Imaging Correlates of Leukocyte Accumulation and CXCR4/CXCL12 in Multiple Sclerosis

Natalia M. Moll, MD, PhD; Michael B. Cossoy, MD; Elizabeth Fisher, PhD; Susan M. Staugaitis, MD, PhD; Barbara H. Tucky, BS; Anna M. Rietsch, BS; Ansi Chang, MD; Robert J. Fox, MD; Bruce D. Trapp, PhD; Richard M. Ransohoff, MD

Objective: To compare leukocyte accumulation and expression of the chemokine receptor/ligand pair CXCR4/CXCL12 in magnetic resonance imaging–defined regions of interest (ROIs) in brains from patients with chronic multiple sclerosis. We studied the following ROIs: normal-appearing white matter (NAWM); regions abnormal only on T2-weighted images (T2 only); and regions abnormal on T2- and T1-weighted images with an abnormal magnetization transfer ratio (T2/T1/MTR).

Design: Case-control study.

Setting: Cleveland Clinic.

Patients: Brain tissue was acquired from 5 patients with secondary progressive multiple sclerosis (MS) and 5 nonneurological controls.

Intervention: Magnetic resonance imaging pathological correlations were performed on the 5 cases. Based on imaging characteristics, 30 ROIs were excised.

Main Outcome Measure: Using immunohistochemical analysis, we evaluated myelin status, leukocyte accumulation, and CXCR4/CXCL12 expression in the MS ROIs and white matter regions from the 5 nonneurological controls.

Results: Eight of 10 T2/T1/MTR regions were chronic active or chronic inactive demyelinated lesions, whereas only 2 of 10 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 with NAWM. Parenchymal T cells were significantly increased in T2/T1/MTR ROIs as compared with T2-only regions and NAWM. Expression of CXCR4 and phospho-CXCR4 were 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 central nervous system inflammation.


©2009 American Medical Association. All rights reserved.
weighted MRI with an abnormal MTR (T2/T1/MTR) often correspond to demyelinated lesions with significantly fewer axons but increased axonal swelling and loss of axonal sodium/potassium adenosine triphosphatase. Areas abnormal on T2-weighted MRI but with a normal MTR and normal T1-weighted imaging results were associated with less severe tissue damage, less axonal loss, and much less axonal swelling and the presence of axonal sodium/potassium adenosine triphosphatase. The majority of regions abnormal only on T2-weighted imaging (T2 only) featured local microglial activation and were characterized as active or chronic active lesions. T2/T1/MTR 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 cells. In vitro studies have shown that microglial activation leads to upregulation of CXCR4. 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-positive glia, representing oligodendrocyte progenitor cells. However, little is known about what roles the CXCR4/CXCL12 system plays during the inflammatory reaction in MS. The CXCL12 level is elevated in the cerebrospinal fluid from patients with MS and other inflammatory neurological disorders. In active MS lesions, CXCL12 is upregulated on astrocytes throughout lesion areas and on some monocytes/macrophages within perivascular cuffs. It has been recently shown that alteration of the pattern of CXCL12 expression at the blood-brain barrier, 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 sections.

In the current study, we evaluated myelin status, inflammatory leukocyte accumulation, and expression of CXCR4 and CXCL12 in MS T2/T1/MTR brain regions compared with T2-only regions and NAWM. Postmortem material was acquired according to an established protocol that has supported our previous reports. Brains from patients with MS were imaged in situ before autopsy and image-to-tissue coregistration was applied to enable MRI pathological correlations.

**METHODS**

**TISSUE AND TISSUE ACQUISITION**

Collection and use of human tissue was approved by the Cleveland Clinic institutional review board. Magnetic resonance imaging pathological correlations were performed on brain tissue from 5 cases with secondary progressive MS as previously described. 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 30 regions of interest (ROIs) were selected and isolated from postmortem MRIs of 5 cases with MS. These ROIs included (1) T2/T1/MTR areas, (2) T2-only areas, and (3) NAWM, which was normal appearing on all images. These patients represent a subset of those previously reported, but tissue ROIs reported herein are distinct from those characterized previously.

Samples of subcortical and periventricular white matter were dissected from each of 5 nonneurological control cases (10 tissue blocks total). The ROIs from MS brains (MRI defined and mapped onto coregistered tissue slice images) and nonneurological control brains were subsequently sectioned and immunostained to evaluate the myelin status, inflammatory activity, and CXCR4/CXCL12 immunoreactivity.

**MRI ACQUISITION AND ANALYSIS**

Magnetic resonance imaging was performed on a 1.5-T magnetic resonance scanner (Siemens, Erlangen, Germany), as described previously. Maps of MS ROIs were generated for each tissue slice from the corresponding MRI planes. A 10-mm 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.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Using MRI region maps, ROIs were identified and isolated from MS brains and nonneurological control tissue. Tissue blocks were cryoprotected overnight in 20% glycerol, embedded in 30% su-
crose, and sectioned 30 µm thick on a freezing sliding micro-
tome. These sections were used for immunoperoxidase histo-
chemical evaluation and double-labeled immunofluorescence.
Sections were pretreated as described previously,22 incu-
bated with primary antibodies for 5 days at 4°C, then incu-
bated with appropriate secondary antibodies and immunos-
tained by the avidin-biotin complex (Vector Laboratories,
Burlingame, California). Diaminobenzidine (Sigma-Aldrich, St
Louis, Missouri) was used as chromogen. Sections for confo-
cal microscopy were incubated with 2 primary antibodies and
non–cross-reacting secondary antibodies conjugated to Alexa
Fluor 488 and Alexa Fluor 594 (Invitrogen, Carlsbad, Califor-
nia). The specificity of immunohistochemical procedures was
tested using antibodies with well-characterized and different
immunoreactivity.

Primary antibodies used for immunostaining in the pres-
ent study are summarized in Table 3.

### Table 3. Primary Antibodies Used for Immunohistochemical Analysis in the Current Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog No.</th>
<th>Clone</th>
<th>Isotype</th>
<th>Host</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>SMI-94</td>
<td>SMI-91</td>
<td>IgG1</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Covance, Emeryville, California</td>
</tr>
<tr>
<td>CXCR4 (CD184)</td>
<td>AHP442</td>
<td></td>
<td>IgG</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Serotec, Oxford, England</td>
</tr>
<tr>
<td>p339 (phospho)-CXCR4</td>
<td>N/A</td>
<td></td>
<td>IgG</td>
<td>Rabbit</td>
<td>1:250</td>
<td>Gift from J. Rubin22</td>
</tr>
<tr>
<td>CD3</td>
<td>VP-C316</td>
<td>PS1</td>
<td>IgG2a</td>
<td>Mouse</td>
<td>1:200</td>
<td>Vector Laboratories, Burlingame, California</td>
</tr>
<tr>
<td>CD20</td>
<td>M0755</td>
<td>L28</td>
<td>IgG2a</td>
<td>Mouse</td>
<td>1:100</td>
<td>DakoCytomation, Glostrup, Denmark</td>
</tr>
<tr>
<td>CD20</td>
<td>ab27093</td>
<td></td>
<td>IgG2a</td>
<td>Mouse</td>
<td>1:100</td>
<td>Abcam, Cambridge, Massachusetts</td>
</tr>
<tr>
<td>CD68</td>
<td>M0814</td>
<td>KPi(4)</td>
<td>IgG1k</td>
<td>Mouse</td>
<td>1:100</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>MHCII</td>
<td>M0775</td>
<td>CR3/43</td>
<td>IgG1k</td>
<td>Mouse</td>
<td>1:100</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>GFAP</td>
<td>Z0334</td>
<td></td>
<td>Ig</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>A0082</td>
<td></td>
<td>Ig</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>DakoCytomation</td>
</tr>
</tbody>
</table>

Abbreviations: GFAP, glial fibrillary acid protein; Ig, immunoglobulin; MBP, myelin basic protein; MHCII, class II major histocompatibility complex.

CONFOCAL MICROSCOPY

Double-labeled MS brain sections were imaged and analyzed on
an SP5 confocal microscope (Leica Application Suite, version 1.6.3;
Leica Microsystems, Wetzlar, Germany). The entire thickness of
the sections (30 µm) was scanned. Fluorescence was collected
in the green, red, and autofluorescence channels. Images pre-
tained 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, anti-
CXCR4, anti-CD3, and anti-CD20 antibodies were digitized
using 20 × objective (Leica DM 4000B and QCapture Pro, version
3.1.1.14; Leica Microsystems), transferred to a worksta-
tion, and manually quantified (Adobe Photoshop CS2; Adobe,
San Jose, California), applying corrections for image size and
resolution. These microphotographs were taken from 9 nonover-
lapping microscopical fields of each MS ROI (5 micrographs
were taken from the middle parts and 4 at the borders of ROIs)
and 5 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 in-
terest in millimeters squared. Quantitative analysis was per-

![Figure 1. Magnetic resonance imaging regional map and corresponding multiple sclerosis brain section with outlined multiple sclerosis regions of interest.](image-url)
formed in a blinded fashion by 2 independent investigators (N.M.M. and A.M.R).

STATISTICAL ANALYSIS

Mixed-model analysis in SAS (SAS Institute Inc, Cary, North Carolina) was applied to determine the statistical significance of the data and compensate for multiple comparisons between varied regions from individual MS brains; \(P < .05\) was considered statistically significant. Results are given as mean (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 of 10) were demyelinated while only 20% of T2-only areas (2 of 10) were demyelinated (Table 4). Areas of NAWM and nonneurological control white matter areas were always myelinated. Some T2/T1/MTR and T2-only areas retaining myelin showed characteristics of intense inflammation on consecutive class II major histocompatibility complex (MHCII)– and CD68-stained sections and were designated myelinated/inflamed (Table 5 and Table 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 mono-

### Table 4. Histopathological Characteristics of Myelin Status and Inflammation in Areas Abnormal on MRI and NAWM

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No./Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T2/T1/MTR</td>
</tr>
<tr>
<td>Myelinated</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>Activated microglia and macrophages, %</td>
<td>100</td>
</tr>
<tr>
<td>Demyelinated</td>
<td>8/10 (80)</td>
</tr>
<tr>
<td>Active</td>
<td>1/8 (12.5)</td>
</tr>
<tr>
<td>Chronic active</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>Chronic inactive</td>
<td>3/8 (37.5)</td>
</tr>
</tbody>
</table>

Abbreviations: MRI, magnetic resonance imaging; NA, not applicable; NAWM, normal-appearing white matter; T2 only, regions abnormal only on T2-weighted imaging; T2/T1/MTR, regions abnormal on T2- and T1-weighted images with an abnormal magnetization transfer ratio.

### Table 5. Quantitative Analysis of CD68+ Cell Density in MS ROIs and Nonneurological Controls

<table>
<thead>
<tr>
<th>MS Case No.</th>
<th>Area</th>
<th>No. of CD68+ Cells/mm², Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Region 1</td>
<td>Region 2</td>
</tr>
<tr>
<td>T2/T1/MTR/Myelin Status</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Border</td>
<td>1639.3 (223.9)</td>
<td>1450 (303.9)</td>
</tr>
<tr>
<td>Middle</td>
<td>344.3 (93)</td>
<td>102.9 (27.9)</td>
</tr>
<tr>
<td>T2-Only/Myelin Status</td>
<td>Reduced</td>
<td>Myelinated/inflamed</td>
</tr>
<tr>
<td>Border</td>
<td>223.0 (67.4)</td>
<td>314.3 (110.5)</td>
</tr>
<tr>
<td>Middle</td>
<td>152.7 (93.1)</td>
<td>381.4 (84.3)</td>
</tr>
<tr>
<td>NAWM/Myelin Status</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Border</td>
<td>1073.2 (111.3)</td>
<td>576.8 (97.1)</td>
</tr>
<tr>
<td>Middle</td>
<td>385.7 (73.7)</td>
<td>297.1 (66.9)</td>
</tr>
<tr>
<td>T2-Only Only</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Border</td>
<td>1073.2 (111.3)</td>
<td>576.8 (97.1)</td>
</tr>
<tr>
<td>Middle</td>
<td>385.7 (73.7)</td>
<td>297.1 (66.9)</td>
</tr>
<tr>
<td>NAWM/Myelin Status</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Border</td>
<td>1073.2 (111.3)</td>
<td>576.8 (97.1)</td>
</tr>
<tr>
<td>Middle</td>
<td>385.7 (73.7)</td>
<td>297.1 (66.9)</td>
</tr>
</tbody>
</table>

Abbreviations: MS, multiple sclerosis; NAWM, normal-appearing white matter; ROI, region of interest; T2 only, regions abnormal only on T2-weighted imaging; T2/T1/MTR, regions abnormal on T2- and T1-weighted images with an abnormal magnetization transfer ratio.

**Table 5.** Quantitative Analysis of CD68+ Cell Density in MS ROIs and Nonneurological Controls

**Table 6.** Quantitative Analysis of CD68+ Cell Density in MS ROIs and Nonneurological Controls

<table>
<thead>
<tr>
<th>Control No.</th>
<th>No. of CD68+ Cells/mm², Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 532</td>
<td>40.2 (4.3)</td>
</tr>
<tr>
<td>A 306</td>
<td>45.6 (10.4)</td>
</tr>
<tr>
<td>A 361</td>
<td>42.4 (6.7)</td>
</tr>
<tr>
<td>A 368</td>
<td>43.6 (8.9)</td>
</tr>
<tr>
<td>A 319</td>
<td>53.8 (8.8)</td>
</tr>
<tr>
<td>Total</td>
<td>45.1 (9.3)</td>
</tr>
</tbody>
</table>

Abbreviations: MS, multiple sclerosis; NAWM, normal-appearing white matter; ROI, region of interest; T2 only, regions abnormal only on T2-weighted imaging; T2/T1/MTR, regions abnormal on T2- and T1-weighted images with an abnormal magnetization transfer ratio.

- \(a P < .05\) for the comparison of CD68+ cell density at the border of T2/T1/MTR with NAWM in MS and nonneurological controls.
- \(b P < .05\) for the comparison of CD68+ cell density at the border of T2 only with NAWM in MS and nonneurological controls.
nuclear phagocytes, the most consistent feature of the MS ROIs. Demyelinated T2/T1/MTR regions exhibited distinctive borders of mononuclear phagocytes (MHCII positive, 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-positive 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 were composed of MHCII-positive and CD68+ cells that were increased at the lesion border and also showed normal or decreased density in the core (Figure 2A and 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 4 of 8 T2/T1/MTR areas and 1 of 2 T2-only demyelinating lesions (Table 4). In this sample, mononuclear phagocyte distribution and number in the T2-only chronic active lesions were similar to those in the 4 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-positive and CD68+ cell density (Figure 2C). This pattern included only T2/T1/MTR areas that were seen as demyelinated areas sharply demarcated from the adjacent myelinated white matter. A subset of the ROIs with abnormal MRI results that retained myelin contained activated inflammatory cells uniformly distributed throughout the areas of interest (Figure 2D and E). Other ROIs showed evenly distributed MHCII-positive and CD68+ cells that revealed features of process-bearing resting microglia. These were myelinated areas that mostly consisted of NAWM and T2-only areas (Figure 2F).

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 = .002) and T2-only areas (P = .01) than in NAWM from MS and nonneurological controls (Table 5). On the other hand, numbers of CD68+ leukocytes did not vary significantly in the interior portions of T2/T1/MTR and T2-only regions and NAWM. Numbers of mononuclear phagocytes did not vary significantly in myelinated T2-only regions (n = 8) as compared with NAWM (n = 10) (P = .06) (Table 5). We were not able to compare quantitatively CD68+ cell number in demyelinated T2/T1/MTR and T2-
only regions because the number of T2-only demyelin-
ated 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\(^+\) lym-
phocytes were preferentially located in perivascular
areas of the MS ROIs (Figure 3A). The mean (SD)
density of CD3\(^+\) cells in perivascular cuffs did not vary
significantly among T2/T1/MTR areas (2065.3 [899.0]
cells/mm\(^2\)), T2-only areas (1906.1 [935.7] cells/mm\(^2\)),
and NAWM (1450.7 [880.6] cells/mm\(^2\)). Additionally,
the mean (SD) number of CD3\(^+\) cuffs was not signifi-
cantly different among T2/T1/MTR areas (27.2 [15.7]
cuffs/mm\(^2\)), T2-only areas (23.1 [14.1] cuffs/mm\(^2\)),
and NAWM (13.3 [10.8] cuffs/mm\(^2\)). Nonneurological
control brains only showed infrequent scattered cuffs.
Parenchymal CD3\(^+\) cells were increased \((P < .05)\) in T2/T1/MTR areas
(mean [SD], 30.1 [14.5] cells/mm\(^2\)) in comparison with
T2-only (mean [SD], 16.3 [5.6] cells/mm\(^2\)) and
NAWM (mean [SD], 12.3 [4.5] cells/mm\(^2\)). CD20\(^+\) cells
were distributed in perivascular spaces in MS tissue
(Figure 3B). CD20\(^+\) cuffs were present in 3 of 5 MS brains.
We did not find any difference in the CD20\(^+\) cuff pres-
ence in T2-only regions as compared with T2/T1/MTR
areas. Where present in cuffs, CD20 cells composed 11% to
16% of lymphocytes, as judged by dual-label immuno-
staining with anti-CD3 and anti-CD20 antibodies.

CXCR4 IS HIGHLY EXPRESSED ON REACTIVE
MHCII-POSITIVE 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 because of its remarkably
broad biology and potential for expression on hemato-
poietic cells (CD3, CD20, CD68) as well as glia, includ-
ing microglia, astrocytes, and NG2-positive glia, and vas-
cular elements.

CXCR4-positive cell density was significantly in-
creased at the borders of T2/T1/MTR \((P = .003)\) and T2-
only \((P = .003)\) areas compared with the central portions
of these areas (Table 6). CXCR4 cell density was also sig-
nificantly higher at the borders of ROIs with abnormal
MRI results \((P \leq .01)\) as compared with NAWM. Non-
neurological control tissue contained only individual ran-
domly distributed CXCR4-positive cells. The distribution
and morphology of CXCR4-positive cells in ROIs
with abnormal MRI results resembled those of CD68\(^+\)
and MHCII-positive microglia and macrophages
(Figure 4A and B). There was a strong correlation be-
tween CXCR4-positive and CD68\(^+\) cells at the borders
of ROIs with abnormal MRI results \((r = 0.91)\) in T2/T1/

Figure 2. Patterns of inflammatory activity within multiple sclerosis lesions. A, Immunostaining for myelin basic protein reveals a demyelinated lesion corresponding to an area abnormal on T2-weighted magnetic resonance imaging only (T2 only) \((\text{bar}=250 \, \mu\text{m})\). B, Consecutive section stained with anti-class II major histocompatibility complex (MHCII) antibodies showing an increased amount of MHCII-positive cells at the border of the lesion and a less cellular central area resembling a chronic active lesion. C, Hypocellular, “burnt-out” region corresponding to a region of interest abnormal on T2- and T1-weighted imaging with an abnormal magnetization transfer ratio \((T2/T1/MTR)\) analyzed by immunostaining with anti-MHCII antibodies. D, Area of reduced myelin staining corresponding to a T2-only region. E, Consecutive section stained with anti-MHCII antibodies illustrating an increased amount and specific morphology of reactive microglia and macrophages in an active multiple sclerosis lesion. F, Immunostaining for MHCII in normal-appearing white matter. G, High magnification of reactive MHCII-positive microglia and macrophages in the area of partial demyelination \((\text{bar}=50 \, \mu\text{m})\). H, High magnification of MHCII-positive cells in a normal-appearing white matter region.
MTR areas and \( r = 0.88 \) in T2-only areas; \( P < .05 \) but a modest correlation in the central portions \( (r = 0.30 \text{ in } \text{T2/T1/MTR areas and } r = 0.52 \text{ in T2-only areas; } P > .05) \). The decreased correlation in the interior was due to the presence of CD68\(^+\) cells that were CXCR4 negative. Dual-label immunohistochemical analysis and confocal microscopy confirmed that the majority of CD68\(^+\) and MHCII-positive microglia and macrophages expressed CXCR4 molecules at the borders of ROIs (Figure 4C and F). MHCII-positive/CXCR4-positive 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–POSITIVE CELLS ARE INCREASED IN NUMBER IN INFLAMED MS LESIONS AND CHARACTERIZE A FUNCTIONALLY ACTIVE POOL OF MICROGLIA AND MACROPHAGES**

Phospho-CXCR4 antibodies recognize an epitope including phosphoserine 339 on the intracellular portion of CXCR4, which is phosphorylated in response to ligand and therefore indicates the presence of recently ligated CXCR4 receptors. Phospho-CXCR4–positive cells represented a subset of CXCR4-positive cells mainly at the borders of chronic active lesions in MS ROIs (Figure 5A and B). There were increased numbers of phospho-CXCR4 cells at the periphery of T2/T1/MTR and T2-only areas compared with the central parts of these areas. The phospho-CXCR4–positive immunostaining was preferentially intracellular, different from membranous and intracellular CXCR4 staining, indicating that the ligated receptor was internalized. Using confocal microscopy, the phospho-CXCR4 marker was colocalized with cells expressing MHCII (data not shown).

**CXCL12 IS UPREGULATED 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-positive astrocytic processes extending toward blood vessels appeared thickened and prominent (Figure 5C). Scattered CXCL12-positive 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-positive blood vessels were numerous in T2/T1/MTR and T2-only areas. Constitutive expression of CXCL12 was also identified in blood vessel walls in NAWM in MS and nonneurological 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 T1-weighted imaging and MTR, along with histopathological examination, to probe the T2 lesion heterogeneity underlying poor correlations between T2-weighted imaging and clinical outcome. For this reason, we compare the extremes of the spectrum evaluating T2-only lesions with T2/T1/MTR lesions.

The current data were obtained by analyzing tissues from a subset of cases previously reported.\(^11\) These studies used a different series of tissues and were evaluated in independent immunostaining experiments. The immunohistochemical and data analysis were conducted by bench scientists (N.M.M., M.B.C., B.H.T., and A.M.R.) who did not participate in the prior study. Therefore, the present results confirmed at a technical level previous observations.
Figure 4. Expression of CXCR4 molecules in a multiple sclerosis (MS) lesion; class II major histocompatibility complex (MHCII)–positive/CXCR4-positive cells in a chronic active MS lesion. A, CXCR4-positive cells at the border of a chronic active lesion corresponding to a region abnormal on T2- and T1-weighted imaging with an abnormal magnetization transfer ratio (bar=250 µm). B, High magnification of CXCR4-positive cells in the lesion showing microglialike morphology of CXCR4-positive cells (bar=50 µm). C-F, Confocal microphotograph of MHCII-positive/CXCR4-positive cells in a chronic active MS lesion. Immunostaining with anti–MHCII-positive antibodies on the green channel (bar length=25 µm) (C), CXCR4 immunoreactivity on the red channel (D), the autofluorescence channel (E), and combined confocal micrograph showing double-positive MHCII/CXCR4 microglia and macrophages in the chronic MS lesion (F).
that T2/T1/MTR regions represent areas of severe tissue destruction and principally correspond to chronic active or chronic inactive demyelinating lesions, whereas T2-only areas delineate regions with less severe damage.

Because imaging results might be affected by increasing postmortem 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 postmortem time, about 5 hours). One cerebral hemisphere was immediately fixed for at least 4 weeks. Even though tissue fixation affects MRI, an adequate postmortem MRI has been reported after 20 years of formalin fixation.25 We found that 80% of T2/T1/MTR regions were demyelinated and were either chronic active or chronic inactive lesions. Only 2 of 10 T2-only regions (20%) 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 with 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/inflamed. No T2-only area was associated with chronic inactive lesions. 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 pathological features and demyelination provide the main pathological discriminators between T2-only and T2/T1/MTR lesions.

Parenchymal T cells were significantly increased in T2/T1/MTR areas as compared with T2-only areas and NAWM, suggestive of greater blood-brain barrier impairment in T2/T1/MTR areas. Consistent with this proposal, serum protein levels in central nervous system (CNS) tissue were previously described in our MRI pathological studies,11 with a higher proportion of intracellular proteins in T2/T1/MTR areas as compared with T2-only areas. Study of early MRI changes in experimental autoimmune encephalomyelitis (EAE) demonstrated that immunoglobulin deposition correlated with MRI signal intensities in lesions with reduced signal intensity on both T1-weighted and T2-weighted imaging and lesions with slightly reduced signal intensity on T1-weighted imaging and increased signal intensity on T2-weighted imaging. B cell–containing cuffs in our material were associated with a subset of demyelinated areas in 3 of 5 MS brains, suggesting regional and interindividual 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 transforming growth factor B1 upregulates CXCR4 expression in primary human monocyte-derived macrophages and enhances CXCL12-stimulated extracellular signal-regulated kinase 1/2 phosphorylation in these cells. In EAE, increased levels of CXCR4 were observed in CNS parenchymal cells. These findings were extended by McCandless and colleagues,21 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. 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 endothelium. 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, and systemic autoimmune and neuroinflammatory disorders (MS, stroke, Alzheimer disease) as well as stem cell biology. Further, given the significant role that the 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, 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 the CXCR4/CXCL12 system in MS brain tissue. Prospective studies of this chemokine/receptor pair in the cerebrospinal fluid and blood, in correlations with MRI and clinical course, might be of potential interest as future routes of this research.

Accepted for Publication: August 29, 2008.

Author Affiliations: Neuroinflammation Research Center (Drs Moll, Cossoy, Staugaitis, Chang, Trapp, and Ransohoff and Mss Tucky and Rietsch) and Departments of Neurosciences (Drs Moll, Cossoy, Staugaitis, Trapp, and Ransohoff and Mss Tucky, Chang, and Rietsch) and Biomedical Engineering (Dr Fisher), Lerner Research Institute, and Department of Anatomic Pathology (Dr Staugaitis), Mellen Center for Multiple Sclerosis Treatment and Research (Drs Fox and Ransohoff), Neurological Institute, Cleveland Clinic, Cleveland, Ohio. Dr Cossoy is now with the Section of Neurology, Health Sciences Institute, Cleveland Clinic, Cleveland, OH 44195 (ransohr@ccf.org).

Correspondence: Richard M. Ransohoff, MD, Neuroinflammation Research Center, NC-30, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH 44195 (ransohr@ccf.org).

Author Contributions: Study concept and design: Moll, Cossoy, Tucky, Trapp, and Ransohoff. Acquisition of data: Moll, Cossoy, Fisher, Staugaitis, Tucky, Chang, Rietsch, and Fox. Analysis and interpretation of data: Moll, Fisher, Rietsch, and Ransohoff. Drafting of the manuscript: Moll, Rietsch, and Ransohoff. Critical revision of the manuscript for important intellectual content: Moll, Cossoy, Fisher, Staugaitis, Tucky, Chang, Fox, and Ransohoff. Administrative, technical, and material support: Moll, Cossoy, Fisher, Tucky, Chang, Rietsch, Fox, Trapp, and Ransohoff. Study supervision: Fisher and Ransohoff.

Financial Disclosure: None reported.

Funding/Support: This study was supported by National Institutes of Health grant P50 NS38667 (Dr Ransohoff).

Additional Contributions: Jar-Chi Lee, MS, Quantitative Health Sciences, Cleveland Clinic, Cleveland, Ohio, assisted with statistical analysis of the research data.

REFERENCES


