Preparation of PBMC, isolation of HLA-G\textsuperscript{pos} T cells and generation of dendritic cells (DC)

PBMC were isolated from peripheral blood of normal healthy volunteers by density gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Linz, Austria). For isolation of HLA-G\textsuperscript{pos} T cells, CD4- or CD8 T cells were first negatively isolated using the corresponding T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated CD4\textsuperscript+ or CD8\textsuperscript+ T cells then were incubated with the HLA-G-specific MEM-G9 biotinylated antibody at a concentration of 5 µg/ml for 25 min. Subsequently, labelled cells were selected using anti biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was determined by addition of a PE labelled goat anti mouse IgG F(c) antibody.

HLA-G\textsuperscript{pos} T cells were also isolated by FACS sorting using the Becton Dickinson FACS Aria (BD). Untouched CD3 T cells were purified using the CD3 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach) and subsequently stained with the antibody combination of CD4 – APC and HLA-G-PE or IgG PE to define the HLA-G-negative population. A gate was set on all non-aggregated cells followed by gating on CD4 T cells. HLA-G positive CD4 T cells were defined by isotype control. Two different populations, CD4 HLA-G\textsuperscript{neg} and HLA-G\textsuperscript{pos}, were sorted.

For the generation of DC, monocytes collected after 1 h adherence were cultured in RPMI 1640 containing 10% FCS supplemented with granulocyte macrophage colony-stimulating factor (100 ng/ml, Leukomax; Sandoz, Basel, Switzerland) and IL-4 (40 ng/ml; PeproTech, Inc., Offenbach, Germany) for 6 days. We induced maturation of the DC by addition of lipopolysaccharide (5 µg/ml, \textit{S. typhi}; Sigma-Aldrich, Taufkirchen, Germany) for 24h.

Antibodies and flow cytometry

The following monoclonal antibodies were used: CD4-FITC/-PE/-PerCP, CD8-FITC/-PE/-PerCP, CD14-APC/-FITC, CD25-APC/-FITC, CD40L-PE, mouse IgG-APC/-biotinylated/-FITC/-PE/-PerCP, rat IgG-PE, goat anti mouse IgG F(c)- PE (all BD Pharmingen Bioscience Heidelberg, Germany), CD45RA-APC (Caltag, Burlingame, CA, USA), CD4-PacificBlue, CD4-APC, CD8-PacificBlue, IgG-PacificBlue (all from Dako, Hamburg, Germany), FOXP3-PE (PCH101), PD1-FITC, ICOS-FITC, Streptavidin- APC (all Ebioscience, San Diego, CA, USA), HLA-G-biotinylated/-FITC/-PE (MEMG9, 87G), HLA-G5-biotinylated (5A6G7) (all Exbio, Praha, Czech Republic), CD95-PE (Beckman Coulter, Krefeld, Germany), CD31-FITC, CD103-FITC (Immunotools, Friesoythe, Germany), CTLA-4-FITC and CCR7-FITC (R&D, Minneapolis, USA).

For flow cytometry, PBMC were washed with PBS (supplemented with 0.1% bovine serum albumin and 0.1% sodium azide) and incubated with the appropriate combination of mAbs for 30 min at 4°C. Cells were analyzed with FACS-Calibur\textsuperscript{TM} using Cell Quest\textsuperscript{TM} software (Becton Dickinson, Heidelberg, Germany) or the CYAN, using Summit software (DakoCytomation, Hamburg Germany). To determine stability of HLA-G expression in culture over time, freshly isolated HLA-G\textsuperscript{pos} T cells were stained as mentioned above. For the following time points, HLA-G expression was determined by addition of a monoclonal anti-HLA-G antibody.
Cells from the CSF were stained as described before 27. For staining of CSF a minimum number of 6000 cells per sample were considered necessary for analysis.

Freshly isolated thymocytes were stained according to FACS staining of PBMC with an anti-CD4/-CD8/-HLA-G or anti-CD4/-CD8/-IgG control combination and analyzed by FACS. Due to the low frequency of HLA-G-positive cells, at least $2 \times 10^5$ thymocytes per test were measured.

For intracellular staining of the isoform HLA-G5 FACS sorted CD4 HLAG$^{\text{pos/neg}}$ T cells were stimulated with CD3/28 beads at ratio 1:1 (beads: cells) for 4 days. Before staining, cells were incubated for 4hrs at 37°C with GolgiStop protein transport inhibitor (BD Pharmingen Bioscience Heidelberg, Germany). After cell surface staining for CD4, cells were fixed and permeabilized with Fixation/Permeabilization solution (BD Pharmingen Bioscience Heidelberg, Germany) according to the manufacturer’s protocol. Staining with biotinylated HLA-G5 antibody or biotinylated mouse IgG1 as isotype control was followed by an incubation with APC- Streptavidin. Cells were analyzed by FACS.

**Immunohistochemistry**

Immunohistochemistry was performed essentially as described 26. Diagnostic muscle biopsy specimens were obtained from patients with inflammatory myopathies (polymyositis, n=5; dermatomyositis, n=5; inclusion body myositis, n=5), degenerative muscle disease (Duchenne muscular dystrophy, DMD, n=3) and non-myopathic controls (n=5). FlashSnap-frozen muscle biopsy specimens were cut into 8- to 10-mm cryostat sections. Acetone-fixed, air-dried sections were blocked and incubated with primary antibodies or corresponding IgG isotype controls. After blocking, secondary antibody was applied, followed by a staining with CD8 or CD3 FITC. Antibody binding was visualized by immunofluorescence microscopy and confocal laser microscopy (Cy3- or FITC-labeled secondary antibody). The following specific antibodies were used: HLA-G (87G, Exbio, Praha, Czech Republic), MHC class I (W6/32), CD3 (OKT3, both Dako, Hamburg, Germany), CD8 (B9.11; Beckman- Coulter, Fullerton, USA), Cy3-labeled goat anti mouse IgG Cy3 labelled (Dako).

**Cytokine Assays**

$1 \times 10^6$ FACS-sorted CD4 HLA-G$^{\text{pos/neg}}$ T cells were stimulated with CD3/CD28 beads (Dynal, Oslo, Norway) for 24 h in a 24 well plate in serum free medium (Ex vivo 15, Cambrex, Verviers, Belgium). Analysis of cytokines in supernatant was performed with a Raybio™ Inflammation Antibody Array I 1.1 (Tebu-bio GmbH, Offenbach, Germany) according to manufacture’s instruction. Briefly, after blocking membranes were incubated with the culture supernatant for 2 h followed by four wash steps. Then membranes were incubated with primary biotinylated antibody mix and subsequently with horseradish peroxidase conjugated streptavidin. Signals were detected by a chemiluminescence imaging system (Amersham Bioscience, Uppsala, Sweden).
ELISA for soluble HLA-G

ELISA for soluble HLA-G (HLA-G5 and shedded HLA-G1) was performed with a commercially available ELISA kit provided by Exbio (Prague, Czech Republic) according to the manufacturer’s instructions. Supernatants of CD4 HLA-G<sup>pos/neg</sup> T cells stimulated for 4 days with CD3/CD28 as well as supernatants from cultures of LCL- HLA-G1 as well as HeLa- HLA-G5 were tested. Concentration of soluble HLA-G is given in international units/ml.

HLA-Gpos T cells are present at sites of inflammation

As shown in Figure 7D of the article, consecutive cryosections of muscle from patients with polymyositis were stained with the HLA-G–specific mAb 87G, the CD8 mAb B9.11 either as single staining (left column) or as double immunofluorescence. Besides some muscle fibers (asterisk), part of the CD8 T cells (arrowhead and arrows), found to surround or even invade muscle fibers, also stains positive for HLA-G. Sections were analyzed using an Axiophot 2 fluorescence microscope (Zeiss, Oberkochen, Germany) and a Plan Neofluar phase-contrast objective lens. Original magnifications were as follows: HLA-G single staining, ×250/0.5 NA; CD8 single staining and CD8/HLA-G double staining, ×500/0.75 NA. Images were captured using a SPOT Insight 4Meg Firewire Color Mosaic camera (Diagnostic Instruments, Sterling Heights, MI) and processed using SPOT software.