doi:10.1093/brain/awy151 BRAIN 2018: 141; 2066–2082 | 2066

BRAIN
A JOURNAL OF NEUROLOGY

# The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells

**Joana Machado-Santos,[1] Etsuji Saji,[2] Anna R. Tröscher,[1] Manuela Paunovic,[1] Roland Liblau,[3] Galina Gabriely,[4] Christian G. Bien,[5] Jan Bauer[1] and Hans Lassmann[1]**

Multiple sclerosis is an inflammatory demyelinating disease in which active demyelination and neurodegeneration are associated with lymphocyte infiltrates in the brain. However, so far little is known regarding the phenotype and function of these infiltrating lymphocyte populations. In this study, we performed an in-depth phenotypic characterization of T and B cell infiltrates in a large set of multiple sclerosis cases with different disease and lesion stages and compared the findings with those seen in inflammatory, non-inflammatory and normal human controls. In multiple sclerosis lesions, we found a dominance of CD8+ T cells and a prominent contribution of CD20+ B cells in all disease courses and lesion stages, including acute multiple sclerosis cases with very short disease duration, while CD4+ T cells were sparse. A dominance of CD8+ T cells was also seen in other inflammatory controls, such as Rasmussen's encephalitis and viral encephalitis, but the contribution of B cells in these diseases was modest. Phenotypic analysis of the CD8+ T cells suggested that part of the infiltrating cells in active lesions proliferate, show an activated cytotoxic phenotype and are in part destroyed by apoptosis. Further characterization of the remaining cells suggest that CD8+ T cells acquire features of tissue-resident memory cells, which may be focally reactivated in active lesions of acute, relapsing and progressive multiple sclerosis, while B cells, at least in part, gradually transform into plasma cells. The loss of surface molecules involved in the egress of leucocytes from inflamed tissue, such as S1P1 or CCR7, and the upregulation of CD103 expression may be responsible for the compartmentalization of the inflammatory response in established lesions. Similar phenotypic changes of tissue-infiltrating CD8+ T cells were also seen in Rasmussen's encephalitis. Our data underline the potential importance of CD8+ T lymphocytes and B cells in the inflammatory response in established multiple sclerosis lesions. Tissue-resident T and B cells may represent guardians of previous inflammatory brain disease, which can be reactivated and sustain the inflammatory response, when they are re-exposed to their specific antigen.

1 Department of Neuroimmunology, Center for Brain Research, Medical University of Vienna, Vienna, Austria
2 Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan
3 INSERM U1043 - CNRS UMR 5282, Centre de Physiopathologie Toulouse-Purpan, Université Toulouse III, Toulouse, F-31000, France
4 Department of Neurology, Anne Romney Center for Neurologic Disease, Harvard Medical School, Boston, USA
5 Epilepsy Center Bethel, Krankenhaus Mara, Bielefeld, Germany

Correspondence to: Prof. Dr Hans Lassmann
Center for Brain Research
Medical University of Vienna
Spitalgasse 4
A-1090 Wien
Austria
E-mail: hans.lassmann@meduniwien.ac.at

Received January 17, 2018. Revised March 22, 2018. Accepted April 15, 2018. Advance Access publication June 4, 2018

**Keywords:** multiple sclerosis; brain lesions; T cells; B cells; immune activation

**Abbreviations:** ADEM = acute disseminated encephalomyelitis; EAE = experimental autoimmune encephalomyelitis

# Introduction

Multiple sclerosis is an inflammatory demyelinating disease of the CNS (Charcot, 1880). Active demyelination and neurodegeneration in the multiple sclerosis brain and spinal cord are consistently associated with the presence of perivascular and parenchymal inflammatory infiltrates, composed of T and B lymphocytes (Frischer *et al.*, 2009). Within the T cell population, MHC class I-restricted CD8+ T cells are the most abundant and preferentially show clonal expansion in the lesions (Booss *et al.*, 1983; Hayashi *et al.*, 1988; Babbe *et al.*, 2000; van Nierop *et al.*, 2017). In addition, clonal expansion of B lymphocytes and plasma cells is reflected by intrathecal immunoglobulin synthesis (Obermeier *et al.*, 2011). Although previous data suggest that CD8+ memory T cells are dominant in lesions of progressive multiple sclerosis (van Nierop *et al.*, 2017), little is known regarding their presence in active lesions at early disease stages as well as their phenotype and activation state in fresh lesions. Also, information on B cells within multiple sclerosis lesions is limited (Frischer *et al.*, 2009), and it is unclear to what extent the inflammatory reaction in the brain is multiple sclerosis specific or reflects a general pattern seen also in other human inflammatory diseases of the CNS. Finally, inflammation in the progressive stage of the disease is, at least in part, trapped within the CNS behind a closed blood–brain barrier (Hochmeister *et al.*, 2006), but little is known about the reasons for its persistence.

Experimental autoimmune encephalomyelitis (EAE) is considered a suitable model for multiple sclerosis and is, thus, frequently used to study molecular mechanisms involved in inflammation and neurodegeneration. EAE can be induced by CD4+ T cells (Ben-Nun *et al.*, 1981), by CD4+ T cells in combination with demyelinating antibodies (Linington *et al.*, 1988) and by CD8+ T cells (Huseby *et al.*, 2001; Saxena *et al.*, 2008). In addition, mechanisms triggered by innate immunity can provoke lesions, which share some pathological features of those present in multiple sclerosis (Felts *et al.*, 2005). Currently available anti-inflammatory or immunomodulatory treatments have in part been developed or tested in EAE models. They are effective in the relapsing stage of multiple sclerosis to a variable degree, but show only a moderate or even no effect, when patients have entered the progressive phase of the disease (Dargahi *et al.*, 2017). Recent clinical trials in multiple sclerosis patients revealed profound anti-inflammatory effects of treatments, targeting all T cells in combination with B cells or targeting B cells alone, and a modest effect of these treatments could even be seen in patients with progressive disease, who still showed evidence for clinical or MRI activity [siponimod (Gajofatto, 2017); and ocrelizumab (Montalban *et al.*, 2017)]. In contrast, other therapeutic strategies, selectively altering/inhibiting the function of MHC class II-restricted T cells (CD4+ T cells) showed disappointing results [anti-CD4 (van Oosten *et al.*, 1997); ustekinumab (Segal *et al.*, 2008)].

These data suggest that there are key differences in the inflammatory response in the CNS between patients with multiple sclerosis and EAE animals. However, information on the phenotype and function of inflammatory cells in different types and stages of multiple sclerosis lesions is sparse. In the present study, we addressed these questions by analysing in detail the phenotype and activation state of T and B lymphocytes in the multiple sclerosis brain in comparison to a very large spectrum of controls with other inflammatory, vascular or neurodegenerative diseases.

# Patients and methods

## Sample characterization and inclusion criteria

Studies were performed on archival formaldehyde-fixed and paraffin-embedded (FFPE) autopsy and, for Rasmussen's encephalitis, on surgical material (Supplementary Table 1) collected at the Center for Brain Research, Medical University Vienna. The clinical course of multiple sclerosis was defined by a certified neurologist. The study included 35 cases of multiple sclerosis comprising acute multiple sclerosis ($n = 11$), relapsing-remitting multiple sclerosis ($n = 1$), secondary progressive multiple sclerosis ($n = 16$) and primary progressive multiple sclerosis ($n = 7$). As controls, cases of acute disseminated encephalomyelitis (ADEM; $n = 1$), Rasmussen's encephalitis ($n = 6$), herpes simplex virus encephalitis ($n = 6$), cytomegalovirus encephalitis ($n = 6$), progressive multifocal leukoencephalopathy ($n = 8$), stroke ($n = 16$), Alzheimer's disease ($n = 14$) and age-matched controls without any detectable brain disease ($n = 10$) were included. In addition, we included a unique case of chronic human autoimmune encephalomyelitis, which occurred in the course of a misguided therapeutic intervention with lyophilized brain cells, and which showed a clinical course and pathological changes, closely similar to those seen in acute multiple sclerosis (Hoftberger *et al.*, 2015). This study was approved by the Ethical Committee of the Medical University Vienna in Vienna, Austria (EK. Nr: 535/2004/2017).

## Neuropathology and lesion selection

All diseased and control autopsy cases underwent detailed neuropathological analysis based on multiple tissue blocks from the brain and the respective blocks that contained the

lesions of interest were selected (Supplementary Table 1). Sections from these blocks were then digitally scanned and lesion maps were created with the respective lesion types and activity stages. Subsequently, all the slides were stained by immunohistochemistry and cells were counted in the previously outlined regions. Cases, blocks and lesions were selected according to the following criteria.

### Multiple sclerosis

Only cases exhibiting inflammation and, at least in a subset of lesions, active demyelinating and neurodegenerative activity were included (Frischer *et al.*, 2009). The samples contained active lesions with initial stages of myelin destruction and areas of early and late active demyelination (Bruck *et al.*, 1995), chronic active or slowly expanding lesions (Frischer *et al.*, 2015) and inactive or remyelinated lesions, all defined according to recently published criteria (Kuhlmann *et al.*, 2017).

### Inflammatory controls

Cases were selected with active lesions characterized by profound brain inflammation and disease-specific tissue injury, including virus-infected cells, when applicable.

### Non-inflammatory disease controls

These cases included patients with acute ischaemic stroke lesions (acute lesions and lesions in the resorption or scar stages) and Alzheimer's disease in the advanced stage of dementia (Braak stages 5 to 6).

### Normal controls

These included brains from patients without neurological disease and absence of neuropathological lesions.

Detailed information on the clinical background of the patients and the type of lesions included into this study are provided in Supplementary Table 1.

## Selection of leucocyte markers

The selection of inflammatory markers was performed according to the following criteria: for determining the basic composition of the inflammatory infiltrates we used antibodies against all T cells (CD3), against MHC class I restricted T cells (CD8α), MHC class II restricted T cells (CD4) and B cells (CD20). Plasma cells were identified by the cytoplasmic immunoglobulin content and by their expression of CD138. When T or B cells recognize their cognate antigen, they become activated and proliferate. We thus determined the proliferation rate of lymphocytes by double staining with the cellular marker (CD3, CD4, CD8α or CD20) and markers for cell proliferation [proliferating cell antigen PCNA, mini-chromosome maintenance protein (MCM2) and Ki67]. Furthermore, T cells transiently express the nuclear factor of activated T cells (NFAT2, encoded by *NFATC1*) after antigen-specific activation (Dietz *et al.*, 2015), and thus, we determined the nuclear translocation of this protein as an additional activation antigen of CD8α- and CD4-positive cells by double staining. Activated cytotoxic T cells were identified by the expression of granzyme B (GZMB). A subset of T cells, which had entered the CNS, were deleted by programmed cell death (apoptosis; Bauer *et al.*, 1998). In our study, we determined the apoptosis rate by identifying apoptotic nuclei in CD4- or CD8α- and β-positive T cells and CD20-positive B cells. We used morphological criteria for apoptosis for the quantitative evaluation, since it is the most sensitive and specific assay. We, however, confirmed programmed cell death by double staining of T cells with the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) technique (Gold *et al.*, 1994) and expression of activated caspase 3 (Stadelmann *et al.*, 1999). In inflammatory lesions, a subset of T cells may become tissue-resident memory cells (Schenkel and Masopust, 2014; Steinbach *et al.*, 2016). A previously-described phenotype of such cells is the loss of expression of CD8β, thus becoming CD8α/α positive and the expression of CD103 and CD69 (Konno *et al.*, 2002; Fan and Rudensky, 2016). Immunohistochemistry for the sphingosine phosphate receptor (S1P1) was used to determine to what extent infiltrating lymphocytes have lost the potential for tissue egress. Additional markers used in our study were CCR5 (a chemokine receptor involved in the recruitment of T cells and their migration in the CNS) (Simpson *et al.*, 2000; Martin-Blondel *et al.*, 2016), OX40 (an antigen expressed on T cells, which in inflammatory conditions of the human brain is expressed on CD4+ T cells) (Pohl *et al.*, 2013) and fosters their survival (Webb *et al.*, 2016), PD1 (an inhibitory receptor involved in downregulation and control of the inflammatory processes) (Legat *et al.*, 2013) and CD27 or CD38 (multifunctional molecules expressed in different leucocyte populations, being also involved in B lymphocyte survival and plasma blast differentiation) (Palanichamy *et al.*, 2014; Claes *et al.*, 2015). To identify a potential regulatory function of the T cells, we analysed the expression of the immunosuppressive cytokines interleukin 10 (IL-10) and transforming growth factor beta (TGF-β). Beforehand, all markers used in this study were tested in paraffin sections of lymph nodes and tonsils. The optimal staining conditions were established in these tissues (Supplementary Fig. 1) and their staining patterns in the inflamed brains are shown in Figs 1 and 5.

## Immunohistochemistry

### Single staining

Consecutive 5-µm thick serial sections were cut and routinely deparaffinized with xylene. Afterwards, endogenous peroxidase was blocked with $H_2O_2$/methanol for 30 min and the slides proceeded to the antigen retrieval step. Antigen retrieval procedures used for immunohistochemistry are listed in Table 1. Non-specific antibody binding was blocked by incubating the sections in 10% foetal calf serum for 20 min. Subsequently, the primary antibodies were applied and the slides were kept at 4°C overnight in a wet chamber. After washing the slides, the secondary antibodies were applied for 1 h at room temperature followed by 1 h incubation in avidin peroxidase in 10% foetal calf serum. Antibody binding was routinely visualized using 3,3′-diaminobenzidine (DAB). For some antibodies, staining was enhanced by biotinylated tyramine amplification (CSA; Bauer and Lassmann, 2016) as presented in Table 1. To increase sensitivity of tissue staining with anti-CD4 antibodies we also used alkaline phosphatase-labelled secondary antibody and developed the sections with MBT/BCIP (Gold *et al.*, 1994). Before mounting the sections, cell nuclei were counterstained with haematoxylin. To control the specificity of immunohistochemistry, sections were either stained in the absence of primary antibodies or after using mouse monoclonal

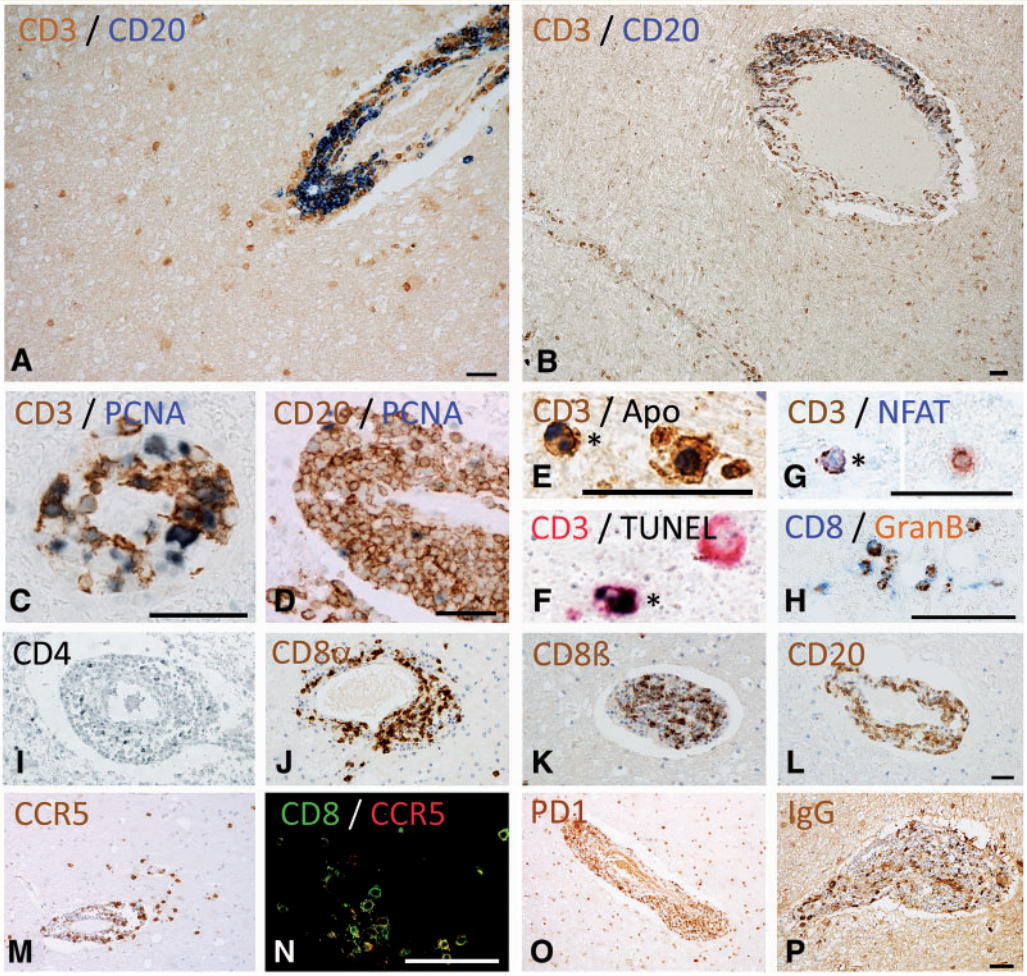


**Figure 1 Examples of the inflammatory response in multiple sclerosis lesions.** **A** and **B** show inflammation in active lesions of acute (**A**) and secondary progressive multiple sclerosis (**B**). CD3+ T cells are stained in brown and B cells in blue. T cells are present in the perivascular space and diffusely disperse into the lesion parenchyme. In contrast, B cells accumulate mainly in the perivascular space of large veins, located in the centre of the lesions. **C** and **D** show proliferating T cells (**C**) and B cells (**D**) expressing PCNA within their nucleus. (**E** and **F**) Apoptosis is seen in T cells (asterisk), labelled with CD3 and identified by the fragmented and condensed nucleus (**E**) or by the positivity for DNA-fragmentation (TUNEL staining; **F**). (**G**) Rare CD3+ T cells, mainly located in the lesion parenchyme show nuclear expression of the transcription factor NFAT (blue nucleus; asterisk) in comparison to an adjacent T cell without nuclear reactivity. (**H**) Clusters of CD8+ T cells (blue) containing GZMB-positive cytoplasmic granules (brown) as a sign of cytotoxic differentiation are seen in the lesions. (**I** to **L**) Perivenous inflammatory cuffs contain only few CD4+ T cells (**I**), while the majority of cells are CD8+ (**J** and **K**) or CD20+ (**L**). (**M** and **N**) A subset of infiltrating lymphocytes express the chemokine receptor CCR5. (**O**) Only a small proportion of lymphocytes express the exhaustion marker PD1. (**P**) In addition to T and B cells a substantial number of immunoglobulin-containing plasma cells is seen in the infiltrates. Scale bars = 100 μm. Images for colourblind readers can be found in Supplementary Fig. 5.

antibodies of irrelevant specificity and normal rabbit serum as primary antibodies.

## Double labelling

In case of antibodies from different species, primary antibodies were incubated simultaneously, followed by simultaneous incubation with a biotin-labelled antibody and an alkaline phosphatase-labelled antibody. The staining was finished by incubation with avidin-peroxidase and sequential development with Fast blue and DAB. For double labelling with antibodies from the same species the same procedure described for the single staining was used until the step of incubation with avidin-peroxidase. At this point, instead, the slides were incubated with avidin-alkaline phosphatase for 1 h at room temperature and developed with Fast blue B salt. After this, to prepare the sections for a new primary antibody and prevent binding of the new antibodies to the primary and secondary antibodies used in the first round, antigen retrieval was performed for 45 min (Bauer and Lassmann, 2016). The sections were then processed as described before for single staining and developed with DAB or 3-amino-9-ethylcarbazole. Alternatively, double staining was performed by immunofluorescence and analysed with a Leica SP2 confocal microscope, using a similar approach as described above, except using fluorescence-labelled secondary antibodies or streptavidin (Bauer and Lassmann, 2016). The following double stainings

**Table 1 Antibodies and immunohistochemistry**

| Primary antibody | Origin | Target | Dilution | Antigen retrieval /amplification | Source |
|---|---|---|---|---|---|
| CD3 | Rabbit (mAB) | T cells | 1:1000 | EDTA/CSA | Neomarkers |
| CD8 | Mouse (mAB) | MHC class I restricted T cells; CD8-$\alpha$ chain | 1:250 | EDTA/CSA | Dako |
| CD20 | Mouse (mAB) | B cells | 1:100 | EDTA | Neomarkers |
| CD4 | Mouse (mAB) | MHC class II restricted T cells | 1:500 + 1:1000 | EDTA/CSA | Dako Acris |
| CD8-$\beta$ | Mouse (mAB) | CD8-$\beta$ chain of T cells | 1:100 | EDTA/CSA | Santa Cruz |
| CD138 | Mouse (mAB) | Syndecan-1, plasma cells | 1:500 | EDTA | Serotec |
| PCNA | Mouse (mAB) | Proliferation cell nuclear antigen | 1:50 000 | Citrate | Dako |
| Mcm2 | Rabbit (pAB) | Mini-chromosome maintenance protein, proliferation marker | 1:200 | EDTA | Abcam |
| Ki67 | Mouse (mAB) | Proliferation marker | 1:500 | Citrate | Dako |
| OX40 | Mouse (mAB) | Tumour necrosis factor receptor superfamily, member 4 | 1:1000 | Citrate/CSA | BD Pharmingen |
| NFAT2 | Mouse (mAB) | Nuclear factor of activated T cells | 1:50 | EDTA | Abcam |
| CD27 | Rabbit (pAB) | TNFRSF7 | 1:1000 | EDTA | Novus Biologicals |
| CD38 | Rabbit (pAB) | ADP-ribosyl cyclase-1 | 1:1000 | EDTA | LifeSpan Biosciences |
| CCR5 | Mouse | C-C chemokine receptor type 5 | 1:250 | EDTA | Dr Matthias Mack |
| PD-1 | Mouse (mAB) | Programmed cell death protein 1 | 1:100 | EDTA | Abcam |
| TGB-$\beta$1 | Mouse (mAB) | Transforming growth factor superfamily | 1:250 | EDTA | Biolegend TW7–28G11 |
| IL-10 | Rabbit (pAB) | Interleukin 10 | 1:100 | EDTA | Bioss |
| Granz B | Mouse (mAB) | Recombinant human granzyme B | 1:1000 | EDTA/CSA | Neomarkers |
| CD103 | Rabbit (mAB) | Integrin protein encoded by the *ITGAE* gene | 1:500 | EDTA/CSA | Abcam ab129202 |
| CD69 | Mouse (mAB) | Transmembrane C-Type lectin protein | 1:200 | EDTA | ThermoFisher |
| S1P1 | Rabbit (pAB) | Sphingosine phosphate receptor | 1:500 | Citrate | PromoKine AB718 |
| CD45RA | Mouse (mAB) | Naïve T cells, B cells | 1:100 | EDTA | Abcam 4KB5 |
| Cleaved Caspase 3 | Rabbit (mAB) | Activated caspase 3 (apoptosis) | 1:750 | Citrate | Cell Signal 5AIE |
| Human Ig | Donkey (pAB) | Human immunoglobulin; plasma cells | 1:1000 | No | Jackson 709–065–149 |

Citrate = antigen retrieval in citrate buffer, pH 5.0; EDTA = antigen retrieval in EDTA buffer, pH 9.0; CSA = biotinylated tyramine amplification; mAB = monoclonal antibody; pAB = polyclonal antibody.
For detailed description of methods see Bauer and Lassmann (2016).

were included in the study: PCNA or MCM2 with CD3, CD8$\alpha$, CD4 and CD20; NFAT2 and CD3; TUNEL and CD3; CD8$\alpha$ and CD8$\beta$, CD8$\alpha$ and CD103, CD8$\alpha$ and GZMB, CD69 and CD8$\alpha$; CD3 and CCR5, CD3 and PD1; CD3 and IL-10 and CD27 or CD38 with CD8$\alpha$, CD20 or CD138, respectively.

## Quantification of immunohistochemistry

Quantification was performed on serial sections of each case and lesion using one section per marker and area of interest. Within each lesion area of appropriate size for quantification and defined activity stage were outlined in sections stained with Luxol fast blue myelin stain and marked in adjacent immunostained sections as areas of interest. For cell counting, a morphometric grid within the ocular lens was used and inflammatory cell numbers were manually counted in 10–50 fields at an objective lens magnification of $\times 20$, depending on the density of inflammatory infiltrates within the tissue and the size of the lesions, covering an area of 2.5 to 12.5 $mm^2$ per area of interest. The inflammatory cells (T and B cells) from perivascular and parenchymal areas were counted separately. Later, these values were pooled for statistical evaluation of global inflammation. All values are expressed as cell counts per square millimetre.

## Statistical analysis

Statistical analysis was performed using Graphpad Prism, and results are presented as box plots showing the median and range of each group. All statistics reporting differences between lesions were calculated from one median value per lesion per patient. Because of the uneven distribution of our data, non-parametric tests were used. Statistical difference between multiple groups was assessed using the Kruskal–Wallis test and followed by Dunn's Multiple Comparison Test. When only two groups were compared the Mann-Whitney test was used. Correlations between different parameters were calculated using Spearman Rank. Only $P$-values $\leqslant 0.05$ were considered as statistically significant.

# Results

First, we determined the extent of T cell and B cell infiltration in a wide spectrum of multiple sclerosis lesions in comparison to that seen in other inflammatory and non-inflammatory diseases of the CNS and in normal controls. In the second step, we aimed to define the functional phenotype of the most abundant lymphocyte population (CD8+ and CD20+) and plasma cells in the multiple sclerosis brain. To understand whether the functional

differentiation of T lymphocytes is specific for multiple sclerosis lesions or a general pattern seen in brain inflammation we analysed a spectrum of inflammatory controls, ranging from a fulminant case of acute disseminated leukoencephalomyelitis with 2 days disease duration (Bauer *et al.*, 1998), a highly active case of human demyelinating autoimmune encephalomyelitis with a pathology mimicking acute multiple sclerosis (Hoftberger *et al.*, 2015) and six patients with Rasmussen's encephalitis, as a CD8+ T cell-mediated inflammatory disease of the CNS (Bien *et al.*, 2002).

## CD8+ T lymphocytes and B cells in inflammatory brain diseases

High numbers of CD3+ T cells were seen in the multiple sclerosis lesions and on average 76% of CD3+ T cells were MHC class I restricted CD8+ cells, while on average only 10% were CD4+ (Figs 1A, B, I–L and 2). This was not only the case in patients with relapsing and progressive disease, but also in patients with fulminant acute multiple sclerosis with a clinical course ranging from 7 days to 7 months (Figs 1A, B and 2). When different multiple sclerosis lesion types were directly compared, numbers of T cells were significantly higher in early stages of multiple sclerosis compared to progressive multiple sclerosis and in active versus inactive lesions (Fig. 2E–L) and this was reflected by a highly significant negative correlation between T cell infiltrates with age and disease duration (Supplementary Fig. 2A and B). The T cells were located in the perivascular space of veins, and they also diffusely infiltrated the lesional parenchyma (Figs 1A and 3A). As described before, diffuse T cell infiltrates were present, but sparse at sites of initial demyelination compared to active lesion areas with accomplished demyelination (Fig. 3A) (Barnett and Prineas, 2004; Marik *et al.*, 2007). No significant differences were seen in the density of T cell infiltrates between acute multiple sclerosis patients following pattern II versus pattern III demyelination (Supplementary Fig. 2C), defined according to Lucchinetti *et al.* (2000).

Overall, the number of CD20+ B cells was highly variable in different multiple sclerosis lesions, ranging from no B cells in some patients with progressive multiple sclerosis to 4 times the T cell numbers (Figs 1A, B and 2). Possible reasons for this high variation between cases and lesions are that B cells were highest in a subset of cases with the most active disease and lesions (Fig. 2 and Supplementary Fig. 2), while in patients at later disease stages plasma cells dominated (Frischer *et al.*, 2009) (Fig. 1P). Furthermore, the vast majority of B cells were focally located in the perivascular space of only one or a few larger veins within the centre of the plaques, but only exceptionally present around small vessels or diffusely in the lesion parenchyma (Fig. 3E). We did not find significant differences in the T and B cell infiltration in the lesions between female and male patients, nor between patients with primary versus secondary progressive multiple sclerosis (data not shown), but acute multiple sclerosis patients with pattern II demyelination according to Lucchinetti *et al.* (2000) had significantly less B cells in their inflammatory infiltrates compared to those with pattern III lesions (Supplementary Fig. 2C).

In other inflammatory diseases of the CNS, we also found a major dominance of CD8+ T lymphocytes in the lesions (86% of CD3+ T cells; Fig. 2A–D). Only small numbers of T cells were present in the brain lesions of non-inflammatory controls, such as stroke or Alzheimer's disease and in normal control brains (Fig. 2) and also in these cases the dominant lymphocyte cell population consisted of CD8$\alpha$+ cells (67% in non-inflammatory controls and 76% on normal controls). In contrast to multiple sclerosis, the number of B cells was very low in the other inflammatory diseases and these cells were nearly absent in non-inflammatory controls. Accordingly, B cell infiltrates were significantly higher in acute multiple sclerosis and the case of relapsing multiple sclerosis ($P < 0.0001$) as well as in primary and secondary progressive multiple sclerosis ($P < 0.01$) in comparison to other inflammatory controls. The only exception was the case of demyelinating human autoimmune encephalitis (not included in Fig. 2), where the infiltrates were composed of 77% B cells, 21% of CD8$\alpha$+ T cells and only exceptional CD4+ T cells (Hoftberger *et al.*, 2015).

## T and B cells are in part activated in active multiple sclerosis lesions and eliminated by programmed cell death

To determine the activation of T and B cells in the lesions we analysed the proliferation rate by double staining with leukocyte markers and proliferation markers (Figs 1C, D and 4C). In a first step we selected from the entire sample 49 cases and lesions with the highest numbers of T and B cells, performed double staining for CD3 with the proliferation markers PCNA, MCM2 and Ki67 and quantified the percentage of double-stained cells. The highest percentage of double-stained cells was seen in a similar range with PCNA and MCM2. The percentage of Ki67 double-positive cells was lower, possibly due to partial loss of this protein in autopsy tissue (Supplementary Fig. 3). For this reason, quantification of proliferating cells in the entire sample was then performed by double-staining with lymphocyte markers (CD3, CD4, CD8$\alpha$ and CD20) and PCNA. The highest proliferation rate of T cells was seen in the case of ADEM with 2 days of CNS disease duration (22.8% of CD3+ cells were PCNA positive). In multiple sclerosis cases, T cell proliferation was higher in lesions of acute and relapsing multiple sclerosis (median: 1.45%, range 0.1–4.8%) compared to progressive multiple sclerosis (median 0.5%, range 0–2.4%). In the progressive stage, proliferating T cells, when present were found in small focal clusters. In the case of human autoimmune encephalitis, with a disease duration that was similar to that seen in

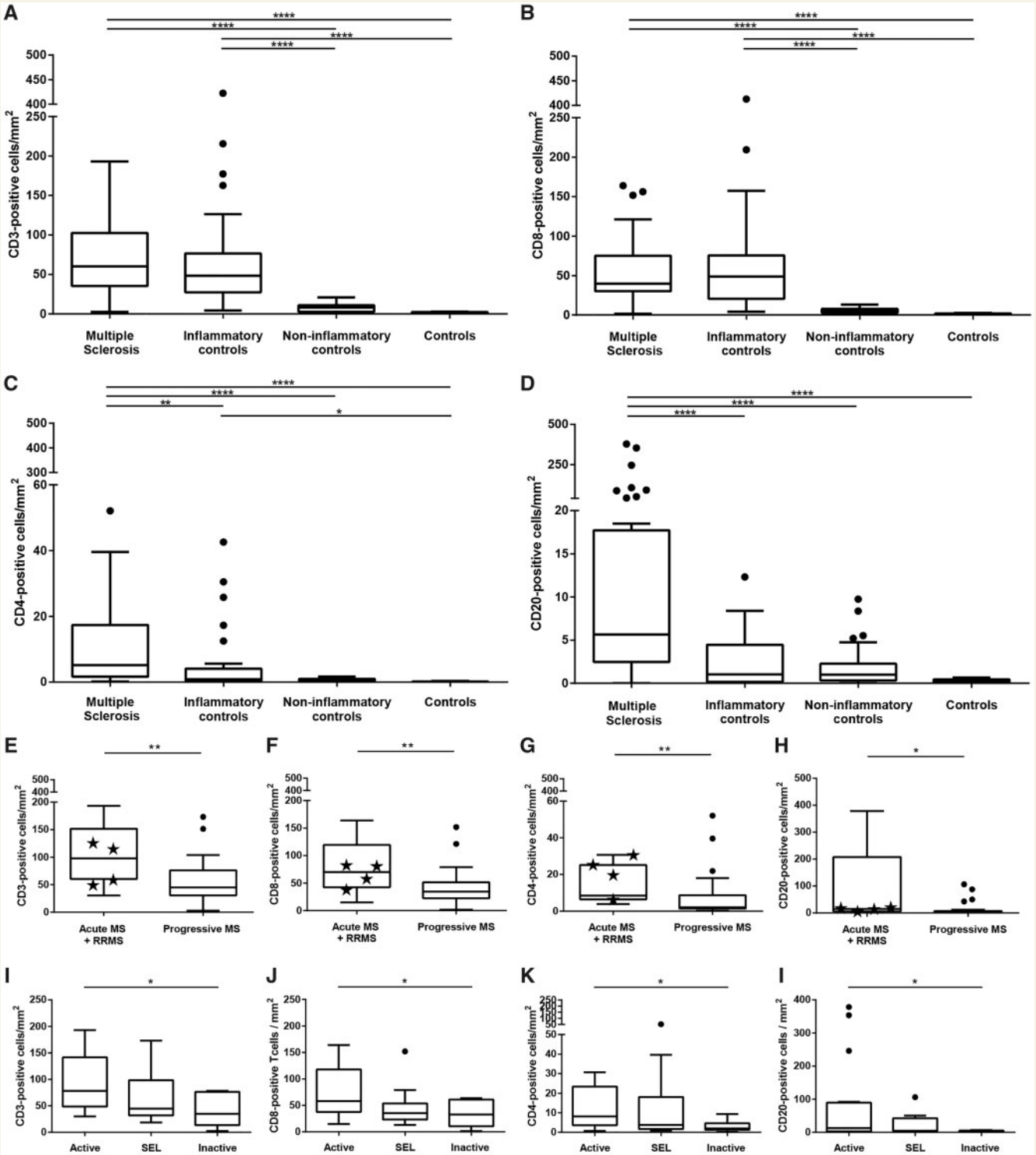


**Figure 2 Characterization of the lymphocyte subsets in multiple sclerosis lesions in comparison to inflammatory and non-inflammatory controls.** (**A**–**D**) The inflammatory response in the lesions is dominated by CD3 + /CD8 + T lymphocytes, while the contribution of CD4 + T cells is minor; the number of CD20 + B cells is highly variable between lesions and cases; while T cells are similar in number and distribution between multiple sclerosis and inflammatory controls, B cells are more selectively enriched in multiple sclerosis lesions in comparison to other inflammatory controls; T and B cell infiltrates are very sparse in non-inflammatory controls. (**E**–**L**) A more detailed analysis shows that T and B cells are significantly more numerous in the lesions of acute and relapsing multiple sclerosis in comparison to those in progressive multiple sclerosis (**E**–**H**). The stars shown in the acute multiple sclerosis and relapsing-remitting multiple sclerosis (RRMS) data indicate those patients, who have not been treated with corticosteroids. In addition, active lesions show the highest degree of inflammation, followed by slowly expanding lesions (SEL) and inactive lesions (**I**–**L**). Significance levels: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

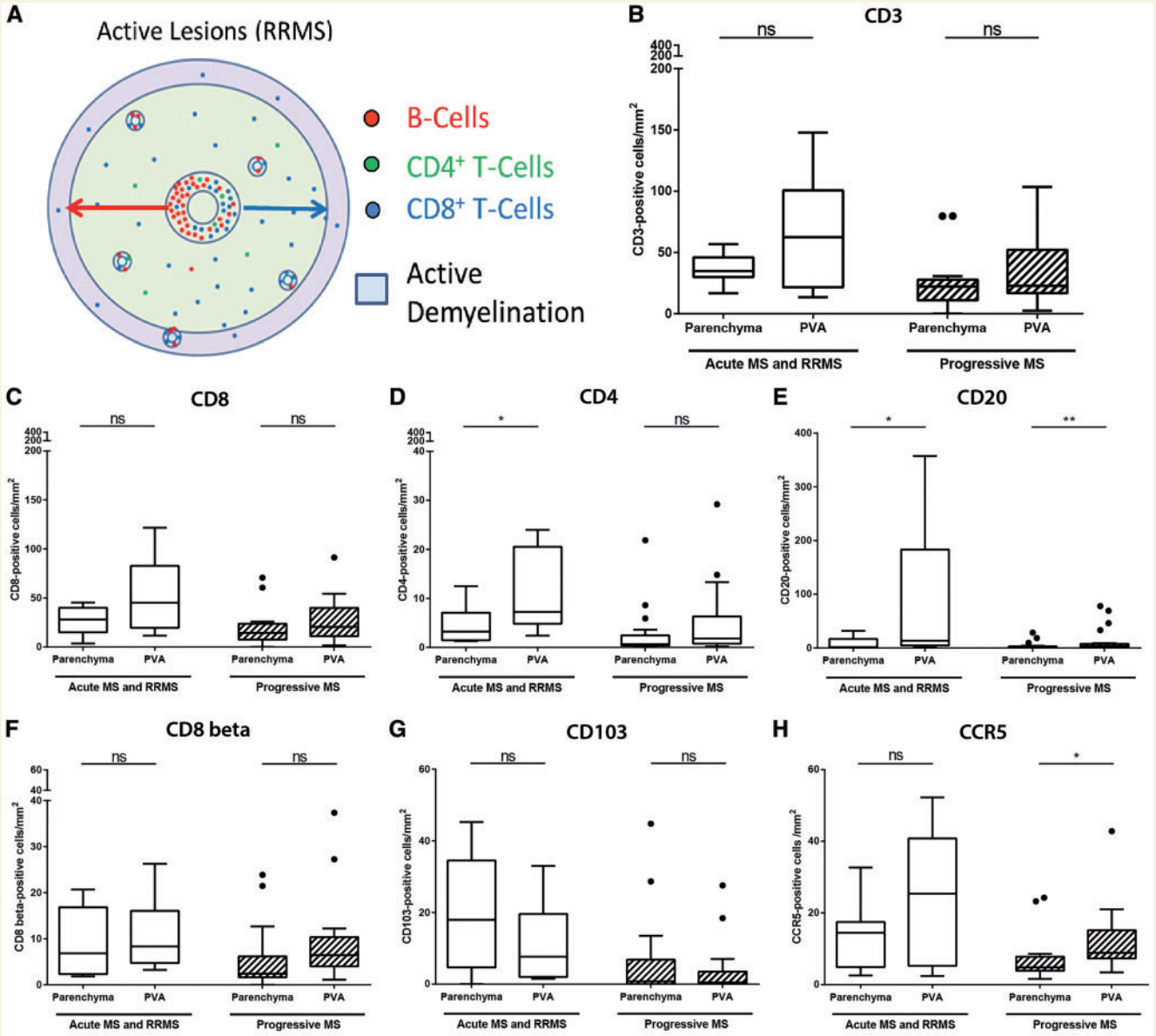


**Figure 3 Distribution of leucocytes within different multiple sclerosis lesion areas.** (**A**) Schematic graph of an active multiple sclerosis lesion with a central large inflamed vein, a demyelinated tissue area, containing macrophages with different stages of myelin degradation (green area) and a rim of initial tissue injury (grey zone) characterized by microglia activation, oligodendrocyte injury (loss of myelin associated glycoprotein) and oligodendrocyte apoptosis ('prephagocytic' lesion areas; Barnett and Prineas, 2004). The highest density of lymphocytes is seen in the perivascular space of the central vein and most of the B cells in the lesion are present at this site. T cells also diffusely infiltrate the lesion parenchyma (green area). They are also present, but in low numbers, at the site of initial demyelination (grey area; Marik *et al.*, 2007). (**B**–**H**) Perivascular (PVA) versus diffuse parenchymal distribution of lymphocyte subsets in multiple sclerosis lesions. When quantification is based on the area of the entire lesion ($mm^2$) most leucocyte subsets (CD3, CD8$\alpha$, CD8$\beta$, CCR5) are present in both compartments without significant preference. Dominant localization in the perivascular space is seen for B cells (CD20) and to a lower extent for CD4+ T cells. In contrast CD103+ T cells show a trend towards accumulation in the lesion parenchyma. Images for colourblind readers can be found in Supplementary Fig. 5. RRMS = relapsing-remitting multiple sclerosis.

the cases of acute multiple sclerosis, the proliferation rate was 2.3%. T cell proliferation was mainly seen in the CD8$\alpha$+ T cell population, while double stained CD4+ cells were exceptional. B cell proliferation was the highest in the case of human autoimmune encephalomyelitis (3.5%). In multiple sclerosis, proliferating B cells were only seen in three cases of acute and two cases of progressive multiple sclerosis, with proliferation rates between 0.8 to 4.3%.

In the next step, we analysed the expression of NFAT2, which is transiently upregulated and translocated into the nucleus when T cells recognize their cognate antigen

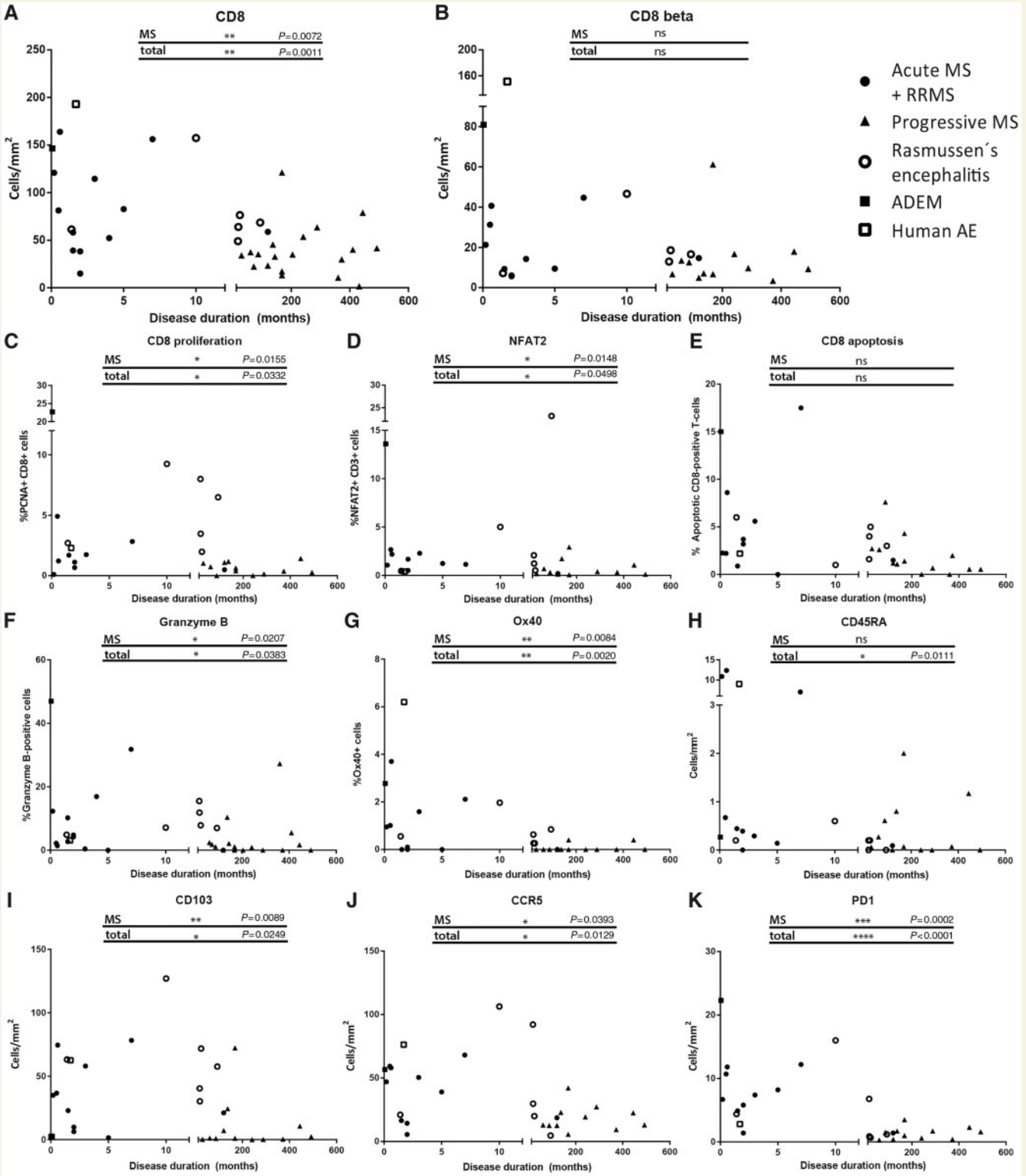


**Figure 4 Phenotypic characterization of CD8+ T lymphocytes in multiple sclerosis lesions from different disease stages in comparison to inflammatory controls.** The values are plotted against disease duration; early stage disease includes a case of ADEM with 2 days disease duration (filled square), of human autoimmune encephalomyelitis (h-AE) with 1.7 months disease duration (open square), of acute multiple sclerosis (disease duration 0.2 to 7 months; filled circles) and of Rasmussen's encephalitis (open circles, disease duration: 1.4 to 93.4 months). Cases of progressive multiple sclerosis are indicated by filled triangles (disease duration of 30 to 492 months). Overall there is a decrease of the inflammatory reaction with disease duration. T cell activation markers (proliferation, NFAT and GZMB expression and apoptosis) are very low in patients with progressive multiple sclerosis, but even in these patients small clusters of activated CD8+ T cells are present. The dominant CD8+ T cell phenotype in all stages is the CD8$\alpha/\alpha$-positive cell, showing an inactive (quiescent) phenotype. MS = multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis.

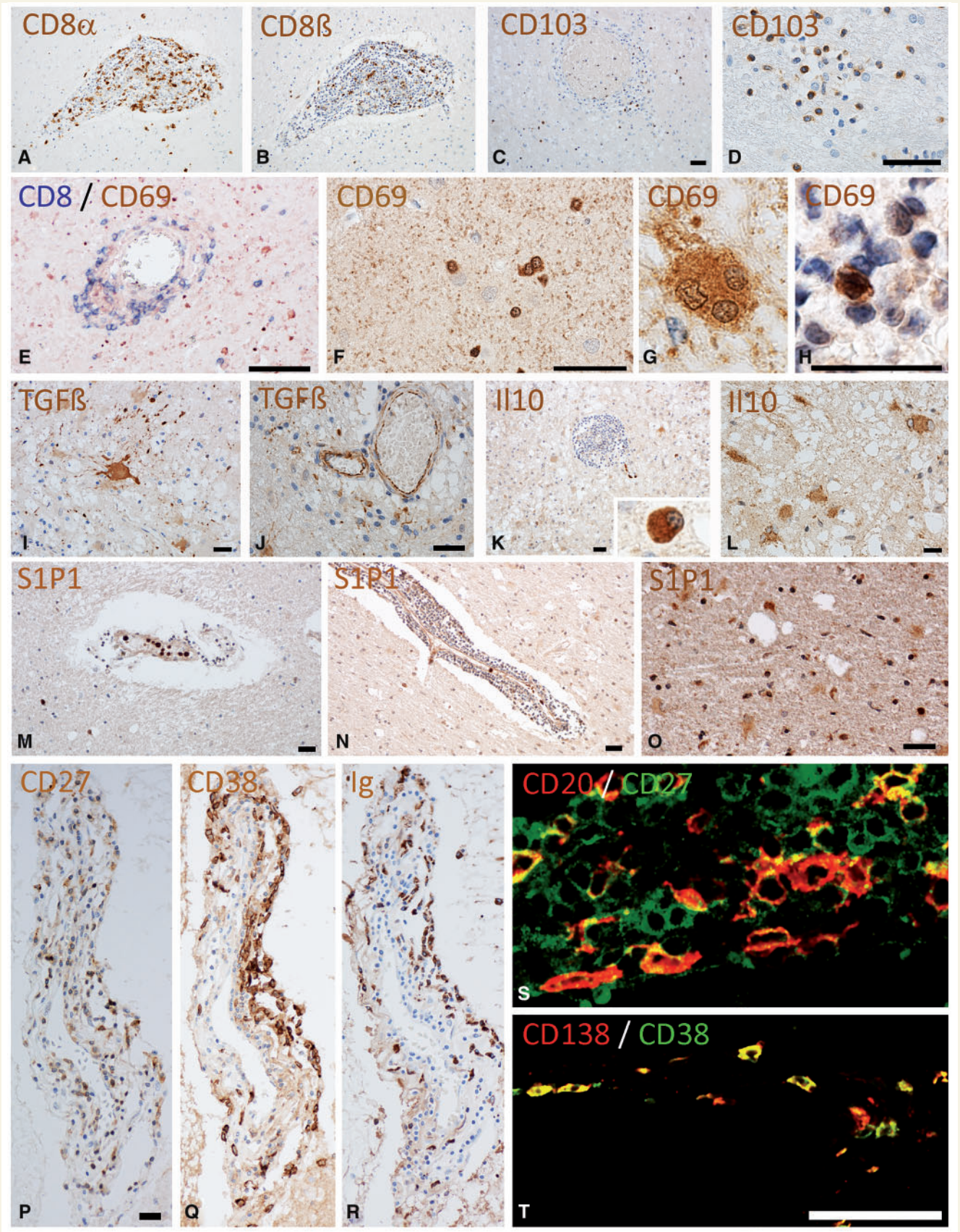


**Figure 5 Phenotypic characterization of CD8+ T cells and B cells in multiple sclerosis lesions.** (**A** and **B**) Only a subset of the CD8$\alpha$ positive cells also express CD8$\beta$ in a lesion of acute multiple sclerosis. (**C** and **D**) CD103 is expressed in a subset of lymphocytes in the lesions; these cells are preferentially located in the lesion parenchyma and less in the perivascular space. (**E**–**G**) CD69 is expressed in multiple

(continued)

(Figs 1G and 4D). As with proliferation markers, nuclear NFAT2 reactivity in T cells was the highest in the case of ADEM (13.6% of all T cells). Expression of NFAT2 in multiple sclerosis lesions was similar compared to the proliferation rate (0 to 2.6% with the highest numbers in cases with acute multiple sclerosis). Another activation marker is OX40, which is expressed in T cells and is associated with T cell survival (Webb *et al.*, 2016). As shown before, only very few T cells in multiple sclerosis lesions express this marker (Fig. 4G), which is different from T cells in the lesions of neuromyelitis optica, where OX40 expression was seen in CD4+ but not in CD8α+ T cells (Pohl *et al.*, 2013). None of the OX40 positive cells showed signs of apoptosis.

A characteristic feature of activated cytotoxic CD8+ T cells is the presence of cytoplasmic granules, containing GZMB (Figs 1H and 4F). As expected, the highest numbers of GZMB positive lymphocytes were seen in the ADEM case (43% of CD8α+ T cells). In multiple sclerosis, the number of GZMB+ lymphocytes was highest in the active lesions of acute/relapsing-remitting multiple sclerosis (median: 4.2 range: 0–31.8%). A similar percentage of GZMB+ CD8α cells (3.1%) was seen in the case of human autoimmune encephalomyelitis (Hoftberger *et al.*, 2015). In the progressive stage, only some focal spots containing granzyme B-positive lymphocytes were encountered in some cases and lesions (median: 1.7; range: 0–27% of CD8+ T cells).

Experimental data have shown that a substantial number of T cells, which infiltrate the CNS tissue during acute inflammation, are locally destroyed by apoptosis (Schmied *et al.*, 1993; Bauer *et al.*, 1998). Analysing the sections, we observed that T cell apoptosis was almost exclusively seen in cells expressing CD8α/β, but not in cells that were CD8α single positive and the highest number of apoptotic CD8+ T cells was seen in ADEM lesions (Figs 1E, F and 4E). Overall, the percentage of apoptotic CD8+ T cells was higher in acute/relapsing-remitting multiple sclerosis lesions (median 3.19; range 0–17%) in comparison to lesions of progressive multiple sclerosis (median: 1.3, range 0–7.6%), although the values did not reach statistical significance. In progressive multiple sclerosis, apoptotic T cells were seen as small focal clusters of cells in a subset of cases, while in acute/relapsing-remitting multiple sclerosis lesions they were more evenly distributed throughout the lesion. Detailed analysis of CD20+ cells did not reveal a single apoptotic B cell.

We then analysed for comparison lymphocyte activation and apoptosis in the lesions of Rasmussen's encephalitis as a CD8+ T cell-mediated inflammatory brain disease (Fig. 4). Overall, T cell activation and apoptosis rates were similar to those seen in acute multiple sclerosis, although the proliferation rate was significantly higher ($P < 0.0001$).

## CD8+ T lymphocytes in the lesions of MS display features of tissue-resident memory cells

Tissue-resident memory cells have been identified and characterized so far in inflammatory lesions of the skin, mucosal surfaces and the brain after viral infection (Mueller and Mackay, 2016). It has been suggested that such cells enter the tissue in acute inflammation as effector T cells, change their phenotype and may then persist within the tissue for prolonged periods of time. In our study, the number of CD45RA positive T cells was very low, with the exception of three cases of acute multiple sclerosis with the highest inflammatory reaction (Fig. 4H). CD8β positive cells were most frequent in early disease stages such as ADEM, human autoimmune encephalomyelitis and early stages of acute multiple sclerosis, while in more chronic stages and in particular in progressive multiple sclerosis, the majority of CD8α-positive cells were CD8β-negative (Figs 4B, 5A and B). CD103, a marker for tissue-resident memory T cells was highest in patients with acute multiple sclerosis (Fig. 5C and D). However, its expression was low in most patients with progressive multiple sclerosis and longstanding disease (Fig. 4I). Another marker suggested to define tissue-resident memory T cells is CD69 (Sathaliyawala *et al.*, 2013). However, in our analysis we found CD69 expression in the brain only in oligodendrocytes, some reactive

**Figure 5** Continued

sclerosis lesions in some macrophages (**E**), in oligodendrocytes and myelin (**F**) and in some reactive astrocytes (**G**), while CD3+ cells (blue cells) invariably were negative. (**H**) In the inflamed mucosa of the tonsil strong CD69 expression is present in a subset of lymphocytes. (**I**–**J**) TGF-β is present in multiple sclerosis lesions in reactive astrocytes and endothelial cells, but absent in perivascular or parenchymal lymphocytes. (**K** and **L**) IL-10 was found at high levels in plasma cells (**K**; see also high magnification insert) and in reactive astrocytes in the lesion (**L**), but not in perivascular or parenchymal lymphocytes (**K**). (**M**–**O**) The S1P1 receptor is highly expressed on circulating leucocytes in the vascular lumen (**M** and **N**), while perivascular and parenchymal lymphocytes are negative; as described before by others (Van Doorn *et al.*, 2010; Brana *et al.*, 2014) we found S1P1 receptor expression in endothelial cells and reactive astrocytes (**N** and **O**). (**P**–**T**) Phenotypic characterization of cells of the B cell lineage. (**P**–**R**) An inflammatory infiltrate in a lesion of secondary progressive multiple sclerosis, which mainly contains plasma cells with only few CD20+ B cells. The number of inflammatory cells expressing CD27 is low (**P**), while there are high numbers of CD38+ cells and immunoglobulin (Ig) containing plasma cells. **S** shows a B cell (red) rich inflammatory infiltrate double stained with CD27 (green). The CD27 expression in the CD20+ cells (double-labelling shown in yellow) ranges from very strong expression to a more restricted punctate expression. **T** shows an inflammatory infiltrate in a lesion from secondary progressive multiple sclerosis mainly containing plasma cells, which are double stained for CD38 and the plasma cell marker CD138. Scale bars = 100 μm. Images for colourblind readers can be found in Supplementary Fig. 5.

astrocytes and macrophages, while it was consistently negative in perivascular inflammatory infiltrates and in CD3+ T cells in double-stained sections (Fig. 5E–G), despite its strong expression in lymphocytes in chronic inflammatory mucosal lesions (Fig. 5H). S1P1 receptor, a key molecule of lymphocyte egress from the tissue was highly expressed on leucocytes within the vascular lumen and moderately expressed in astrocytes and endothelial cells of the brain tissue. However, infiltrating leucocytes in the perivascular space and in the parenchyma of the lesions were S1P1-negative (Fig. 5M–O). About half of the T cells within the lesions expressed the chemokine receptor CCR5, while the expression of PD1 was much lower and mainly present in patients with acute or relapsing multiple sclerosis (Figs 1M–O and 4J and K). Only a very small subset of CD8α+ T cells in the lesions was double-stained with CD27 or CD38.

TGF-β and IL-10 are key cytokines mediating anti-inflammatory effects. By investigating these molecules we thus tried to address the question whether the CD8+ cells, which accumulate in chronic multiple sclerosis lesions, could be regulatory (anti-inflammatory) T cell populations. We found TGF-β expression mainly in astrocytes, endothelial cells and some macrophages in active lesions of patients with acute and relapsing disease, and we found a significant correlation between astrocytic TGF-β expression and the presence of CD103 on T cells ($P = 0.0002$), possibly related to the observation that CD103 is induced by TGF-β (Zhang and Bevan, 2013). We did not observe any TGF-β expression in perivascular or parenchymal lymphocytes (Fig. 5K). IL-10 was prominently expressed within a subset of immunoglobulin containing plasma cells in the lesions, and in reactive astrocytes (Fig. 5K and L), but was not seen in lymphocytes in the inflammatory infiltrates.

The comparison of the phenotypic profile between multiple sclerosis, ADEM, human autoimmune encephalitis and Rasmussen's encephalitis provided similar results, with some additional insights (Fig. 4). In contrast to the other diseases, in the ADEM case, the majority of CD8α+ cells was also CD8β+ and did not express CD103, indicating that loss of CD8β and induction of CD103 in T cells requires at least several days of disease duration. In human autoimmune encephalitis the majority of CD8 cells were also CD8β+ and the number of CD103+ cells was high. Brain lesions in patients with Rasmussen's encephalitis showed a phenotypic profile of CD8+ T cells similar to that seen in acute/relapsing multiple sclerosis with a high percentage of CD103+ CD8+ T cells, as shown before by others (Owens *et al.*, 2016).

## Phenotype of cells of the B cell lineage

As described before, CD20+ B lymphocytes were particularly numerous in patients with acute multiple sclerosis, in the patient with relapsing-remitting multiple sclerosis and in the rare classical active lesions of patients with progressive multiple sclerosis (Fig. 2). In contrast, the numbers of plasma cells and the plasma cell/B cell ratio were significantly higher in lesions from patients with progressive multiple sclerosis in comparison to those in acute multiple sclerosis (Frischer *et al.*, 2009) (Supplementary Fig. 4). This suggests that infiltrating B cells gradually transform into plasma cells with disease chronicity. In line with this concept, we found the expression of markers, associated with B-cell survival and plasma blast differentiation (CD27 and CD38) in the lesions (Fig. 5P–T).

## Effect of multiple sclerosis-related treatment on the phenotype of inflammatory cells in lesions

As our study was performed on archival material from patients, who in their vast majority died before the implementation of disease-modifying immunomodulatory multiple sclerosis treatments, only a single patient (Patient 22, who had secondary progressive multiple sclerosis) received interferon-β. The phenotype of inflammatory cells in the lesions of this patient was similar to that in other patients with progressive multiple sclerosis. Eight of 12 patients with acute or relapsing multiple sclerosis received steroids in the last month before death. Thus, we compared the inflammatory profile between the four patients without steroid treatment with that seen in treated patients and found no difference for all phenotypic markers with the exception of the number of CD4+ T cells. Three of four untreated patients revealed CD4+ T cell counts, which were in the upper range of those seen in steroid-treated patients (Fig. 2E–H). We did not observe a difference in the percentage of proliferating or apoptotic T cells or of GRZB expression in T cells between patients with or without corticosteroid treatment.

# Discussion

In this study we performed a detailed phenotypic characterization of T and B lymphocytes in different types and activity stages of multiple sclerosis lesions and compared the findings with lymphocytic infiltrates in inflammatory, non-inflammatory and normal controls. We have shown before that active demyelination and neurodegeneration in multiple sclerosis are associated with T cell, B cell and plasma cell infiltrates (Frischer *et al.*, 2009). However, there is a subset of multiple sclerosis cases, in particular of patients in the late stage of progressive disease, where the lymphocytic inflammatory response has declined to levels seen in age-matched controls. In these patients, no active demyelination was present and acute axonal degeneration was similar in extent to the one seen in the ageing brain of controls (Frischer *et al.*, 2009). These data indicate that T and B lymphocytes play an important role in the induction of active multiple sclerosis-related tissue injury.

Our study shows that substantial inflammation by T and B cells is present in patients with active early (acute/relapsing) as well as progressive disease, but that the overall extent of inflammation is lower in the progressive stage compared to that in the early stage. A limitation of our study is that only a single case of relapsing multiple sclerosis is included and that the results related to early disease stages are, thus, biased by our findings in acute multiple sclerosis. However, classical active lesions, which are present, but rare in progressive multiple sclerosis (Frischer *et al.*, 2015), also showed profound inflammation, composed of T and B cells, suggesting that the degree of inflammation is mainly associated with the activity stage of the lesions rather than with the clinical phenotype of the patients. We did not find significant differences in the inflammatory reaction between patients with primary or secondary progressive disease. While T cells in similar number and phenotype were also present in the inflammatory controls, lesions of acute multiple sclerosis patients and less prominently those from patients with progressive multiple sclerosis revealed a significantly higher B cell infiltration in comparison to ADEM, Rasmussen's encephalitis or virus induced inflammatory diseases of the CNS (Fig. 2). In contrast to other controls, extremely high numbers of B cells were present in the case of multiple sclerosis-like human demyelinating autoimmune encephalomyelitis (Hoftberger *et al.*, 2015). Immunization in humans with brain tissue or antigens in the vast majority of cases results in inflammatory polyradiculoneurits or ADEM, which are believed to be T cell-mediated autoimmune diseases (Stuart and Krikorian, 1928; Alvord, 1970). However, a small subset of patients develop inflammatory demyelinating lesions, which closely reflect those seen in acute multiple sclerosis (Uchimura and Shiraki, 1957; Hoftberger *et al.*, 2015). Such a case has been included in our present study. Overall, our data show that CD8+ T cells and CD20+ B cells dominate in inflammatory demyelinating lesions of multiple sclerosis in all disease stages.

## Potential contribution of different lymphocyte subsets to the pathogenesis of multiple sclerosis

### MHC class II restricted CD4+ T cells

Based on the human leucocyte antigen (HLA) association of multiple sclerosis and experience with the model of autoimmune encephalomyelitis, MHC class II-restricted CD4+ T cells, polarized towards Th17 cells, are believed to be major drivers of the inflammatory reaction in multiple sclerosis (Hohlfeld *et al.*, 2016*b*). This is in contrast to the observation from pathology that CD4+ T cells are only a quantitatively minor population and show little clonal expansion in the multiple sclerosis lesions (Booss *et al.*, 1983; Babbe *et al.*, 2000; van Nierop *et al.*, 2017). It has been argued that the massive skewing of the inflammatory cells towards CD8+ T cells may be due to the fact that in most studies only patients from late stages of relapsing or progressive disease were included. However, in our study we have analysed a substantial number of patients with acute multiple sclerosis and a clinical duration from 7 days to a few months. In these patients, too, CD8+ T cells invariably dominated the lesions and the CD4+ T cell component was minor. One can argue that CD4+ T cells may initiate fresh lesions and are quickly eliminated from the tissue by apoptosis, as has been shown in EAE models (Schmied *et al.*, 1993). However, very few, if any, apoptotic CD4+ T cells could be detected in multiple sclerosis lesions in the current study. Results from clinical trials, selectively targeting CD4 cells were, so far, negative (van Oosten *et al.*, 1997; Segal *et al.*, 2008) and key cytokines, defined in the inflammatory process in CD4+ T cell-mediated EAE, showed in part opposite roles in patients with multiple sclerosis (Panitch *et al.*, 1987; Arnason, 2011). Nevertheless, our present results do not rule out a role of these cells in the induction phase of the disease or the initiation of fresh lesions.

### MHC class I restricted CD8+ T cells

On a quantitative basis, CD8+ T cells were the dominant lymphocyte population in all stages of disease and lesions of multiple sclerosis patients. This dominance of CD8 cells is not specific for multiple sclerosis, but was observed to a similar extent in patients with Rasmussen's encephalitis or virus induced inflammatory brain diseases. Our data suggest that these cells are in part activated within the CNS lesions, reflected by proliferation and expression of the activation marker NFAT2. In addition, the cytotoxic potential of these cells is reflected in the expression of GZMB. Notably, the CD8+ cells are in part destroyed locally by programmed cell death (apoptosis). These phenotypic characteristics of infiltrating T cells were most prominent in the patient with post-infectious ADEM and a clinical disease history of only 2 days. They were much less pronounced in patients with acute multiple sclerosis, in patients with disease duration of 7 days to 7 months, and even less in patients with progressive multiple sclerosis with disease duration of several years. However, even in these patients, focal clusters of activated T cells were seen, possibly reflecting their antigen-specific activation (Iijima and Iwasaki, 2015).

The majority of CD8+ T cells showed features described for tissue-resident memory cells, such as being CD8$\alpha/\alpha$ double positive (Konno *et al.*, 2002; Fan and Rudensky, 2016). One reason for their persistence in the tissue may be that they downregulate the expression of S1P1 and CCR7 receptors, which are instrumental for the egress of lymphocytes from tissue sites, and which are downregulated when CD103 is induced (Iijima and Iwasaki, 2015). We have shown before that lymphocytes in multiple sclerosis lesions do not express CCR7, which differs from the strong expression of this chemokine receptor in lymphocytes of the CSF (Kivisakk *et al.*, 2004). In our present study, we confirm previously published data on S1P1

expression in astrocytes and endothelial cells (Van Doorn *et al.*, 2010; Brana *et al.*, 2014). Importantly, we now show the lack of S1P1 expression on leucocytes within established multiple sclerosis lesions. The cells in part expressed CD103 mainly in active lesions of acute multiple sclerosis. CD103 has been suggested to be involved in the downregulation of the molecules involved in lymphocyte egress from the tissue, such as S1P1 (Schenkel and Masopust, 2014; Steinbach *et al.*, 2016). One local cue stimulating the expression of CD103, and possibly the local development of tissue-resident memory T cells (Zhang and Bevan, 2013), is TGF-β (Koyama and Podolsky, 1989; Iijima and Iwasaki, 2015), which we found to be highly expressed in astrocytes in active lesions in early stages of multiple sclerosis but not in slowly expanding lesions of progressive multiple sclerosis. Thus, CD103 may be involved in the induction of a tissue-resident phenotype, but its expression may be lost at late stages of chronic inflammation. However, we did not detect an expression of CD69 in the T cells. CD69 was highly expressed in oligodendrocytes, as shown before in rodents (Miyamoto *et al.*, 2016) and in a subset of macrophages. This is different from results of a recent study (van Nierop *et al.*, 2017), which described that T cells in lesions of patients with late progressive multiple sclerosis are CD69+ but lack CD103 expression. Both markers, however, are not necessarily expressed in tissue-resident memory T cells in chronic inflammation (Steinert *et al.*, 2015).

In virus-induced skin or brain disease effector T cells migrate into the lesions in the acute stage and eliminate the pathogen. Following this early phase of invasion, the cells become tissue-resident memory cells, which persist within the tissue for long times in an inactive state but can be reactivated when they become re-exposed to their specific antigen (Konno *et al.*, 2002; Wakim *et al.*, 2010; Mueller and Mackay, 2016). Thus, these cells play a central role in the establishment of a tissue-specific immunological memory. Their activation after new exposure to their cognate antigen may locally propagate chronic compartmentalized inflammation and tissue damage in the multiple sclerosis brain.

### CD20+ B cells

Our study shows a prominent contribution of CD20 positive B cells to the inflammatory response in multiple sclerosis, which was particularly high in patients at early stages of multiple sclerosis and even in progressive multiple sclerosis significantly higher compared to that seen in the other inflammatory controls. In contrast to CD20+ cells, infiltration with plasma cells in the meninges and perivascular space is highest in patients with progressive disease (Frischer *et al.*, 2009), suggesting a gradual differentiation of infiltrating B cells into a stable plasma cell population. This view is further supported by the expression of CD27 and CD38 in cells of the B cell lineage in the lesions. Although the overall contribution of B cells to the inflammatory infiltrates in multiple sclerosis was lower compared to that of CD8+ T cells, the extent of B cell infiltration in the lesions may be underestimated in our study. In contrast to T cells, B cells are mainly seen in the perivascular space of medium-sized veins and restricted to a small number of these vessels located in the plaque centre. Although we have used large numbers of serial sections, single vessels with prominent B cell infiltration may have been missed, in particular in patients with progressive multiple sclerosis and large lesions. Recent observations of a prominent effect of therapies, targeting B cells in multiple sclerosis patients, suggest their major role in the inflammatory process in this disease (Hauser *et al.*, 2008; Montalban *et al.*, 2017). The role of B cells in multiple sclerosis lesions is currently unresolved and may include direct pro-inflammatory effects, indirect effects on T cells through antigen presentation or the infection with Epstein-Barr virus in the peripheral immune system or the brain (Lehmann-Horn *et al.*, 2017). In addition, CD20+ T cells may play a role in the pathogenesis of the disease (Schuh *et al.*, 2016). It is noteworthy that, similar to what has been described in experimental models, plasma cells appear to be a prominent source of the anti-inflammatory cytokine IL-10 within the CNS (Fillatreau, 2015).

## Relation of lymphocyte infiltration with tissue injury

We have shown previously that in the late stages of progressive multiple sclerosis inflammation composed by T and B cells may decline to levels seen in age-matched controls (Frischer *et al.*, 2009). In these patients, no evidence for ongoing demyelination was seen and also acute axonal injury declined to the levels seen in age-matched controls. These data suggest that lymphocytic inflammation is an important driver for multiple sclerosis-related active demyelination and tissue injury (Frischer *et al.*, 2009). The pathological data presented here, however, argue against a direct cell contact-mediated active demyelination. At the sites of active myelin destruction, the number of tissue infiltrating T or B cells was very low in comparison to that seen in more advanced lesion stages (Barnett and Prineas, 2004; Marik *et al.*, 2007) and the majority of T and B cells were present in the perivascular cuffs, distant from the sites of initial myelin and axonal injury. A similar pattern of inflammation in relation to active tissue injury has been described in cortical lesions (Kutzelnigg *et al.*, 2005; Magliozzi *et al.*, 2010; Howell *et al.*, 2011). These data suggest that demyelination and acute neurodegeneration are induced by soluble factors, produced by lymphocytes, which diffuse into the tissue and trigger demyelination either directly or indirectly through microglia activation. This view is supported by the presence of a multiple sclerosis-specific soluble factor in the serum and CSF, which may induce demyelination

and neurodegeneration in tissue culture *in vitro* (Bornstein and Appel, 1965). In EAE following immunization with myelin oligodendrocyte glycoprotein (MOG) specific demyelinating autoantibodies have been identified (Linington *et al.*, 1988), but such demyelinating anti-MOG antibodies appear to be a feature of MOG antibody-associated inflammatory demyelinating disease, but not of multiple sclerosis patients (Jarius *et al.*, 2016). The nature of the demyelinating or neurotoxic factor in multiple sclerosis patients is currently undefined. Recent studies suggest that they are not immunoglobulins, but other soluble factors produced by B lymphocytes of multiple sclerosis patients (Lisak *et al.*, 2012, 2017).

# Conclusions

The results of our study underline the importance of T and B lymphocytes in the pathogenesis of multiple sclerosis lesions. The dominance of tissue-resident CD8+ T cells indicates that these cells survive and persist in the CNS, becoming trapped or compartmentalized in the brain and spinal cord. The function of these cells is so far undefined. As in virus-induced brain or skin diseases, they may be quiescent resident memory T cells, which become activated and promote inflammatory tissue damage, when confronted with their cognate antigen (Iijima and Iwasaki, 2015). Alternatively, they may be regulatory T cells, which protect the brain against further immune-mediated damage. As classical effector T cells, regulatory T cells may also become activated, proliferate and by using GZMB-related cytotoxic mechanisms can eliminate pathogenic effector T cells or produce anti-inflammatory cytokines (Cao *et al.*, 2007; Hogan, 2017). However, we did not find evidence for the production of the key anti-inflammatory cytokines TGF-β and IL-10 in these cells. Further studies aiming to decipher the antigen-specificity of tissue infiltrating lymphocytes in multiple sclerosis patients will be essential to clarify their role in multiple sclerosis (Hohlfeld *et al.*, 2016*a*).

# Acknowledgements

We thank Marianne Leiszer, Ulrike Köck and Angela Kury for expert technical assistance.

# Funding

The study was funded by the Austrian Science Fund, Project I-2114-B27 (ERA-Net: MELTRA-BBB), I 3335-B27 and the European Union, Marie Curie Actions – ITN; Project NEUROKINE 316722; E.S. was supported by postdoctoral fellowships for research abroad from the Uehara Memorial Foundation and the Kanae Foundation for the Promotion of Medical Science.

# Supplementary material

Supplementary material is available at *Brain* online.

# References

Alvord EC. Acute disseminated encephalomyelitis and "allergic" neuroencephalopaties. New York, NY: Elsevier; 1970.

Arnason B. Tumour necrosis factor neutralization in MS: a cautionary tale. Int MS J 2011; 17: 63–8.

Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med 2000; 192: 393–404.

Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. Ann Neurol 2004; 55: 458–68.

Bauer J, Bradl M, Hickley WF, Forss-Petter S, Breitschopf H, Linington C, et al. T-cell apoptosis in inflammatory brain lesions: destruction of T cells does not depend on antigen recognition. Am J Pathol 1998; 153: 715–24.

Bauer J, Lassmann H. Neuropathological techniques to investigate central nervous system sections in multiple sclerosis. Methods Mol Biol 2016; 1304: 211–29.

Ben-Nun A, Wekerle H, Cohen IR. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. Eur J Immunol 1981; 11: 195–9.

Bien CG, Bauer J, Deckwerth TL, Wiendl H, Deckert M, Wiestler OD, et al. Destruction of neurons by cytotoxic T cells: a new pathogenic mechanism in Rasmussen's encephalitis. Ann Neurol 2002; 51: 311–18.

Booss J, Esiri MM, Tourtellotte WW, Mason DY. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. J Neurol Sci 1983; 62: 219–32.

Bornstein MB, Appel SH. Tissue culture studies of demyelination. Ann N Y Acad Sci 1965; 122: 280–6.

Brana C, Frossard MJ, Pescini Gobert R, Martinier N, Boschert U, Seabrook TJ. Immunohistochemical detection of sphingosine-1-phosphate receptor 1 and 5 in human multiple sclerosis lesions. Neuropathol Appl Neurobiol 2014; 40: 564–78.

Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, et al. Monocyte/macrophage differentiation in early multiple sclerosis lesions. Ann Neurol 1995; 38: 788–96.

Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. Immunity 2007; 27: 635–46.

Charcot J. Leçons sur les maladies du systeme nerveux faites à la Salpetriere, 4th ed. Paris: A. Delahaye; 1880.

Claes N, Fraussen J, Stinissen P, Hupperts R, Somers V. B cells are multifunctional players in multiple sclerosis pathogenesis: insights from therapeutic interventions. Front Immunol 2015; 6: 642.

Dargahi N, Katsara M, Tselios T, Androutsou ME, de Courten M, Matsoukas J, et al. Multiple sclerosis: immunopathology and treatment update. Brain Sci 2017; 7.

Dietz L, Frommer F, Vogel AL, Vaeth M, Serfling E, Waisman A, et al. NFAT1 deficit and NFAT2 deficit attenuate EAE via different mechanisms. Eur J Immunol 2015; 45: 1377–89.

Fan X, Rudensky AY. Hallmarks of tissue-resident lymphocytes. Cell 2016; 164: 1198–211.

Felts PA, Woolston AM, Fernando HB, Asquith S, Gregson NA, Mizzi OJ, et al. Inflammation and primary demyelination induced by the

intraspinal injection of lipopolysaccharide. Brain 2005; 128(Pt 7): 1649–66.
Fillatreau S. Regulatory plasma cells. Curr Opin Pharmacol 2015; 23: 1–5.
Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. Brain 2009; 132(Pt 5): 1175–89.
Frischer JM, Weigand SD, Guo Y, Kale N, Parisi JE, Pirko I, et al. Clinical and pathological insights into the dynamic nature of the white matter multiple sclerosis plaque. Ann Neurol 2015; 78: 710–21.
Gajofatto A. Spotlight on siponimod and its potential in the treatment of secondary progressive multiple sclerosis: the evidence to date. Drug Des Devel Ther 2017; 11: 3153–7.
Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, et al. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. Lab Invest 1994; 71: 219–25.
Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med 2008; 358: 676–88.
Hayashi T, Morimoto C, Burks JS, Kerr C, Hauser SL. Dual-label immunocytochemistry of the active multiple sclerosis lesion: major histocompatibility complex and activation antigens. Ann Neurol 1988; 24: 523–31.
Hochmeister S, Grundtner R, Bauer J, Engelhardt B, Lyck R, Gordon G, et al. Dysferlin is a new marker for leaky brain blood vessels in multiple sclerosis. J Neuropathol Exp Neurol 2006; 65: 855–65.
Hoftberger R, Leisser M, Bauer J, Lassmann H. Autoimmune encephalitis in humans: how closely does it reflect multiple sclerosis? Acta Neuropathol Commun 2015; 3: 80.
Hogan PG. Calcium-NFAT transcriptional signalling in T cell activation and T cell exhaustion. Cell Calcium 2017; 63: 66–9.
Hohlfeld R, Dornmair K, Meinl E, Wekerle H. The search for the target antigens of multiple sclerosis, part 1: autoreactive CD4+ T lymphocytes as pathogenic effectors and therapeutic targets. Lancet Neurol 2016a; 15: 198–209.
Hohlfeld R, Dornmair K, Meinl E, Wekerle H. The search for the target antigens of multiple sclerosis, part 2: CD8+ T cells, B cells, and antibodies in the focus of reverse-translational research. Lancet Neurol 2016b; 15: 317–31.
Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, Gentleman SM, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain 2011; 134(Pt 9): 2755–71.
Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. J Exp Med 2001; 194: 669–76.
Iijima N, Iwasaki A. Tissue instruction for migration and retention of TRM cells. Trends Immunol 2015; 36: 556–64.
Jarius S, Ruprecht K, Kleiter I, Borisow N, Asgari N, Pitarokoili K, et al. MOG-IgG in NMO and related disorders: a multicenter study of 50 patients. Part 2: epidemiology, clinical presentation, radiological and laboratory features, treatment responses, and long-term outcome. J Neuroinflammation 2016; 13: 280.
Kivisakk P, Mahad DJ, Callahan MK, Sikora K, Trebst C, Tucky B, et al. Expression of CCR7 in multiple sclerosis: implications for CNS immunity. Ann Neurol 2004; 55: 627–38.
Konno A, Okada K, Mizuno K, Nishida M, Nagaoki S, Toma T, et al. CD8alpha alpha memory effector T cells descend directly from clonally expanded CD8alpha + beta high TCRalpha beta T cells *in vivo*. Blood 2002; 100: 4090–7.
Koyama SY, Podolsky DK. Differential expression of transforming growth factors alpha and beta in rat intestinal epithelial cells. J Clin Invest 1989; 83: 1768–73.
Kuhlmann T, Ludwin S, Prat A, Antel J, Bruck W, Lassmann H. An updated histological classification system for multiple sclerosis lesions. Acta Neuropathol 2017; 133: 13–24.
Kutzelnigg A, Lucchinetti CF, Stadelmann C, Bruck W, Rauschka H, Bergmann M, et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain 2005; 128(Pt 11): 2705–12.
Legat A, Speiser DE, Pircher H, Zehn D, Fuertes Marraco SA. Inhibitory receptor expression depends more dominantly on differentiation and activation than "exhaustion" of human CD8 T cells. Front Immunol 2013; 4: 455.
Lehmann-Horn K, Kinzel S, Weber MS. Deciphering the role of B cells in multiple sclerosis-towards specific targeting of pathogenic function. Int J Mol Sci 2017; 18.
Linington C, Bradl M, Lassmann H, Brunner C, Vass K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol 1988; 130: 443–54.
Lisak RP, Benjamins JA, Nedelkoska L, Barger JL, Ragheb S, Fan B, et al. Secretory products of multiple sclerosis B cells are cytotoxic to oligodendroglia *in vitro*. J Neuroimmunol 2012; 246: 85–95.
Lisak RP, Nedelkoska L, Benjamins JA, Schalk D, Bealmear B, Touil H, et al. B cells from patients with multiple sclerosis induce cell death via apoptosis in neurons *in vitro*. J Neuroimmunol 2017; 309: 88–99.
Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol 2000; 47: 707–17.
Magliozzi R, Howell OW, Reeves C, Roncaroli F, Nicholas R, Serafini B, et al. A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. Ann Neurol 2010; 68: 477–93.
Marik C, Felts PA, Bauer J, Lassmann H, Smith KJ. Lesion genesis in a subset of patients with multiple sclerosis: a role for innate immunity? Brain 2007; 130 (Pt 11): 2800–15.
Martin-Blondel G, Brassat D, Bauer J, Lassmann H, Liblau RS. CCR5 blockade for neuroinflammatory diseases—beyond control of HIV. Nat Rev Neurol 2016; 12: 95–105.
Miyamoto Y, Torii T, Tanoue A, Yamauchi J. VCAM1 acts in parallel with CD69 and is required for the initiation of oligodendrocyte myelination. Nat Commun 2016; 7: 13478.
Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, et al. Ocrelizumab versus Placebo in primary progressive multiple sclerosis. N Engl J Med 2017; 376: 209–20.
Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol 2016; 16: 79–89.
Obermeier B, Lovato L, Mentele R, Bruck W, Forne I, Imhof A, et al. Related B cell clones that populate the CSF and CNS of patients with multiple sclerosis produce CSF immunoglobulin. J Neuroimmunol 2011; 233: 245–8.
Owens GC, Chang JW, Huynh MN, Chirwa T, Vinters HV, Mathern GW. Evidence for resident memory T cells in rasmussen encephalitis. Front Immunol 2016; 7: 64.
Palanichamy A, Apeltsin L, Kuo TC, Sirota M, Wang S, Pitts SJ, et al. Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis. Sci Transl Med 2014; 6: 248ra106.
Panitch HS, Hirsch RL, Haley AS, Johnson KP. Exacerbations of multiple sclerosis in patients treated with gamma interferon. Lancet 1987; 1: 893–5.
Pohl M, Kawakami N, Kitic M, Bauer J, Martins R, Fischer MT, et al. T cell-activation in neuromyelitis optica lesions plays a role in their formation. Acta Neuropathol Commun 2013; 1: 85.
Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJ, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. Immunity 2013; 38: 187–97.

Saxena A, Bauer J, Scheikl T, Zappulla J, Audebert M, Desbois S, et al. Cutting edge: multiple sclerosis-like lesions induced by effector CD8 T cells recognizing a sequestered antigen on oligodendrocytes. J Immunol 2008; 181: 1617–21.

Schenkel JM, Masopust D. Tissue-resident memory T cells. Immunity 2014; 41: 886–97.

Schmied M, Breitschopf H, Gold R, Zischler H, Rothe G, Wekerle H, et al. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. Am J Pathol 1993; 143: 446–52.

Schuh E, Berer K, Mulazzani M, Feil K, Meinl I, Lahm H, et al. Features of human CD3 + CD20 + T cells. J Immunol 2016; 197: 1111–17.

Segal BM, Constantinescu CS, Raychaudhuri A, Kim L, Fidelus-Gort R, Kasper LH. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. Lancet Neurol 2008; 7: 796–804.

Simpson J, Rezaie P, Newcombe J, Cuzner ML, Male D, Woodroofe MN. Expression of the beta-chemokine receptors CCR2, CCR3 and CCR5 in multiple sclerosis central nervous system tissue. J Neuroimmunol 2000; 108: 192–200.

Stadelmann C, Deckwerth TL, Srinivasan A, Bancher C, Bruck W, Jellinger K, et al. Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. Am J Pathol 1999; 155: 1459–66.

Steinbach K, Vincenti I, Kreutzfeldt M, Page N, Muschaweckh A, Wagner I, et al. Brain-resident memory T cells represent an autonomous cytotoxic barrier to viral infection. J Exp Med 2016; 213: 1571–87.

Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyarto BZ, et al. Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. Cell 2015; 161: 737–49.

Stuart G, Krikorian KS. The neuro-paralytic accidents of anti-rabies treatment. Ann Trop Med Parasitol 1928; 22: 327–77.

Uchimura I, Shiraki H. A contribution to the classification and the pathogenesis of demyelinating encephalomyelitis; with special reference to the central nervous system lesions caused by preventive inoculation against rabies. J Neuropathol Exp Neurol 1957; 16: 139–203; discussion, 208.

Van Doorn R, Van Horssen J, Verzijl D, Witte M, Ronken E, Van Het Hof B, et al. Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. Glia 2010; 58: 1465–76.

van Nierop GP, van Luijn MM, Michels SS, Melief MJ, Janssen M, Langerak AW, et al. Phenotypic and functional characterization of T cells in white matter lesions of multiple sclerosis patients. Acta Neuropathol 2017; 134: 383–401.

van Oosten BW, Lai M, Hodgkinson S, Barkhof F, Miller DH, Moseley IF, et al. Treatment of multiple sclerosis with the monoclonal anti-CD4 antibody cM-T412: results of a randomized, double-blind, placebo-controlled, MR-monitored phase II trial. Neurology 1997; 49: 351–7.

Wakim LM, Woodward-Davis A, Bevan MJ. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. Proc Natl Acad Sci USA 2010; 107: 17872–9.

Webb GJ, Hirschfield GM, Lane PJ. OX40, OX40L and autoimmunity: a comprehensive review. Clin Rev Allergy Immunol 2016; 50: 312–32.

Zhang N, Bevan MJ. Transforming growth factor-beta signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. Immunity 2013; 39: 687–96.