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 fluorescent 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 – closely ensheathed by split layers of the vascular basal lamina 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 immunocytochemical differentiation between the resident SPM (mab ED2+, mab ED1+) and the extremely 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 TMBPGFP 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 TMBPGFP 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 perivasculat 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 $5 \times 10^6$ TMBP-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 TMBP-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.
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 TMBPGFP 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 TMBPGFP 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.

**Immunological 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-
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 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].

CD40, anti-CD80, anti-CD86 (data not shown; see however the right column of Fig. 3 in Walther et al., 2001) [4].
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 traffickling 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 immunocytochemical 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 Commitee (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 (ivc) 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 $5 \times 10^6$ 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 preceding 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<sub>MBP</sub>GFP) lymphocytes.

**Labeling of SPM employing intracerebroventricular (ivc) injections**

After an intraperitoneal injection of Ketamin plus Xylazin (100 mg Ketanest® plus 5 mg Rompun® per kg body

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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 ivc 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 transcardiac 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) H2O2 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% OsO4 + 1.5% K3[HFe(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, beam splitter 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 co-stimulatory 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 IgM (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$_2$O$_2$ 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$^\text{TM}$ Cy$_2^\text{TM}$-Streptavidin (Amersham Life Science, PA 42001) or FluoroLink$^\text{TM}$ Cy$_3^\text{TM}$-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 TBP400/495/580, 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.

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