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Imaging correlates of leukocyte accumulation and CXCR4/ CXCR12 in multiple sclerosis

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Abstract

OBJECTIVE—To compare leukocyte accumulation and expression of the chemokine receptor/ligand pair, CXCR4/CXCL12, in MRI-defined regions of interest (ROIs) from chronic multiple sclerosis (MS) brains. We studied the following ROIs: NAWM (normal appearing white matter); T₂-only (regions abnormal only on T₂-WI); T₂/T₁/MTR (regions abnormal on T₂-weighted, T₁-weighted images (-WI) and magnetization transfer ratio (MTR)).

DESIGN—MRI-pathology correlations were performed on five secondary progressive MS (SPMS) cases. Based on imaging characteristics, thirty ROIs were excised. Using immunohistochemistry, we evaluated myelin status, leukocyte accumulation and CXCR4/CXCL12 expression in the MS ROIs and white matter regions from five non-neurological control cases.

RESULTS—Eight of ten T₂/T₁/MTR regions were chronic-active or chronic-inactive demyelinated lesions, whereas only two of ten T₂-only regions were demyelinated and characterized as active or chronic active lesions. Equivalent numbers of CD68⁺ leukocytes (the predominant cell type) were present in myelinated T₂-only regions as compared to NAWM. Parenchymal T-cells were significantly increased in T₂/T₁/MTR ROIs as compared to T₂-only regions and NAWM. Expression of CXCR4 and phospho-CXCR4 was found on reactive microglia and macrophages in T₂-only and T₂/T₁/MTR lesions. CXCL12 immunoreactivity was detected in astrocytes, astrocytic processes and vascular elements in inflamed MS lesions.

CONCLUSIONS—Inflammatory leukocyte accumulation was not increased in myelinated MS ROIs with abnormal T₂ signal as compared with NAWM. Robust expression of CXCR4/CXCL12 on inflammatory elements in MS lesions highlights a role of this chemokine/receptor pair in CNS inflammation.

Keywords

multiple sclerosis; MRI; inflammation; CXCR4; CXCL12; microglia

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INTRODUCTION

Conventional magnetic resonance imaging (cMRI) is frequently used to confirm clinical diagnosis of MS, monitor disease evolution, and assess response to treatment. cMRI evaluations show several advantages over clinical assessments, including their more objective nature and increased sensitivity to MS-related changes^{1, 2}. However, there are only weak or modest correlations between the clinical manifestations of the disease and cMRI measures of the disease burden especially in progressive MS³⁻⁵. T₂-weighted sequences depict a wide spectrum of pathologic changes in brain tissue and therefore lack specificity. Moreover, T₂-weighted images (T₂-WI) do not show tissue damage occurring in normal-appearing white and grey matter which is known to be extensively injured in the late stages of MS⁶. More pathologically specific brain imaging methods include MR spectroscopy (MRS), T₁-WI, diffusion tensor imaging (DTI) and magnetization transfer ratio (MTR). Hypointense lesions on T₁-WI ("black holes") correspond to areas of more significant demyelination, axonal loss and axonal pathology in MS tissue⁷⁻¹⁰. The pathological substrates of MR-related changes are currently of high interest, in the effort to understand the pathogenesis of disability in MS patients. Our recent MRI-pathological study of MS brain tissue showed that areas abnormal on T₂-, T₁-WI and MTR often correspond to demyelinated lesions with significantly fewer axons but increased axonal swelling and loss of axonal Na⁺/K⁺ ATPase^{7, 11}. Areas abnormal on T₂-WI but normal on MTR and T₁-WI were associated with less severe tissue damage, less axonal loss and much less axonal swelling and the presence of axonal Na⁺/K⁺ ATPase. The majority of T₂-only regions featured local microglial activation and were characterized as active or chronic active lesions. T₂/T₁/MTR abnormal regions, which had the most axonal degeneration, were principally chronic inactive lesions. The current study was undertaken to extend these findings, first by detailed analysis of inflammatory elements in the lesions and second by characterizing the CXCR4/CXCL12 system.

Focal areas of myelin destruction observed in MS often occur on a background of inflammation dominated by T-lymphocytes, hematogenous macrophages, microglial activation, and the presence of few B-lymphocytes and plasma cells¹²⁻¹⁴. *In vitro* studies have shown that microglial activation leads to up-regulation of CXCR4^{15, 16}. CXCR4 and its ligand, CXCL12, are also associated with migration, proliferation, survival and effector functions and can be present on astrocytes, microglia, oligodendroglia and subsets of NG2⁺ glia, representing oligodendrocyte progenitor cells¹⁷. However, little is known about what roles the CXCR4 / CXCL12 system plays during the inflammatory reaction in MS. CXCL12 is elevated in the cerebrospinal fluid (CSF) from patients with MS and other inflammatory neurological disorders^{18, 19}. In active MS lesions, CXCL12 is up-regulated on astrocytes throughout lesion areas and on some monocytes/macrophages within perivascular cuffs^{19, 20}. It has been recently shown that alteration of the pattern of CXCL12 expression at the blood-brain barrier (BBB) including CXCL12 redistribution toward vessel lumina was associated with CXCR4 activation (indicated by the presence of phosphorylated epitopes) on infiltrating leukocytes and demyelination and inflammation in MS tissue sections²¹.

In the current study, we evaluated myelin status, inflammatory leukocyte accumulation and expression of CXCR4 and CXCL12 in MS brain regions which were abnormal on T₂-, T₁-WI and MTR as compared to regions abnormal only on T₂-WI but normal on T₁-WI and MTR images and NAWM. Postmortem material was acquired according to an established protocol which has supported our previous reports. MS brains were imaged *in situ* before autopsy and image-to-tissue co-registration was applied to enable MRI-pathological correlations.

MATERIALS AND METHODS

Tissue and tissue acquisition

Collection and use of human tissue was approved by Cleveland Clinic Institutional Review Board. MRI-pathological correlations were performed on brain tissue from five patients with SPMS as previously described¹¹. The demographic and clinical details on these cases are shown in Table 1. Control tissues were obtained from autopsies of patients without neurological disease performed at Cleveland Clinic (Table 2). These tissues were not subjected to postmortem MRI, but were otherwise processed identically to MS tissue.

For the current study, a total of thirty regions of interest (ROIs) were selected and isolated from postmortem MRIs of five MS patients. These ROIs included i) areas abnormal on T₂-, T₁-WI and MTR images (T₂/T₁/MTR), ii) areas abnormal on T₂-WI but normal on T₁-WI and MTR (T₂-only), and iii) NAWM which was normal-appearing on all images. These patients represent a subset of those previously reported¹¹ but tissue ROIs reported here were distinct from those characterized previously.

Samples of subcortical and periventricular white matter were dissected from each of five non-neurological control cases (total 10 tissue blocks). ROIs from MS brains (MRI-defined and mapped onto co-registered tissue slice images) and non-neurological control brains were subsequently sectioned and immunostained to evaluate the myelin status, inflammatory activity and CXCR4/CXCL12 immunoreactivity.

MRI: acquisition and analysis

MRI was performed on 1.5-Tesla MR scanner (Siemens, Erlangen, Germany), as described previously¹¹. Maps of MS ROIs were generated for each tissue slice from the corresponding MRI planes. A 10mm grid was overlaid on the image planes to provide a frame of reference. The outlines of ROIs that corresponded to each region type (T₂/T₁/MTR and T₂-only) and NAWM were transferred to the region maps (Figure 1). These maps were then used to guide tissue sampling for the histological analysis.

Immunohistochemistry

Using MRI region maps, ROIs were identified and isolated from MS brains and non-neurological control tissue. Tissue blocks were cryoprotected overnight in 20% glycerol, embedded in 30% sucrose, and sectioned 30µm thick on a freezing-sliding microtome. These sections were used for immunoperoxidase histochemistry and double-label immunofluorescence.

Sections were pretreated as described previously²², incubated with primary antibodies for 5 days at 4°C, then incubated with appropriate secondary antibodies and immunostained by the avidin-biotin complex (Vector Laboratories, Burlingame, CA). Diaminobenzidine (Sigma-Aldrich, St Louis, MO) was used as chromogen. Sections for confocal microscopy were incubated with two primary antibodies, and non-cross-reacting secondary antibodies conjugated to Alexa Fluor® 488 and Alexa Fluor® 594 (Invitrogen, Carlsbad, CA). The specificity of immunohistochemical procedures was tested using antibodies with well-characterized and different immunoreactivity.

Antibodies—Primary antibodies used for immunostaining in the present study are summarized in Table 3.

Confocal microscopy

Double-label MS brain sections were imaged and analyzed on SP5 confocal microscope (Leica Microsystems, Germany; Leica Application Suite, version 1.6.3). The entire thickness of the sections (30µm) was scanned. Fluorescence was collected in the green, red and autofluorescence channels. Images presented in this study consisted of 30 to 60 optical serial sections combined to form a “through-focus” image.

Quantitative analysis

Multiple micrographs of each ROI, including NAWM, and non-neurological control brains stained with anti-CD68, -CXCR4, -CD3 and -CD20 antibodies were digitized using 20x objective (Leica DM 4000B, Germany; QCapture Pro, version 5.1.1.14), transferred to a workstation and manually quantified (Adobe Photoshop CS2), applying corrections for image size and resolution. These microphotographs were taken from nine nonoverlapping microscopical fields of each MS ROI (five micrographs were taken from the middle parts and four at the borders of ROIs) and five nonoverlapping fields from the white matter of each non-neurological control section. Cell densities were calculated as previously described²³ as a ratio of cell number to area of interest in mm². Quantitative analysis was performed in blinded fashion by two independent investigators (N.M.M. and A.M.R.).

Statistical analysis

Mix model analysis in SAS software package was applied to determine statistical significance of the data and compensate for multiple comparisons between varied regions from individual MS brains; $p < 0.05$ was considered statistically significant. Results are given as mean $\pm$ SD. Pearson correlation coefficients (r) were calculated to determine the relation between CD68 and CXCR4⁺ cells in MS ROIs.

RESULTS

T₂/T₁/MTR areas primarily represent demyelinating MS lesions, whereas T₂-only areas are variably demyelinated

We found that 80% of T₂/T₁/MTR areas (8/10) were demyelinated while only 20% of T₂-only areas (2/10) were demyelinated (Table 4). Areas of NAWM and non-neurological control white matter areas were always myelinated. Some T₂/T₁/MTR and T₂-only areas retaining myelin showed characteristics of intense inflammation on consecutive MHCII and CD68 stained sections and were designated myelinated/inflamed (Tables 5-6).

Mononuclear phagocyte accumulation at the borders of chronically inflamed T₂-only and T₂/T₁/MTR ROIs

Initial characterization of inflammation was performed by establishing number and distribution of mononuclear phagocytes, the most consistent feature of the MS ROIs. Demyelinated T₂/T₁/MTR regions exhibited distinctive borders of mononuclear phagocytes (MHCII⁺, CD68⁺), indicating a long-lasting pathological process. On the other hand, T₂-only regions were histopathologically diverse, ranging from highly inflamed lesions to myelinated areas with normal density of MHCII⁺ and CD68⁺ cells.

Based on the distribution of MHCII and CD68 immunoreactivity, several patterns of inflammatory activity were observed in T₂/T₁/MTR as compared with T₂-only areas (Figure 2). Some ROIs comprised MHCII⁺ and CD68⁺ cells which were increased at the lesion border and also showed normal or decreased density in the core (Figure 2 A, B). Morphologically the majority of the cells at the lesion borders resembled activated microglia and macrophages showing enlarged round cell bodies and short processes. This pattern was observed in four of

eight T₂/T₁/MTR areas and one of two T₂-only demyelinating lesions (Table 4). In this sample, mononuclear phagocyte distribution and number in the T₂-only chronic active lesion was similar to that in the four chronic active lesions which corresponded to T₂/T₁/MTR regions. Other ROIs (3 of 8 T₂/T₁/MTR lesions) were associated with hypocellular, “burnt-out” areas with significant reduction in MHCII⁺ and CD68⁺ cell density (Figure 2 C). This pattern included only T₂/T₁/MTR areas which were seen as demyelinated areas sharply demarcated from the adjacent myelinated white matter. A subset of the MRI-abnormal ROIs which retained myelin contained activated inflammatory cells uniformly distributed throughout the areas of interest (Figure 2 D, E). Other ROIs showed evenly distributed MHCII⁺ and CD68⁺ cells which revealed features of process-bearing resting microglia. These were myelinated areas which mostly consisted of NAWM and T₂-only areas (Figure 2 F). CD68⁺ cells were quantitated in all ROIs and confirmed these descriptive categories. The density of CD68⁺ microglia and macrophages was significantly higher at the border of T₂/T₁/MTR areas ($p=0.002$) and T₂-only areas ($p=0.01$) than in NAWM from MS and non-neurological control brains (Table 5). On the other hand, numbers of CD68⁺ leukocytes did not vary significantly in the interior portions of T₂/T₁/MTR, T₂-only regions and NAWM. Numbers of mononuclear phagocytes did not vary significantly in myelinated T₂-only regions ($n=8$) as compared to NAWM ($n=10$) ($p=0.06$, Table 5). We were not able to compare quantitatively CD68⁺ cell number in demyelinated T₂/T₁/MTR and T₂-only regions as the number of T₂-only demyelinated lesions was too low (Table 5).

CD3⁺ and CD20⁺ cells are increased in both parenchymal and perivascular compartments in T₂/T₁/MTR areas

CD3 and CD20 immunoreactivity was studied in ROIs of MS and control tissue. We found that CD3⁺ lymphocytes were preferentially located in perivascular areas of the MS ROIs (Figure 3 A). The density of CD3⁺ cells in perivascular cuffs did not vary significantly among T₂/T₁/MTR areas (2065.3 ± 899.0 cells/mm²), T₂-only (1906.1 ± 935.7 cells/mm²) and NAWM (1450.7 ± 880.6 cells/mm²). Additionally, the number of CD3⁺ cuffs was not significantly different among T₂/T₁/MTR (27.2 ± 15.7 cuffs/mm²), T₂-only (23.1 ± 14.1 cuffs/mm²) and NAWM (13.3 ± 10.8 CD3⁺ cuffs/mm²). Non-neurological control brains only showed infrequent scattered cuffs. Parenchymal CD3⁺ cells were increased ($p<0.05$) in T₂/T₁/MTR areas (30.1 ± 14.5 cells/mm²) in comparison with T₂-only (16.3 ± 5.6 cells/mm²) areas and NAWM (12.3 ± 4.5 cells/mm²). CD20⁺ cells were distributed in perivascular spaces in MS tissue (Figure 3 B). CD20⁺ cuffs were present in three of five MS brains. We did not find any difference in the CD20⁺ cuff presence in T₂-only regions as compared with T₂/T₁/MTR. Where present in cuffs, CD20 cells comprised 11-16% of lymphocytes, as judged by dual-label immunostaining with anti CD3 and CD20 antibodies.

CXCR4 is highly expressed on reactive MHCII⁺ microglia in MS lesions

We defined the distribution of the chemokine/receptor pair, CXCR4/CXCL12, in these brain regions. The CXCR4/CXCL12 system was chosen due to its remarkably broad biology and potential for expression on hematopoietic cells (CD3, CD20, CD68) as well as glia including microglia, astrocytes and NG2⁺ glia as well as vascular elements.

CXCR4⁺ cell density was significantly increased at the borders of T₂/T₁/MTR ($p=0.003$) and T₂-only areas ($p=0.003$) compared to the central portions of these areas (Table 6). CXCR4 cell density was also significantly higher at the borders of MRI-abnormal ROIs ($p \leq 0.01$) as compared to NAWM. Non-neurological control tissue contained only individual randomly distributed CXCR4⁺ cells. The distribution and morphology of CXCR4⁺ cells in MRI-abnormal ROIs resembled those of CD68⁺ and MHCII⁺ microglia and macrophages (Figure 4 A,B). There was a strong correlation between CXCR4⁺ and CD68⁺ cells at the borders of MRI-abnormal ROIs ($r=0.91$ in T₂/T₁/MTR areas and $r=0.88$ in T₂-only, $p<0.05$) but modest

in the central portions ($r=0.30$ in T_2/T_1 /MTR areas and $r=0.52$ in T_2 -only, $p>0.05$). The decreased correlation in the interior was due to the presence of $CD68^+$ cells that were CXCR4-negative. Dual-label immunohistochemistry and confocal microscopy confirmed that majority of $CD68^+$ and $MHCII^+$ microglia and macrophages expressed CXCR4 molecules at the borders of ROIs (Figure 4 C,F). $MHCII^+/CXCR4^+$ cells in these areas showed an appearance typical for activated microglial cells, with enlarged rounded cell bodies and short, thick processes. Although CXCR4 was mainly expressed on activated microglial cells in MS lesions, isolated cortical neurons and astrocytes also expressed this chemokine receptor (data not shown).

Phospho-CXCR4⁺ cells are increased in number in inflamed MS lesions and characterize a functionally active pool of microglia and macrophages

Phospho-CXCR4 (pS³³⁹-) antibodies recognize an epitope including phospho-serine 339 on the intracellular portion of CXCR4 which is phosphorylated in response to ligand and therefore indicates the presence of recently ligated CXCR4 receptors²⁴. Phospho-CXCR4⁺ cells represented a subset of CXCR4⁺ cells mainly at the borders of chronic active lesions in MS ROIs (Figure 5 A, B). There were increased numbers of phospho-CXCR4 cells at the periphery of T_2/T_1 /MTR and T_2 -only areas compared to the central parts of these areas. The phospho-CXCR4⁺ immunostaining was preferentially intracellular, different from membranous and intracellular CXCR4 staining indicating that the ligated receptor was internalized. Using confocal microscopy phospho-CXCR4 marker was co-localized with cells expressing MHCII (data not shown).

CXCL12 is up-regulated on astrocytes and endothelium of blood vessels in MS chronic lesions

The presence of phospho-CXCR4 implies the proximity of the ligand, CXCL12. CXCL12 immunoreactivity was found in astrocytes and astrocytic processes in inflamed MS lesions. CXCL12⁺ astrocytic processes extending towards blood vessels appeared thickened and prominent (Figure 5 C). Scattered CXCL12⁺ astrocytes were also identified in NAWM regions.

Double-label immunostaining for von Willebrand factor and CXCL12 revealed abundant CXCL12 immunoreactivity associated with vascular elements. CXCL12⁺ blood vessels were numerous in T_2/T_1 /MTR and T_2 -only areas. Constitutive expression of CXCL12 was also identified in blood vessel walls in NAWM in MS and non-neurological control tissues.

COMMENT

The present study extends our characterization of imaging-pathological correlates in chronic lesions of MS^{7, 11}. This research uses additional MRI sequences such as T_1 -WI and MTR along with histopathology, to probe the T_2 lesion heterogeneity underlying poor correlations between T_2 -WI and clinical outcome. For this reason, we compare the extremes of the spectrum evaluating lesions abnormal only on T_2 -WI with those abnormal on T_2 -WI, T_1 -WI and MTR images.

The current data were obtained by analyzing tissues from a subset of cases previously reported¹¹. These studies utilized a different series of tissues, and were evaluated in independent immunostaining experiments. The immunohistochemistry and data analysis were conducted by bench scientists (NMM; MBC; BHT; AMR) who did not participate in the prior study. Therefore, the present results confirmed at a technical level previous observations that T_2/T_1 /MTR regions represent areas of severe tissue destruction and principally correspond to chronic active or chronic inactive demyelinating lesions, whereas T_2 -only areas delineate regions with less severe damage.

As imaging results might be affected by increasing *post-mortem* time and as a result of tissue fixation, we endeavored to minimize the impact of these factors on MRI in our study. Patients were rapidly transported to the imaging facility for *in situ* MRI immediately followed by autopsy (mean *post-mortem* time was about 5 hours). One cerebral hemisphere was immediately fixed for at least four weeks. Even though tissue fixation affects MRI, an adequate post-mortem MRI has been reported after 20 years of formalin fixation²⁵.

We found that 80% of T₂/T₁/MTR regions were demyelinated and were either chronic active or chronic inactive lesions. Only two of ten (20%) T₂-only regions in this study corresponded to demyelinated lesions highlighting heterogeneity of T₂-only regions. The smaller proportion of demyelinated T₂-only regions in the current study as compared to previously published data might be explained by sampling variability. The majority of T₂-only regions revealed microglial activation and were myelinated. One region showed intense microglial activation without increased CD68⁺ cell number and was designated myelinated/infamed. No T₂-only area was associated with chronic inactive lesional pathology. Where active or chronic active T₂-only and T₂/T₁/MTR lesions could be directly compared, they appeared similar in regard to mononuclear phagocyte number and distribution (Table 5). Therefore, we tentatively propose that axonal pathology and demyelination provides the main pathological discriminator between T₂-only and T₂/T₁/MTR lesions.

Parenchymal T-cells were significantly increased in T₂/T₁/MTR areas as compared to T₂-only areas and NAWM suggestive of greater BBB impairment in T₂/T₁/MTR areas. Consistent with this proposal, serum proteins in CNS tissue were previously described in our MRI-pathology studies¹¹ with a higher proportion of intracellular proteins in T₂/T₁/MTR areas as compared with T₂-only areas. Study of early MRI changes in EAE demonstrated that Ig deposition correlated with MRI signal intensities in lesions with reduced signal intensity on both T₁- and T₂-WI and lesions with slightly reduced signal intensity on T₁-WI and increased signal intensity on T₂-WI²⁶. B-cell containing cuffs in our material were associated with a subset of demyelinated areas in three of five MS brains suggesting regional and inter-individual variability in the pathogenic cascade that underlies tissue injury in MS.

Reactive microglia and macrophages in areas corresponding to active or chronic active MS lesions expressed CXCR4 and the presence of phospho-CXCR4 epitopes indicated the ligated receptor. The regulatory cytokine TGFβ-1 up-regulates CXCR4 expression in primary human monocyte-derived macrophages and enhances CXCL12-stimulated ERK1,2 phosphorylation in these cells²⁷. In EAE, increased levels of CXCR4 were observed in CNS parenchymal cells^{28, 29}. These findings were extended by McCandless and colleagues who showed that inhibiting CXCR4 activation during EAE induction leads to loss of the typical intense perivascular cuffs, which are replaced by widespread leukocyte infiltration and worsened EAE severity.

We found that expression of CXCL12 correlated with increased inflammatory activity in MS lesions. CXCL12 immunostaining was found in cells exhibiting astrocytic morphology and associated with vascular elements in MS tissue. Previously, CXCL12 expression was reported in blood vessels and astrocytes in active and inactive MS lesions and occasionally on a few cells in perivascular infiltrates^{19,20}. In “silent” MS lesions, CXCL12 immunoreactivity was less than that observed in active MS lesions. Previous studies demonstrated CXCL12 immunoreactivity on both parenchymal and luminal sides of CD31⁺ endothelial cells in active MS lesions, whereas in NAWM and in non-MS tissues CXCL12 expression was only localized to the parenchymal side of the endothelium²¹. This redistribution of CXCL12 was correlated with extent of inflammation, demyelination and macrophage infiltration in MS lesions. In the current study, CXCR4/CXCL12 distribution was tightly associated with the presence of activated microglia and macrophages, but did not correlate with MRI indicators of tissue injury.

CXCR4 and its ligand, CXCL12 are currently of high interest as therapeutic targets in different pathological conditions including cancers, AIDS, systemic autoimmune and neuroinflammatory disorders (MS, stroke, Alzheimer's disease) as well as stem cell biology. Further, given the significant role that CXCR4/CXCL12 system plays in hematopoiesis including hematopoietic cell maturation and survival as well as homing of hematopoietic progenitors to the bone marrow and regulation of neuronal progenitor cell migration in the CNS^{17, 30}, it remains to be determined whether functions of this chemokine/receptor pair could be pharmacologically manipulated to treat inflammation in the CNS.

The current study represents a cross-sectional analysis of CXCR4/CXCL12 system in MS brain tissue. Prospective studies of this chemokine/receptor pair in the CSF and blood, in correlations with MRI and clinical course might be of potential interest as future routes of this research.

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Figure 1. MRI regional map and corresponding MS brain section with outlined MS ROIs. (A) – MRI image with marked T₂/T₁/MTR, T₂-only and NAWM regions; (B) – Corresponding brain section with the ROIs outlined in black.

Figure 2.

Patterns of inflammatory activity within MS lesions.

(A) – Immunostaining for MBP reveals demyelinated lesion corresponding to T₂-only region on MRI (bar length – 250μm), (B) – Consecutive section stained with anti-MHCII antibodies showing increased amount of MHCII⁺ cells at the border of the lesion and less cellular central area resembling chronic active lesion; (C) – Hypocellular, “burnt out” region corresponding to T₂/T₁/MTR ROI analyzed by immunostaining with anti-MHCII antibodies; (D) – Area of reduced myelin staining corresponding to T₂-only region, (E) – Consecutive section stained with anti-MHCII antibodies illustrating increased amount and specific morphology of reactive microglia and macrophages in an active MS lesion, (G) – High magnification of reactive MHCII⁺ microglia and macrophages in the area of partial demyelination (bar length – 50μm); (F) – Immunostaining for MHCII in NAWM; H – High magnification of MHCII⁺ cells in NAWM region.

Figure 3.

CD3⁺ and CD20⁺ lymphocytes in chronic MS brains.

(A) – Distribution of CD3⁺ lymphocytes in T₂/T₁/MTR region (bar length – 250μm); (B) – Dual staining with anti-CD3 (brown, black arrows) and anti-CD20 (red, red arrows) antibodies showing distribution of T- and B-lymphocytes in perivascular area in MS lesion (bar length – 50μm).

Figure 4.

Expression of CXCR4 molecules in MS lesion; MHCII⁺/CXCR4⁺ cells in chronic active MS lesion.

(A) – CXCR4⁺ cells at the border of chronic active lesion corresponding to T₂/T₁/MTR region (bar length – 250μm); (B) – High magnification of CXCR4⁺ cells in the lesion showing microglia-like morphology of CXCR4⁺ cells (bar length – 50μm); (C), (D), (E), (F)– Confocal microphotograph of MHCII⁺/CXCR4⁺ cells in chronic active MS lesion, (C) –Immunostaining with anti-MHCII⁺ antibodies, green channel (bar length – 25μm), (D) – CXCR4 immunoreactivity on red channel, (E) – Autofluorescence, (F) – Combined confocal

micrograph showing double positive MHCII/CXCR4 microglia and macrophages in the chronic MS lesion.

Figure 5.

CXCR4, phospho-CXCR4 and CXCL12 immunoreactivity in chronic MS lesions.

(A) and (B) – Consecutive sections immunostained with anti-CXCR4 (A) and anti-phospho-CXCR4 (B) antibodies illustrating similar distribution and morphology of CXCR4⁺ and phospho-CXCR4⁺ cells in chronic active MS lesion (bar length – 100µm); (C) –

Immunostaining with anti-CXCL12 antibodies in a chronic MS lesion showing astrocytic morphology of CXCL12⁺ cells and abundant CXCL12 immunoreactivity in blood vessels (bar length – 50µm).

Table 1

Demographic and clinical data on MS cases.

MS cases ID #	Gender	Age at death	MS type at last follow-up	EDSS at last follow-up	MS duration (years)
25	M	56	SPMS	9.5	33
37	M	65	SPMS	8.5	46
41	F	46	SPMS	8.0	14
42	F	53	SPMS	9.0	19
44	M	52	SPMS	8.5	30
n=5	M=3 / F=2	54.4±6.9	SP=100%	8.7±0.6	28.4±12.5

SPMS – secondary progressive MS.

Table 2

Demographic and clinical data on non-neurological controls.

Case ID #	Sex	Age, years	Postmortem time, hours	Cause of Death
A 523	F	96	9	Withdrawal of care; chronic obstructive pulmonary disease
A 306	F	65	7	Acute myocardial infarction
A 361	F	81	22	Cardiopulmonary arrest
A 368	M	65	11	Acute pericardial hemorrhage
A 319	F	53	36	Cardiopulmonary arrest

Table 3

Primary antibodies used for immunohistochemistry in the current study.

Antibody	Cat#	Clone	Isotype	Host	Dilution	Company
MBP	SM1-94	SM1-91	IgG1	mouse	1:1000	Covance, Emeryville, CA
CXCR4 (CD184)	AHP442	-/-	IgG	rabbit	1:200	Serotec, Oxford, UK
pS339 (phospho)- CXCR4	N/A	-/-	IgG	rabbit	1:250	a generous gift from Dr. J. Rubin ²²
CD3	VP-C316	PS1	IgG2a	mouse	1:200	Vector Laboratories, Burlingame, CA
CD20	M0755	126	IgG2a	mouse	1:100	DakoCytomation, Golstrup, Denmark
CD20	ab27093	-/-	IgG	rabbit	1:100	Abcam, Cambridge, MA
CD68	M0814	KPI(4)	IgG1k	mouse	1:100	DakoCytomation, Golstrup, Denmark
MHCII	M0775	CR3/43	IgG1k	mouse	1:100	DakoCytomation, Golstrup, Denmark
GFAP	Z0334	-/-	Ig	rabbit	1:5000	DakoCytomation, Golstrup, Denmark
von Willebrand Factor	A0082	-/-	Ig	rabbit	1:1000	DakoCytomation, Golstrup, Denmark

Table 4

Histopathologic characteristics of myelin status and inflammation in areas abnormal on MRI and NAWM.

Characteristics	T ₂ T ₁ MTR	T ₂ -only	NAWM
Myelinated	2/10 (20%)	8/10 (80%)	10/10 (100%)
activated microglia and macrophages	100%	100%	30%
Demyelinated	8/10 (80%)	2/10 (20%)	0
active	1/8 (12.5%)	1/2 (50%)	n/a
chronic active	4/8 (50%)	1/2 (50%)	n/a
chronic inactive	3/8 (37.5%)	0	n/a

Table 5

Quantitative analysis of CD68⁺ cell density in MS ROIs and non-neurological controls.

CD68/mm ²		T ₂ T ₁ MTR / Myelin status				T ₂ -only / Myelin status				NAWM / Myelin status		CD68/mm ²
MS ID #	Area	Region 1	Region 2	Region 1	Region 2	Region 1	Region 2	Region 1	Region 2	Region 1	Region 2	Non-neurol Cntr
25	Border	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	Myelinated	Myelinated	Myelinated	Myelinated	A 532
	Middle	1639.3±223.9 344.3±93	1450±303.9 102.9±27.9	1364.2±189.4 327.2±89.7	1483.9±84.8 865.7±133.8					518.5±66.7	507.1±10.1	40.2±4.3
37	Border	Reduced	Myelinated/Inflamed	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	A 306
	Middle	223.0±67.4 152.7±93.1	314.3±110.5 381.4±84.3	203.6±64.5 217.1±48.8	316.1±22.1 304.3±104.2			384.3±51.6	340.0±18.6			45.6±10.4
41	Border	Reduced	Myelinated/Inflamed	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	A 361
	Middle	1108.9±102.2 414.7±53.7	678.6±65.2 370.0±43.6	703.6±66.6 738.6±142	471.4±98.1 634.3±77.5			294.3±53	410.0±12.0			42.4±6.7
42	Border	Reduced	Reduced	Myelinated	Myelinated	Myelinated	Myelinated/Inflamed	Myelinated	Myelinated	Myelinated	Myelinated	A 368
	Middle	453.6±96.6 354.9±32.6	403.6±50.3 462.8±80.7	421.4±93.9 382.9±70.1	371.4±69.3 374.9±89.8			342.8±41.3	351.4±77.7			43.6±8.9
44	Border	Reduced	Reduced	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	A 319
	Middle	1073.2±111.3 385.7±73.7	576.8±97.1 297.1±66.9	478.5±85.1 431.4±38.7	371.4±46.3 437.2±28.3			341.4±21.1	355.7±27.8			53.8±9.8
TOTAL	Border	792.1±492.3 ^a	618.6±434.6 ^β									45.1±9.3
	Middle	326.5±125.7	471.3±214.6									

^a $p < 0.05$ for the comparison of CD68⁺ cell density at the border of T₂T₁/MTR with NAWM in MS and non-neurological control

^β $p < 0.05$ for the comparison of CD68⁺ cell density at the border of T₂-only with NAWM in MS and non-neurological control.

Table 6

Quantitative analysis of CXCR4⁺ cell density in MS ROIs and non-neurological controls.

Patient ID	Area	T ₂ T ₁ MTR / Myelin status			T ₂ -only / Myelin status			NAWM / Myelin status		CXCR4
		Region 1	Region 2		Region 1	Region 2		Region 1	Region 2	
25	Border	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	Myelinated	Myelinated	A 532 0-2cells/ROI
	Middle	919.6±54.0 411.4±62.0	605.3±34.4 168.6±17.9		919.6±96.7 250.0±114.4	1057.1±289.3 532.8±247.5		190.0±50.2	252.9±99.9	
37	Border	Reduced	Myelinated/Inflamed	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	A 306 4-5cells/ROI
	Middle	276.8±57.5 174.3±73.6	414.3±21.0 280.0±117.4		291.1±46.8 55.7±30.1	273.2±49.6 282.8±37.6		314.7±76.9	150.0±30.7	
41	Border	Reduced	Myelinated/Inflamed	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	A 361 0-1cells/ROI
	Middle	685.7±58.0 135.7±29.0	450.0±56.3 470.0±84.1		726.8±48.5 180.0±64.5	632.1±123.8 342.9±97.0		207.1±42.9	282.9±53.1	
42	Border	Reduced	Reduced	Myelinated/Inflamed	Myelinated	Myelinated/Inflamed	Myelinated/Inflamed	Myelinated	Myelinated	A 368 3-6cells/ROI
	Middle	285.7±65.7 50.0±8.7	219.6±54.5 121.4±25.7		333.9±91.2 118.6±17.2	250.0±28.0 150.0±29.4		219.6±92.9	91.4±41.8	
44	Border	Reduced	Reduced	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	Myelinated	A 319 5-8cells/ROI
	Middle	794.6±82.8 345.7±45.1	357.1±94.9 31.4±20.6		255.3±77.7 268.6±63.6	476.8±67.3 567.2±100.0		42.26±13.5	25.7±13.0	
TOTAL	Border	500.9±235.8 ^{α,β}			521.6±307.7 ^{α,β}			177.6±108.6		3.4cells/ROI*
	Middle	218.9±153.9			274.9±185.9					

* ROI size average in non-neurological controls was 28.8 mm²

^α p<0.01 - for the comparison of CXCR4⁺ cell density at the border of T₂T₁/MTR and T₂-only regions with the central portions of these regions

^β p<0.01 - for the comparison of the cell density at the border of T₂T₁/MTR and T₂-only areas with NAWM in MS and in non-neurological control.