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Research article

Increased expression of ICAM-1, VCAM-1, MCP-1, and MIP-1α by spinal perivascular macrophages during experimental allergic encephalomyelitis in rats

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Abstract

Background: T-cells extravasation and CNS parenchyma infiltration during autoimmune neurodegenerative disease can be evoked by local antigen presenting cells. Studying the chemoattracting potential of spinal perivascular macrophages (SPM) during experimental allergic encephalomyelitis (EAE), we observed numerous infiltrates of densely-packed mononuclear cells. Apart from the poor spatial and optical resolution, no differentiation between the resident SPM (mabs ED1+, ED2+) and the just recruited monocytes/macrophages (mab ED1+) was possible.

Results: This is why we labeled SPM by injections of different fluoresecent dyes into the lateral cerebral ventricle before induction of active EAE. Within an additional experimental set EAE was induced by an intraperitoneal injection of T-cells specifically sensitized to myelin basic protein (MBP) and engineered to express the green fluorescent protein (GFP). In both experiments we observed a strong activation of SPM (mabs OX6+, SILK6+, CD40+, CD80+, CD86+) which was accompanied by a consistently increased expression of ICAM-1, VCAM-1, and the chemokines MCP-1 and MIP-1α.

Conclusion: These observations indicate that SPM play a role in promoting lymphocyte extravasation.

Background

Antigen specificity during an autoimmune attack is affected by antigen presentation and recognition, antigen expression and the response of target organs [1]. Accordingly, accumulating evidence shows that the extravasation of lymphocytes into the CNS perivascular (Virchow-Robin) space in the course of progressing autoimmune disease may be initiated by resident antigen presenting cells [2–4].

It is generally acknowledged that, under conditions of a structurally intact blood-brain barrier, the cerebral/spinal perivascular macrophages (CPM/SPM) are the antigen presenting cells of the brain [5–9]. CPM/SPM exhibit mor-

phological features consistent with macrophages [10], express the scavenger receptor [8], the Major Histocompatibility Complex (MHC) class II glycoproteins on their surface [6], and act as scavengers in the cerebral blood-brain interface zone [11]. CPM/SPM retain the phagocytosed material and remain within the perivascular space for up to 2 years [11], with a rather slow turnover rate of about 6% per month [12]. Due to presence of Fc and complement receptors on their surface and expression of macrophage specific antigens [5], CPM/SPM are considered to be "the only macrophages found in the tissues of the CNS" [13].

CPM/SPM differ from pericytes with respect to their morphology and anatomic localization in the perivascular space [14]. Pericytes are – like elsewhere in the body – completely ensheathed by split layers of the vascular basal lamina and separated from the CNS tissue by the *membrana limitans gliae perivascularis*. CPM/SPM, on the contrary, are never enclosed within the basal lamina of the blood vessels. In this way, placed along the lymphatic drainage pathways of the brain, CPM/SPM are in a prime position not only for antigen presentation, but also for influencing endothelial cells and leukocyte migration from the blood [15].

This is why we decided to study their role in experimental allergic encephalomyelitis (EAE), which is considered to represent an animal model for multiple sclerosis [16]. Our special interest was focused on the immunocytochemically detectable expression of cell adhesion molecules and chemokines by CPM/SPM.

Observation of spinal-cord sections from rats with EAE, however, revealed numerous densely-packed mononuclear cells in the Virchow-Robin space, which seriously jeopardized the imunocytochemical differentiation between the resident SPM (mab ED2$^+$, mab ED1$^+$) and the recently extravasated monocytes/macrophages (mab ED1$^+$, mab ED2$^-$) [6,14,17].

To circumvent this poor spatial and optical resolution we labeled SPM by intracerebroventricular (*icv*) injections of different fluorescent dyes before induction of active EAE (injection with myelin basic protein in complete Freund's adjuvant). In additional experiments EAE was induced by the intraperitoneal injection of T-cells specifically primed to myelin basic protein (MBP) and engineered to express the green fluorescent protein (GFP) [18,19].

In both sets of experiments we observed a strong activation of SPM (mabs OX6$^+$, SILK6$^+$, CD40$^+$, CD80$^+$, CD86$^+$) which was accompanied by a consistently increased expression of ICAM-1, VCAM-1, and the chemokines MCP-1 and MIP-1α. We conclude that, indicating the

sites for lymphocytic extravasation, SPM play an important role in the initiation of local inflammatory processes.

Results

SPM in intact control animals

There was no diffuse staining indicative of free marker substance in the spinal cord of both rat strains after injection of horseradish peroxidase (HRP), Fluoro-Emerald (FE), or Fluoro-Ruby (FR) in the right lateral ventricle: only the perivascular macrophages were labeled (*arrows* in Fig. 1).

SPM in rats with active EAE at the peak of paraparesis

The SPM-labeling turned out to be extremely irregular after *icv* injection of any label in LEW/Han Rij Hsd rats at the peak of EAE (severe paraparesis). In 11 out of 21 paraplegic animals we found no labeling in the spinal cord 24 hours after icv application (Fig. 2A). Since tracers could be detected in the brain parenchyma around the injection site, we attributed their lack in the lumbar spinal cord to a blockade of the cerebrospinal-fluid circulation by the inflammatory oedema.

Observation of vibratome sections from the other 10 paraplegic rats revealed successful intensive labeling of the perivascular macrophages in both brain as well as spinal cord (*arrows* in Fig. 2B). Obviously in these animals the CSF-circulation was not so strongly affected by the inflammatory oedema.

Unfortunately these sections failed to provide clearcut information about the relationship between SPM and lymphocytes during extravasation. The presence of well-advanced lymphocytic infiltrates in the spinal cord parenchyma indicated that the extravasation had already occurred. No spatial resolution of the various cellular elements within these "perivascular cuffs" by conventional microscopy was possible (Fig. 2C, 2D). The electron microscopic analysis showed that a large portion of the HRP-labeled SPM contained shrunken nuclei and vacuolated scanty cytoplasm, i.e. displayed signs of degeneration (Fig. 2E).

SPM in rats with transfer EAE at the peak of paraparesis

Following injection of T$_{MBP}$GFP cells the syngenic LEW/CRL BR rats developed the typical monophasic course of EAE. The peak of paraparesis and incontinence was observed 5 days after injection of T$_{MBP}$GFP cells. The histological examination at this period revealed a massive infiltration of the CNS with green fluorescent mononuclear cells (Fig. 3A, 3B). Their density was extremely irregular. Injection of 5% FR at the peak of paraparesis into the lateral ventricle labeled the SPM in red (Fig. 3B) and allowed unbiased observations of the relationship between SPM and infiltrating lymphocytes.
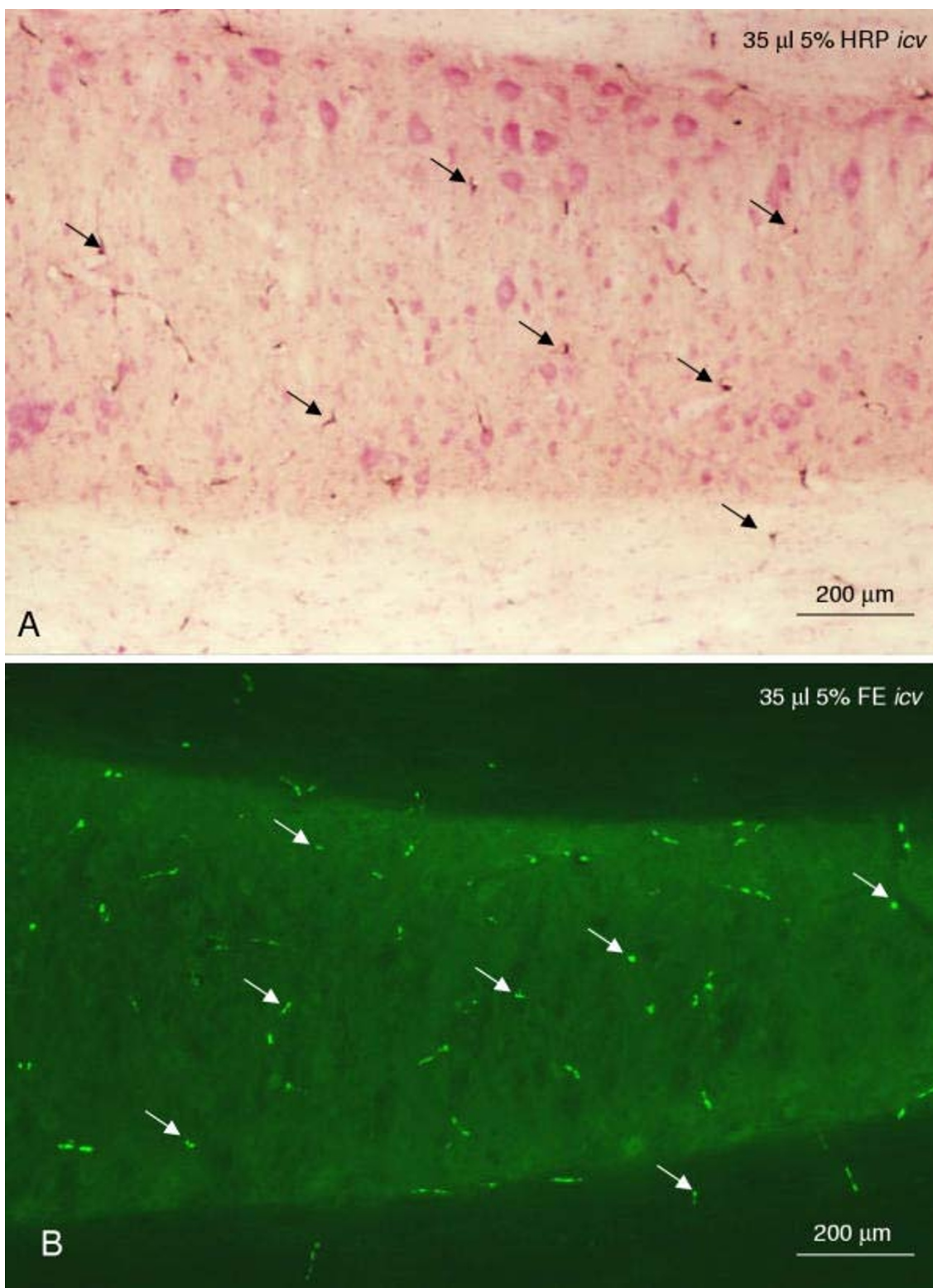
Figure 1
Labeling of spinal perivascular macrophages (SPM) after *icv* injection of horseradish peroxidase (HRP) and Fluoro-Emerald (FE). Longitudinal section through the lumbar spinal cord of intact control animals showing SPM (*arrows*) labeled by HRP **(A)** and by FE **(B)**. 50 μm thick vibratome sections.
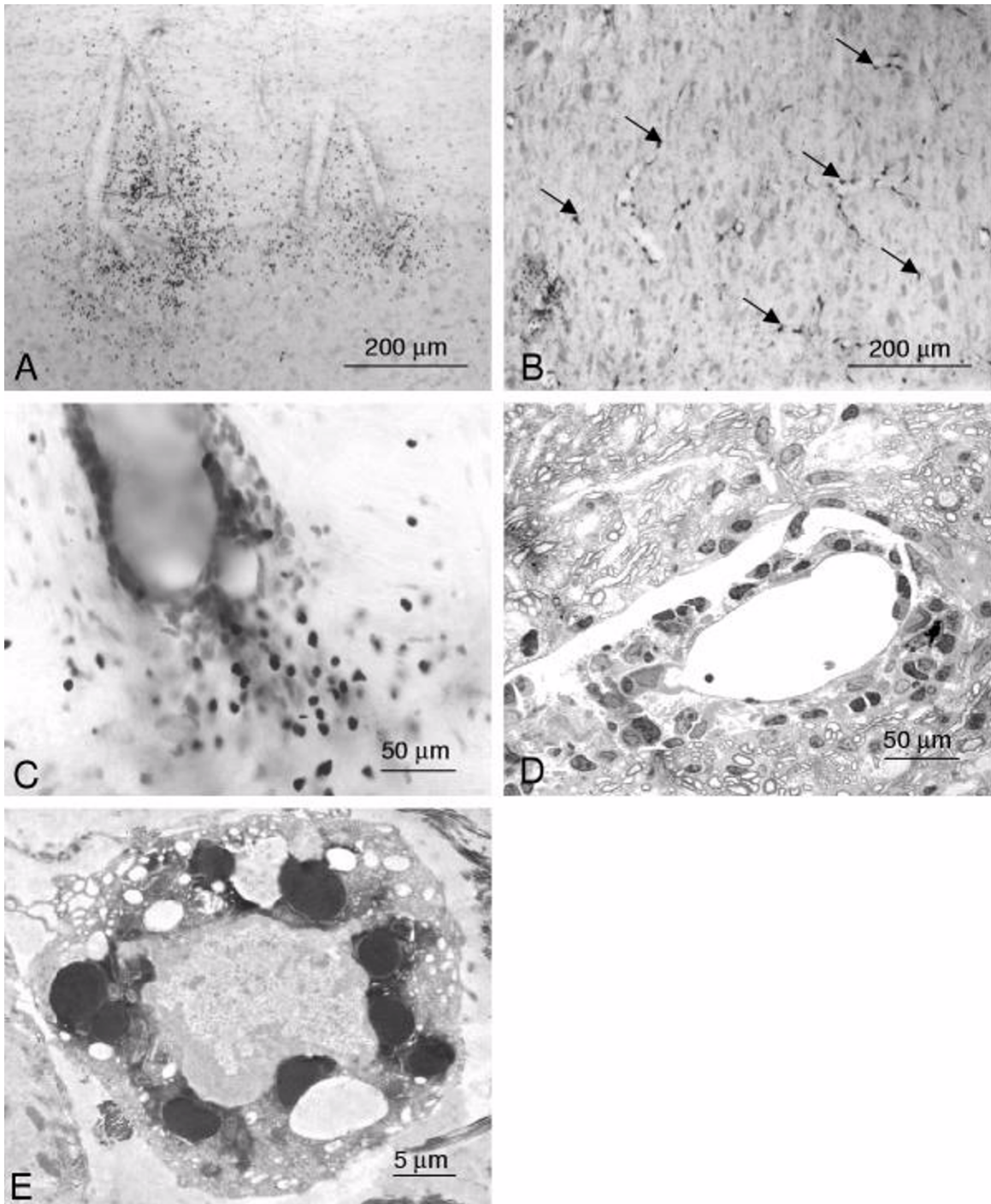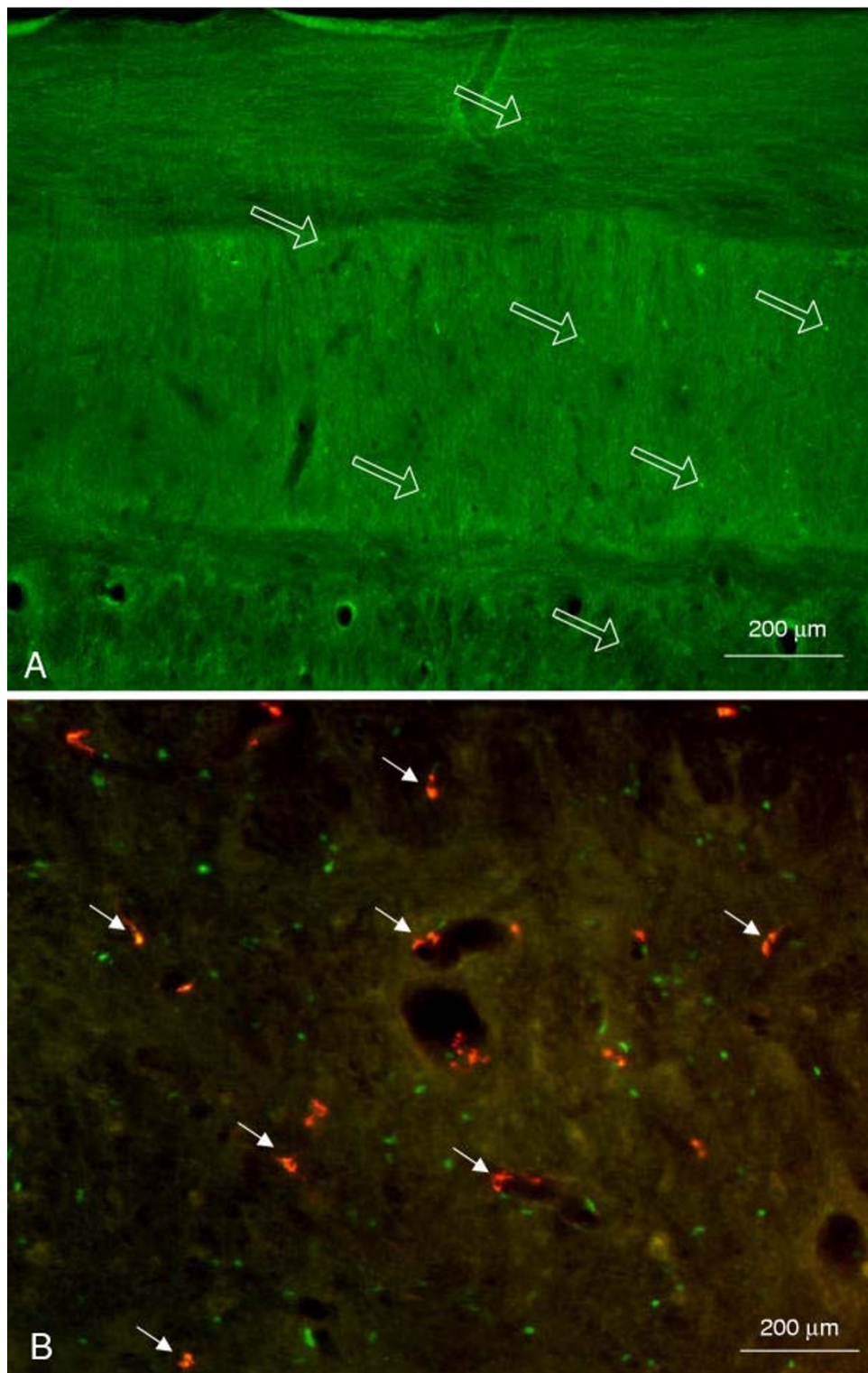
Figure 2
Labeling of spinal perivascular macrophages (SPM) after *icv* injection of HRP in rats with EAE. **A:** Spinal cord of a rat with a severe paraparesis showing large erythrocytic infiltrates but no labeling of SPM; 50 μm vibratome section. **B:** The successful HRP-DAB labeling of SPM (*arrows*) in rats with EAE reveals more labeled SPM than in normal control animals; 50 μm vibratome section. **C:** The dense perivascular leukocytic infiltrates in the spinal cord parenchyma of rats with severe paraparesis allow only poor spatial resolution between the endothelial and extravasated cells in 50 μm thick vibratome sections. **D:** No differentiation among the various types of extravasated cells filling the periavscular space was possible also in plastic semithin sections (0.5 μm thick) counterstained with toluidine blue. **E:** Rat with severe paraparesis. Electron micrograph showing a HRP-labeled SPM with vacuolated cytoplasm. Counterstaining with uranyl acetate and lead citrate.

Figure 3
Vibratome section from the lumbar spinal cord of a rat in which EAE was induced by an intraperitoneal injection of 5×10^6 T_{MBP}GFP cells. **A:** Five days post application (peak of paraparesis) the green fluorescent autoaggressive T-lymphocytes (*empty arrows*) have infiltrated both white and grey matter. **B:** The assumption that the bulk of T_{MBP}GFP cells would stay in contact with the red (FR-labeled) SPM (*arrows*) was wrong: most lymphocytes succeeded to find a way towards the CNS parenchyma.
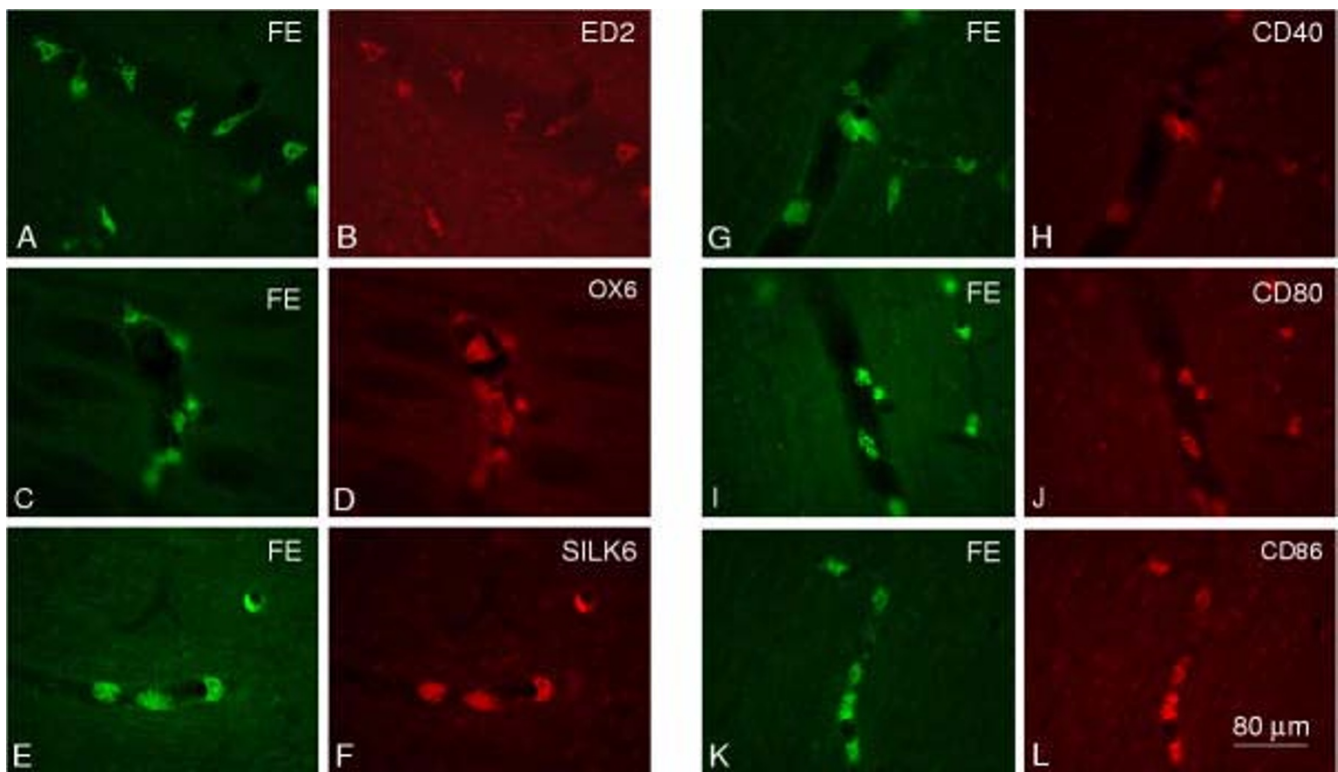
Figure 4
Activation of SPM in 50 μm vibratome sections from the spinal cord of rats with severe EAE. All cells which have been labeled by *icv* application of FE (*green* in **A,C,E**) are ED2-positive (**B**), OX6-positive (**D**), SILK6-positive (**F**). **G, H:** Almost all FE-labeled SPM express the CD40 molecule (CY3). **I, J, K, L:** FE-labeled SPM (**I, K**) express also the B7-1 (**J**) and the B7-2 (**L**) molecule as detected by immunofluorescence with anti-CD80, anti-CD86, and CY3.

These new data falsified one expectation that had been based on earlier work. Our hypothesis, that most (if not all) of the red-fluorescent SPM would be approached and contacted by the extravasated autoagressive lymphocytes, turned out to be wrong. Despite a general impression that some T_{MBP}GFP cells adhered to SPM, most lymphocytes found a way into the CNS parenchyma through the walls of capillaries and venules which do not possess a SPM lining.

Thus, the invasion of leukocytes (non-labeled as well as T_{MBP}GFP cells) occurred despite the barrier role of SPM to CNS infiltration which we had previously shown in a different kind of experiment [4]. Being a component of a neuroprotective mechanism of extraparenchymal antigen presentation and prevention of infiltrates, activated SPM can impede blood cells from crossing the lamina superficialis gliae limitans and infiltrate CNS parenchyma *under conditions of a non-inflammatory immune response to exogenous proteins*[4]. During EAE, however, SPM fail to stop the infiltration of CNS parenchyma by highly activated lymphocytes (primed against MBP). Obviously the degree of T cell activation, the number of activated T cells, and the

localization of the target antigen in the CNS are all important factors in determining the extent of intraparenchymal T cell infiltration.

Immunlogical pertinence of the non-phagocytic cells

Many non-phagocytic cells in the spinal perivascular space, which were not labeled by the *icv* applied fluorescent dyes, could be stained by the lymphocyte-recognizing antibodies R73, W3/25, and OX-50 (data not shown; see however Fig. 4 in Walther et al., 2001) [4].

Activation of SPM during EAE

Estimating the degree of activation of SPM during EAE, we found that the bulk of SPM, which were labeled and thus identified by by *icv* injection with FE (Fig. 4A,4C,4E,4G,4I,4K) were ED2-positive [20], expressed MHC class II glycoproteins, IL1-β, CD40-, and B7-molecules (Figs. 4B,4D,4F,4H,4J,4L). Whereas the immunoreactivity for both the ED2-antigen and MHC class II glycoproteins is constitutive and thus present also in unaffected rats, there was no staining of SPM in spinal cord sections from intact rats after incubation with SILK6, anti-

CD40, anti-CD80, anti-CD86 (data not shown; see however the right column of Fig. 3 in Walther et al., 2001) [4].

Expression of cell adhesion molecules by SPM during EAE

To elucidate the putative chemoattracting potential of SPM, which were labeled green by an icv injection of FE, we investigated whether they expressed molecules promoting extravasation of leukocytes. Despite the common view that during ongoing inflammation the expression of adhesion molecules by the cerebral endothelium is not sufficient to support leukocyte recruitment [21–23], we compared the expression of cell adhesion molecules between unaffected rats and animals with EAE. Following incubation with polyclonal anti-ICAM-1 and anti-VCAM-1 and visualization of the immunoreaction product by the red fluorescence of Cy3, we observed much more immunopositive SPM (orange-yellow fluorescence) in the paraplegic rats (Fig. 5A,5C) than in the intact controls (Fig. 5B,5D). Although the numerous and densely-packed cells in and around the Virchow-Robin space rendered difficult the discrimination between endothelial cells, recruited macrophages and activated SPM, these results are in line with the earlier findings demonstrating expression of VCAM-1 mRNA in cerebral perivascular macrophages in the acute phase of immune-mediated injury [24].

Production of chemokines by SPM during EAE

Chemokines are proposed to play a role in CNS inflammatory disease at the stage of leukocyte recruitment in the perivascular space in response to activated antigen-specific T-lymphocytes [25,26]. Together with their receptors the chemokines build a complex and sophisticated biochemical signaling system involved in the regulation of leukocyte and lymphocyte trafficking [27,28]. Both, adhesion molecule expression as well as production of chemokines within the CNS have been reported (reviewed in Prat et al., 2001) [29]. However, sound morphological evidence elucidating mechanism(s) by which precisely identified SPM (but not perivascular glial cells or perivascular astrocytes) could promote extravasation of leukocytes into the Virchow-Robin space and thus initiate inflammatory relapses, is still missing.

The chemokines *MCP-1* (monocyte chemoattractant protein) and MIP-1α (macrophage inflammatory protein 1α) are known to play crucial roles in recruiting macrophages and monocytes [30–32]. Using monoclonal mouse antibodies we observed more MCP-1 and MIP-1α positive SPM (Fig. 5E,5G) in animals with EAE than in intact control rats (Fig. 5F,5H).

Discussion

Clinical importance of T-cell extravasation

A major issue in the pathogenesis of any autoimmune neurological disease is *how* autologous proteins, that had been pathologically altered "behind" an intact blood-brain barrier (BBB) can mediate T-lymphocyte entry into the central nervous system (CNS) and trigger inflammatory relapses [33]. These relapses consist of (i) early proinflammatory response, (ii) recruitment of antigen specific T-lymphocytes, and (iii) invasion of leukocytes (monocytes/macrophages, T- and B-lymphocytes) into the brain parenchyma [34–38].

The last process listed, i.e. the accumulation of immune effectors around the cerebral microvasculature ("perivascular lymphocytic cuffs") is considered to initiate or exacerbate an inflammatory process, which is accompanied by a localized breakdown of the blood-brain barrier to serum proteins, leading to focal oedema [39,40]. The "bystander demyelination" [41,42], together with the subsequent interruption of axons [43,44], provide the pathologic correlate of irreversible neurologic impairment [45].

In contrast, the molecular mechanisms which rule the first two processes, i.e. the proinflammatory response and the subsequent CNS-recruitment of T-lymphocytes, remain unclear. This lack of sound knowledge is very disappointing because in the course of many immune-mediated diseases such as multiple sclerosis, postinfectious encephalomyelitis, viral encephalitis, the entry of T-lymphocytes into the CNS is one of the earliest events in their pathogenesis [46–48]. Morphologically, T-lymphocytes have been shown to cross the BBB via a transendothelial route, preferentially in parajunctional areas of endothelial cells [49]. Interestingly, there appears to be a particularly high density of endothelial adhesion molecule expression in these areas [50]. Although the large number of T-lymphocytes seen in specific regions or plaques in the acute stage of multiple sclerosis suggests that there is specific targeting in the immune response [15], the basic mechanisms ruling the extravasation of lymphocytes into the perivascular (Virchow-Robin) space and the subsequent infiltration of the brain-parenchyma have not been entirely elucidated yet.

Possible role of T-cell receptors (TCR)

The generally accepted current theory is based on the fact that CNS is continuously patrolled by activated T-lymphocytes [49–51]. Upon antigenic stimulation to a blast phase [52] these lymphocytes which carry T cell receptors (TCR) for CNS autoantigens initiate an "encephalitogenic immune response", i.e. if these "antigen seeking" T-cells encounter their antigen in the CNS [46,52–54] they could promote an inflammatory reaction through the secretion of pro-inflammatory cytokines [55–58]. This leads to the upregulation of endothelial adhesion molecules and the local production of chemokines, which in concert facilitate the entry of circulating B- and T-lymphocytes [59] and inflammatory effector cells into the local lesion sites [60].

This "direct" or "chemotaxis-based" theory of extravasation and infiltration acknowledges the "site-specific lymphocyte entry" as a key event for the progression of disease and outcome [21,36,61–63].

Possible role(s) of the resident antigen presenting cells

Since, however, the inflammation-triggering antigen recognition by T-lymphocytes can take place only after presentation of antigen in association with the MHC class II molecule, the role of the resident antigen presenting cells (APC) during lymphocyte extravasation is a matter of continuously expanding interest [53,64–67]. Earlier work has shown that the most probable candidate for antigen presentation within the rat CNS are the ED2-positive CPM/SPM [reviewed in [68]].

Due to their strategic location distal to the blood-endothelial interface and proximal to the glia limitans, CPM/SPM are able to phagocytize neuronal debris in the cerebral interstitium and to contact extravasated T-lymphocytes [9,69]. CPM/SPM synthesize MHC class II glycoproteins constitutively and, following neuronal death, transform into IL-1β secreting neuronophages [70,71]. Recent evidence also implicates SPM as primary targets of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infection in the CNS of humans and macaques [72]. Anyway, these cells must not be mixed up with other "sentinels" of the immune system within the brain, e.g. the ED2-negative dendritic cells in the meninges and choroid plexus [73].

Accordingly, current knowledge shows that since the synthesis of T-cell receptors (TCR) is directly dependent on the concentration of MHC class II peptides expressed by APC [74], the latter are able to mediate the trafficking of specific antigen-primed T-lymphocytes [75] or B-lymphocytes [76,77] into the cerebral area. Thus, the capacity to activate lymphocytes for transfer of severe experimental autoimmune encephalomyelitis has been attributed to cerebral APC [2,75,78]. Furthermore, activating memory/blast CD4+ T-lymphocytes through secretion of cytokines [79,80] and co-stimulatory molecules, APC could play a key role in the repetitive lymphocytic invasions and leukocyte recruitment [81–83]. Finally, selective depletion of blood-borne or resident perivascular macrophages during EAE causes suppression of clinical symptoms [84–89].

Conclusions

In the present study we used a combination of a technique for invasive, but very reliable labeling of SPM plus an immunocytochemcal analysis of their activity status. Studying the reactivity of identified SPM in very small details, this report provides evidence for increased expression of cell adhesion molecules (ICAM-1, VCAM-1) and intensified production of chemokines (MCP-1, MIP-1α) by SPM

during EAE. Since these findings, which could enhance T-cell extravasation, were observed in both "antigen-induced or active" and "T cell transfer-induced" forms of EAE, we suppose that they reflect a common neurobiological response of antigen-presenting SPM upon interaction with primed T-lymphocytes.

Materials and Methods
Animals

Young adult (3 months of age) male Lewis rats were used: Twenty-six animals were from the strain LEW/Han Rij Hsd (purchased from Harlan Winkelmann, D-33176 Borchen, Germany) and 16 rats from the strain LEW/CRL BR (purchased from Charles River Deutschland, D-97633 Sulzfeld, Germany). Before and after experiments all rats were kept on standard laboratory food and tap water *ad libitum* with an artificial light-dark cycle of 12 hours light on, 12 hours light off. All experiments were conducted in accordance with the German law on Animal Protection and all procedures were approved by the Local Animal-Protection Committe (Bezirksregierung Köln, Az. 23.203.2-K 35, 33/98).

Overview of experiments

Sixteen LEW/Han Rij Hsd animals comprised the **intact control group** and were used to optimize the labeling of spinal perivascular macrophages (SPM) by intracerebroventricular (*icv*) injections of horseradish peroxidase (HRP; 4 rats) or fluorochrome-conjugated dextrans (4 rats). Other 8 rats were used to immunocytochemically demonstrate the basic expression of molecules promoting SPM activation and lymphocyte extravasation.

The **antigen-induced EAE (active EAE) group** consisted of 10 rats which were used to immunocytochemically demonstrate the reactive changes in SPM during EAE.

The **T cell transfer-induced EAE (transfer EAE) group** consisted of 16 LEW/CRL BR rats. Eight animals were used as intact controls. The other 8 rats received an intraperitoneal injection of 5×10^6 autoaggressive MBP-specific T-lymphocytes in 3 ml 10% FCS-containing medium. Since these cells had been genetically engineered to express the green fluorescent protein (GFP), they were readily detectable within the heterogenous perivascular infiltrates ("cuffs") in the spinal cord of rats with EAE [18]. A preceeding labeling of the SPM in red fluorescence (Fluoro-Ruby) revealed the complex relationships between the resident SPM and the extravasated GFP-transduced, MBP-specific (T_{MBP}GFP) lymphocytes.

Labeling of SPM employing intracerebroventricular (icv) injections

After an intraperitoneal injection of Ketamin plus Xylazin (100 mg Ketanest® plus 5 mg Rompun® per kg body
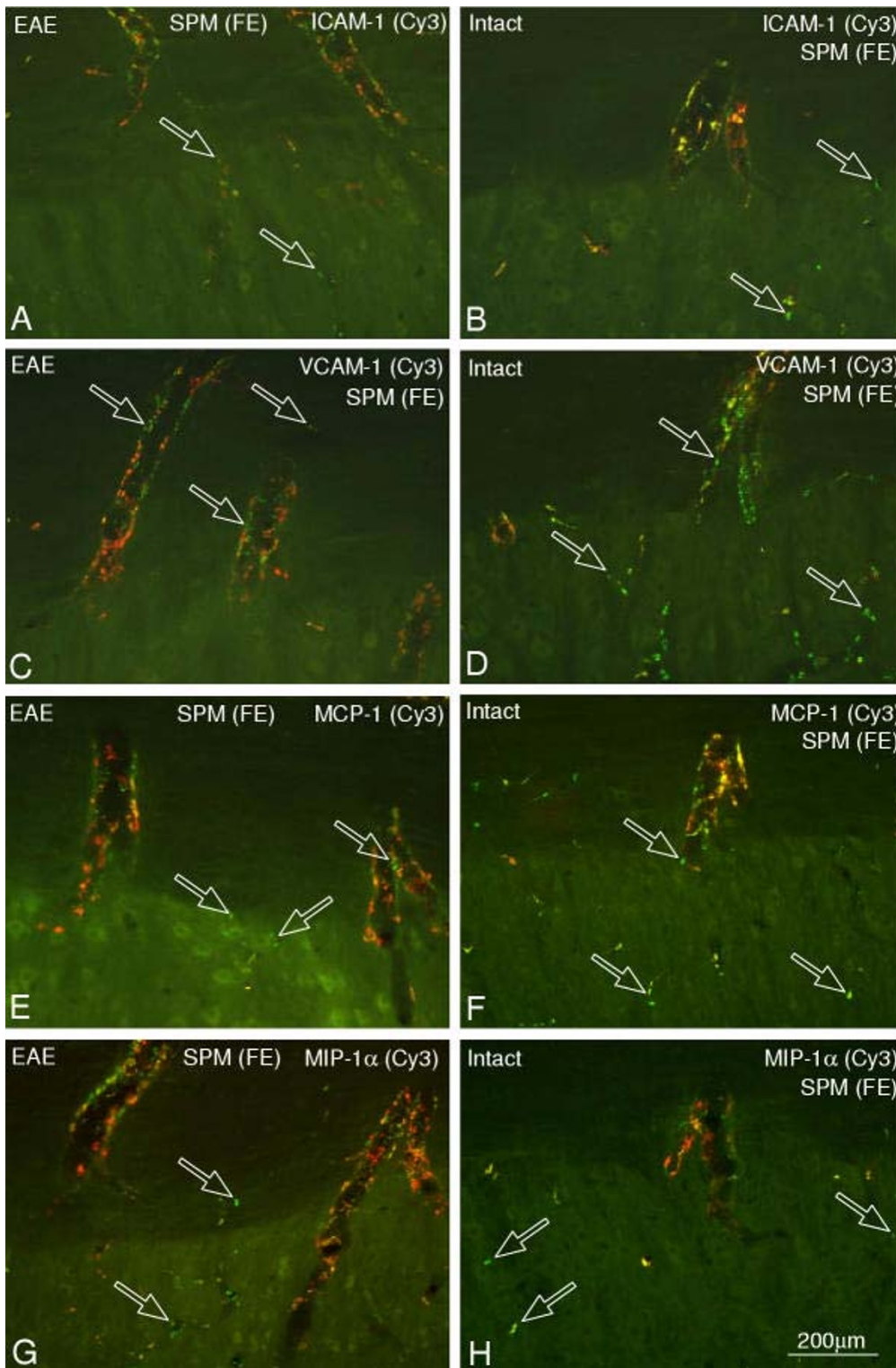
Figure 5
Expression of cell adhesion molecules and chemokines as revealed by double exposure pictures taken from in the lumbar spinal cord of animals with EAE (**A, C, E, G**) and in intact rats (**B, D, F, H**). SPM have been labeled and identified by *icv* injection of 5% FE and fluoresce in green. The reaction product expressed by SPM has been visualized by FluoroLink™ Cy3™-Streptavidin, which yields an orange-yellow fluorescence from the immunopositive SPM.

weight) the rats were fixed in a stereotactic apparatus and received 35 µl of a 5% solution of horseradish peroxidase (HRP, Type VIA, Sigma, No. P 6782), fluorescein-dextran (Fluoro-Emerald, FE; Molecular Probes, Cat. No. D-1820) or rhodamine-dextran (Fluoro-Ruby, FR; Molecular Probes D-1817) in 0.9% NaCl saline into the right lateral cerebral ventricle. All solutions were injected over a period of 5 min [90] using a very thin (10 µm thick) glass pipette to minimize tissue damage [91].

Induction of experimental allergic encephalomyelitis (EAE)

EAE was induced in 37 LEW/Han Rij Hsd rats by a single intradermal (hind footpad) injection containing 25 µg myelin basic protein from guinea pig (MBP; Sigma M2295), 100 µg Mycobacterium tuberculosis H37 RA (Difco Laboratories, Cat. Nr. 3114-33-8) in 50 µl Complete Freund's Adjuvant (CFA; Difco Laboratories, Cat. Nr. 0638-60-7).

Tissue processing

Fixation

All rats were anesthetized with ether, their vascular system rinsed for 60 sec with 0.9% NaCl saline and fixed for at least 40 min by transcardial perfusion with 1.0 litre of periodate-lysine-paraformaldehyde (PLP) fixative [92]. The PLP fixative was separately mixed from premade stem stock solutions immediately prior to the perfusion of each animal. The CNS (cerebrum, cerebellum, brainstem, spinal cord) was removed and stored in a 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7,4.

Visualizing of HRP-labeled SPM

In contrast to our previous work [4] we focused our observations on the lumbar spinal cord only: Since the major visible symptom during EAE is the severe paraparesis of the hind limbs, most of the pathological alterations should be expected, localized and observed in this region. Longitudinal vibratome sections (50 µm thick) of the spinal cord were incubated for 20 min in 0.75% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (150 mg in 200 ml buffer; DAB, Sigma, D 5637) plus 0.01% (v/v) H_2O_2 plus 0.075% (w/v) nickel chloride in 0.05 M Tris-HCl buffer, pH 7.6. This procedure yielded a very intensive dark-purple to black reaction product exclusively in the cytoplasm of the SPM. Control tissue (sections from brains which had not received HRP injections) contained no reaction product. For electron microscopy some of the sections were postfixed in 1% OsO_4 + 1.5% $K_3^{III}Fe(CN)_6$[93], dehydrated in graded acetones, and embedded flat in Araldite CY212 (Fluka, No. 44610).

Visualizing of FE- and FR-labeled SPM

To detect the green fluorescence of FE or the red fluorescence of FR vibratome sections (50 µm thick) of the spinal

cord were illuminated through filter set 10 (excitation BP 450-490, beamsplitter FT 510, emission BP 515-565) or filter set 15 (Excitation BP 546/12, Emission LP 590) of a Carl Zeiss microscope.

Immunocytochemistry

Primary antibodies

(1) Mouse monoclonal OX-6 (Serotec, MCA 46, 1:1000) recognizing rat MHC class II (Ia) antigen. (2) Mouse monoclonal SILK 6 (Serotec, MCA-1397, 1:500) recognizing recombinant rat IL-1β. (3) Armenian hamster monoclonal anti-mouse CD40 (BD PharMingen, 09401A, 1:100) to test for presence of the costimulatory molecule CD40 on SPM. (4) Mouse monoclonal CD80 (BD PharMingen, 22661D, 1:100) to test for presence of the co-stimulatory molecule B7-1 on rat SPM. (5) Mouse monoclonal CD86 (BD PharMingen, 22671D, 1:100) to test for presence of the co-stimulatory molecule B7-2 on rat SPM. (6) Monoclonal mouse anti-rat TCR alpha/beta (clone R73, Serotec MCA 453G, 1:20) recognizing a constant determinant of the α/β T cell receptor. (7) Monoclonal mouse anti-rat CD4 (clone number W3/25, Serotec MCA55G, 1:50) recognizing the CD4 cell surface glycoprotein expressed by helper T cells and thymocytes. (8) Monoclonal mouse anti-rat CD44 (clone OX-50, Serotec MCA643XZ, 1:50) recognizing the CD44 cell surface antigen expressed by T cells, B cells, macrophages, and thymocytes. (9) Polyclonal goat anti-rat ICAM-1(M-19; Santa Cruz Biotechnology Inc., sc-1511, 1:50) recognizing rat intercellular adhesion molecule-1. (10) Polyclonal goat anti-rat VCAM-1(C-19; Santa Cruz Biotechnology Inc., sc-1504, 1:50) recognizing rat vascular cell adhesion molecule-1. (11) Polyclonal rabbit anti-rat MCP-1 (Cedarlane, No. CL9576AP, 1:500). recognizing the CC-chemokine "monocyte chemoattractant protein-1" which promotes the directed migration of inflammatory cells. (12) Polyclonal rabbit anti-rat MIP-1α (Cedarlane, No. CL9577AP, 1:2000) recognizing the chemokine "macrophage inflammatory protein-1α ".

Secondary antibodies

(1) Biotinylated goat anti-rabbit IgG (DAKO, No. E0432). (2) Biotinylated goat anti-mouse IgG (Fab-specific, Sigma, B-0529). (3) Biotinylated goat anti-mouse IgG (Fc specific, pre-absorbed with human IgG and rat serum proteins, Sigma, No. B-9904). (4) Biotinylated rabbit anti-goat IgG (DAKO, No. E0466). In order to minimize the background staining of neurons the biotinylated IgGs were always pre-absorbed with the individual rat's spleen protein [70]. (5) Biotin-conjugated mouse anti-hamster IgG (cocktail; PharMingen, 12102D).

Standard incubation protocol

Free floating vibratome sections were immunostained on a shaker at room temperature through the following steps:

(1) 0.6% (v/v) H_2O_2 in buffer (to block the endogenous peroxidase activity) for 30 min; (2) 5.0% (w/v) bovine serum albumin (BSA, Sigma No. A-9647) in 0.1 M Tris-buffered saline (TBS) pH 7.6 for 60 min; (3) primary antibody (see above) diluted in TBS plus 0.8% (w/v) BSA for 2 h; (4) 5.0% (v/v) normal goat serum (NGS, Vector No. S-1000) or normal rabbit serum (NRS, DAKO, No. X0902) plus 0.8% BSA in TBS for 15 min. (5) 1:400 dilution of the corresponding biotinylated secondary antibody in TBS for 1 h; (6) 1:100 diluted FluoroLink™ Cy2™-Streptavidin (Amersham Life Science, PA 42001) or FluoroLink™ Cy3™-Streptavidin (Amersham Life Science, PA 43001) for 1 h. Steps (1) and (5) were followed by two 10 min washes in TBS. Steps (3) and (6) were followed by four 10 min washes in TBS. Finally, sections were dehydrated with ethanol and Histoclear (a nontoxic xylene-substitute) and coverslipped.

Controls
Omission of the primary or secondary antibody yielded blank sections in which only the green SPM fluoresced. Incubation of sections with non-relevant biotinylated secondary antibodies (e.g. goat anti-rabbit IgG for recognition of mouse primary antibodies) yielded also blank sections.

Fluorescence microscopy
FE-labeled SPM and Cy2-immunopositive cells were visualized through filter set 10 of a Carl Zeiss fluorescence microscope. FR-labeled SPM and Cy3-immunopositive cells were observed through Zeiss filter set 15 (excitation BP546/12, beamsplitter FT580, emission LP590). Both FE-labeled SPM and CY3-immunopositive cells could be simultaneously observed employing Zeiss filter set 25 (excitation TBP400/495/570, beamsplitter FT410/505/585, emission TBP460/530/610).

Authors' contributions
N.H. participated in the *icv* labelings and counts of the SPP. N.L. performed the immunocytochemistry. M.S. participated in the icv labelings. B.W. performed the intraperitoneal injections of T_{MBP}GFP lymphocytes. W.F.N. participated in the coordination of the study. O.G.L. participated in the icv labelings. D.N.A. designed the study, induced EAE, participated in the *icv* labelings and perfused the animals.

All authors read and approved the final manuscript.

References
1. Marrack P, Kappler J, Kotzin B: **Autoimmune disease:why and where it occurs.** *Nature Medicine* 2001, **7**:899-905
2. Perry VH: **A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation.** *J Neuroimmunol* 1998, **90**:113-121
3. Archelos JJ, Hartung H-P: **Pathogenetic role of autoantibodies in neurological disease.** *TINS* 2000, **23**:317-327
4. Walther M, Popratiloff AS, Lachnit N, Hofmann N, Streppel M, Guntinas-Lichius O, Neiss WF, Angelov DN: **Exogenous antigen containing perivascular phagocytes induce a nonencephalitogenic extravasation of primed lymphocytes.** *J Neuroimmunol* 2001, **117**:30-42
5. Hickey WF, Kimura H: **Perivascular microglial cells of the CNS are bone marrow derived and present antigen in vivo.** *Science* 1988, **239**:290-292
6. Graeber MB, Streit WJ, Kreutzberg GW: **Identity of ED2-positive perivascular cells in rat brain.** *J Neurosci Res* 1989, **22**:103-106
7. Graeber MB, Streit WJ, Büringer D, Larry Sparks D, Kreutzberg GW: **Ultrastructural location of major histocompatibility complex (MHC) class II positive perivascular** *J Neuropathol* 1992, **cells in histologically normal human brain.**:303-311
8. Mato N, Ookara S, Sakamoto A, Aikawa E, Ogawa T, Mitsuhashi U, Masuzawa T, Suzuki H, Honda M, Yazaki Y, Watanabe E, Luoma J, Yla-Herttuala S, Fraser I, Gordon S, Kodama T: **Involvement of specific macrophage-lineage cell surrounding arterioles in barrier and scavenger function in brain cortex.** *Proc Natl Acad Sci USA* 1996, **93**:3269-3274
9. Angelov DN, Walther M, Streppel M, Guntinas-Lichius O, Neiss WF: **The cerebral perivascular cells.** *Advances in Anatomy, Embryology and Cell Biology* Springer Verlag Berlin 1998, **147**:
10. Mori S, Leblond CP: **Identification of microglia in light and electron microscopy.** *J Comp Neuol* 1969, **135**:57-80
11. Kida S, Steart PV, Zhang ET, Weller RO: **Perivascular cells act as scavengers in the cerebral perivascular spaces and remain distinct from pericytes, microglia and macrophages.** *Acta Neuropathol* 1993, **85**:646-652
12. Bechmann I, Kwidzinski E, Kovac AD, Simbürger E, Horvath T, Gimsa U, Dirnag U, Priller J, Nitsch R: **Turnover of rat brain perivascular cells.** *Exp Neurol* 2001, **168**:242-249
13. Dijkstra CD, Damoiseaux JGMC: **Macrophage heterogeneity established by immunocytochemistry.** *Progr Histochem Cytochem* 1993, **27**:1-65
14. Graeber MM, Streit WJ, Kiefer R, Schoen S, Kreutzberg GW: **New expression of myelomonocytic antigens by microglia and perivascular cells following lethal motor neuron injury.** *J Neuroimmunol* 1990, **27**:121-132
15. Weller RO, Engelhardt B, Phillips MJ: **Lymphocyte targeting of the central nervous system: A review of afferent and efferent CNS-immune pathways.** *Brain Pathol* 1996, **6**:275-288
16. Raine CS: **The Dale E. McFarlin memorial lecture: The immunology of the multiple sclerosis lesion.** *Ann Neurol* 1994, **36**:S61-S72
17. Dijkstra CD, Döpp EA, van den Berg TK, Damoiseaux JGMC: **Monoclonal antibodies against rat macrophages.** *J Immunol Methods* 1994, **174**:21-23
18. Flügel A, Willem M, Berkowicz T, Wekerle H: **Gene transfer into CD4$^+$ T lymphocytes: green fluorescent protein-engineered, encephalitogenic T cells illuminate brain autoimmune responses.** *Nature Medicine* 1999, **5**:843-847
19. Flügel A, Bradl M: **New tools to trace populations of inflammatory cells in the CNS.** *Glia* 2001, **36**:125-136
20. Dijkstra CD, Döpp EA, Joling P, Kraal G: **The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3.** *Immunology* 1985, **54**:589-599
21. Engelhardt B, Conley FK, Butcher EC: **Cell-adhesion molecules on vessels during** *J Neuroimmunol* 1994, **inflammation in the mouse central nervous system:**199-208
22. Bell MD, Perry VH: **Adhesion molecule expression on murine cerebral endothelium following the injection of a proinflammogen or during acute neuronal** *J Neurocytol* 1995, **degeneration.**:695-710
23. Perry VH, Bell MD, Brown HC, Matyszak MK: **Inflamation in the nervous system.** *Curr Opin Neurobiol* 1995, **5**:636-641

24. Jander S, Pohl J, Gillen C, Schroeter M, Stoll G: **Vascular cell adhesion molecule-1 mRNA is expressed in immune-mediated and ischemic injury of the rat nervous system.** *J Neuroimmunol* 1996, **70**:75-80

25. Ransohoff RM, Glabinski AR, Tani M: **Chemokines in immune-mediated inflammation of the central nervous system.** *Cytokine and Growth Factor Rev* 1996, **7**:35-46

26. Ransohoff RM, Tani M, Glabinski AR, Chernosky A, Krivacic K, Peterson JW, Chien H-F, Trapp BD: **Chemokines and chemokine receptors in model neurological pathologies: Molecular and immunohistochemical approaches.** *Methods Enzymol* 1997, **287**:319-348

27. Aloisi F: **Immune function of microglia.** *Glia* 2001, **36**:165-179

28. Baggiolini M: **Chemokines in pathology and medicine.** *J Intern Med* 2001, **250**:91-104

29. Prat A, Biernacki K, Wosik K, Antel JP: **Glial cell influence on the human blood-brain-barrier.** *Glia* 2001, **36**:145-155

30. Calvo CF, Yoshimura T, Gelman M, Mallat M: **Production of Monocyte Chemotactic Protein-1 by rat brain macrophages.** *Eur J Neurosci* 1996, **8**:1725-1734

31. Glabinski AR, Tuohy VK, Ransohoff RM: **Expression of chemokines RANTES, MIP-1alpha and GRO-alpha correlates with inflammation in acute experimental autoimmune encephalomyelitis.** *Neuroimmunomodulation* 1998, **5**:166-171

32. Rezaie P, Trillo-Pazos G, Everall IP, Male DK: **Expression of β-chemokines and chemokine receptors in human fetal astrocyte and microglial co-cultures: potential role of chemokines in the developing CNS.** *Glia* 2002, **37**:64-75

33. Wekerle H, Linington C, Lassmann H, Meyermann R: **Cellular immune reactivity within the CNS.** *TINS* 1986, **9**:271-277

34. Hickey WF, Kimura H: **Graft-vs-host disease elicits expression of class I and class II hostocompatibility antigens and the presence of scattered T lymphocytes in rat central nervous system.** *Proc Natl Acad Sci USA* 1987, **84**:2082-2086

35. Paterson PY, Day ED, Whitacre CC: **Neuroimmunologic diseases: Effector cell responses and immunoregulatory mechanisms.** *Immunol Rev* 1981, **55**:89-120

36. Raivich G, Jones LL, Kloss CUA, Werner A, Neumann H, Kreutzberg GW: **Immune surveillance in the injured nervous system: T-lymphocytes invade the axotomized mouse facial motor nucleus and aggregate around sites of neuronal degeneration.** *J Neurosc* 1998, **18**:5804-5816

37. Stoll G, Jander S: **The role of microglia and macrophages in the pathophysiology of the CNS.** *Progr Neurobiol* 1999, **58**:233-247

38. Flügel A, Schwaiger F-W, Neumann H, Medana I, Willem M, Wekerle H, Kreutzberg GW, Graeber MB: **Neuronal FasL induces cell death of encephalithogenic T lymphocytes.** *Brain Pathol* 2000, **10**:353-364

39. Brosnan CF, Claudio L, Tansey FA, Martiney J: **Mechanism of autoimmune neuropathies.** *Ann Neurol* 1990, **27(suppl)**:75-79

40. Ptak W, Herzog WR, Askenase PW: **Delayed-type hypersensitivity initiation by early-acting cells that are antigen mismatched or MHC incompatible with late-acting, delayed-type hypersensitivity effector T cells.** *J Immunol* 1991, **146**:469-75

41. Bauer J, Sminia T, Wouterlood FG, Dijkstra CD: **Phagocytic activity of macrophages and microglial cells during the course of acute and chronic relapsing experimental autoimmune encephalomyelitis.** *J Neurosci Res* 1994, **38**:365-375

42. Wisniewski HM, Bloom BR: **Primary demyelination as a non-specific consequence of a cell-mediated immune reaction.** *J Exp Med* 1975, **141**:346-359

43. Ferguson B, Matyszak MK, Esiri MM, Perry VH: **Axonal damage in acute multiple sclerosis lesions.** *Brain* 1997, **120**:393-399

44. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L: **Axonal transection in the lesions of multiple sclerosis.** *N Engl J Med* 1998, **338**:278-285

45. Kiefer R, Kieseier BC, Stoll G, Hartung H-P: **The role of macrophages in the immune-mediated damage to the peripheral nervous system.** *Progr Neurobiol* 2001, **64**:109-127

46. Hickey WF, Hsu BL, Kimura H: **T-lymphocyte entry into the central nervous system.** *J Neurosci Res* 1991, **28**:254-260

47. Lassmann H, Zimprich F, Vass K, Hickey WF: **Microglial cells are a component of the perivascular glia limitans.** *J Neurosci Res* 1991, **28**:236-243

48. Muhallab S, Lidman O, Weissert R, Olsson T, Svenningsson A: **Intra-CNS activation by antigen-specific T lymphocytes in experimental autoimmune encephalomyelitis.** *J Neuroimmunol* 2001, **113**:201-211

49. Raine CS, Cannella B, Duijvestijn AM, Cross AH: **Homing to central nervous system vasculature by antigen-specific lymphocytes. II. Lymphocyte/endothelial cell adhesion during the initial stages of autoimmune demyelination.** *Lab Invest* 1990, **63**:476-489

50. Lassmann H, Zimprich F, Rössler K, Vass K: **Inflammation in the nervous system.** *Rev Neurol (Paris)* 1991, **147**:763-781

51. Lassmann H, Vass K, Brunner C, Seutelberger F: **Characterization of inflammatory infiltrates in experimental allergic encephalomyelitis.** *Progr Neuropathol* 1986, **6**:33-62

52. Hickey WF: **Basic principles of immunological surveillance of the normal central nervous system.** *Glia* 2001, **36**:118-124

53. Cross AH, Cannella B, Brosnan CF, Raine CS: **Homing to central nervous system vasculature by antigen specific lymphocytes. I. Localization of 14C-labeled cells during acute, chronic and relapsing experimental allergic encephalomyelitis.** *Lab Invest* 1990, **63**:162-170

54. Krakowsky ML, Owens T: **Naive T lymphocytes traffic to inflamed central nervous system, but require antigen recognition for activation.** *Eur J Immunol* 2000, **30**:1002-1009

55. Male D, Pryce G, Rahman J: **Comparison of the immunological properties of rat cerebral and aortic endothelium.** *J Neuroimmunol* 1990, **30**:167-172

56. Berger T, Weerth S, Kojima K, Linington C, Wekerle H, Lassmann H: **Experimental autoimmune encephalomyelitis: the antigen specificity of T-lymphocytes determines the topography of lesions in the central and peripheral nervous system.** *Lab Invest* 1997, **76**:355-364

57. Serpe CJ, Kohm AP, Huppenbauer CB, Sanders VM, Jones KJ: **Exacerbation of facial motoneuron loss after facial nerve transection in severe combined immunodeficient (scid) mice.** *J Neurosci* 1999, **19RCN**:1-5

58. Delves PJ, Roitt IM: **The immune system.** *N Engl J Med* 2000, **343**:37-49

59. Knopf PM, Harling-Berg CJ, Cserr HF, Basu D, Sirulnick EJ, Nolan SC, Park JT, Keir G, Thompson EJ, Hickey WF: **Antigen-dependent intrathecal synthesis in the normal rat brain: tissue entry and local retention of antigen specific B cells.** *J Immunol* 1998, **161**:692-701

60. Lassmann H: **Basic mechanisms of brain inflammation.** *J Neural Transm Suppl* 1997, **50**:183-190

61. Engelhardt JI, Tajti J, Appel SH: **Lymphocytic infiltrates in the spinal cord in amyotrophic lateral sclerosis.** *Arch Neurol* 1993, **50**:30-36

62. Kawamata T, Akiyama A, Yamada T, McGeer PL: **Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord.** *Am J Pathol* 1992, **140**:691-707

63. Schluesener HJ, Seid K, Kretzschmar J, Meyermann R: **Leukocyte chemotactic factor, a natural ligand to CD4, is expressed by lymphocytes and microglial cells of the MS plaque.** *J Neurosci Res* 1996, **44**:606-611

64. Cross AH, O'Mara T, Raine CS: **Chronologic localization of myelin-reactive cells in the lesions of relapsing EAE: implications for the study of multiple sclerosis.** *Neurology* 1993, **43**:1028-1033

65. Morimoto K, Craig Hooper D, Bornhorst A, Corisdeo S, Bette M, Fu ZF, Schäfer MK-H, Koprowsky H, Weihe E, Dietzschold B: **Intrinsic responses to Borna disease virus infection of the central nervous system.** *Proc Natl Acad Sci USA* 1996, **93**:13345-13350

66. Hu P, Pollard J, Hunt N, Chan-Ling T: **Microvascular and cellular responses in the retina of rats with acute experimental allergic encephalomyelitis (EAE).** *Brain Pathol* 1998, **8**:487-498

67. Deininger MH, Zhao Y, Schluesener HJ: **CP-10, a chemotactic peptide, is expressed in lesions of experimental autoimmune encephalomyelitis, neuritis, uveitis and in C6 gliomas.** *J Neuroimmunol* 1999, **93**:156-163

68. Raivich G, Bohatscheck M, Kloss CUA, Werner A, Neumann H, Jones LL, Kreutzberg GW: **Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function.** *Brain Res Rev* 1999, **30**:77-105

69. Lawrence JM, Morris RJ, Wilson DJ, Raisman G: **Mechanisms of allograft rejection in the rat brain.** *Neuroscience* 1990, **37**:431-462

70. Angelov DN, Neiss WF, Streppel M, Walther M, Guntinas-Lichius O, Stennert E: **ED2-positive perivascular cells act as neuronophages during delayed neuronal loss in the facial nucleus of the rat.** *Glia* 1996, **16:**129-139

71. Angelov DN, Streppel M, Walther M, Guntinas-Lichius O, van Dam AM, Stennert E, Neiss WF: **ED2-positive perivascular neuronophages produce Interleukin-1β during delayed neuronal loss in the facial nucleus of the rat.** *J Neurosci Res* 1998, **54:**820-827

72. Williams KC, Corey S, Westmoreland SV, Pauley D, Knight H, deBakker C, Alvarez X, Lackner AA: **Perivascular macrophages are the primary cell type productively infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS.** *J Exp Med* 2001, **193:**905-915

73. McMenamin PG: **Distribution and phenotype of dendritic cells and resident tissue macrophages in the dura mater, leptomeninges, and choroid plexus of the rat brain as demonstrated in wholemount preparations.** *J Comp Neurol* 1999, **405:**553-562

74. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML: **The immunological synapse: a molecular machine controlling T cell activation.** *Science* 1999, **285:**207-208

75. Matsumoto Y, Hara N, Tanaka R, Fujihara M: **Immunohistochemical analysis of the rat central nervous system during experimental allergic encephalomyelitis, with special reference to Ia-positive cells with dendritic morphology.** *J Neuroimmunol* 1986, **136:**3668-3676

76. Litzenburger T, Fässler R, Bauer J, Lassmann H, Linington C, Wekerle H, Iglesias A: **B lymphocytes producing demyelinating autoantibodies: development and function in gene-targeted transgenic mice.** *J Exp Med* 1998, **188:**169-180

77. Cross AH, Trotter JL, Lyons J-A: **B cells and antibodies in CNS demyelinating disease.** *J Neuroimmunol* 2001, **112:**1-14

78. Gautam AM, Glynn P: **Lewis rat lymphoid dendritic cells can efficiently present homologous myelin basic protein to encephalitogenic lymphocytes.** *J Neuroimmunol* 1989, **22:**113-121

79. Martin M, Resch K: **Interleukin 1: more than a mediator between leukocytes.** *TINS* 1988, **9:**171-177

80. Croft M, Dubey C: **Accessory molecule and costimulation requirements for CD4 T cell response.** *Crit Rev Immunol* 1997, **17:**89-118

81. Sun D, Wekerle H: **Ia restricted encephalitogenic T lymphocytes mediating EAE lyse autoantigen-presenting astrocytes.** *Nature* 1986, **320:**70-72

82. Matsumoto Y, Ohmori J, Fujihara M: **Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T-cells under in vivo mimicking conditions.** *Immunology* 1992, **76:**209-216

83. Brosnan CF, Raine CS: **Mechanisms of immune injury in multiple sclerosis.** *Brain Pathol* 1996, **6:**243-257

84. Brosnan C, Bornstein MB, Bloom BR: **The effect of macrophage depletion on clinical and pathological expression of experimental allergic encephalomyelitis.** *J Immunol* 1981, **126:**614-620

85. Bauer J, Huitinga I, Zhao W, Lassmann H, Hickey W, Dijkstra CD: **The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis.** *Glia* 1995, **15:**437-446

86. Huitinga I, van Rooijen N, de Groot CJA, Uitdehaag BMJ, Dikstra CD: **Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages.** *J Exp Med* 1990, **172:**1025-1033

87. Huitinga I, Ruuls SR, Jung S, van Rooijen N, Hartung H-P, Dikstra CD: **Macrophages in T cell line-mediated, demyelinating, and chronic relapsing experimental autoimmune encephalomyelitis in Lewin rats.** *Clin Exp Immunol* 1995, 344-351

88. Tran EH, Hoekstra K, van Rooijen N, Dijkstra CD, Owens T: **Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage depleted mice.** *J Immunol* 1998, **161:**3767-3775

89. Polfliet MMJ, van de Veerdonk F, Döpp E, van Kesteren-Hendrikx EM, van Rooijen N, Dijkstra CD, van der Berg T: **The role of perivascular and meningeal macrophages in experimental allergic encephalomyelitis.** *J Neuroimmunol* 2002, **122:**1-8

90. Wagner HJ, Pilgrim C, Brandl J: **Penetration and removal of horseradish peroxidase injected into the cerebrospinal fluid: role of cerebral perivascular spaces, endothelium and microglia.** *Acta Neuropathol* 1974, **27:**299-315

91. Mingheti L, Walsh DT, Levi G, Perry VH: **In vivo expression of cyclooxygenase-2 in rat brain following intraparenchymal injection of bacterial endotoxin and inflammatory cytokines.** *J Neuropathol Exp Neurol* 1999, **58:**1184-1191

92. McLean IW, Nakane PK: **Periodate-lysine-paraformaldehyde fixative a new fixative for immunoelectron microscopy.** *J Histochem Cytochem* 1974, **22:**1077-1087

93. Langford LA, Coggeshall RE: **The use of potassium ferricyanide in neural fixation.** *Anat Rec* 1980, **197:**297-303