CCR5 in multiple sclerosis : expression, regulation and modulation by statins

Kuipers, H.F.

Citation

Kuipers, H. F. (2007, March 28). *CCR5 in multiple sclerosis : expression, regulation and modulation by statins*. Retrieved from https://hdl.handle.net/1887/11460

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CCR5 expression in MS

Enhanced expression of the chemokine receptor CCR5 in Multiple Sclerosis lesions.

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Hedwich F. Kuipers, Elise S. Van Haastert, H. Maria Feenstra, Peter J. van den Elsen and Paul van der Valk

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Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. There are strong indications that chemokines, small proteins that mediate the movement of cells towards sites of inflammation, play an important role in the pathogenesis of MS. In the current study we have examined the expression of the chemokine receptor CCR5 in various stages of MS lesions. In control brain tissue we found CCR5 expression mainly on microglia and astrocytes. In normal appearing white matter of MS patients CCR5 expression on astrocytes was slightly enhanced, whereas CCR5 expression on microglia did not appear altered. In preactive lesions however, activated microglia displayed strong CCR5 immunoreactivity and phagocytic macrophages in active demyelinating lesions expressed even higher levels of CCR5. In addition, high levels of CCR5 expression were also found on hypertrophic astrocytes inside and at the border of active lesions. Astrocytes populating the center of chronic active and chronic inactive lesions displayed the same high level of CCR5 expression, whereas macrophages at the rim of chronic active lesions appeared to express lower levels of CCR5 than macrophages inside active lesions. Taken together, these data indicate that CCR5 expression is mainly confined to the two immunocompetent cell types of the brain, microglia and astrocytes. Furthermore, we show that CCR5 expression on these cell types changes during MS lesion development, suggesting a role for this chemokine receptor in the pathogenesis of MS.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), hallmarked by inflammatory lesions throughout the brain and spinal cord, and is the major cause of neurological disability among young adults in Western countries. The pathology of MS is characterized by infiltration of inflammatory cells, localized destruction of myelin, oligodendrocyte death and axonal degeneration [1]. Depending on the location of the lesion in the CNS, loss of myelin and axonal transection result in various neurological impairments. Microglia and macrophages are thought to play an important role in demyelination by producing toxic agents and phagocytosing myelin proteins [1,2]. In addition, astrocytes have also been implicated in the pathogenesis of MS [1]. Within normal appearing white matter (NAWM) of MS patients clusters of activated microglia can be observed, which might represent a very early stage of MS lesions [3]. In addition, active demyelinating lesions are occupied by large numbers of macrophages, and at later stages astrocytes. Thus it seems that MS lesion development is accompanied by enhanced motility of glial cells.

Chemokines are a group of secreted proteins that are able to attract various cell types towards sites of inflammation. These small proteins exert their action through binding to G protein-coupled receptors, which leads to activation and migration of the responding cell. Interactions between chemokines and chemokine receptors are promiscuous, i.e. most chemokines activate more than one receptor and most chemokine receptors can bind several chemokines [4,5]. On the basis of their structure, chemokines (and their receptors) have been classified into four subfamilies: C, CC, CXC and CX_3C, of which the CC and CXC subfamilies contain the most members. The CC chemokine receptor 5 (CCR5) is one of the best described chemokine receptors and mainly regulates trafficking and effector functions of memory/effector T-lymphocytes, macrophages, and immature dendritic cells by binding to the chemokines CCL5 (RANTES), CCL3 (MIP-1α) and CCL4 (MIP-1β) [4-6]. Because of its important immune regulatory role, CCR5 is implicated in the pathogenesis of various inflammatory diseases [7].

Several neurodegenerative diseases, including MS, are associated with (enhanced) expression of chemokines and chemokine receptors in the CNS [8,9], making these molecules interesting targets for further investigation in the pathogenesis and possible treatment of these diseases. A number of groups have studied chemokine levels in the cerebrospinal fluid of MS patients and chemokine expression in demyelinating MS lesions. These studies show that, amongst others, CCL5 and CCL3 levels are elevated in MS CSF [10,11]. In addition, enhanced expression of CCL3, -4 and -5 has been found in MS lesions [12-14], strongly implicating an involvement of these chemokines and their responding receptor, CCR5, in the pathogenesis of MS.

A number of groups have reported expression of CCR5 in the brain, in particular on microglia [15]. In addition, increased CCR5 expression has been found on microglia and macrophages in active demyelinating MS lesions [10,12]. This current study is the first

to analyze CCR5 expression in various lesion stages, including very early lesions, in a large panel of MS brains. We show that CCR5 is expressed by microglia/macrophages as well as by astrocytes. In addition, our data indicate that CCR5 expression is enhanced in various stages of MS lesions, revealing two consecutive patterns of CCR5 upregulation. Therefore, we hypothesize that the chemokine receptor CCR5 and its respective chemokines are important mediators in the pathogenesis of MS.

Materials & Methods

Human Brain Tissue

Human brain tissue was obtained by rapid autopsy according to standardized procedures under the management of the Netherlands Brain Bank, Amsterdam, The Netherlands. All patients or their next of kin had given written consent for autopsy and use of their brain tissue for research purposes. Brain samples were obtained from 6 patients without neurological complications and 19 MS patients. In MS cases, tissue samples were taken from lesions located in the brain. In most cases lesions were located in the subcortical and periventricular white matter. Tissue samples from control cases were taken from the subcortical white matter or corpus callosum. Sampling of MS tissue was done using a magnetic resonance imaging (MRI)-guided protocol [16]. For the purpose of this study, brain tissue samples were snap-frozen and stored in liquid nitrogen. The clinical diagnosis of MS was established by reviewing available clinical data and confirmed neuropathologically by an experienced neuropathologist (Dr. W. Kamphorst, Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands) using paraffin sections stained with hematoxylin/eosin (H&E) and Luxol Fast Blue.

Neuropathological Evaluation

Lesion stages in sampled tissue were classified using the staging system described previously by Van der Valk and De Groot [3], distinguishing preactive lesions, active (demyelinating) lesions, chronic active demyelinating lesions and chronic inactive lesions. For this, brain sections were immunohistochemically stained for myelin proteins, major histocompatibility complex class II (MHC-II), CD45, CD68 and GFAP. The different lesion stages present were determined on the basis of parameters such as the presence of inflammatory cells and myelin containing macrophages, enhanced expression of MHC-II, CD45 and CD68 antigen expression on microglia, the extent of astrogliosis and morphological criteria such as cellularity of the center and rim of the lesion.

Immunohistochemical Staining

Serial 5 μm thick cryostat section of MS brain tissue and normal control brain tissue

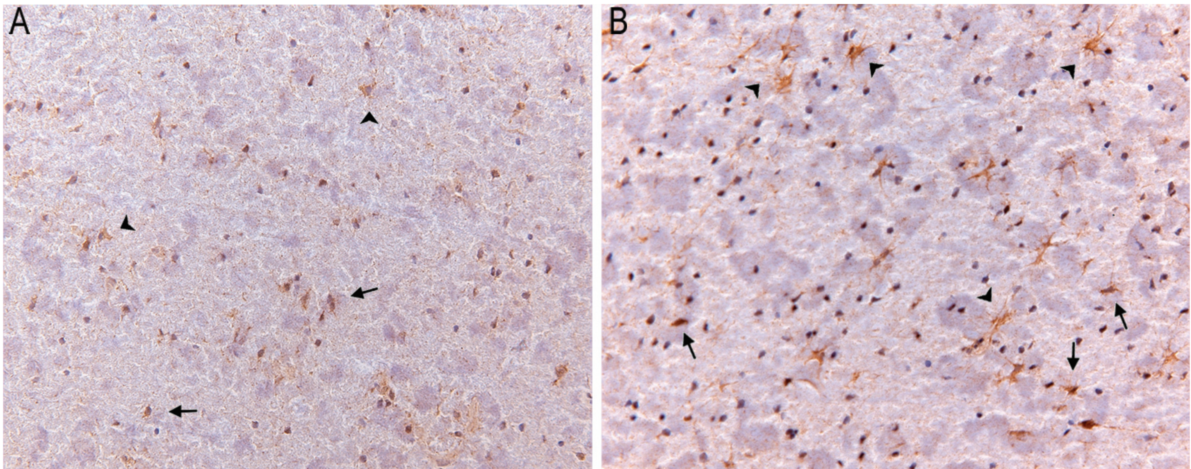

Figure 1. Microglia and astrocytes in normal (appearing) white matter express CCR5. Immunohistochemical staining for CCR5 in (A) normal control white matter and (B) normal appearing white matter from a MS patient. **A** Moderate staining of CCR5 on resting microglial cells (arrows) and astrocytes (arrowheads). **B** Moderate staining on a number of microglial cells (arrows) and moderate to strong staining on astrocytes (arrowheads). Nuclei were counterstained with hematoxilin. Magnifications: A-B ×200.

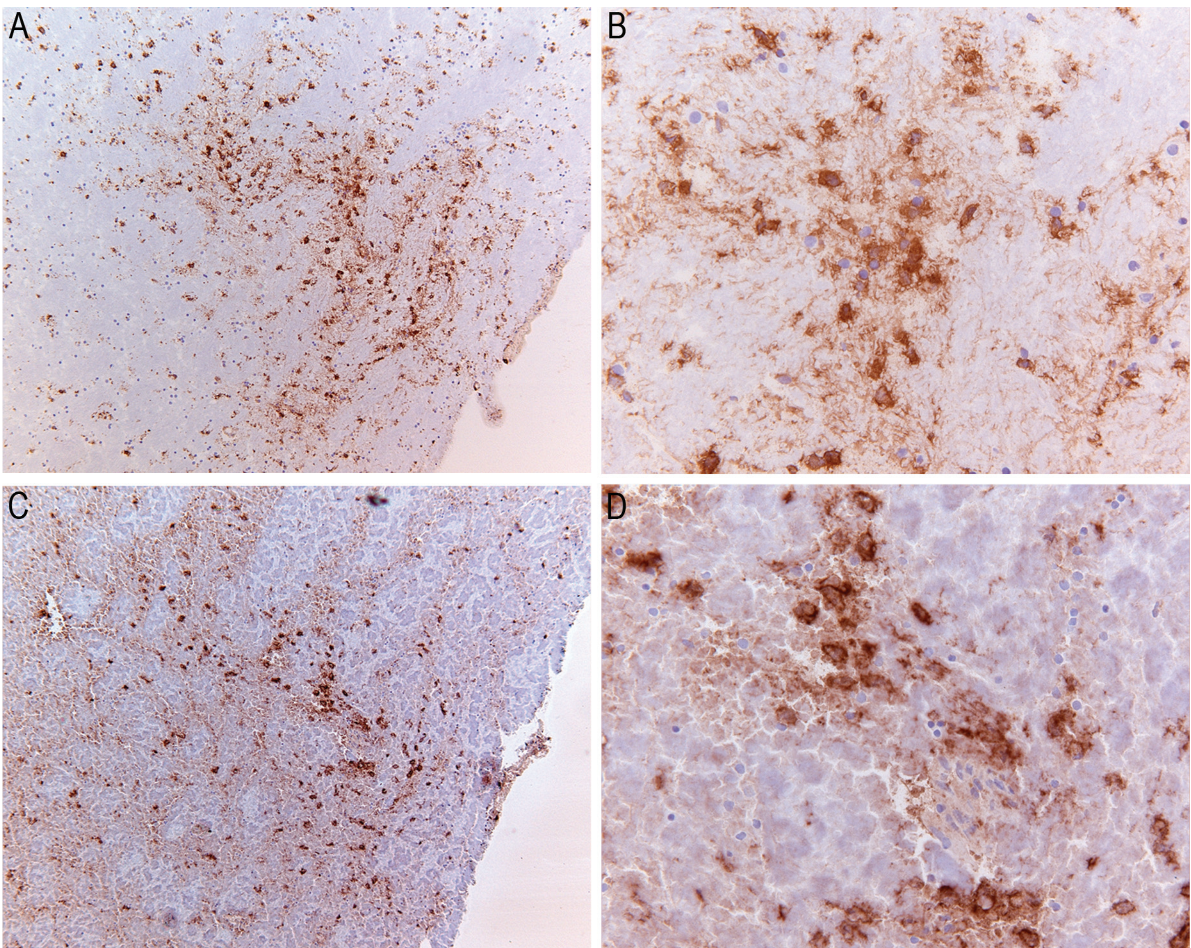

Figure 2. Microglia in preactive lesions express high levels of CCR5. Immunohistochemical staining on a preactive lesion. **A-B** Clusters of activated microglia in the NAWM of a MS patient are identified by their strong staining for MHC-II (LN3). **C-D** Activated microglia in the preactive lesion display a strong expression of CCR5. Nuclei were counterstained with hematoxilin. Magnifications: A and C ×50, B and D ×200.

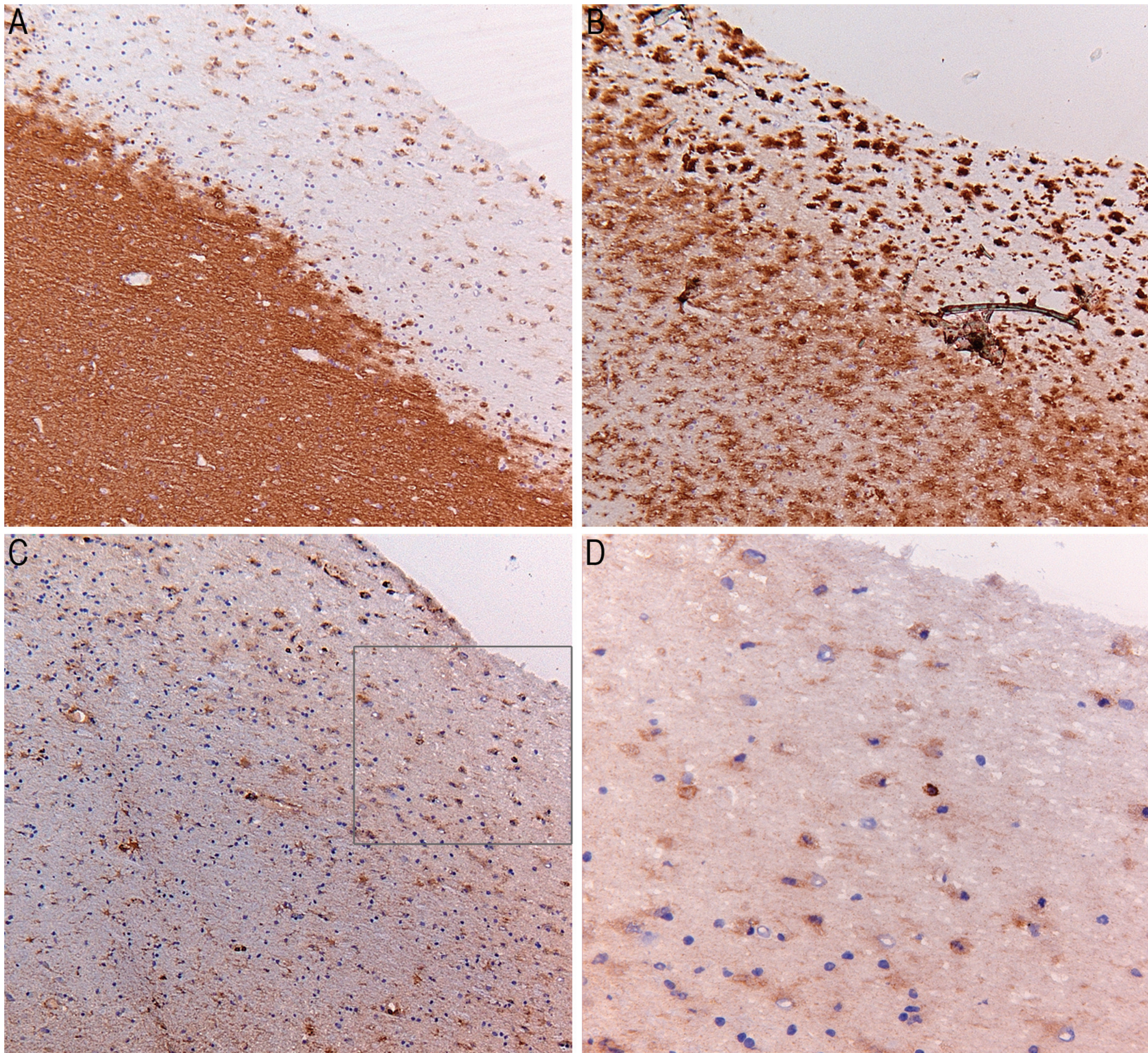

Figure 3. CCR5 is expressed in active demyelinating lesions. Immunohistochemical analysis of an active demylinating lesion. **A** The lesion is identified by loss of myelin as revealed by lack of PLP staining. Phagocytic (foamy) macrophages inside the lesion contain myelin degradation products. **B** MHC-II immunostaining reveals intense staining for MHC-II on foamy macrophages inside the lesion and activated microglia around the lesion. **C-D** CCR5 is strongly expressed by phagocytic macrophages inside the lesion and hypertrophic astrocytes in and around the lesion. The box in C depicts the area of the magnification shown in D. Nuclei were counterstained with hematoxilin. Magnifications: A-C ×50, D ×200.

were collected onto 0.1% poly-L-lysine (PLL)-coated Superfrost glass slides or SuperFrost Plus glass slides (Menzel Gläser, Braunschweig, Germay) and dried overnight at room temperature. The sections were fixed with acetone for 10 minutes or paraformaldehyde for 15 minutes. All washes were carried out with phosphatase buffered saline (PBS; pH 7.4) or PBS containing 0.1% saponine (PBS-S) for paraformaldehyde-fixed sections and antibodies were diluted in PBS containing 1% bovine serum albumine (PBS-BSA). Sections were immunostained at room temperature using the streptavidin-biotin complex (sABC) procedure. Briefly, the sections were preincubated with 2% normal

rabbit serum for 10 minutes, followed by incubation with primary antibodies (Table 1) for 1 hour at RT. The slides were then washed and incubated with biotinylated rabbit anti-mouse IgG $F(ab')_2$ (1:500; Dako) for 30 minutes and washed again. The slides were incubated with horseradish peroxidase (HRP)-linked sABC (sABC-HRP; Dako) for 1hour at RT and after washing peroxidase activity was demonstrated with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) in PBS containing 0.03 % H_2O_2 for 5 minutes. Finally, the sections were counterstained with hematoxyline. Stainings omitting the primary antibodies, isotype control stainings and stainings with an irrelevant antibody were included as negative controls.

Sections were evaluated by light microscopy by at least two independent observers. The extent of immunoreactivity for CCR5 was determined using an arbitrary scale of – to +++, reflecting the extent and intensity of immunoreactivity.

Double-immunofluorescence Staining

Serial 5 µm thick cryostat sections of MS brain tissue were collected onto SuperFrost Plus glass slides and dried overnight. Aceton-fixed sections were preincubated with PBS-5% BSA for 10 minutes. All antibodies were diluted in PBS-BSA and incubated for 1 hour, unless noted otherwise. Sections were first stained with a primary antibody against CCR5 (R&D, clone 45549) and subsequently with a biotinylated goat anti mouse IgG2b antibody (1:100; Dako) diluted in PBS-10% normal goat serum / 10% normal human serum for 30 minutes. Biotin was visualized by incubation with Alexa-488-conjugated streptavidin (1:750; Molecular Probes, Leiden, The Netherlands). After this, sections were stained with antibodies against HLA-DR and GFAP and subsequently incubated with an Alexa-594-conjugated goat anti mouse IgG2b or IgG1antibody (1:400; Molecular Probes) for 30 minutes. Finally, sections were mounted with glycerol/50 mM Tris buffer (pH 7.6) containing DAPI. Stainings omitting the primary antibodies were included as negative controls.

Table 1. Primary antibodies used for immunohistochemical staining

Antibody	Isotype	Specificity	Dilution	Source*
PLP	IgG2a	myelin proteolipid protein	1:500	Serotec
HLA-DR clone (LN3)	IgG2b	HLA-DR, DP, DQ	1:200	VUMC
GFAP	IgG1	glial fibrillary acidic protein	1:10-20	Monosan
CCR5 (clone 45531)	IgG2b	CCR5	1:50	R&D
CCR5 (clone 45549)	IgG2b	CCR5	1:50	R&D
CCR5 (MC-5)	IgG2a	CCR5	1:10	Mack

* Serotec, Oxford, UK; VUMC: Dr. J.H.M Hilgers, VU University Medical Center, Amsterdam, The Netherlands; Monosan, Uden, The Netherlands; R&D Systems, Minneapolis, MN/USA; Mack: Prof. M. Mack, Klinikum, University of Regensburg, Regensburg, Germany.

Fluorescence was visualized with a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Heidelberg, Germany), equipped with excitation laser lines of 488 nm and 568 nm, closely matching the fluophores used (Alexa-488 and -594). The confocal laser scanning microscope was fitted with a ×40/1.25 NA oil immersion objective. In each field of vision, image stacks of approximately 20 2D digital images (512 × 512 pixels) were acquired, depending on the effective thickness of the tissue sections. The bottom and top of the stack were identified interactively until no tissue could be detected anymore, after which image acquisition started with the lowest slice. Voxel size at the specimen level was 0.49 × 0.49 × 0.49 μm^3 (NB: resolution in z-direction is lower than in x–y direction). The dynamic range was 8 bits. Sequential sections were made to minimize possible leak through of the different fluorophores during imaging.

Results

Neuropathology of MS lesions

Brain tissue samples containing the different lesion types characterized according to the criteria of Van der Valk and De Groot [3] were selected and evaluated for CCR5 expression by immunohistochemistry. According to these criteria, four stages of MS lesions are classified: preactive lesions, active (demyelinating) lesions, chronic active demyelinating lesions and chronic inactive lesions. Preactive lesions consist of clusters of activated microglial cells within the white matter. These microglia display enhanced expression of MHC-II, CD45 and CD68. In this type of lesion no signs of demyelination and only occasionally perivascular infiltrates are present. Active demyelinating lesions, on the other hand, are characterized by demyelinated areas abundant in MHC-II expressing macrophages containing phagocytosed myelin. GFAP-positive reactive, hypertrophic astrocytes are distributed evenly throughout the demyelinated region. Chronic active demyelinating lesions are demyelinated hypocellular areas containing only a small number of macrophages with some residual myelin components, but are surrounded by a border of MHC-II expressing macrophages of which a variable number contain myelin degradation products. In these lesions astrocytes are predominantly located at the edge of the lesion center and within the hypercellular rim. Chronic inactive lesions are characterized by hypocellular demyelinated regions with low numbers of inflammatory cells, widespread astrogliosis and enlarged extracellular spaces. In addition to these lesions, normal appearing white matter (NAWM) from areas adjacent to lesions and normal white matter from control patients was evaluated for CCR5 expression. Cell types expressing CCR5 were distinguished and the extent and intensity of expression was determined using an arbitrary scale of – to +++. The results of these analyses are summarized in Table 2.

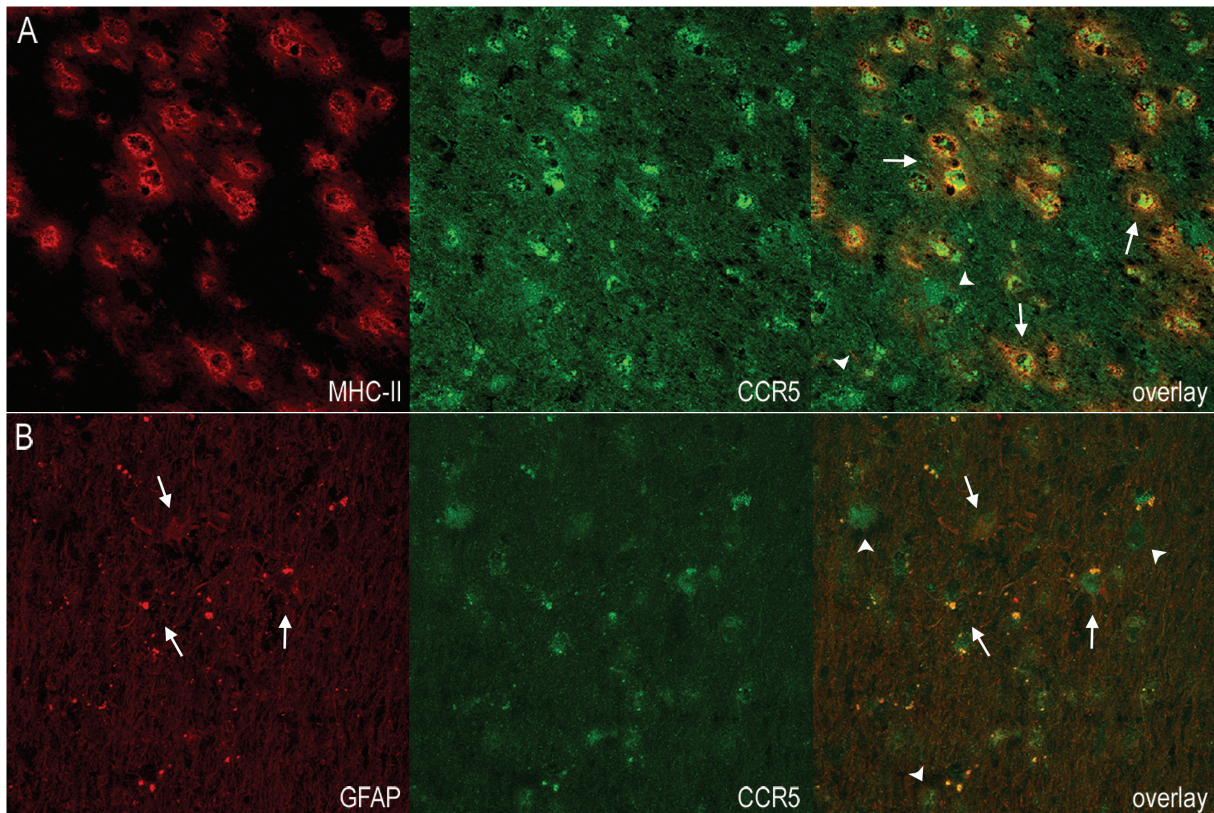

Figure 4. CCR5 in active MS lesions is expressed by macrophages and astrocytes. Confocal immunofluorscence analysis of an active demyelinating lesion using antibodies against CCR5 and the cell specific markers MHC-II and GFAP to distinguish macrophages and astrocytes, respectively. Left panels depict staining for (A) MHC-II or (B) GFAP, middle panels depict CCR5 staining and right panels are overlays of both stainings. **A** Doublestaining for MHC-II (red) and CCR5 (green) reveals that most phagocytic macrophages inside the lesion are immunoreactive for CCR5 (arrows), whereas there are also some cells present that are immunoreactive for CCR5 only (arrowheads). **B** Cell bodies of astrocytes present in the lesion, distinguished by red GFAP staining, are CCR5 immunoreactive (arrows). In addition, several cells only immunoreactive for CCR5 are present (arrowheads). Images for the different fluorophores were obtained sequentially to eliminate bleed-through.

CCR5 expression in control brain white matter and NAWM

In brain sections from normal control patients CCR5 expression is found mainly on resting microglia, distributed evenly throughout the white matter (Figure 1A). These cells are characterized by a small cell body with numerous, often ramified, cytoplasmic processes. We found that CCR5 expression on these cells is moderate (Table 2). In addition to this we detected also moderate CCR5 expression on astrocytes in control white matter. Similar to control brain tissue, a number of resting microglia in the NAWM of MS patients express CCR5 moderately (Figure 1B). Again we also found CCR5 expression on astrocytes. However, compared to control white matter, the expression of CCR5 on astrocytes is enhanced in MS NAWM (Table 2).

CCR5 expression in preactive MS lesions

In the NAWM of MS patients abnormal clusters of microglia without signs of

demyelination can be found. These abnormalities are termed preactive lesions and could represent very early stages of MS lesions. In comparison to resident microglial cells in the NAWM or control white matter, microglia in preactive lesions display an activated phenotype, characterized by macrophage-like morphology and high expression of MHC-II (Figure 2A and B). We found that CCR5 staining is enhanced in these preactive lesions (Figure 2C and D). The cells expressing high levels of CCR5 in these lesions display a morphology of activated microglia (Table 2, Figure 2B and D). Therefore we conclude that CCR5 expression is increased in activated microglia of preactive lesions.

CCR5 expression in active demyelinating MS lesions

Active demyelinating MS lesions are hallmarked by the presence of foamy macrophages that contain phagocytosed myelin debris and display an activated phenotype, illustrated by their high expression of MHC-II (Figure 3A and B). In general, we detected more CCR5 staining in active lesions than in the surrounding

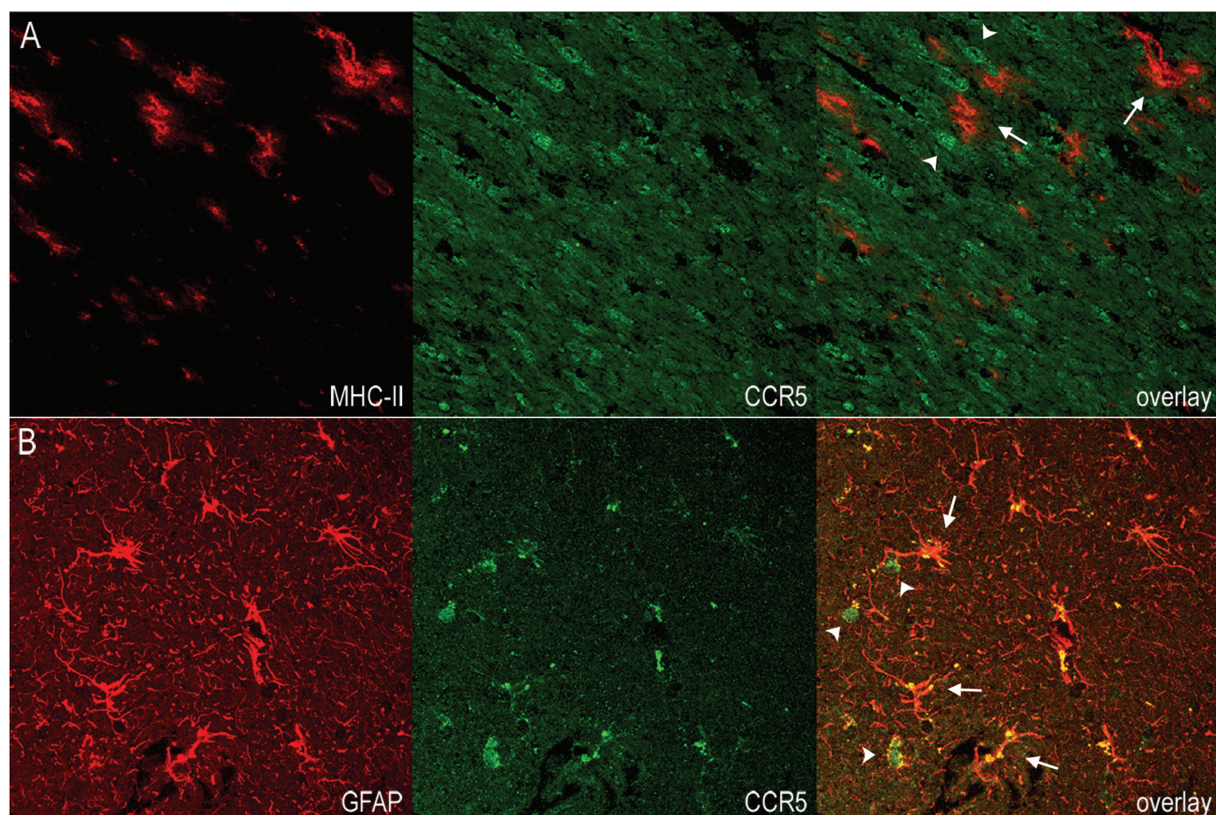

Figure 5. Astrocytes at the border of active demyelinating lesions express CCR5. Confocal immunofluorscence analysis of CCR5 expressing cells around an active demyelinating lesion. **A** Double staining for MHC-II (red) and CCR5 (green). Only few MHC-II positive macrophages / microglia around the lesion are immunoreactive for CCR5 (arrows), whereas there are several other CCR5 expressing cells present (arrowheads). **B** Double staining for GFAP (red) and CCR5 (green). Astroyctes around the lesion display immunoreactivity for both GFAP and CCR5 (arrows). In addition, some single positive cells with macrophage morphology are present (arrowheads). Images for the different fluorophores were obtained sequentially to eliminate bleed-through.

Table 2. Summary of CCR5 immunohistochemical staining patterns in control and MS brain tissue

	Control white matter	NAWM	Preactive lesions	Active lesions	Chronic active lesions	Chronic inactive lesions
Microglia	+*	- / +*	++	n.p.	n.p.	n.p.
Foamy macrophages	n.p.	n.p.	n.p.	++/+++	+/++	n.p.
Astrocytes	+	++	++	++/+++	+++	+++
Oligodendrocytes	-	-	-	-	-	-
Endothelium	+	+/++	+/++	+	- / +	+

NAWM: normal-appearing white matter; - no staining; + moderate staining; ++ strong staining; +++ intense staining; n.p. not present; * some cells are positive

NAWM (Figure 3C). Inside active lesions CCR5 expression was largely found on cells with foamy macrophage morphology (Figure 3D). In addition, we could also detect CCR5 expressing cells inside and around the lesion with astrocyte morphology.

To further determine the origin of the CCR5 expressing cells present in active lesions we performed fluorescent double staining with MHC-II and GFAP antibodies. Double staining for MHC-II, as a marker for macrophages and microglia, and CCR5 confirmed that the CCR5 expressing cells inside active demyelinating lesions consist mainly of macrophages (Figure 4A). Almost no CCR5 negative macrophages were observed, whereas some single CCR5 positive cells were present, which most likely represent astrocytes. Likewise, double staining with GFAP, a marker for astrocytes, revealed that astrocytes inside the lesion also express CCR5 (Figure 4B). We observed that GFAP expression in astrocytes inside active lesions is localized mainly in the cell extensions, whereas CCR5 expression is confined to the astrocyte cell body. In addition, hypertrophic astrocytes around the lesion clearly express CCR5, whereas only a number of macrophages/microglia in these areas are immunoreactive for CCR5 expression (Figure 5A and B).

CCR5 expression in chronic active lesions

Whereas large numbers of activated macrophages are present in active demyelinating lesions, only few macrophages can be found in the center of chronic active MS lesions. In contrast, astrocytes populate the center of these lesions. These astrocytes express high levels of CCR5, staining of which resembles GFAP staining (Figure 6A and B). In addition, the rim of chronic active lesions consists largely of activated macrophages with elevated MHC-II expression (Figure 6C). We could detect only moderate to strong CCR5 expression on these macrophages (Table 2). In contrast, the astrocytes present at the lesion rim did show strong immuoreactivity for CCR5 (Figure 6D).

CCR5 expression in chronic inactive lesions

Chronic inactive lesions consist of large demyelinated areas, devoid of macrophages, but filled with extensive astrogliosis, visible as a network of astrocyte processes with large gaps. Like lesion morphology, CCR5 expression in chronic inactive lesions resembles that of the center of chronic active lesions. Extensive expression of CCR5 can be found on astrocytes (Figure 7A). Not only the cell body of these astrocytes is immunoreactive for CCR5, but CCR5 is also expressed on astrocyte processes, closely resembling GFAP staining (Figure 7B).

Discussion

In the current study we have examined the expression of CCR5 in various stages of MS lesions and in control white matter. On basis of morphology and double staining with cell specific markers we identified the cell types expressing CCR5 during lesion development. Taken together, our findings indicate that CCR5 expression is mainly confined to microglia/macrophages and astrocytes, and that this expression is upregulated at various stages during MS lesion development.

Expression of CCR5 and other chemokine receptors on microglia in control and MS brain has been reported earlier [10,12]. These reports describe enhanced expression of CCR5 on activated microglia near the edges of active demyelinating lesions and on phagocytic macrophages inside active and chronic active MS lesions, corroborating our findings. In addition, enhanced expression of CCR5 on reactive microglia in Alzheimer's disease brain has been shown previously [15], emphasizing the involvement of this receptor in neurodegenerative diseases. However, few reports describe CCR5 expression on astrocytes. CCR5 expressing reactive astrocytes have been reported only in a number of MS cases and CCR5 expression was detected only on astrocytes processes around blood vessels [12]. Therefore, this is the first report showing extensive expression of CCR5 on astrocytes in control and MS brain tissue. Furthermore, this is first study describing CCR5 expression in various MS lesion stages, from very early lesions to chronic inactive lesions, enabling elucidation of chemokine receptor expression during the development of MS lesions.

Examining CCR5 expression in various lesion stages revealed two consecutive waves of CCR5 upregulation: first, microglia/macrophages start to upregulate CCR5 expression and at a later stage CCR5 expression on astrocytes is enhanced. The observed pattern of cell migration also follows this scheme: preactive lesions consist mainly of clustered (activated) microglia and the major cell type in early active lesions is the phagocytic macrophage, whereas at later stages, in chronic active and inactive lesions, astrocytes populate the lesion. It is known that microglia are the first cell type to respond to immunological stimuli in the brain, and that astrocytes respond at a later stage [17]. Therefore, it seems that there is a pattern of general activation of microglia

and astrocytes during MS lesion formation, of which enhanced CCR5 expression is one of the features. This might implicate that CCR5 expression is just a random feature of glial activation during disease progression and not functional. However, it seems that recruitment of microglia and, at a later stage, astrocytes is an important aspect in lesion formation and development, regardless of the question whether this phenomenon is harmful (initiating and/or exacerbating demyelination), or beneficial (removing debris and limiting lesion progression). In any case, aberrant expression of chemokine receptors such as CCR5 most likely underlies the migration of glial cells to lesion sites and therefore plays an important role in MS pathogenesis.

It has been proposed that microglial activation is one of the first events in MS lesion formation [2,3,18]. In this study we show that microglial activation in MS brain tissue is accompanied by upregulation of CCR5, as observed in preactive lesions. As discussed above, this elevated expression of CCR5 might actually trigger clustering of these

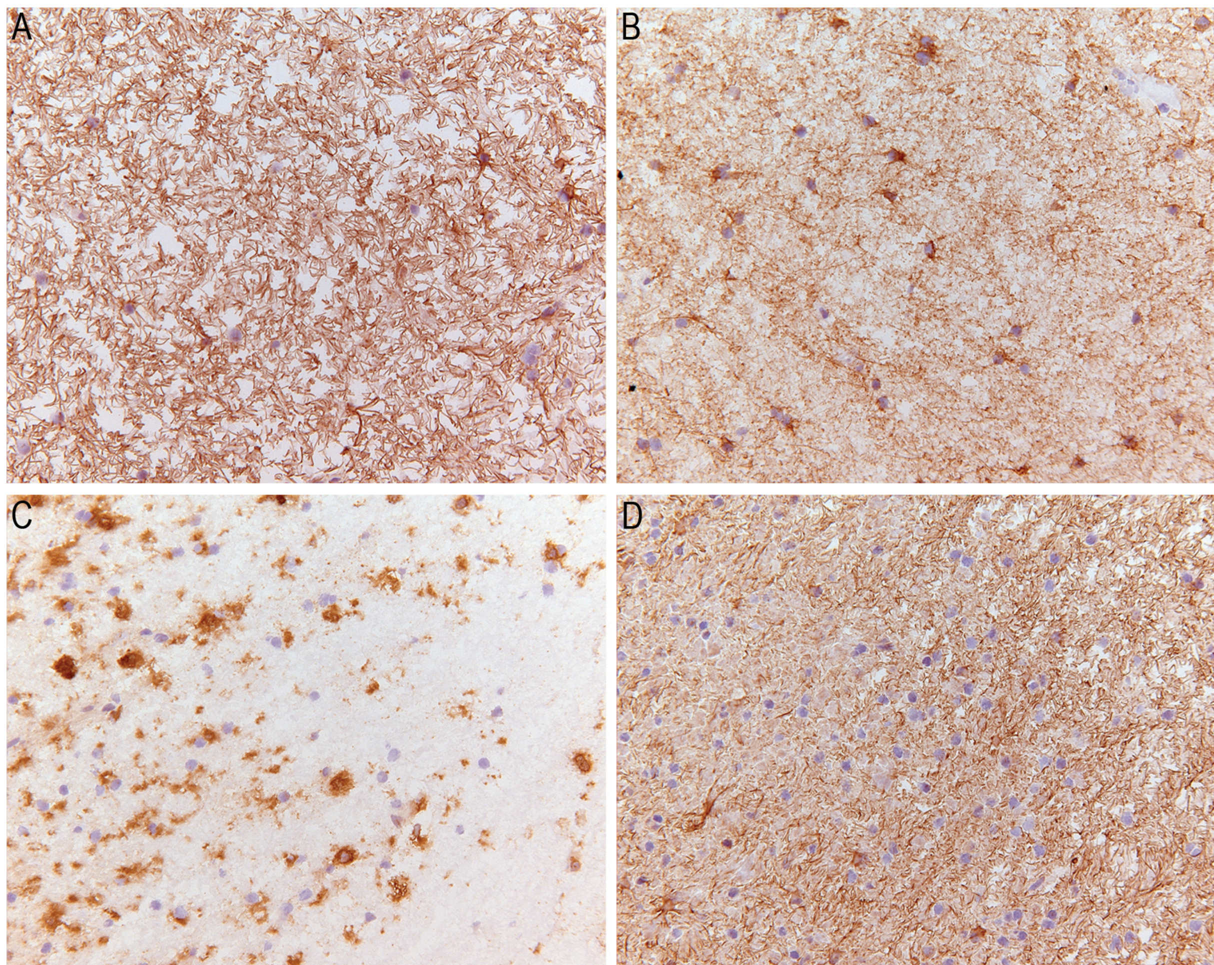

Figure 6. Astrocytes at the center and rim of chronic active lesion express CCR5. Immunohistochemical staining of a chronic active lesion. **A-B** CCR5 staining (A) inside in the lesion center closely resembles staining of GFAP (B) on astrocytes. **C** Active macrophages at the hypercellular rim of the lesion are strongly immunoreactive for MHC-II. **D** At the lesion rim CCR5 is mainly expressed by hypertrophic astrocytes. Nuclei were counterstained with hematoxilin. Magnifications: A-B ×200, C-D ×100.

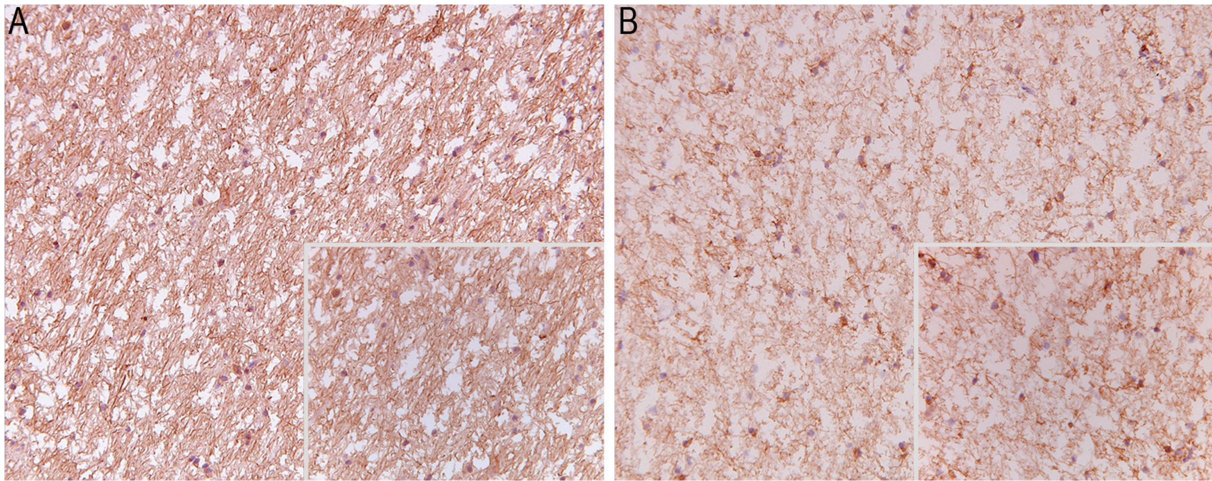

Figure 7. Astrocytes in chronic inactive lesions express CCR5. Immunohistochemical staining of a chronic inactive lesion. **A-B** Strong immunoreactivity for CCR5 (A) is found on astrocyte processes filling the lesion, visualized by GFAP staining (B). Nuclei were counterstained with hematoxilin. Magnifications: A-B ×100, inserts ×200.

cells to sites were preactive lesions are formed. Subsequently, it is thought that during the development of active demyelinating lesions microglia transform into foamy macrophages upon phagocytosis of myelin [1,19]. Indeed, microglia present in preactive lesions acquire the morphology of amoeboid cells, resembling foamy macrophages. In addition, our immunohistochemical data further support this hypothesis, considering the observation that foamy macrophages inside active lesions display the same level of CCR5 expression as activated microglia in preactive lesions. In late lesions we observe less CCR5 expressing macrophages, which suggests that either CCR5 expression on macrophages is lost, or these cells are lost themselves. The first theory is supported by the lower intensity of CCR5 immunoreactivity on macrophages in chronic active lesions. Alternatively, a possible explanation for the loss of CCR5 expressing macrophages during late lesion development is that microglia-derived macrophages, present in early active lesions, are replaced by infiltrating blood-derived monocytes or macrophages, as has been described by Li *et al*. [19].

At later lesion stages, we find astrocytes with high expression of CCR5 present at lesion sites. Similar to CCR5 expression on microglia, we propose that CCR5 expression on astrocytes is upregulated during cell activation. We find considerable levels of CCR5 expression on astrocytes in NAWM and in early lesion stages. However, this expression is dramatically enhanced on astrocytes around active demyelinating lesions and inside chronic active and inactive lesion. These astrocytes have a hypertrophic morphology, which is characteristic of their activated state.

Although the current study is not a quantative analysis of CCR5 expression, comparison of immunoreactivity for microglia/macrophage markers with that for CCR5 reveals that during MS lesion development not all microglia/macrophages present in white matter and in lesions express CCR5. For example, it seems that microglial

CCR5 expression is less in NAWM than in control brain tissue. A possible explanation for this observation could be that in MS brain tissue CCR5 expressing microglia have migrated to lesion sites (and subsequently transformed into macrophages), resulting in a lower amount of CCR5 positive microglia in not affected white matter. However, it also appears that not all activated microglia in preactive lesions are immunreactive for CCR5. Therefore, it is very likely that other chemokine – chemokine receptor systems are also activated and involved in disease development, possibly in other lesion/disease stages than the CCR5 system, or in overlapping stages. These could include the chemokines CCL2, -8 and –7 (MCP-1, -2 and –3, respectively), and CXCL10 (IP-10) and their responding receptors [20-23].

Obviously, an important question remaining is which source produces the chemokines to which CCR5 expressing cells in MS tissue migrate. In this matter there are two major suspects: infiltrating inflammatory cells or astrocytes. In the first case immune cells from the blood, for example autoreactive T lymphocytes or monocytes/macrophages, infiltrate into the brain parenchyma where they produce proinflammatory cytokines and chemokines, initiating local inflammation and demyelination. However, this scenario is not very likely, because in early lesions and in preactive lesions only few perivascular inflammatory cells can be found ([3] and own observations). In addition, in order for these cells to migrate across the blood-brain-barrier into the brain parenchyma a lymphocyte-attracting milieu should already be present and thus be produced by resident cells. In contrast, astrocytes are a more likely candidate. Astrocytes have been shown to produce either *in vitro* or *in vivo* several chemokines, including CCL5 and CCL4 [24,25]. Furthermore, reactive astrocytes in active lesion express enhanced levels of CCL3 and CCL5, of which the latter seems to be expressed almost exclusively by astrocytes [13,21]. In addition, microglia and macrophages in MS lesions have also been shown to express CCL3 and CCL4 [13,21]. It would seem contradictory that astrocytes and microglia simultaneously produce chemokines and express their responding receptor. However, this phenomenon has been observed before, which suggests that, in addition to paracrine signaling, production of chemokines by astrocytes and microglia provides autocrine loops leading to activation of microglia and reactive astrogliosis [23].

In conclusion, although it has been suggested before that CCR5 might have a role in lesion progression [10], we conclude from the data obtained in the current study that CCR5 could also play a role in early stages of MS lesion formation. Furthermore, our data indicate that not only microglia, but also astrocytes might play an important role in the development of MS lesions.

Acknowledgements

We would like to thank the Netherlands Brain Bank for supplying the human brain tissue, Prof. Dr. M. Mack for providing the MC-5 antibody, and Dr. J.A. Beliën and

Drs. T. Hazes for their technical assistance with fluorescent confocal microscopy. This research was financially supported by the Dutch MS Research Foundation (grants MS 00-407 and MS 04-543).

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