Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis

Verena Brucklacher-Waldert,1,2,3 Klarissa Sturner,1 Manuela Kolster,1 Julia Wolthusen4 and Eva Tolosa1,2

1 Institute for Neuroimmunology and Clinical Multiple Sclerosis Research, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany
2 Department of Immunology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany
3 Graduate School of Cellular and Molecular Neuroscience, University of Tuebingen, Germany
4 Department of Neurology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany

Correspondence to: Eva Tolosa,
Department of Immunology,
University Medical Centre Hamburg-Eppendorf,
Martinistr. 52,
20246 Hamburg,
Germany
E-mail: etolosa@uke.uni-hamburg.de

Multiple sclerosis is a T cell-mediated demyelinating disease of the central nervous system. Interleukin-17-producing T helper cells, named Th17 cells, represent a novel CD4+ T cell effector subset involved in the response against extracellular pathogens. In addition, Th17 cells are pathogenic in several animal models of autoimmune disease, including the animal model for multiple sclerosis, but their function in multiple sclerosis remains to be elucidated. In this study, we analysed the frequency and the phenotype of Th17 cells in the cerebrospinal fluid and peripheral blood of multiple sclerosis patients. We show that the frequency of Th17 cells is significantly higher in the cerebrospinal fluid of patients with relapsing-remitting multiple sclerosis during relapse, in comparison to relapsing-remitting patients in remission or to patients with other non-inflammatory neurological diseases. Similarly, in patients with clinically isolated syndrome during their first neurological episode, Th17 cells are more abundant than in clinically isolated syndrome patients with no acute symptoms. Patients with inflammatory neurological diseases other than multiple sclerosis also showed increased frequency of Th17 cells compared to patients with no inflammatory diseases. To assess a potential pathological impact of Th17 cells in disease, we generated T cell clones from the cerebrospinal fluid and peripheral blood of patients with multiple sclerosis. We found that Th17 clones expressed higher basal levels of the activation markers CD5, CD69, CD2 and human leukocyte antigen-DR as well as of the CD28-related family of co-stimulatory molecules, when compared to Th1 clones, and confirmed these findings with ex vivo human T cells. Molecules involved in T cell adhesion to endothelium, such as CD49d, CD6 and the melanoma cell adhesion molecule, were also more abundant on the Th17 than on the Th1 cells. Furthermore, functional assays showed that Th17 clones were more prone than Th1 clones to melanoma cell adhesion molecule-mediated adhesion to endothelial cells, and that Th17 cells had a higher proliferative capacity and were less susceptible to suppression than Th1 cells. Altogether our data suggest that Th17 cells display a high pathogenic potential and may constitute a relevant pathogenic subset in multiple sclerosis.

Keywords: multiple sclerosis; T cells; IL-17

Abbreviations: CFSE = carboxyfluorescein succinimidyl ester; CIS = patient group with clinically isolated syndrome and neurologically stable; CIS/R = patient group showing signs of their first neurological event; CTLA = cytotoxic T-lymphocyte antigen; ICOS = inducible T-cell costimulator; IFN-γ = gamma interferon; IL = interleukin; MCAM = melanoma cell adhesion molecule; PBMC = peripheral blood mononuclear cells; PD-1 = programmed death-1 receptor; RR-MS = patient group in remission phase of
relapsing-remitting multiple sclerosis; RR-MS/R = patient group with relapsing-remitting multiple sclerosis showing acute relapse; TCC = T cell clone; Th = T helper

**Introduction**

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system. Studies on experimental autoimmune encephalomyelitis and immunological assessments of multiple sclerosis patients have supported the concept of multiple sclerosis as a CD4+ T helper (Th) 1 cell driven autoimmune disease that develops in genetically susceptible individuals (Sospedra and Martin, 2005). Th1 cells are defined by their ability to secrete gamma interferon (IFN-γ) and by the expression of T-bet. However, many features of multiple sclerosis are not reflected merely by Th1-mediated brain inflammation, but rather point to a more complex pathogenesis including different T cell populations. Th17 cells have recently been discovered as a further T helper subtype, producing the proinflammatory cytokine Interleukin (IL)-17A (Harrington et al., 2005; Park et al., 2005) and expressing RORyt as a lineage-specific transcription factor (Ivanov et al., 2006). In the case of experimental autoimmune encephalomyelitis, mice lacking components of the IL-12/Th1 axis were not protected from disease development (Becher et al., 2002; Zhang et al., 2003), but rather developed more severe disease; whereas mice lacking components of the IL-23/Th17 axis were resistant to experimental autoimmune encephalomyelitis (Cua et al., 2003; Langrish et al., 2005). A recent study has shown that, regardless of the cytokine expression of the myelin-specific T cells, it is the transcription factor T-bet that determines pathogenicity of both Th1 and Th17 cells in the mouse model of multiple sclerosis (Yang et al., 2009).

In humans, Th17 cells are increased in the blood of patients with uveitis and scleritis (Amadi-Obi et al., 2007) and in the intestine of patients with Crohn’s disease (Annuziato et al., 2007). Recently, data have emerged linking the IL-23/Th17 pathway to multiple sclerosis: monocyte-derived dendritic cells from multiple sclerosis patients produce more IL-23 but equivalent amounts of IL-12 compared to healthy controls, and peripheral blood CD3+ T cells from multiple sclerosis patients produce significantly more IL-17 than T cells from healthy donors (Vakin-Dembinsky et al., 2006). Multiple sclerosis patients have increased numbers of IL-17A mRNA-positive mononuclear cells both in peripheral blood and in the cerebrospinal fluid (CSF) compared to healthy controls (Matusевичius et al., 1999). Microarray analysis of multiple sclerosis plaques isolated at autopsy demonstrated an increase in IL-17 mRNA compared to the amount found in brains from control patients without nervous system pathology (Lock et al., 2002). Recently, IL-17-producing cells were detected in lesions of multiple sclerosis brain sections, but not in normal appearing white matter or non-inflamed brain specimens (Kebir et al., 2007; Tzartos et al., 2008). Interestingly, human Th17 lymphocytes were also shown to migrate more efficiently across the blood–brain barrier than Th1 cells (Kebir et al., 2007). Altogether, these studies point strongly towards a potentially pathogenic role of IL-17-producing CD4+ T cells in multiple sclerosis.

However, while the treatment with an antibody against the common subunit of IL-12 and IL-23 (IL-12 p40) could prevent experimental autoimmune encephalomyelitis in rodents and non-human primates (Brok et al., 2002), the use of ustekinumab, a neutralizing antibody for the same subunit of the human cytokine, was not effective in a trial on patients with relapsing-remitting multiple sclerosis (Segal et al., 2008), although it improved the condition of patients with Crohn’s disease and psoriasis (Leonardi et al., 2008; Sandborn et al., 2008).

To address the relevance of Th17 cells in multiple sclerosis, we quantified the frequency of ex vivo IL-17A-producing CD4+ T cells in peripheral blood and CSF of multiple sclerosis patients and found a higher Th17 frequency in the CSF of patients during relapses compared to patients in remission. Characterization of human T cell clones derived from multiple sclerosis patients showed that Th17 T cell clones were constitutively more activated and expressed higher levels of co-stimulatory and adhesion molecules than Th1 T cell clones. Functional experiments with ex vivo Th17 cells demonstrated a higher proliferative capacity and a reduced susceptibility to suppression compared to Th1 cells. Altogether, our data suggest that Th17 cells have a high pathogenic potential and may therefore contribute to the pathology of multiple sclerosis.

**Materials and methods**

**Patients**

Our study included patients with relapsing-remitting multiple sclerosis, meeting the McDonald criteria (McDonald et al., 2001), individuals with clinically isolated syndrome (CIS) and controls. The relapsing-remitting multiple sclerosis group included patients that were either neurologically stable for at least 30 days before sampling (remission phase; RR-MS, n = 7), or patients that showed mono-/multifocal neurological episode lasting more than 24 h at the time of sampling after being neurologically stable for more than 30 days and excluding an acute systemic infection (acute relapse; RR-MS/R, n = 12). The CIS group was subdivided into patients examined at time of the first neurological episode lasting more than 24 h, caused by inflammation and being indicative of multiple sclerosis (CIS/R, n = 9) and patients being neurologically stable for at least 30 days and showing no acute symptoms at the time of examination (CIS, n = 8). Controls included patients with inflammatory neurological disease other than multiple sclerosis (n = 9) and patients with non-inflammatory neurological diseases (n = 12). The characteristics of multiple sclerosis and control patients are listed in Supplementary Tables 1 and 2, respectively (online supplementary data). Only multiple sclerosis patients for whom all clinical and paraclinical data were available (MRI, CSF counts, neurological status scored on the Expanded Disability Status Scale) participated in this study. Patients had not received any glucocorticoid steroids or other immunomodulatory drugs within the last 6 months prior to sampling, except Patient 23 (marked as ‘‘) in Supplementary Table 1 and Patient 31 (marked as ‘‘) in the same table, who each suffered a relapse whilst being treated with glatiramer.
acetate and beta-interferon-1a treatment, respectively. All patients were recruited from the University Medical Centre Hamburg-Eppendorf. Healthy controls were recruited from the Blood Bank at the University Medical Centre Hamburg-Eppendorf. This study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg, No. 2758) and informed consent was obtained from all study subjects.

Primary cells and T cell clones

CSF samples were collected during diagnostic procedures and processed within 30 min. CSF-derived mononuclear cells were expanded by stimulation with PHA and 60 Gray-irradiated feeders in the presence of 20U/ml human recombinant IL-2 (Tecin). On day 14, CSF cells were cloned under limiting dilution (0.3 cells/well) (Sospedra et al., 2006). T cell clones derived from peripheral blood mononuclear cells were cloned directly after isolation under limiting dilution (1 cell/well and 3 cells/well).

Th1 and Th17 T cell clones were also generated on the basis of surface expression of IL-17, as described previously (Brucklacher-Waldert et al., 2009). Briefly, CD4⁺ T cells were stimulated for 6 h with PMA/ionomycin and subsequently stained with anti-IL-17A Alexa647. Surface IL-17Alow and IL-17Ahigh were sorted at 1 cell/well onto 96 well culture plates containing IMDM supplemented with 5% human serum, 20 U/ml hrIL-2 and irradiated autologous peripheral blood mononuclear cells (FACSaria, BD Biosciences). Cells were re-stimulated every second week. Under these culture conditions, in the presence of IL-2 and without polarizing cytokines, Th1 and Th17 T cell clones maintain their phenotype over repeated rounds of stimulation (Brucklacher-Waldert et al., 2009). In all cases clonality was assessed by T cell receptor Vβ-chain staining (Muraro et al., 2000).

Flow cytometry analysis

A list of the antibodies used in this study can be found in the online supplementary data. For intracellular cytokine staining, mononuclear cells were cultured in X-VIVO15 medium, supplemented with PMA (50 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) in the presence of Brefeldin A (10 μg/ml, eBioscience) for 5 h, fixed and permeabilized with the corresponding buffers (eBioscience) and stained for CD4, IFN-γ and IL-17A at room temperature. For phenotype analysis of the T cell clones, cells were stained on the surface 10 days after re-stimulation. Ex vivo peripheral blood mononuclear cells and CSF cells were stained on the surface for CD49d, melanoma cell adhesion molecule (MCAM)/CD146 and CD28 after 6 h of PMA/ionomycin treatment in the presence of Brefeldin A, and then fixed and permeabilized previous to staining for CD4, IFN-γ and IL-17A. Analysis of CD3, inducible T-cell costimulator (ICOS), cytotoxic T-lymphocyte antigen (CTLA)-4 and programmed death-1 receptor (PD-1) expression was performed in magnetically isolated CD4⁺ T cells (CD4 II isolation kit from Miltenyi Biotec), stimulated for 18 h with PMA/ionomycin in order to allow the surface expression, and in the presence of Brefeldin A for the last 8 h to keep enough cytokine for detection inside the cell. All analyses were done on an LSRII (BD Biosciences) flow cytometer. Data analysis was performed with the FlowJo software. To analyse surface markers in combination with intracellular staining of IFN-γ and IL-17A, a first gate was set up on live cells in the FSC/SSC plot, followed by a gate on CD4⁺ cells. Two further gates on IFN-γ⁺ and IL-17A⁺positive cells defined the Th1 and Th17 populations, respectively. The surface markers were subsequently analysed on the Th1 and Th17 populations.

Reverse transcription–polymerase chain reaction and enzyme-linked immunosorbent assay

The primer and probe sets TBX21 Hs00203436_m1 and RORC Hs01076112_m1 for T-bet and RORC, respectively, were purchased from Applied Biosystems. 18S rRNA was used as endogenous control and the relative gene expression was calculated by the ∆∆ Ct method using peripheral blood mononuclear cells as calibrator. Culture supernatants were collected on day 2 after PMA/ionomycin stimulation and IL-17A measured using the human IL-17A enzyme-linked immunosorbent assay kit (eBioscience).

Adhesion assay

Cells from Th1 or Th17 T cell clones, derived from peripheral blood of a healthy donor, were stained with 10 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes). The human brain endothelial cell line hCMEC/D3 (Wekslers et al., 2005), generously provided by Drs B. Weksler, I. Romero and P.O. Couraud (Inst. Cochin, Paris, France), was grown to confluency in 100 μg/ml collagen type I (BD Biosciences) and coated on 4-well plates in EBM-2 medium (Lonza) supplemented with 5% foetal calf serum, 1 ng/ml basic fibroblast growth factor and 1.4 μM hydrocortisone (both Sigma). hCMEC/D3 cells were washed once with phosphate buffered saline and 15 × 10⁶ CFSE-labelled cells were added to each well in 500 μg/ml IMDM/10% foetal calf serum. A total of 20 μg/ml anti-CD146 (clone P1H12, BD) was added where indicated. After 2 h of incubation at 37 °C, non-adherent cells were washed as described (Tolosa and Shaw, 1996). Briefly, wells were gently filled with phosphate buffered saline and plates were then inverted onto a vessel containing phosphate buffered saline. Unbound cells were left to sediment by gravity for 10 min. Cells remaining bound to the endothelial monolayer were fixed with 4% paraformaldehyde and analysed under the fluorescence microscope (Zeiss) using the 20× objective and the appropriate fluorescence filter. A minimum of six fields per condition were photographed and counted using Image J software.

Proliferation and suppression assays

Purified CD4⁺ T cells were labelled with 5 μM CFSE and stimulated with different concentrations of anti-CD3 and in presence of 35 Gray-irradiated allogeneic feeder cells for 5 days. For the suppression assays, CFSE-labelled CD4⁺CD25⁺ T-responder cells and CD4⁺CD25high regulatory T cells obtained by magnetic cell sorting (Miltenyi Biotec) were co-cultured as above. Proliferating cells were determined in the Th1 and Th17 gates as described.

Statistical analysis

Unpaired Student’s t-test was used to compare Th1 and Th17 frequencies between patients and controls. Th1 and Th17 frequencies in CSF and blood samples of patients and controls were compared by two-tailed paired Student’s t-tests. Differences were considered significant if P ≤ 0.05.
Results

The frequency of Th17 cells is increased in the CSF of patients with multiple sclerosis and is higher during relapses

The frequency of Th17 cells was assessed in blood and CSF of our patient cohort, which included the following groups of patients: (i) patients with the first manifestation of multiple sclerosis but not yet overt disease analysed during the acute phase (clinically isolated syndrome in relapse, CIS/R); (ii) patients who had suffered the first manifestation but, at the time of sampling, had been neurologically stable (in remission) for at least 30 days (CIS); (iii) patients with well established relapsing-remitting disease at the moment of a relapse (RR-MS/R); or (iv) patients with well established relapsing-remitting disease during a remission phase (RR-MS). As controls we used patients with neurological diseases without brain inflammation (other neurological diseases) and patients with inflammatory diseases of the brain other than multiple sclerosis (other inflammatory neurological diseases). After gating on CD4+ T cells, Th17 cells were identified as IL-17A+ IFN-γ− cells and Th1 cells as IL-17A− IFN-γ+ cells (Fig. 1A). In peripheral blood, the percentage of Th17 cells was low (ranging from 0.9 ± 0.13% in patients with other neurological diseases to maximum of 1.5 ± 0.29% in patients with other inflammatory neurological diseases), and there were no significant differences among the different groups (Fig. 1B). By contrast, in the CSF, when we compared samples of the different groups we observed that patients with other inflammatory neurological diseases (other inflammatory neurological diseases; n = 9), CIS/R (n = 9), CIS (n = 8), RR-MS/R (n = 12) and RR-MS (n = 7) patients. (C) Paired frequencies of Th17 cells in peripheral blood and CSF of patients with other neurological diseases, RR-MS/R and RR-MS in relapse. Results are represented as the mean ± SEM. Statistical significance is indicated if: *P ≤ 0.05, **P ≤ 0.005 and ****P ≤ 0.00005.

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When comparing paired CSF and blood samples from multiple sclerosis patients the frequency of Th17 cells in the CSF was two times higher compared to peripheral blood both in patients during relapse and in remission (Fig. 1C).
We did not observe any correlation between Th17 frequency in the CSF and disability status or disease duration in our patient cohort. In addition to Th17 cells, in all patients we detected a minor population of CD4+ T cells simultaneously producing IL-17A and IFN-\(\gamma\) (IFN-\(\gamma\)+ IL-17A+, double producers), always two- to five-fold higher in the CSF than in peripheral blood but with no differences in the various disease groups (Supplementary Fig. 1). Notably, IL-17A-producing CD8+ T cells were either not detected at all or present at extremely low frequencies (data not shown). IFN-\(\gamma\)-producing CD4+ T cells were much more abundant than Th17 cells, both in the peripheral blood and in the CSF of all patients and controls tested. No change in the frequency of IFN-\(\gamma\)-producing cells in the peripheral blood was detected in the different samples and, in the CSF, only relapsing-remitting multiple sclerosis patients showed a significant decrease when compared to patients with other neurological diseases (44.5 ± 3.0% and 58.5 ± 3.7%, respectively, \(P < 0.001\)) (Fig. 1D). Our data show that, even if the majority of the CSF infiltrating cells belongs to the Th1 subset, only the Th17 cells were elevated in the CSF of patients during relapses.

**Generation and characterization of human Th17 T cell clones**

We circumvented the lack of a unique surface cell marker and the paucity of Th17 cells by generating human Th17 clones to investigate the phenotypic and functional features of Th17 cells. We established T cell clones from the CSF of a relapsing-remitting multiple sclerosis patient in relapse (00647), from peripheral blood of a patient with relapsing-remitting multiple sclerosis during remission (00483), and from the CSF and peripheral blood of a patient with the first episode of disease (CIS/R, 01792). The nature of the clones was revealed by intracellular cytokine staining for IL-17A and IFN-\(\gamma\) (Fig. 2A). Seventy-six T cell lines were obtained from peripheral blood (PB) mononuclear cells of a relapsing-remitting multiple sclerosis patient (00483, left) and 593 T cell lines from the CSF of a relapsing-remitting multiple sclerosis patient in relapse (00647, right). Cell lines were considered Th1, Th17 or IFN-\(\gamma\)+ IL-17A+ when the proportion of cells positive for the relevant cytokine was 20% or higher. (C) Representative IL-17A production of a Th17 (black bar) and Th1 T cell clone (grey bar) from donor 00483. (D) Reverse transcription-PCR analysis for transcription factors ROR\(\gamma\) and T-bet of Th17 T cell clones (black bars, \(n = 4\)) and Th1 T cell clones (grey bars, \(n = 4\)) from patient 00647. Values are relative expression compared to peripheral blood mononuclear cells (calibrator = 1). Results show the mean ± SEM of four different clones; \(**P \leq 0.005\).
IL-17A or IFN-γ by Vβ staining, and 12 Th17 and 14 Th1 clones were chosen for further studies. The ability to secrete IL-17A and to express RORγt and T-bet mRNA was used to confirm the nature of the selected clones (Fig. 2C and D). The Th17 clones secreted IL-17A, which was barely detectable in the Th1 clone. RORγt mRNA was clearly expressed in Th17 clones (n = 4), but not in Th1 clones (n = 4) (Fig. 2C). In contrast, T-bet could be detected in both, although at higher levels in Th1 clones.

**Th17 clones express constitutively high levels of activation markers and adhesion molecules**

The expression of surface markers was assessed in 14 Th1 and 12 Th17 T cell clones from patient 00647. We first analysed the chemokine receptors CCR6 and CXCR3, which have been associated with T cell homing. As already reported, CCR6 was expressed at higher levels by Th17 clones (P < 0.001), and was only weakly positive in the Th1 clones. In contrast, CXCR3 was expressed moderately in both Th1 and Th17 clones, with no significant differences (Fig. 3A). Next, we analysed molecules involved in T cell adhesion to the endothelium. Th17 clones expressed higher surface levels of CD49d (P = 0.007), the ligand for vascular cell adhesion molecule-1 and of CD6 (P = 0.019), a ligand for activated leukocyte cell adhesion molecule, than Th1 clones (Fig. 3B). MCAM (CD146), which binds to the same molecule on the endothelium, was negative in all but one of the Th1 clones analysed, while it was heterogeneously expressed in the Th17 clones, from negative (30% of the clones) to very high levels (Fig. 3C). To understand the requirements for expansion of Th17 cells, we analysed the expression of cytokine receptors. IL-23 is an important cytokine for the expansion and maintenance of Th17 cells (Stritesky et al., 2008), while the role of IL-2 in the growth of Th17 clones is still controversial (Amadi-Obi et al., 2007; Laurence et al., 2007). Our data show that IL-23R is significantly more highly expressed on the Th17 clones compared to the Th1 clones (P < 0.001). In contrast, expression of the IL-2Rα (CD25) and IL-7Rα (CD127) was similar in all clones tested, probably indicating that these IL-2 and IL-7 are not specifically promoting one of the two lineages. The IL-2Rβ (CD122) and the common γ chain (CD132) of the IL-2R were expressed at slightly higher levels in Th17 T cell clones compared to Th1 T cell clones (Fig. 3D). Finally, we assessed molecules that are functionally relevant for supporting T cell stimulation and proliferation. We focused on the CD28 family members, and surprisingly, both the stimulatory CD28 (P < 0.001) and ICOS (P < 0.001), but also the inhibitory receptors CD152 (CTLA-4; P < 0.001) and CD279 (PD-1; P = 0.013) were expressed at higher levels in Th17 than in Th1 clones (Fig. 3E). CD28 was especially over-represented in the Th17 subset, with most clones being highly positive (Fig. 3F). Notably, CD28 was also found at much higher levels in the Th17 clones.
cell clones than on Th1 clones derived from patient 00483 (data not shown), whereas ICOS and PD-1 were expressed at low levels in these clones, but still significantly higher than in the Th17 ones (data not shown). With regard to T cell activation molecules, early activation markers CD5 (P = 0.038) and CD69 (P = 0.009), and the T cell-APC contact promoting CD2 (P = 0.022) were expressed at much higher levels in Th17 clones than Th1. Human leukocyte antigen-DR, known on T cells as an activation marker, was also highly expressed in Th17 clones (P = 0.002), and only modestly in Th1 clones. Ectoenzymes CD26 and CD38 (P = 0.050), usually upregulated in activated T cells, showed no differences or even higher expression in the Th1 clones (Fig. 3G). The higher expression of adhesion molecules CD49d, CD6, MCAM and of co-stimulatory molecules CD28, ICOS and PD-1 was confirmed in Th17 clones generated from the CSF of a patient during acute disease (Patient 01792, Supplementary Fig. 2). Interestingly, clones raised from peripheral blood of this same patient showed a similar tendency, but the differences between Th1 and Th17 cells were less obvious, and CD49d was expressed at the same levels in the Th1 and in the Th17 T cell clones derived from peripheral blood. Altogether, our results show that Th17 clones display higher levels of basal activation and express more co-stimulatory molecules and adhesion receptors in the surface than Th1 clones.

Expression of adhesion molecules in ex vivo Th17 and Th1 cells

To discard that the phenotypic features of Th17 clones result from artefacts introduced during cloning or long in vitro culture procedures, we analysed freshly isolated ex vivo Th17 cells from normal donors and from multiple sclerosis patients. CSF cells and peripheral blood from four patients with relapsing-remitting multiple sclerosis and peripheral blood mononuclear cells from three healthy donors were stimulated with PMA/ionomycin for 6 h in the presence of Brefeldin A and then stained, first for the surface markers CD49d or MCAM/CD146 and then permeabilized and stained for CD4, IL-17A and IFN-γ. Gates were set on IFN-γ- and on IL-17A-producing cells for Th1 and Th17 cells, respectively, and the expression of adhesion molecules was assessed within these gates (Fig. 4A). We did not detect significant differences in CD49d expression between Th17 and Th1 cells in the peripheral blood of healthy donors or relapsing-remitting multiple sclerosis patients (Fig. 4B). In the CSF of multiple sclerosis patients, by contrast, CD49d expression was two-fold higher than in peripheral blood, and in the four healthy donors analysed, IL-17A-producing T cells always showed higher levels of CD49d than IFN-γ-producing cells (P = 0.017). The expression of MCAM/CD146 on primary T cells was bimodal (Fig. 4C). Th1 cells from peripheral blood of healthy donors showed a major MCAM/CD146low and a minor MCAM/CD146high peak. In contrast, in all cases tested, more than half of the cells in the Th17 population are MCAM/CD146high (P < 0.001) (Fig. 4D). The higher expression of CD49d on ex vivo Th17 cells in the CSF of relapsing-remitting multiple sclerosis patients and the high proportion of ex vivo Th17 cells expressing MCAM/CD146 in the peripheral blood of healthy donors confirm the high expression of adhesion molecules previously seen in Th17 T cell clones.

Melanoma cell adhesion molecule/CD146 is involved in the adhesion of Th17 cells to endothelial cells

To assess if MCAM/CD146 is involved in the adhesion of Th17 to endothelium, we used the human brain endothelial cell line
hCEMC/D3 (Weksler et al., 2005). This cell line has the functional properties of brain endothelial cells, and constitutes a reliable in vitro model of the human blood–brain barrier. Since MCAM binds homotypically, we first checked for expression of MCAM in hCEMC/D3 cells. We observed that MCAM is constitutively expressed at very high levels by these endothelial cells (data not shown). For the adhesion experiments we raised new Th1 and Th17 T cell clones from peripheral blood of a healthy donor and analysed their MCAM expression. None of the 36 Th1 clones evaluated expressed MCAM, while 9 of the 13 Th17 clones (69%) were MCAM-positive (Fig. 5A). For the adhesion assays, we chose six MCAM-positive Th17 and compared them to six Th1 clones (which are MCAM-negative). A higher percentage of cells from the Th17 clones was bound to the hCEMC/D3 endothelial cells when compared to Th1 cells (Fig. 5B and C). We wondered if Th17 cells were per se more prone to adhesion than Th1 or if it was just the presence of melanoma cell adhesion molecule that made the difference. First, addition of anti-MCAM blocking antibodies reduced the binding of Th17 cells but had no effect on the binding of the Th1 cells (Fig. 5C). Second, we compared MCAM-positive and MCAM-negative Th17 T cell clones, and found that, within Th17 clones of the same individual, those expressing MCAM adhered slightly better than those that were negative (Supplementary Fig. 3). These data suggest that MCAM/CD146 contributes to the adhesion of Th17 cells to brain endothelium.

**Higher expression of co-stimulatory molecules in Th17 cells**

To assess the expression levels of CD28 ex vivo, CSF cells and peripheral blood mononuclear cells from four patients with relapsing-remitting multiple sclerosis and peripheral blood mononuclear cells from three healthy donors were stimulated and then surface stained for CCR7, CD45RA and CD28, and subsequently permeabilized and stained for CD4, IL-17A and IFN-γ. As expected, the vast majority of Th17 and Th1 cells in the peripheral blood belong to the effector memory (CD45RA-CCR7+) compartment (data not shown). The expression of CD28 was evaluated in both healthy controls and multiple sclerosis patients. In peripheral blood, CD28 was again expressed at higher levels in the Th17 cells (Fig. 6A), but no significant differences between healthy donors and multiple sclerosis patients were observed. In contrast, in the CSF, CD28 was expressed at higher levels than in peripheral blood, with Th17 cells expressing twice as much CD28 as Th1 cells. The analysis of IFN-γ+ IL-17A+ double producer cells showed a high CD28 expression, indicating that they seem to be phenotypically closer to Th17 than to Th1 cells. The expression of CTLA-4, ICOS and PD-1 was analysed in a similar way in peripheral blood mononuclear cells from healthy donors (n = 4), but since these molecules are expressed only after T cell activation, CD4+ T cells were stimulated for 18 h and Brefeldin A was added for the last 8 h. In this way, intracellular cytokine expression could still be detected without compromising the de novo surface expression of the co-stimulatory molecules. Under these conditions, Th17 cells expressed significantly higher amounts of ICOS on the surface compared to Th1 cells. After this activation period, CTLA-4 and PD-1 expression showed bimodal distribution, and thus were analysed as percentage of positive cells instead of fluorescence intensity. Significantly more CTLA-4 (P = 0.001) and PD-1-positive (P = 0.001) cells were observed in the Th17 than in the Th1 subset. CD3, by contrast, was expressed at similar levels in both cell types (Fig. 6B). Thus, co-stimulatory molecules, independent of their stimulatory or inhibitory nature, are also expressed at higher levels in ex vivo analysed Th17 cells. In the case of CD28, this difference is more pronounced in the CD4+ T cells infiltrating the target organ in multiple sclerosis patients.

**Higher proliferation and reduced susceptibility to suppression of Th17 cells**

The percentage of IL-17A-producing T cell lines obtained by our cloning procedure was higher than expected (15%) given the
starting population in the CSF (6%), even though no polarizing cytokines were added to the cultures to promote Th17 differentiation or expansion. This fact, together with the high expression of co-stimulatory molecules both in the Th17 clones and the ex vivo Th17 cells, prompted us to compare the proliferation ability of Th1 and Th17 cells. We first compared the capacity of both cells types to proliferate to polyclonal stimuli. For this, CD4+ T cells from healthy donors were labelled with CFSE and stimulated with increasing amounts of anti-CD3 monoclonal antibody in the presence of irradiated allogeneic feeder cells, which provide the required array of co-stimulatory ligands. After 5 days, cells were incubated with PMA/ionomycin in the presence of Brefeldin A, permeabilized and stained for IL-17A and IFN-γ. The percentage of proliferating cells was analysed for both Th1 and Th17 subsets after gating on IFN-γ- and IL-17A-producing CD4+ cells, respectively (Fig. 7A). Th17 cells proliferated more readily and to lower concentrations of anti-CD3 and feeder cells, with 36.5% (±3.3) of dividing cells with 30 ng/ml anti-CD3, and reaching a proliferation plateau at 60 ng/ml (76.7% ± 3.3). In contrast, at the same time point, less than 11.9% (±3.8) of the Th1 cells were dividing with 30 ng/ml and the maximum of Th1 cells proliferating did not exceed 39.8% ± 10.5 (Fig. 7A). Since Th17 cells are more prone to proliferation, we wondered if both cell types were equally susceptible to suppression by regulatory T cells. For this,
we stimulated CFSE-labelled CD4+ T cell responders with anti-CD3 in the presence of allogeneic peripheral blood mononuclear cells, and added graded amounts of autologous naturally occurring regulatory T cells (Fig. 7B). After 6 days of co-culture, we confirmed that Th17 cells proliferated at higher rates than Th1 cells (91% versus 76% in this particular example) and we observed that Th17 cells were highly resistant to suppression: at the maximum ratio of 1:1 (responder T cells:regulatory T cells), proliferation of Th1 cells was reduced from 76% proliferating cells to 20%, while in Th17 cells proliferation was reduced from 91% to 76%. Suppression was proportional to the amount of regulatory T cells added to the system, but in all cases it was only marginal for Th17 cells added to the system, but in all cases it was only marginal for Th17 cells (Fig. 7B, right panel). IFN-γ+ IL-17A+ double producers were as resistant as Th17 cells to suppression by regulatory T cells (not shown). Therefore, ex vivo Th17 cells show a high proliferative capacity and are less susceptible to regulatory T cells mediated suppression than Th1 cells from the same donor.

**Discussion**

In the CSF of subjects with inflammatory neurological diseases, including multiple sclerosis, an increased number of IFN-γ-producing T cells has been described (Giunti et al., 2003), suggesting that CSF is enriched in Th1-polarized memory T cells capable of differentiating into effector cells upon antigen encounter. However, the discovery of Th17 cells and their pathogenic role in experimental autoimmune encephalomyelitis forced a revision of effector subsets in multiple sclerosis patients. Nearly 10 years ago, higher numbers of IL-17-expressing mononuclear cells in the CSF of multiple sclerosis patients were documented (Matusevicius et al., 1999), although at that time there was no evidence for the relevance of such cells in multiple sclerosis. We show here at a single cell level the presence of IL-17A-producing CD4+ T cells in the CSF of both non-inflamed and inflamed CSF. In the first case, the percentage of IL-17A-producing cells is similar to that of peripheral blood, while in patients with inflammatory diseases it is increased, and this is more pronounced in multiple sclerosis patients during relapse. Compared to the high frequency of IFN-γ-producing cells in the CSF (more than 50% of the CD4+ T cells even in patients with no inflammatory disease), Th17 cells, with percentages ranging from two to six of the CD4+ T cell subset, might appear irrelevant. However, it is remarkable that the frequency of Th17 cells is higher during relapses and in later stages of disease, while Th1 cells remain stable, with similar
percentages in inflamed and non-inflamed CSF, and even decreased in samples from patients obtained during relapse. Although interesting, a longitudinal study investigating the frequency of Th17 cells in the CSF of multiple sclerosis patients during disease exacerbation and remission is not feasible due to difficulties in repeatedly tapping patients within short time intervals. In addition to the classical Th17 cells, a further subset simultaneously producing IL-17A and IFN-γ was always present in the CSF, representing 0.5–1% of the total CSF CD4+ T cells. This population, however, remained relatively stable in all samples tested, with no significant differences between the various disease stages. It is still unknown whether these double producers represent yet another subset of helper T cells that require differential signals for differentiation, or reflect a degree of plasticity between Th1 and Th17 cells.

In the brain of multiple sclerosis patients, CD4+ T cells are distributed mostly in the perivascular spaces and meninges, while CD8+ T cells are found mainly in the parenchyma (Gay et al., 1997). A recent histopathological report showed increased labeling for IL-17 in both CD4+ and CD8+ T cells in active areas of multiple sclerosis lesions (Tzartos et al., 2008), with evidence of active synthesis rather than uptake. In our study we could not detect IL-17A-producing CD8+ T cells in the CSF, and none of the 60 CD8+ T cell lines obtained produced IL-17A. In peripheral blood, a few CD8+ T cells were reportedly IL-17A+. This discrepancy with the data from Tzartos et al. (2008) might be explained by the fact that the parenchymal lesions analysed in that paper and the CSF are obviously two different compartments, and even if CSF is in contact with all brain surfaces and interstitial fluid drains into it, the movement of cells is still limited. It may also reflect different stages of the disease, since the samples studied by Tzartos et al. (2008) were autopsy tissue from patients with a disease duration of many years, in most cases, and already in the progressive phase of the disease, while our patients were either in the first episodes or in the relapsing-remitting phase of the disease.

While raising Th17 and Th1 T cell clones we found that higher percentages of the Th17 clones were obtained in comparison to the starting Th17 frequency, suggesting the possibility that these cells have a proliferation advantage over the Th1 counterparts. Our experiments using ex vivo T cell confirmed that Th17 cells proliferate more readily and are less susceptible to suppression than Th1 cells. Higher resistance to suppression by regulatory T cell clones of Th17 clones raised from patients with Crohn’s disease had already been reported (Annunziato et al., 2007). A higher expression of co-stimulatory molecules could be the reason for the elevated proliferation rates of Th17 cells. While CD28 is constitutively present on most T cells, CTLA-4, ICOS and PD-1 are upregulated after activation. Expression of the positive stimulators CD28 and ICOS is higher in Th17 cells, but the inhibitory molecules CTLA-4 and PD-1 were also expressed at higher levels, at least under the conditions tested (18 h of stimulation). Given that CTLA-4 and PD-1 are only expressed after stimulation, an elevated constitutive expression of CD28 might be the determinant for the higher proliferation of Th17 cells. It is likely that ICOS plays a role in promoting Th17 expansion, given that splenocytes from mice deficient in ICOS produced less IL-17 even through they produced normal levels of tumor necrosis factor-α and IFN-γ (Dong and Nurieva, 2003). In addition, the higher expression of CD2 in Th17 cells might help to maintain the immunological synapse more efficiently, and thus result in better conditions for induction of proliferation.

The expression of adhesion molecules for T cell binding to endothelium is a key factor for T cells to extravasate and find their way into the brain. Blocking very late antigen-4 results in amelioration of experimental autoimmune encephalomyelitis (Kent et al., 1995), and a humanized antibody against CD49d has proved efficient in reducing lesion load and symptoms in multiple sclerosis patients (O’Connor et al., 2005). There is much less known about MCAM/CD146 in multiple sclerosis, but MCAM/CD146 is expressed in a subset of activated T cells, and is involved in the initial steps of lymphocyte–endothelium interaction (Guzguez et al., 2007), and thus in the recruitment of activated T cells to the sites of inflammation. CD49d is slightly more expressed on Th17 cells compared to Th1, especially in the CSF, but the percentage of MCAM/CD146-positive cells was much higher within the Th17 compartment, both in ex vivo cells and in the T cell clones. Of all Th1 clones analysed, we found only one expressing MCAM/CD146, while most Th17 clones do so. Moreover, Th17 cells adhere better to a brain endothelial cell line than Th1, and this is at least in part due to the presence of MCAM. Th17 cells also express higher levels of CCR6 and CD6, which facilitate entry of infiltrating T cells into the central nervous system and play a role in the development of experimental autoimmune encephalomyelitis and probably multiple sclerosis (Cayrol et al., 2008; Reboldi et al., 2009). Altogether, it seems that Th17 cells have an advantage for their entry into the central nervous system, as already shown by Keib et al. (2007).

Even with the higher migratory and proliferative potentials, we cannot ignore the fact that Th17 cells are, in comparison to Th1 cells, a minority in the CSF of multiple sclerosis patients. However, IL-17 is a potent proinflammatory cytokine involved in the coordination of local tissue inflammation: it indirectly promotes the recruitment of monocytes and neutrophils (Fossiez et al., 1996; Keib et al., 2007) and the expansion of myeloid cells (Linden et al., 2000). IL-17 also drives T cell responses by chemokine (C-C motif) ligand-20 induction (Kao et al., 2005), which recruits dendritic and T cells. CCR6, the receptor for chemokine (C-C motif) ligand-20, is highly upregulated in Th17 cells (Singh et al., 2008), and thus could be involved in amplifying IL-17 immunity by recruiting more Th17 cells to the inflamed tissue via a positive feedback loop. IL-17 also promotes the transmigration of human CD4+ lymphocytes through blood–brain barrier endothelial cells by disrupting the tight junctions (Keib et al., 2007) and synergizes with IFN-γ in inducing intercellular adhesion molecules-1 expression on endothelium (Albanesi et al., 1999). Thus, a combined role of Th1 and Th17 cells seems to confer maximum possibilities to amplify the immune response in the brain, although this view is currently challenged by the results of the ustekinumab clinical trial in multiple sclerosis patients.

In summary, the presence of high numbers of IL-17-producing cells at the lesion site (Tzartos et al., 2008), an increased frequency in the CSF during relapses, their higher capacity to proliferate and to adhere to endothelium and transmigrate, together
with their capacity to elicit a powerful inflammatory response, support a role for this subset in the pathogenesis of multiple sclerosis, most likely together with Th1 cells.

Note added in proof

After we had submitted our manuscript, Durelli et al. (2009) reported that RR-MS patients with active disease show increased frequency of Th17 cells in peripheral blood. Our data (Fig. 1B) show the same tendency, although the difference was not statistically significant probably due to a smaller cohort of patients analyzed.

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Supplementary material

Supplementary material is available at Brain online.

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