Expression of the immune-tolerogenic major histocompatibility molecule HLA-G in multiple sclerosis: implications for CNS immunity

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HLA-G is a non-classical major histocompatibility complex (MHC) class I antigen with highly limited tissue distribution under non-pathological conditions. Although capable of acting as a peptide-presenting molecule, its strong immune-inhibitory properties identify HLA-G as a mediator of immune tolerance with specific relevance at immune-privileged sites such as trophoblast or thymus. To assess the role of HLA-G in CNS immunity, we investigated its expression in brain specimens from patients with multiple sclerosis ($n = 11$), meningitis ($n = 2$) and Alzheimer's disease ($n = 2$) and non-pathological CNS controls ($n = 6$). Furthermore, cultured human microglial cells and CSF of patients with multiple sclerosis and controls were assessed. Furthermore, CSF from MS patients and controls, as well as cultured human microglial cells were assessed. Using several HLA-G specific mAb and immunohistochemistry, HLA-G protein was found strongly expressed in brain specimens from patients with multiple sclerosis while it was rarely detectable in the non-pathological control specimens. In multiple sclerosis brain specimens, HLA-G immunoreactivity was observed in acute plaques, in chronic active plaques, in perilesional areas as well as in normal appearing white matter. In all areas microglial cells, macrophages, and in part endothelial cells were identified as the primary cellular source of expression. HLA-G was also found in other disease entities (meningitis, Alzheimer's specimens) where expression correlated to activation and MHC class II expression on microglial cells. Importantly, ILT2, a receptor for HLA-G, was also found in multiple sclerosis brain specimens thus emphasizing the relevance of this inhibitory pathway *in vivo*. HLA-G mRNA and protein expression and regulation could also be corroborated on cultured human microglial cells *in vitro*. Further, expression of HLA-G in the CSF of multiple sclerosis patients and controls was analysed by flow cytometry and ELISA. Monocytes represented the main source of cellular HLA-G expression in the CSF. Corresponding to the observations with the tissue specimens, CSF mean levels of soluble HLA-G were significantly higher in multiple sclerosis than in non-inflammatory controls (171 ± 31 versus 39 ± 10 U/ml; $P = 0.0001$). The demonstration of HLA-G and its receptor ILT2 on CNS cells and in areas of microglia activation implicate HLA-G as a contributor to the fundamental mechanisms regulating immune reactivity in the CNS. This pathway may act as an inhibitory feedback aimed to downregulate the deleterious effects of T-cell infiltration in neuroinflammation.

Introduction

HLA-G is a non-classical HLA class I antigen that is characterized by a tissue-restricted expression, a low polymorphism and seven isoforms (HLA-G1 to -G7) (Carosella *et al.*, 2003). HLA-G1 has a structure similar to that of classical HLA class I molecules: a heavy chain non-covalently associated with β2-microglobulin and a nonameric peptide. HLA-G has a highly limited tissue distribution under non-pathological conditions (Kovats *et al.*, 1990; Crisa *et al.*, 1997). Although in principle capable of presenting antigens to CD8 cells, HLA-G exerts overall negative immune regulatory functions: HLA-G inhibits allogeneic proliferation of T cells (Riteau *et al.*, 1999; Bainbridge *et al.*, 2000; Lila *et al.*, 2001; Wiendl *et al.*, 2003*a*), natural killer cell cytotoxicity (Rouas-Freiss *et al.*, 1997*a, b*; Khalil-Daher *et al.*, 1999; Riteau *et al.*, 2001), as well as antigen-specific T-cell cytotoxicity (Le Gal *et al.*, 1999; Wiendl *et al.*, 2003*a*). HLA-G exerts its direct immune-inhibitory function through three yet identified inhibitory receptors, ILT2 = CD85j, ILT4 = CD85d and KIR2DL4 = CD158d (Colonna *et al.*, 1998; Navarro *et al.*, 1999; Rajagopalan and Long, 1999).

Although under physiological conditions, the expression of HLA-G is very limited (Kovats *et al.*, 1990; Crisa *et al.*, 1997), HLA-G molecules are induced after allograft transplantation (Lila *et al.*, 2000, 2002; Rouas-Freiss *et al.*, 2003), during mixed lymphocyte reaction (Lila *et al.*, 2001) and may be one of the mechanisms used by tumour cells to escape immune surveillance (Paul *et al.*, 1998; Ibrahim *et al.*, 2001; Wiendl *et al.*, 2002). There is accumulating evidence demonstrating that HLA-G is induced in the course of inflammatory pathologies such as myositic lesions (Wiendl *et al.*, 2000), psoriatic skin lesions (Aractingi *et al.*, 2001) or in atopic dermatitis (Khosrotehrani *et al.*, 2001), and seems to play important functions at immunologically privileged sites, such as the thymus (Crisa *et al.*, 1997; Mallet *et al.*, 1999) or the cornea (Le Discorde *et al.*, 2003). Therefore, HLA-G has been proposed as a mechanism to protect target tissues from auto-aggressive inflammation and to serve as a fundamental mechanism of immune surveillance (Carosella *et al.*, 2001; Wiendl *et al.*, 2003*c*).

The brain has long been considered an immunologically privileged site. This idea is based on the observation that tissue transplants in the CNS are not commonly rejected by the immune system (Medawar, 1948; Barker and Billingham, 1977). An anti-inflammatory and, with regard to invading immune cells, pro-apoptotic environment in the brain, the limited access of brain-derived antigens to the lymphoid organs, the presence of the blood–brain barrier, low major histocompatibility complex (MHC) expression in the brain parenchyma and the absence of dendritic cells are used to explain the lack of an effective immune response to antigens in the brain. However, numerous studies in infectious, autoimmune and tumour models have challenged this view by showing that potent immune reactions can and do occur in the CNS (Hickey, 2001). Multiple sclerosis is the prototypic autoimmune inflammatory disorder of the CNS. T cells play a pivotal role in orchestrating the complex cascade of events hallmarked by chronic inflammation, primary demyelination and axonal damage (Hohlfeld, 1997; Noseworthy *et al.*, 2000; Lassmann *et al.*, 2001; Hemmer *et al.*, 2002).

Here, we assessed the role of the immune-tolerogenic MHC molecule HLA-G in CNS immunity by studying the expression of HLA-G on CSF cells, cultured microglial cells and in tissue sections from individuals with multiple sclerosis and various controls. The demonstration of HLA-G and its receptor ILT2 on CNS antigen-presenting cells and in areas of CNS inflammation suggests that this pathway may act as an inhibitory feedback aimed at downregulating the deleterious effects of T-cell infiltration under neuroinflammatory conditions. Further, upregulated expression of HLA-G on CNS monocytes implies an important function of HLA-G in the basic immune surveillance mechanisms of the CNS.

Materials and methods
Antibodies and reagents

The following monoclonal antibodies were used: anti-HLA-G mAb 87G and O1G (both specific for HLA-G1 and -G5), anti-HLA-G mAb 16G1 (specific for the soluble HLA-G5 isoform) (these antibodies being kindly provided by D. Geraghty, Seattle, WA); MEM-G9, Exbio, Praha, Czech Republic); anti-MHC I (TP25.99; kindly provided by S. Ferrone, Buffalo, NY); anti-ILT2 (GH75) (kindly provided by M. Colonna, Washington University, St Louis, MO), anti-MHC-II (L243; ATCC, Rockville, MD); anti-CD3 (PS1; Novocastra, Newcastle, UK), anti-GFAP (DAKO, Glostrup, Denmark), anti-lysozyme (DAKO), anti-CD14-PerCP (MΦP-9), anti-CD16-PE (3G8), anti-CD19-APC (HIB19), streptavidin-FITC (all BD Pharmingen, Heidelberg, Germany), peroxidase-labelled rabbit anti-human β2-microglobulin (DAKO); unlabelled and PE-labelled goat anti-mouse IgG (H + L) F(ab)2 fragment (Dianova, Hamburg, Germany). All antibodies were titrated for optimized concentrations in flow cytometry and immunohistochemistry. Normal goat serum was from Dianova, human IgG (Alphaglobin®) was obtained from Grifols (Langen, Germany). For immunofluorescence double-labelling studies, primary antibodies against HLA-G (87G, 01G, 16G1, all mouse monoclonals), GFAP (rabbit polyclonal; DAKO) and lysozyme (rabbit polyclonal; DAKO) were used followed by appropriate Cy2- and Cy3-conjugated secondary antibodies (Dianova).

Biotinylation of anti-HLA-G mAb MEM-G9

Anti-HLA-G mAb MEM-G9 was biotinylated using the commercially available EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce, Rockford, IL). Briefly, 2 mg of MEM-G9 antibody were incubated with an excess of biotin (2.66×10^{-4} nmol biotin) for 30 min. Thereby, the NHS-activated biotins react efficiently with the primary amino groups ($-NH_2$) of the antibody to form stable amide bonds. Biotinylation was tested via flow cytometry using HLA-G1 transfectants (Wiendl *et al.*, 2002).

Brain tissue specimens and pathological classification

We investigated CNS tissue samples from 11 patients with clinical and histopathological diagnosis of multiple sclerosis. These were obtained from the Human Brain and Spinal Fluid Resource Center, Los Angeles, CA ($n = 5$, post-mortem material, patients' mean age 55.4, range 50–60 years, 3 female, 2 male), the UK Multiple Sclerosis Tissue Bank ($n = 5$, post-mortem material, mean age 56.4, range 38–73, 4 female, 1 male), and the Department of Neuropathology, University of Goettingen ($n = 1$, biopsy material, 54 years, female). As controls, we investigated CNS specimens from patients with meningitis ($n = 2$, 1 male, 1 female, age 1–2 years), specimens from 2 patients with Alzheimer disease ($n = 2$, 1 male, 73 years, CERAD C, Braak & Braak V and 1 female, 80 years, CERAD C, Braak and Braak VI), and specimens classified as non-pathological CNS controls. The latter were obtained from the Human Brain and Spinal Fluid Resource Center, Los Angeles, CA ($n = 5$, post-mortem material, patients mean age 78.6; range 54–91, 4 female, 1 male) and the Brain Bank of the Institute of Brain Research Tübingen ($n = 1$, 33 years, male).

Concerning the multiple sclerosis specimens, lesions were classified depending on the presence of macrophages containing oil-red-O positive degradation products, perivascular inflammatory infiltrates and demyelination (Bruck *et al.*, 1995). Based on these criteria, acute and chronically active as well as inactive plaques with pathological features of demyelination and/or inflammation could be defined. In addition, tissue samples from macroscopically normal appearing white matter (NAWM) from each multiple sclerosis case were studied. Unfortunately, information concerning the underlying disease course was not available in most of the cases studied.

For this study, only frozen sections were used. To establish the immunohistochemical stainings for HLA-G and ILT2, frozen human tissue specimens from pathological cases or organs previously described as immunoreactive for HLA-G were investigated (Blaschitz *et al.*, 1997; Wiendl *et al.*, 2002). These tissues were from the Brain Bank of the Institute of Brain Research (University of Tübingen) and included human glial neoplasms (1 astrocytoma, WHO grade II and 1 glioblastoma, WHO grade IV), 2 human placenta specimens and 3 normal CNS control specimens obtained from deeper biopsies when the surface regions were not involved in pathological processes.

Immunohistochemistry

The tissue specimens were snap-frozen in liquid nitrogen. The samples were cut in 10 μm slices and stored at $-20°C$. Slides were fixed in acetone for 10 min. Histological stainings for haematoxylin and eosin (HE), Luxol-fast blue (LFB) and periodic acid–Schiff (PAS) were done according to routine protocols. Immunohistochemical tissue labelling was performed by using the Benchmark immunohistochemistry system (Ventana, Strasbourg, France). For antibody labelling of HLA-G, endogenous peroxidase of the tissue sections was blocked with 3% H_2O_2 in methanol for 14 min. To establish HLA-G immunohistochemistry, anti-HLA-G-antibody (87G) was applied in dilutions between 1 : 10 and 1 : 100, finally showing best results for a dilution of 1 : 100 for 32 min. The two other HLA-G antibodies were applied at a dilution of 1 : 250 (01G and 16G1). Dilution for MHC-II staining was 1 : 100, for ILT2 it was 1 : 3. An avidin and a biotin blocker were applied to the samples for 4 min, respectively, followed by an 8-min incubation with one drop of I-View-Biotin

Ig (Ventana). For DAB visualization, the sections were incubated with one drop of I-View SA-HRP for 8 min and then with DAB/H_2O_2 for additional 8 min. Finally, the sections were incubated with a copper enhancer (Ventana) for 4 min, washed, counterstained with haematoxylin and mounted. Evaluation of the immunohistochemical stainings and photographic documentation was performed using an Olympus Vanox AH-3 light microscope (Hamburg, Germany). Double-labelling immunohistochemistry was performed with antibodies against HLA-G (87G) and macrophages (lysozyme) and astrocytes (GFAP) on fresh frozen multiple sclerosis biopsy tissue. Briefly, sections were fixed in ice-cold acetone, air-dried and incubated with antibodies against HLA-G at a dilution of 1 : 50. Biotinylated anti-mouse secondary antibodies and avidin-Cy2 were applied sequentially for 1 h each and followed by antibodies against GFAP (rabbit polyclonal; 1 : 50) or lysozyme (rabbit polyclonal; 1 : 200), respectively, overnight. Cy3 coupled to anti-rabbit antisera was used to visualize bound primary antibody. Sections were viewed and analysed using an Olympus BX51 fluorescence microscope equipped with a Color View Soft Imaging Systems© digital camera. Fluorescent images were superimposed with AnalySIS® software.

Isolation and culture of adult human microglial cells, monocytes and T cells

The studies on human glial cells were performed in accordance with the guidelines set by the Ethics Review Board of the Montreal Neurological Institute, McGill University (Montreal, Canada), according to the guidelines of the Canadian Institutes for Health Research (CIHR). Primary adult human glial cells were obtained from surgical resections performed for the treatment of non-tumour-related intractable epilepsy. Tissue was obtained from regions requiring resection to reach the precise epileptic focus and was distant from the main electrically active site. Dissociated cultures of microglia were prepared as previously described, based on the differential adherence of the glial cells (Yong *et al.*, 1992). Briefly, brain tissue was subjected to enzymatic dissociation with trypsin (0.025%) and DNase I (25 μg/ml) (Boehringer Mannheim, Laval, QC) for 30 min at 37°C, followed by mechanical dissociation by passage through a 132 μm nylon mesh (Industrial Fabrics Corporation, Minneapolis, MN). Cells were further separated on a linear 30% Percoll density gradient (Pharmacia LKB, Baie D'Urfé, QC) and centrifuged at 15 000 r.p.m. at 4°C for 30 min. The cells recovered from the interface contained a mixed glial cell population consisting of ∼65% oligodendroglia, 30% microglia and 5% astrocytes. To enrich for microglia, the mixed cell population was suspended in minimal essential culture medium (MEM), supplemented with 5% FCS, 2.5 U/ml penicillin, 2.5 μg/ml streptomycin, 2 mM glutamine and 0.1% glucose (Life Technologies, Burlington, ON) and left overnight in 12.5 cm^2 tissue culture flasks (Falcon, Fisher Scientific, Montréal, QC) in a humid atmosphere at 37°C with 5% CO_2. The non-adherent oligodendroglia were removed by gentle pipetting and the remaining adherent microglia were allowed to develop morphologically for 3 days. Remaining microglia were of 95% purity as assessed by immunocytochemistry and flow cytometry (Yong *et al.*, 1992; Becher and Antel, 1996). Microglia were cultured for ∼7 days and then harvested using trypsin (0.25%)–EDTA.

For monocyte isolation, blood was obtained by venipuncture and PBMC were isolated by density gradient centrifugation using Lymphocyte Separating Solution (PAA Laboratories GmbH, Linz,

Austria). Monocytes were enriched by 1 h adherence to plastic flasks at 37°C in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% FCS (Biochrom, Berlin, Germany) and penicillin (100 IU/ml)/streptomycin (10 μg/ml) (Life Technologies). Non-adherent cells were removed. Adherent cells were detached using cell dissociation buffer and purity was analysed by flow cytometry (>90% CD14 positive cells). Where indicated, monocytes were stimulated with supernatants from Th (T-helper) 1 or Th2 cells as described (Kim *et al.*, 2004).

Th1 and Th2 polarized glatiramer acetate (GA)-reactive T cells were generated from normal individuals by combining an IL-7 based assay with a traditional split-well paradigm as previously described (Kim *et al.*, 2004). Briefly, PBMC were isolated as described above and plated at 2×10^5 per well in 96-well plates in medium containing 10 ng/ml of IL-7 (PeproTech Inc., Rocky Hill, NJ) without antigen, or with GA, a four-amino acid copolymer known to generate high-frequency T-cell proliferation in a TCR-mediated and MHC-restricted fashion. Th1 and Th2 polarization was achieved by concurrent addition of either IL-12 (2.5 ng/ml) and anti-IL-4 blocking antibody (2.5 μg/ml), or the addition of IL-4 (50 ng/ml) and anti-IL-12 blocking antibody (5 μg/ml, all from R&D Systems, Minneapolis MN), respectively. After 7 days, 100 μl of supernatant were removed from each well and replaced with fresh medium enriched with rhIL-2 (20 U/ml final concentration). On day 11, each well was evaluated for specificity to antigen in a split-well assay: equal aliquots of cells from each well were restimulated with or without antigen, with freshly isolated autologous irradiated PBMC (3000 rad). Three days after restimulation (Day 14), supernatants were removed and 18 h [³H]-thymidine uptake was measured (beta scintillation counter) on equal portions of the cells to confirm antigen-reactive wells. A 'positive' (confirmed GA-reactive) well was stringently defined as an antigen-pulsed well with an SI >3 compared with its corresponding 'no antigen' well, and with absolute proliferation of at least 3 SD above the mean of all 'no antigen' wells. The remaining cells of confirmed antigen-reactive wells were resuspended in fresh medium for an additional 3–10 days (total of 6–13 days after stimulation). Supernatants were then removed and assayed for IFN-γ and IL-5 by ELISA to confirm their Th1 or Th2 profile. Supernatants from Th1-polarized GA-reactive lines typically produced 2000–4000 pg/ml of IFN-γ and <100 pg/ml of IL-5. In contrast, supernatants from Th2-polarized GA-reactive lines typically produced <300 pg/ml of IFN-γ and 1500–3500 pg/ml of IL-5.

Isolation and characterization of fetal microglia

Human CNS tissue (cerebral hemispheres) from fetuses at 17–23 weeks of gestation was obtained from the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY), following CIHR-approved guidelines. As previously described (D'Souza *et al.*, 1995), mixed glial cultures were obtained by dissociation of the fetal CNS with trypsin and DNase I followed by mechanical dissociation. After washing, the cell suspension was plated at a concentration of $3–5 \times 10^6$ cells/ml in DMEM supplemented with 10% FCS, antibiotics, glutamine and glucose. To isolate microglia, the mixed CNS cell culture (containing astrocytes, neurons and microglia) was allowed to differentiate for 2 weeks. The flasks were shaken at 180 r.p.m., and the floating microglia were subsequently harvested from the supernatant. Fetal microglia were plated in flasks, and after 2 h, contaminating non-adherent cells were removed and phenotypic and functional assays were initiated within 48 h.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from all cell types, following lysis with TRIzol (Invitrogen) using the Qiagen RNeasy mini kit (Qiagen, Mississauga, Ontario) according to manufacturer's instructions. For microglia, Qiagen Minelute columns were used to concentrate RNA in a small volume (12 μl). All RNA samples isolated were treated with DNase (Qiagen). Reverse transcription was performed on 3 μg RNA, except for microglia where total RNA (<3 μg) was used. Briefly, cDNA was generated using random hexaprimers (Roche) with the Moloney murine leukaemia virus (MMLV)-RT enzyme (Invitrogen) at 42°C. Quantitative real-time PCR (QRT–PCR) was performed as described (Wiendl *et al.*, 2003*b*) utilizing the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Data were analysed with the ABI Prism® Detection System using the comparative C_T (threshold cycle) method (Applied Biosystems, User Bulletin). Samples were normalized to 18S rRNA, to account for the variability in the initial concentration of the total RNA and the conversion efficiency of the RT reaction. Standard curves were generated for each gene using 18S, HLA-G1 and HLA-G5 plasmids. Normalization of samples was performed by dividing the copies of the gene of interest by copies of the reference gene, 18S rRNA. Oligonucleotides for the detection of HLA-G1/2 and HLA-G5/6 mRNA have been described (Mitsdoerffer *et al.*, 2005).

CSF and blood samples

Paired blood and CSF samples were obtained from 21 patients (8 female, 13 male) who were referred to our centre (University of Tübingen) for diagnostic lumbar puncture [14 patients with multiple sclerosis, mean age 36 years (28–54); 7 patients with other neurological diseases (OND), mean age 56 years (20–79)] (Table 1). Patients with multiple sclerosis were diagnosed according to criteria of McDonald *et al.* (2001). Patients recruited for the study were without corticosteroids for at least 2 months, and had never been on immune-modulatory therapy. Controls included patients with OND such as dementia, normal pressure hydrocephalus or vertigo, who underwent spinal fluid analysis for differential diagnostic reasons (Table 1). All patients gave informed consent according to a protocol approved by the local ethics committee. CSF (8–20 ml) was obtained by lumbar puncture from all patients. At the same time, 10 ml peripheral blood was collected by venous puncture.

Flow cytometry analysis

Peripheral blood and CSF were analysed by multicolour flow cytometry (Cepok *et al.*, 2001). Briefly, fresh blood was diluted 1 : 1 with PBS supplemented with 2% FCS. For each staining, 200 μl of the mixture was added to a well of a round-bottom 96-well plate, spun down (220 *g* for 5 min) and the supernatant discarded. Fresh CSF was immediately spun down (220 *g* for 10 min), the supernatant was removed and the pellet was resuspended in 25 μl of PBS (containing 2% FCS) per staining with a minimum number of 6000 cells necessary for each staining. Pre-prepared antibody mixes (5 μl) were added to blood and CSF samples. CSF cells were stained with anti-HLA-G-biotinylated-streptavidin-FITC (MEM-G9), anti-CD14-PerCP and anti-CD16-PE and anti-CD19-APC or the respective isotype controls. After incubation (30 min, 4°C), erythrocyte lysis was performed for the blood stainings, followed by washing steps. No lysis was necessary for CSF samples. Cell numbers (per microlitre) in

Table 1 Characteristics of patients with multiple sclerosis (MS) and other neurological diseases (OND)

Number	Sex	Age (years)	Diagnosis/ classification	Acute relapse
MS				
MS1	M	42	RRMS	Yes
MS2	M	37	RRMS	No
MS3	M	38	RRMS	Yes
MS4	F	40	CIS	Yes
MS5	M	37	CIS	Yes
MS6	M	29	CIS	No
MS7	M	41	CIS	No
MS8	F	54	SPMS	Yes
MS9	M	50	RRMS	Yes
MS10	F	39	RRMS	Yes
MS11	F	28	RRMS	No
MS12	F	42	RRMS	Yes
MS13	F	32	RRMS	Yes
MS14	F	37	RRMS	No
OND				
OND1	M	24	Benign paroxysmal vertigo	
OND2	M	67	Nystagmus of unknown origin	
OND3	F	20	Pseudotumour cerebri	
OND4	M	56	Polyneuropathy	
OND5	M	75	Syncope	
OND6	M	79	Dementia	
OND7	M	76	Dementia	

RRMS: relapsing remitting disease; CIS: clinically isolated syndrome; SPMS: secondary progressive disease.

the CSF ranged from 1 to 15. Surface expression of immune molecules was quantified by flow cytometry on a fluorescent activated cell sorter (FACSCalibur cytometer, Becton Dickinson, Heidelberg, Germany) using standard methods (Wiendl *et al.*, 2002). Histograms of stained monocytes were analysed by calculating the specific fluorescence index (SFI: specific geometric mean: geometric mean of unspecifc isotype). For dot plots quadrant analysis was performed.

For analysis of HLA-G expression on cultures of human microglia, Fc receptors were blocked overnight with 10% human serum in PBS supplemented with 1% FCS and sodium azide (0.1%). HLA-G was detected using the 87G mouse IgG2b antibody in combination with PE-conjugated goat anti-mouse IgGs. FITC-conjugated CD14 was used to double stain for the microglial cell surface marker. Isotypes matched for concentration of the primary antibodies were used for all stainings. Cells were fixed in 1% formaldehyde and were acquired on a FACSCalibur (Becton Dickinson) and analysed using FlowJo software (Treestar, Ashland, OR).

ELISA for soluble HLA-G

ELISA for soluble HLA-G was performed with a commercially available ELISA kit provided by Exbio according to the manufacturer's instructions. Samples were concentrated by lyophilization prior to analysis. As an internal standard for the detection threshold and the amount of HLA-G, we used supernatants from HLA-G5 transfectants (Wiendl *et al.*, 2002).

Statistical analysis

For statistical analysis of the blood and CSF, we used paired *t*-tests. Each paired *t*-test was conducted for multiple sclerosis patients as well as the OND controls. For comparison of acute versus stable disease, unpaired *t*-tests were applied. The *P* values were calculated two-tailed in all cases and considered statistically significant at $P < 0.05$.

Results

Expression of HLA-G in the CNS

We analysed the presence and distribution of HLA-G immunoreactivity in sections from patients with multiple sclerosis ($n = 11$). To characterize the lesions, LFB (Fig. 1A), MHC-class II (Fig. 1B), PAS (Fig. 1C) and HE stainings (not shown) were performed. In chronic active plaques, strongest HLA-G immunoreactivity was detected in the transition zone between the plaque centre and the perilesional areas (Fig. 1D–F). This pattern could be observed for three HLA-G antibodies used for this analysis, namely mAb OG1 (Fig. 1D), mAb 87G (Fig. 1E) (both detecting HLA-G1 as well as HLA-G5), as well as mAb 16G1 (detecting only soluble HLA-G5) (Fig. 1F). A sharp decline of HLA-G immunoreactivity was observed in the junction zone between white and grey matter, with HLA-G-reactivity virtually restricted to the white matter. In the vicinity of MS lesions, the adjacent normal appearing grey matter remained predominantly negative for HLA-G whereas HLA-G expression in adjacent normal appearing white matter was similar to the expression levels of the lesion borders (data not shown). In early and highly inflammatory MS lesions, HLA-G expression was abundant and detected on macrophages/activated microglia cells (Fig. 1G–I). Similarly, perilesional activated microglia cells were immunoreactive for HLA-G (Fig. 1G). In contrast, HLA-G-immunoreactivity was very low in the non-pathological control specimens: in five of the six control cases, HLA-G immunoreactivity was virtually undetectable (Fig. 1J). One control case showed foci of HLA-G immunoreactive areas (data not shown). However, these were less intense and less frequent as compared with brain tissue specimens of patients with multiple sclerosis. Here, frequency and quantity of HLA-G expressing microglia cells was within the range of 'constitutively' MHC-II expressing microglial cells that have been described to occur in the CNS under physiological conditions (Mittelbronn *et al.*, 2001). Of note, endothelial cells (Fig. 1K) and meningeal vessels as well as arachnoidal cap cells (Fig. 1L) show HLA-G immunoreactivity. Of note, immunoreactivity of endothelial cells was not significantly different between the non-pathological and the pathological samples.

In order to delineate whether HLA-G expression in the CNS is a phenomenon specifically related to multiple sclerotic lesion pathogenesis or rather a basic mechanism of immunoregulation in the CNS, we assessed other disease entities characterized by primary or secondary CNS inflammation. In specimens from patients with Alzheimer's disease ($n = 2$) as well as in meningitis ($n = 2$), we could also detect strong
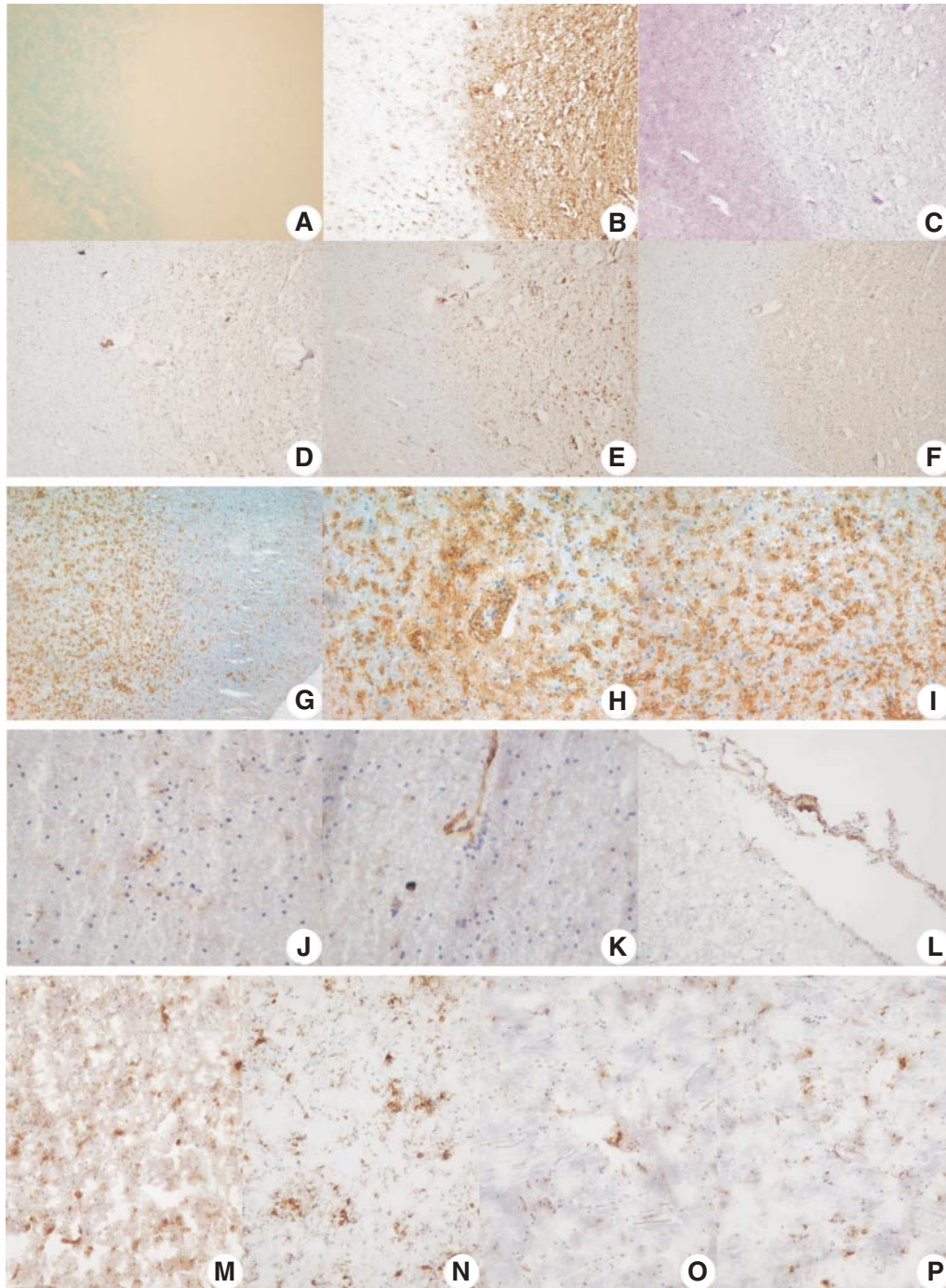
Fig. 1 HLA-G immunoreactivity in the normal and pathological CNS. (**A**–**I**) Presence of HLA-G immunoreactivity in multiple sclerosis brain specimens: serial sections of a chronic active MS lesion were characterized with LFB [**A**; original magnification (OM) ×10], MHC-class II (**B**; OM ×10), and PAS (**C**; OM ×10) and stained for HLA-G using 3 different monoclonal antibodies [mAb 01G (**D**), mAb 87G (**E**), mAb 16G1 (**F**) OM ×10]. Strong HLA-G immunoreactivity in association to the plaque border zone is observed (**D**–**F**). The pattern of HLA-G immunoreactivity is similar for the 3 anti-HLA-G mAbs and parallels immunoreactivity for MHC-class II antigens (**B**). In an early inflammatory multiple sclerotic lesion (**G** and **H**–**I**), HLA-G immunoreactivity (mAb 87G) is clearly associated with the lesion and decreases with distance to the lesion centre (**G**; OM ×4). HLA-G immunoreactivity is observed in areas with lymphocytic (**H**; OM ×10) and monocytic (**I**; OM ×10) inflammation. (**J**–**L**) Presence of HLA-G immunoreactivity in non-pathological brain specimens: HLA-G expression (mAb 01G) is rarely detectable in non-pathological CNS specimens (**J**; OM ×40). However, endothelial cells (**K**; OM ×40) as well as meningeal vessel and arachnoidal cap cells (**L**; OM ×20) show HLA-G expression under non-pathological conditions (**K** and **L**). (**M**–**P**) Presence of HLA-G immunoreactivity in other CNS diseases. CNS tissue specimens with primary or secondary inflammatory components [meningitis (**O** and **P**) and Alzheimer's disease (**M** and **N**)] were analysed. HLA-G immunoreactivity (here shown mAb 01G) is detectable in Alzheimer's disease (**M**; OM ×20) and in meningitis (**O**; OM ×20) and correlates with the distribution patterns of MHC-class II staining (**N** and **P**).

HLA-G immunoreactivity (Fig. 1M and O). HLA-G reactivity correlated to the expression of MHC-II (Fig. 1N and P), indicating a state of activation on (micro)glial cells in the CNS.

In the hypocellular gliotic centre of demyelinated plaques in chronic active plaques, the main source of HLA-G were macrophages, showing small excentric nuclei within a large foamy cytoplasm with a low nuclear to cytoplasmic ratio (Fig. 2A and B). Encompassing the plaque in both chronic active and inactive multiple sclerosis cases, a strong circular area of HLA-G-immunoreactive microglial cells was detected (Fig. 2C; chronic active plaque). Almost all cells in this area presented with a rod- to oval-shaped nucleus and multiple thin or coarse cytoplasmic ramifications, demonstrating their microglial origin and different stages of activation (Fig. 2C). In contrast, oligodendroglial or astroglial cells did not show

HLA-G immunoreactivity. Interestingly, in the normal appearing white matter of multiple sclerosis brain specimens, HLA-G expression was comparable to the expression in periplaque white matter areas showing comparable strong HLA-G staining patterns for ramified and activated microglia (Fig. 2D). In contrast, in the grey matter located near the multiple sclerotic lesions, neither neuronal cells nor glial cells showed HLA-G-immunoreactivity (Fig. 2E).

To corroborate our findings on the cellular source of HLA-G expression, we performed additional double-labelling experiments. We analysed an acutely inflamed early multiple sclerotic lesion characterized by the presence of abundant MHC class II-positive microglia/macrophages in the lesion centre. HLA-G-positive cells in the plaque stained negative for GFAP (Fig. 2F) but positive for lysozyme (Fig. 2G), therefore underlining their monocytic origin and excluding
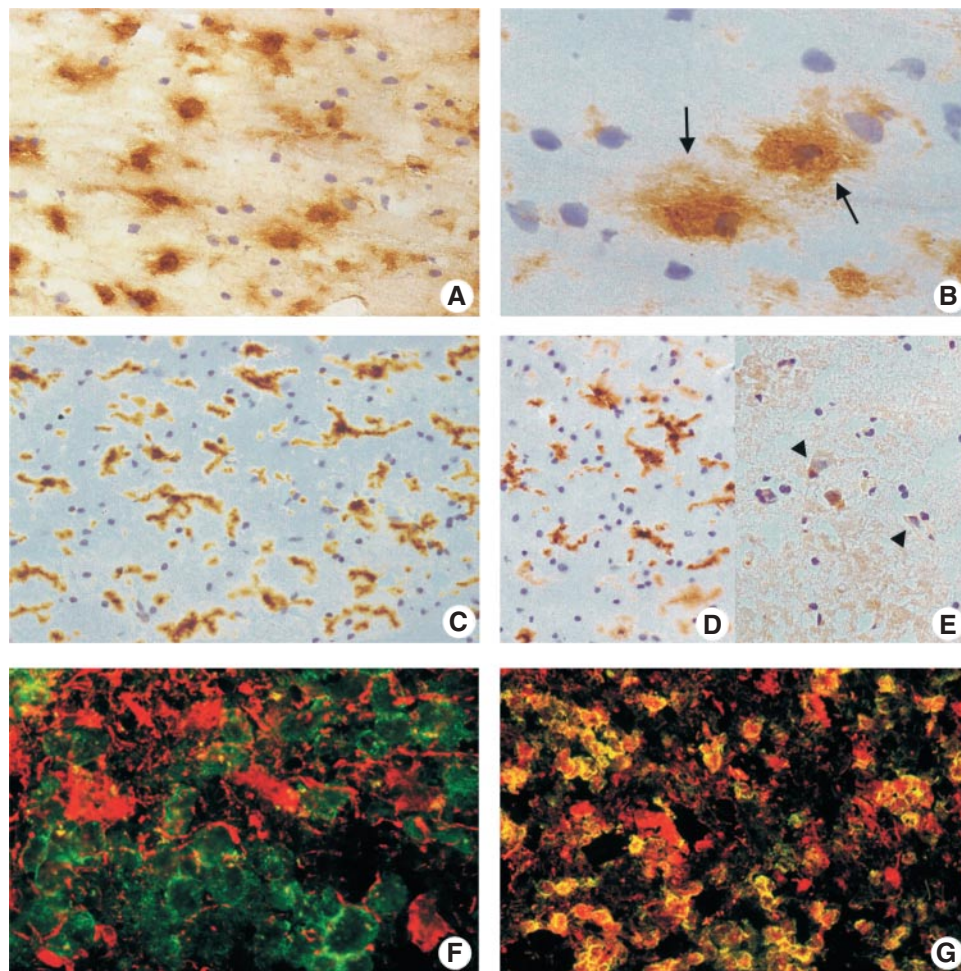
Fig. 2 Macrophages and microglial are the main cellular source of HLA-G-expression in the CNS. (**B**) In chronic active MS plaque centres, HLA-G reactive cells mainly represented macrophages (**A**, OM ×40; **B**, arrows; higher magnification OM ×100; mAb 87G). (**C**) In the periplaque areas, ramified and activated microglia displayed strong HLA-G immunoreactivity (OM ×20). (**D**) In normal appearing white matter of multiple sclerosis brain specimens, strong HLA-G expression predominantly could be attributed to ramified and activated microglia comparable to periplaque areas (×20). (**E**) In grey matter areas of multiple sclerosis, no HLA-G immunoreactivity could be observed (neurons indicated by arrowheads) (×20). (**F**) Double fluorescence labelling for HLA-G (FITC, green) and GFAP (Cy3, red) excluded astrocytes as source for HLA-G expression in active multiple sclerosis plaques. (**G**) Double fluorescence labelling for HLA-G (FITC, green) and lysozyme (Cy3, red) corroborates microglia/macrophages as main source of HLA-G expression in multiple sclerosis brain specimens.

astrocytes as the source for HLA-G expression. Taken together, HLA-G immunoreactivity was strongly upregulated in multiple sclerotic lesions while virtually absent in the normal brain. Microglia, macrophages as well as endothelial cells were identified as cellular sources of HLA-G expression in the inflamed CNS.

Presence of the HLA-G-receptor ILT2 in the CNS under normal and pathological conditions

The members of the immunoglobulin-like transcript family ILT2 and ILT4 have been described as inhibitory receptors for HLA-G. To further validate the relevance of HLA-G expression *in vivo*, we next assessed the expression of ILT2 in pathological and non-pathological CNS specimens. In the normal human CNS, ILT2 immunoreactive cells were rarely detectable (data not shown). In chronic active MS plaques, ILT2 immunoreactivity (Fig. 3B) could be observed in the plaque centre and the plaque border and paralleled HLA-G immunoreactivity (Fig. 3A). The main cellular sources for both molecules were macrophages and microglia (Fig. 3C). In actively demyelinating plaques, occasionally ILT2 was also found to be expressed in lymphocytic infiltrates (Fig. 3D). Of note, ILT2 reactivity was only occasionally found on CNS lymphocytic infiltrates. It is important to note that the expression of ILT2 on the cell surface is depending on the activation status and differs within different lymphocytic subpopulations. In our view, this is the most probable explanation for the pattern of ILT2-immunoreactivity that we observed.

HLA-G mRNA and protein expression by cultured human microglial cells: upregulation in response to inflammatory stimuli

To corroborate the results achieved by immunohistochemistry, we examined cultured human adult microglial cells for the expression of transcripts encoding the main transmembranous (HLA-G1/2) as well as the main soluble (HLA-G5/6) HLA-G isoforms by QRT–PCR. In parallel, we examined human peripheral blood monocytes, which were previously described to constitutively express HLA-G mRNA and protein (Yang *et al.*, 1996; Lozano *et al.*, 2002; Mitsdoerffer *et al.*, 2005). In order to assess whether HLA-G expression is differentially regulated by soluble products secreted by T-cell lines characterized as Th1 or Th2-type (based on high levels of IFN-γ or IL-5, respectively), cultures of microglia were exposed to Th1/2 supernatants for 48 h. mRNA transcripts of HLA-G1/2 as well as HLA-G5/6 were expressed both by monocytes and microglial cells under basal culture conditions (Fig. 4). Exposure to distinct inflammatory environments, either Th1 or Th2 supernatants, led to the upregulation of microglial HLA-G mRNA, which was stronger with Th1 than with Th2 supernatants. Monocytes showed the same pattern of upregulation in response to Th1/2 stimuli; the proinflammatory Th1 supernatants induced higher levels of HLA-G expression than Th2 supernatants.

We next assessed HLA-G expression on the protein level. We found that human microglia, isolated from both the adult ($n = 1$) and fetal CNS ($n = 2$), can express HLA-G at the protein level, especially under IFN-γ-treated conditions, as
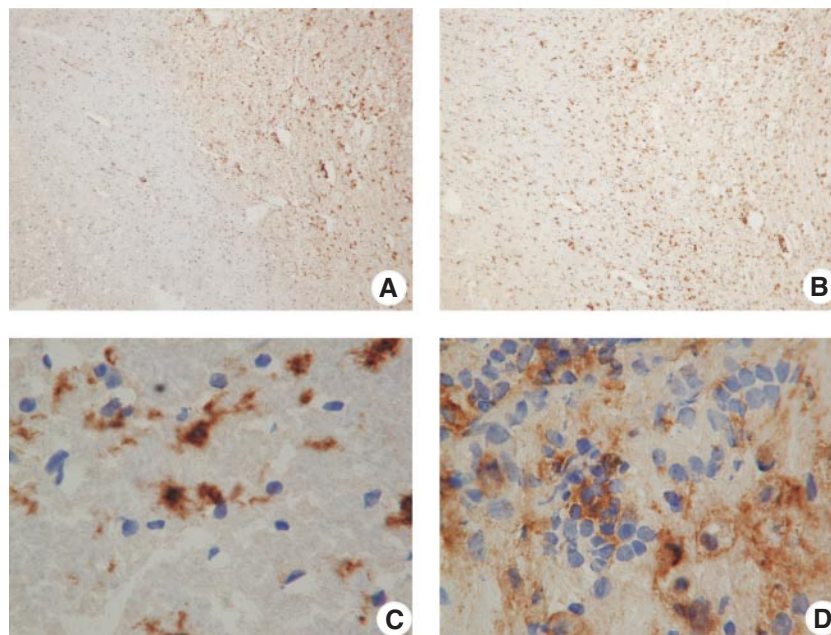
Fig. 3 Expression of the HLA-G receptor ILT2 in human multiple sclerosis brain specimens. In multiple sclerotic lesions, immunoreactivity for the HLA-G receptor ILT2 is detectable. The expression of ILT2 (**B**; OM ×10; chronic active plaque) correlated to the distribution pattern of HLA-G (**A**; OM ×10; mAb 87G). Microglia and macrophages were identified as main source of ILT2 expression (**C**; OM ×100). Furthermore, also in acute lymphocytic infiltrates, clear ILT2 immunoreactivity was detected (**D**; OM ×100).
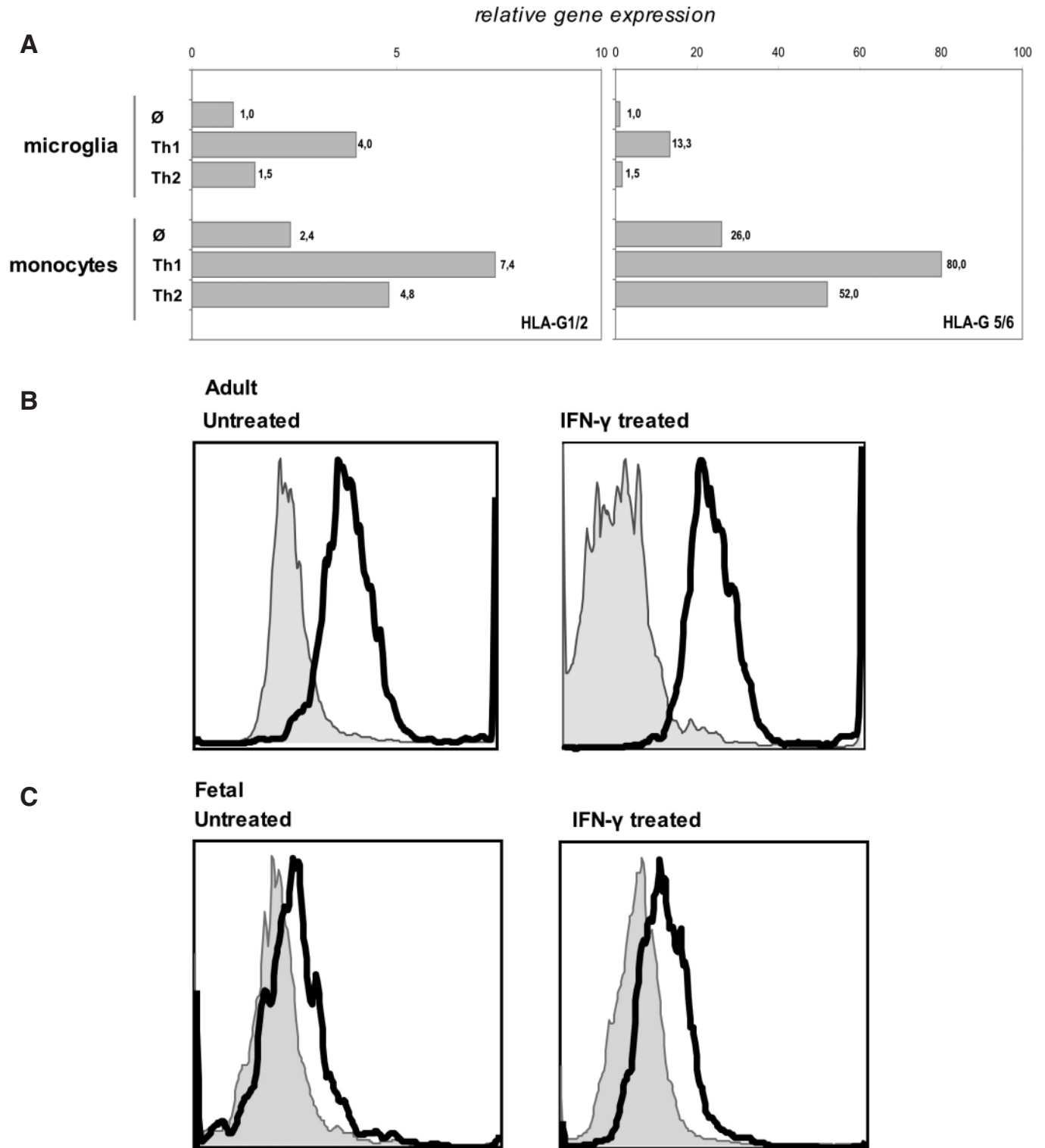
Fig. 4 Expression and modulation of HLA-G1/2 and HLA-G5/6 mRNA and HLA-G protein in human microglial cells. Human microglial cells were cultured in the absence or presence of Th1 or Th2 supernatants, or IFN-γ (500 U/ml), harvested after 48 h of induction and analysed for the expression of HLA-G (mAb 87G) at the mRNA in **A** and at the protein level in **B** and **C**. HLA-G1/2 (**A**, left) and HLA-G5/6 (**A**, right) expressions were determined by QRT–PCR in adult microglia and, in parallel, in monocytes from an independent donor. Bars and numbers represent the relative gene expression of indicated molecules calculated in relation to unstimulated microglia (set to 1). Flow cytometry was used to detect cell surface expression of HLA-G (mAb 87G) in cultures of untreated (left) or IFN-γ treated (right) microglia isolated from adult (**B**) or fetal (**C**) brain. Cells were double-stained for the marker CD14 and histograms represent staining for HLA-G (thick black line) over the isotype control antibody (grey-filled histograms) on CD14-gated cells.

detected by flow cytometry (Fig. 4B and C). In two out of three preparations (both fetal microglial preparations), IFN-γ treatment led to an increase in the HLA-G stain over the isotype control antibody, in comparison with the untreated microglia (Fig. 4C). In one preparation, no HLA-G protein was detectable under basal conditions, but was strongly upregulated in the presence of IFN-γ treatment (SFI from 5.8 to 48.5) (data not shown). Thus, under basal culture conditions (which vary in microglial cells *in vitro*), there is a variable level of HLA-G expression, but under proinflammatory/activated conditions HLA-G is consistently detected. In other words, HLA-G can be upregulated by IFN-γ *in vitro* if microglia are not basally activated. If there is a basal level of activation, HLA-G is expressed but upregulation seems little or absent.

Expression of HLA-G in the CSF of patients with multiple sclerosis and controls

Expression of HLA-G on CSF monocytes

The CSF compartment has been proposed to partially constitute a functional equivalent of the lymphatic system for the CNS (reviewed by Ransohoff *et al.*, 2003). There is accumulating evidence that the extent to which the CSF mirrors the immune repertoire regulating CNS immunity and inflammation is greater than previously appreciated (Kivisakk *et al.*, 2004; Skulina *et al.*, 2004). Therefore, we next investigated the expression of HLA-G on CSF cells in comparison to peripheral blood in patients with multiple sclerosis ($n = 14$) and various other neurological controls ($n = 7$). We observed that levels of HLA-G on CD14$^+$ monocytes were significantly elevated in the CSF, compared with peripheral blood, of patients with multiple sclerosis (SFI CSF = 59.36 $\pm$ 23.80, versus SFI blood = 1.83 $\pm$ 0. 65, $P = 0.028$; frequency of HLA-G positive monocytes as calculated by quadrant analysis: 74.24% $\pm$ 5.81 in CSF versus 7.79% $\pm$ 3.43 in blood, $P < 0.0001$) (Fig. 5A and B). While B cells (CD19) and NK cells (CD56) were negative for HLA-G, we found a small number of T cells (CD4 as well as CD8) in patients with multiple sclerosis who expressed HLA-G in the CSF (data not shown). Notably, higher frequencies of HLA-G expressing monocytes in the CSF as compared with peripheral blood were also found in patients with OND (frequency of HLA-G positive monocytes as calculated by quadrant analysis: 69.00% $\pm$ 11.74 in CSF versus 9.43% $\pm$ 3.54 in blood of OND patients; $P = 0.002$).

Distribution of the CD14$^+$ CD16$^-$ and CD14$^+$ CD16$^+$ monocyte subsets in CSF and peripheral blood and its correlation to HLA-G expression

The pattern of HLA-G expression is known to be tightly regulated due to cell-specific transcriptional control. Gene expression can be increased by type I and type II interferons (Yang *et al.*, 1996; Lefebvre *et al.*, 2001; Mitsdoerffer *et al.*,

2005), IL-10 (Moreau *et al.*, 1999) or glucocorticoids (Moreau *et al.*, 2001). We therefore assessed whether the elevated expression of HLA-G by CSF monocytes reflects a more activated state of monocytes or rather correlates with either of the two principal peripheral monocyte subsets (CD14$^+$ CD16$^-$ versus CD14$^+$ CD16$^+$) (Geissmann *et al.*, 2003). Although peripheral blood monocytes are heterogeneous, they all express the CD14 molecule. A subset co-expresses CD16, a low-affinity Fc-gamma type III receptor. This population is attributed to a more proinflammatory phenotype, shows some characteristics of tissue macrophages and expands greatly in acute and chronic infections or systemic inflammatory syndromes. First, we analysed the distribution of CD14$^+$ CD16$^+$ versus CD14$^+$ CD16$^-$ monocytes in the CSF compartment in comparison to peripheral blood. While CD14$^+$ CD16$^+$ cells represented 29.87% $\pm$ 6.84 of CD14$^+$ monocytes in peripheral blood of patients with multiple sclerosis ($n = 14$), 77.10% $\pm$ 5.12 of CD14 positive monocytes co-stained with CD16 in the CSF (Fig. 6A). This situation was similar in patients with OND (81.54 % $\pm$ 4.85 CD14$^+$ CD16$^+$ cells in the CSF versus 34.72 % $\pm$ 7.65 in peripheral blood, $n = 7$) (Fig. 6A), indicating that the proportion of CD14$^+$ CD16$^+$ monocytes is elevated in the CSF compartment when compared with peripheral blood. This probably reflects the accumulation of monocytes with distinct migratory properties in the CNS compartment independent of any inflammatory situation.

Next, we analysed the co-expression of HLA-G on these monocyte subsets. Analysis of patients with multiple sclerosis showed that only a small percentage of CD14$^+$ CD16$^+$ monocytes in peripheral blood co-expressed HLA-G (10.33% $\pm$ 3.44; $n = 14$). In contrast, HLA-G was expressed on 77.34% $\pm$ 5.28 of the CD14$^+$ CD16$^+$ monocytes in the CSF (Fig. 6B). Similar results were found for OND patients, with 14.27% $\pm$ 3.71 HLA-G-positive CD14$^+$ CD16$^+$ monocytes in peripheral blood versus 75.26 $\pm$ 10.15% HLA-G-positive CD14$^+$ CD16$^+$ monocytes in the CSF ($n = 7$) (Fig. 6C). When analysing the CD14$^+$ CD16$^-$ monocytes in the CSF, HLA-G was expressed on 52.39% in multiple sclerosis and 41.36% in OND patients. In contrast, only 5.51% $\pm$ 2.13 and 6.76% $\pm$ 3.01 of the CD14$^+$ CD16$^-$ monocytes were HLA-G positive in the blood of patients with multiple sclerosis and OND, respectively. A total of 9 out of 14 patients with multiple sclerosis had an acute relapse or experienced a first demyelinating episode. No significant differences in HLA-G expression between patients with acute relapse and patients with stable disease were found (data not shown).

Detection of soluble HLA-G in the CSF

To further validate our findings concerning HLA-G expression in neuroinflammation, we also investigated the presence of soluble HLA-G molecules in the CSF of multiple sclerosis patients ($n = 8$) in comparison with OND ($n = 6$) by ELISA. Soluble HLA-G molecules were detectable in both groups. However, CSF mean levels were significantly higher in
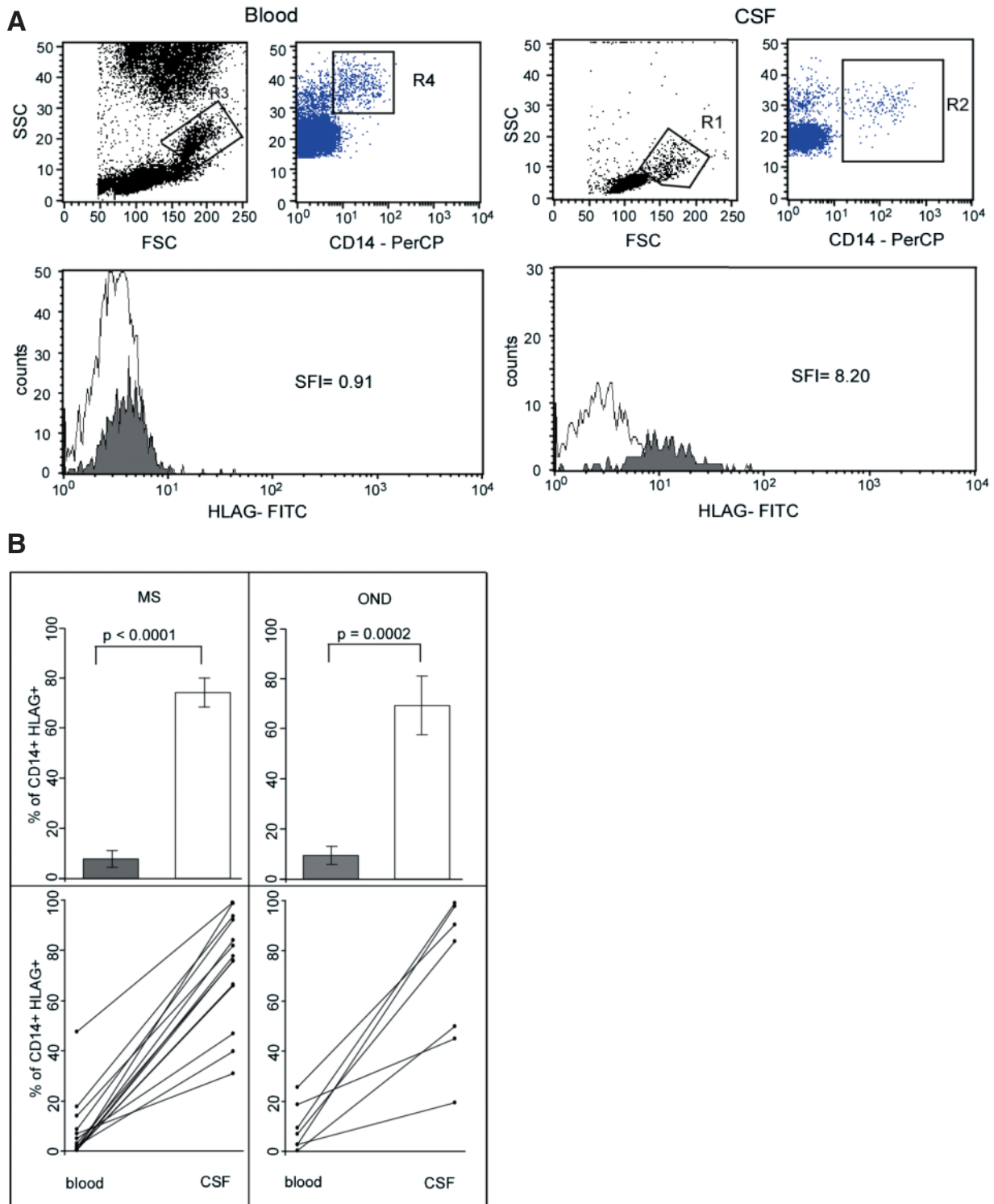
Fig. 5 Detection of HLA-G expression on monocytes in the CSF of patients with MS and OND. (**A**) Monocytes were identified based on their size in the FSC/SSC and on their expression of CD14 using flow cytometry (upper panels) in the peripheral blood (left) and in the CSF (right). Isotype matched antibodies were used in each patient to define background fluorescence (a, lower panels, open histogram). Figure shows HLA-G staining using mAb MEM-G/9 in one representative CSF sample from a multiple sclerosis patient (filled histogram). (**B**) Analysis of HLA-G expression on CD14$^+$ monocytes in multiple sclerosis and patients with OND. Percentages of HLA-G-expressing monocytes were compared between peripheral blood and CSF. The bars in the upper panel represent the mean frequency of HLA-G positive CD14$^+$ monocytes in peripheral blood (grey bars) versus CSF (open bars). The difference, indicated by the mean $\pm$ SEM is highly significant for multiple sclerosis ($n = 14$; $P < 0.0001$) and for OND ($n = 7$; $P = 0.0002$). The lower panels directly compare the frequencies of HLA-G positive monocytes in blood (each left) and CSF (each right) from multiple sclerosis patients ($n = 14$, left) and OND ($n = 7$, right).
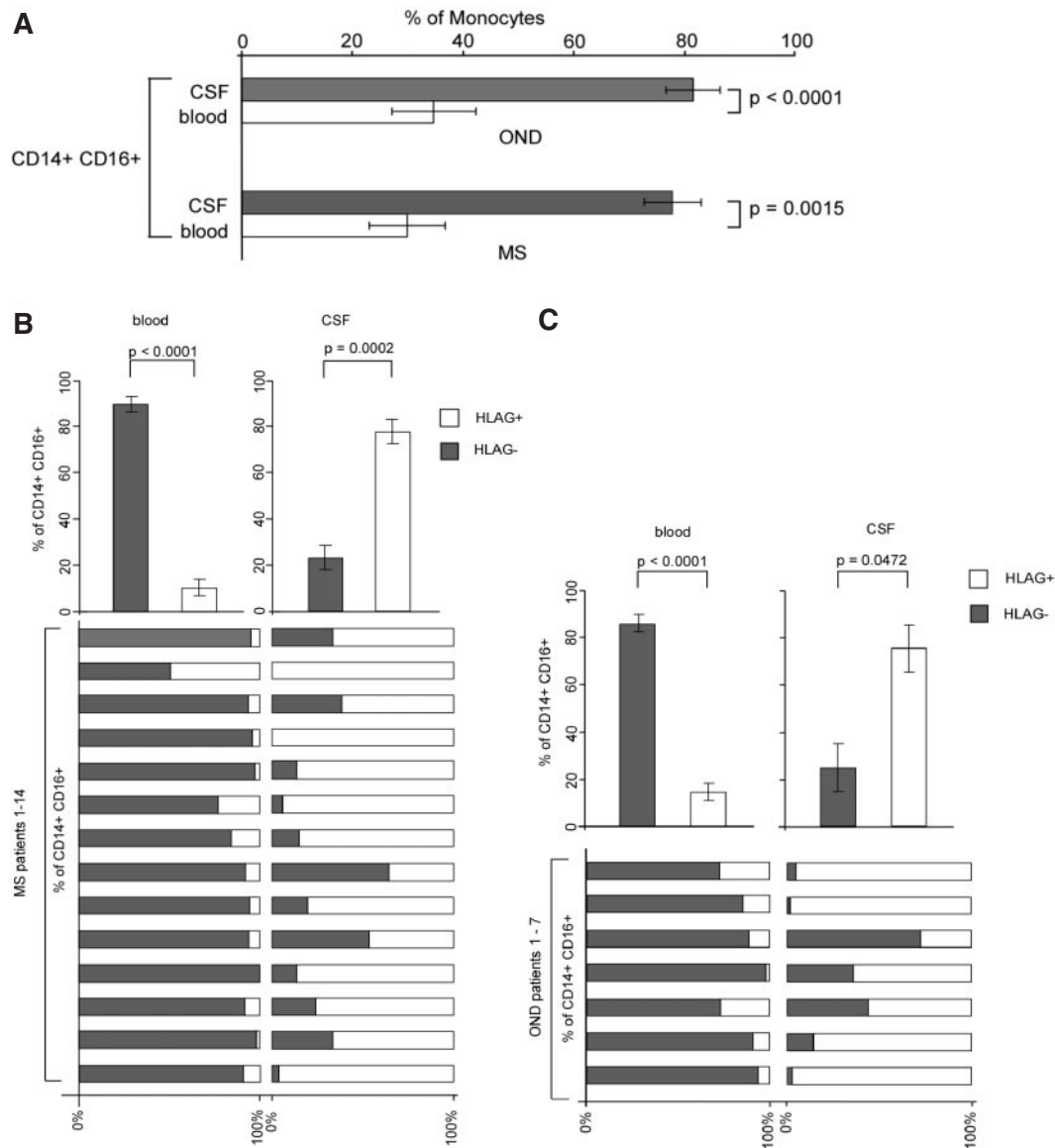
Fig. 6 Distribution of the CD14$^+$ CD16$^-$ and the CD14$^+$ CD16$^+$ monocyte subsets in the CSF and peripheral blood, and correlation to HLA-G expression. (**A**) The frequency of CD14$^+$ CD16$^+$ monocytes was compared between peripheral blood and CSF in patients with multiple sclerosis ($n = 14$) and OND ($n = 7$). The bars represent the percentage of CD14$^+$ CD16$^+$ monocytes in all CD14$^+$ monocytes in peripheral blood (open bars) versus CSF (filled bars). The difference indicated by the mean $\pm$ SEM is highly significant both for patients with multiple sclerosis ($n = 14$; $P < 0.0015$) and for OND ($n = 7$; $P = 0.0001$). Analysis of HLA-G expression on CD14$^+$ CD16$^+$ monocytes in patients with multiple sclerosis in **B** and OND in **C**. Percentage of HLA-G positivity on CD14$^+$ CD16$^+$ monocytes was calculated in peripheral blood in comparison to the CSF. The bars in each upper panel (**B** and **C**) represent the mean frequency of HLA-G-positive (grey open bars) versus HLA-G-negative (filled bars) CD14$^+$ CD16$^+$ monocytes in the peripheral blood (left) versus CSF (right). The lower panels directly compares the frequencies of HLA-G-positive CD14$^+$ CD16$^+$ monocytes in blood and CSF in the individual patients [(**B**): patients with multiple sclerosis, $n = 14$; (**C**) patients with OND, $n = 7$].

multiple sclerosis than in OND (171.5 $\pm$ 31.0 U/ml versus 38.8 $\pm$ 9.6 U/ml; $P = 0.0001$) (Fig. 7). No significant differences in HLA-G expression between patients with acute relapse and patients with stable disease were found (data not shown).

Discussion

Our study investigated the expression of the immune-tolerogenic non-classical MHC class Ib molecule HLA-G in

the CNS. We found that HLA-G and its receptor ILT2, while virtually absent under normal non-pathological conditions in the CNS parenchyma, are abundantly expressed in MS lesions and in the periplaque white matter (Figs 1–3). As a cellular source of HLA-G expression *in situ*, we identified macrophages, microglial cells and endothelial cells, but not astrocytes, oligodendroglial cells or neurons (Fig. 1,2). HLA-G was also found in other CNS disease entities with primary or secondary inflammatory components (meningitis, Alzheimer's disease) and expression correlated with the
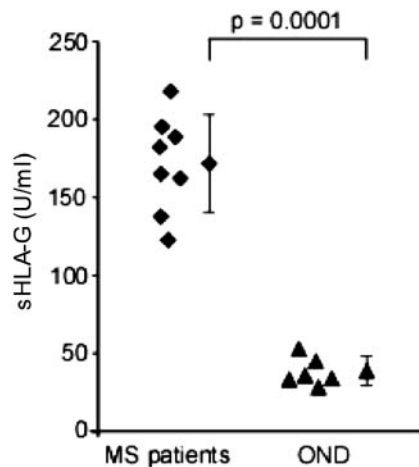
Fig. 7 Levels of soluble HLA-G in the CSF of patients with multiple sclerosis and OND. CSF samples were assessed for the presence of soluble HLA-G by ELISA. Results of individual samples are given in U/ml. Values were compared between patients with multiple sclerosis ($n = 8$) and OND ($n = 6$). The difference, indicated by the mean $\pm$ SEM, is highly significant ($P = 0.0001$).

state of activation and MHC class II expression of microglial cells. These findings were corroborated *in vitro* by demonstrating that adult and fetal human microglial cells express HLA-G mRNA and protein, and upregulate these when exposed to inflammatory conditions simulated by the addition of T-cell (particularly Th1) supernatants or IFN-γ (Fig. 4). Finally, HLA-G was found upregulated on monocytes in the CSF relative to peripheral blood, which represent the main source of HLA-G in the CSF (Figs 5 and 6). Multiple sclerosis patients exhibit significantly higher CSF levels of soluble HLA-G as compared with OND, thus further emphasizing the immunobiological relevance of this pathway for neuroinflammation (Fig. 7).

Our study identifies microglial cells/macrophages as the main source of HLA-G in the human brain. In addition, our data strengthen the hypothesis that inflammatory processes, such as multiple sclerosis, regulate HLA-G expression, for which cellular sources are mainly antigen-presenting cells (APCs).

The CNS has historically been considered an immunologically privileged site, but should be more accurately viewed as an immunologically specialized site (Ransohoff *et al.*, 2003). The CNS is constantly patrolled by activated T cells which may induce profound damage if they identify their specific or a cross-recognized antigen in the context of appropriate MHC restriction elements. The specialized anatomic barriers, such as the blood–brain barrier (Fabry *et al.*, 1994) and the peculiarities of the lymphatic drainage do not necessarily guarantee the integrity of this organ. As such, limiting the local inflammatory response is crucial for an organ as vulnerable as the CNS. A critical player in the immune homeostasis of the CNS environment is the microglial cell. Considering microglial cells as the major APC in the CNS, immune cell–microglia interactions may have a critical impact on the outcome of brain-derived or brain-directed

immune responses. Although the dichotomous nature of microglia/macrophages in modulating CNS immune responses is well appreciated, previous studies have so far focused primarily on the relevance of stimulatory signals on microglial cells/macrophages, such as classical MHC class I and class II molecules (e.g. Perry, 1998; Hoftberger *et al.*, 2004) or costimulatory molecules (CD80, CD86, CD40), including their contribution in modulating or amplifying acute/chronic neuroinflammation (e.g. Becher and Antel, 1996; Aloisi, 1999; Zehntner *et al.*, 2003). Demonstrating the expression and regulation of the immune-tolerogenic molecule HLA-G on macrophages and microglial cells, we provide novel insights into the complex immunobiology of these CNS APCs. HLA-G proteins inhibit NK cell- as well as T cell-mediated cytolysis (Le Gal *et al.*, 1999; Riteau *et al.*, 2001; Wiendl *et al.*, 2002, 2003*a*) and suppress proliferation of CD4 T cells (Bainbridge *et al.*, 2000; Lila *et al.*, 2001). Soluble HLA-G molecules have similar immune-inhibitory properties and may induce apoptosis in activated CD8 T cells (Fournel *et al.*, 2000). Recently, HLA-G expressed by monocytes was identified as an important negative immune-regulatory principle downregulating the production of Th1 as well as Th2 cytokine productions and, inhibiting antigen-specific and autologous CD4 T-cell activation and inducing anergic T cells (LeMaoult *et al.*, 2004; Mitsdoerffer *et al.*, 2005). HLA-G exerts its inhibitory functions by interaction with inhibitory receptors such as members of the immunoglobulin-like transcript family ILT2 and ILT4. ILT2 immunoreactivity was found in inflamed areas of multiple sclerotic lesions and strongly paralleled the expression pattern of HLA-G (Fig. 3). Since ILT2 is broadly expressed on cells of the lymphoid or myeloid lineage (Fig. 3), it is tempting to speculate that HLA-G could exert its inhibitory functions via APC–APC interactions or APC–T-cell interactions (Colonna *et al.*, 1998; McIntire *et al.*, 2004).

HLA-G was also found on CSF cells, where monocytes represented the main source of expression. Interestingly, monocytic HLA-G was significantly upregulated in the CSF compartment as compared to the peripheral blood (Fig. 5). This upregulation in the CSF compartment was observed in patients with multiple sclerosis as well as in patients with OND including patients with neurological disorders lacking any CNS damage (see Table 1). Most of the CSF monocytes (77.81% in MS patients, 81.54% in OND patients), belong to the CD14$^+$ CD16$^+$ monocyte population, while they represent only ~30% of peripheral monocytes (Fig. 5A). Thus, one could assume that the accumulation of CD14$^+$ CD16$^+$ monocytes in the CSF most probably reflects the distinct composition of these cells in the CSF compartment, the CSF containing more monocytes that have (i) more macrophage characteristics and (ii) a distinct migratory behaviour (Geissmann *et al.*, 2003).

In correspondence to our data showing upregulated HLA-G expression in multiple sclerosis brain specimens, we found significantly elevated soluble HLA-G in the CSF of multiple sclerosis patients in comparison with OND.

This observation thus confirms and extends data of recent work by Fainardi and colleagues that demonstrated a possible link between levels of soluble HLA-G5 in serum or CSF with disease activity in multiple sclerosis (Fainardi *et al.*, 2003). Although these observations emphasize the possible relevance of HLA-G in regulating pathogenic CNS inflammation, our patient sample is too small to comment on the relation of HLA-G levels with disease activity or the disease course. Further studies are warranted also to address the question, to what extent peripheral or CSF HLA-G levels may reflect the therapeutic effects of immunomodulatory/immunosuppresunosuppressive therapies (Mitsdoerffer *et al.*, 2005) or even represent a useful marker for delineating treatment responders, as has been suggested for soluble (classical) HLA class I molecules (Fainardi *et al.*, 2004).

As for the role of HLA-G in the regulation of CNS inflammation, it could be argued that the proinflammatory activity of T cells might be regulated via a negative feedback loop by inducible HLA-G expression on resident microglia/macrophages (and invading peripheral monocytes/macrophages): in the presence of an inflammatory milieu, the proinflammatory activity of encephalitogenic T cells might be counterregulated via a negative feedback loop, such that activated Th1 T cells producing high levels of IFN-γ are forced to reduce their cytokine levels via inducible HLA-G expression. Since microglial cells are capable of quickly migrating to the site of inflammation or injury (Kreutzberg, 1996), they could participate in the very early modulation of inflammation. The idea of local immunosuppression by HLA-G possibly affecting disease activity and progression is appealing, both from an immunopathogenic and a therapeutic view and perspective. Parenchymal HLA-G, while being upregulated on macrophages and microglial cells in response to inflammatory stimuli, might represent a regulatory principle balancing and controlling antigen-specific as well as autologous T-cell activation *in vivo*. Of note, although we use IFN-γ as a marker of the 'Th1 environment', it is clear that IFN-γ is not the only molecule present within the Th1 milieu that contributes to the observed HLA-G induction on monocyte/microglia differentiation. It is likely that multiple molecules in the Th1 environment (including but not limited to IFN-γ) are likely to contribute to the modulation of the human monocyte/microglia (Kim *et al.*, 2004).

Taken together, our data provide insights into the significance of HLA-G in CNS immunity. HLA-G represents an inducible molecule that may be important for maintaining an 'antiinflammatory milieu' in the CNS. Since HLA-G downregulates immune responses by interacting with virtually all cytotoxic cellular immune effectors and since only low numbers of HLA-G-expressing cells are required to convey significant immune inhibitory effects (Wiendl *et al.*, 2003*a*), this molecule represents an appealing self-derived antiinflammatory principle, possibly applicable as a strategy against the aggressive inflammatory responses that occur in the CNS of patients with multiple sclerosis.

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