6 HLA-G^{high} DC-10 and one out of 8 HLA-G^{low} DC-10 independent donors tested are presented. Numbers represent the percentages of anergy of T(DC-10) compared to T(mDC) calculated as follows: 100-[(T(mDC cpm)-T(DC-10 cpm)/T(mDC cpm)*100].

Figure S2. Quantification of HLA-G mRNA transcripts in DC-10. DC-10 were differentiated in the presence of IL-4, GM-CSF and IL-10 for 7 days. On day 7, total mRNA was extracted from DC-10 and expression of HLA-G gene was evaluated by RT-PCR. Following normalization to GAPDH, relative mRNA amounts from DC-10 cells were adjusted to corresponding expression levels of a calibrator (JEG-3 cell line). Numbers represent arbitrary units. DC-10 were obtained from the indicated 3'UTR genotyped donors.



Amodio et al., Supplementary Figure 1



Amodio et al., Supplementary Figure 2