J Neuropathol Exp Neurol
Vol. 72, No. 12
December 2013
pp. 1135–1144

ORIGINAL ARTICLE

The Regulation of Reactive Changes Around Multiple Sclerosis Lesions by Phosphorylated Signal Transducer and Activator of Transcription

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Abstract

Activation of signal transducer and activator of transcription 3 (STAT3) by phosphorylation is thought to mediate anti-inflammatory responses to CNS injury. Several studies have reported an increase in phosphorylated STAT3 (pSTAT3) in peripheral T cells and monocytes from patients with multiple sclerosis (MS) during relapses, suggesting that pSTAT3 might represent an inflammatory marker. Here, we examined immunoreactivity for pSTAT3 in brain tissue samples from MS patients and controls. Phosphorylated STAT3 immunoreactivity was sparse within lesions, with no difference between active and inactive lesions. It was, however, significantly greater in white matter (WM) adjacent to active and inactive lesions; moreover, it was significantly greater in WM adjacent to active versus inactive lesions. Phosphorylated STAT3–positive cells were identified as astrocytes and macrophages/microglia. Phosphorylated STAT3 expression was also detected by Western blotting in WM of patients with MS. In comparison, pSTAT3 immunoreactivity was either rare or found focally in brain tissue samples from patients with other neurologic diseases. Our findings show that pSTAT3 does not correlate with inflammatory activity in MS lesions, but that it may play an important role in regulating reactive changes proximal to MS lesions.

Key Words: Astrocytes, Gliosis, Immunoreactivity, Macrophages/Microglia, Multiple sclerosis, STAT3, White matter.

INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) is a cytoplasmic transcription factor that induces expression of responsive genes (1, 2). It can be activated by tyrosine phosphorylation after the binding of cytokines and/or growth factors to cell surface receptors (2, 3). After tyrosine phosphorylation and subsequent dimerization, STAT3 rapidly translocates to the nucleus and binds to specific DNA sequences in the promoters of genes to regulate their expression (4, 5). Signal transducer and activator of transcription 3 activation is mediated by a number of cytokines that act on different cell types and has been implicated in injury responses (6, 7). In the CNS, STAT3 is expressed by astrocytes, microglia, neurons, and other cell types (8–12), and activation of STAT3 by phosphorylation increases markedly after CNS insults (7–10, 12–14).

Signal transducer and activator of transcription 3 plays critical roles in cell growth and survival (15, 16). Dysregulation of the STAT3 pathway has been implicated in the development of chronic inflammatory and neurodegenerative diseases, as well as in several malignant tumors (17). In glial tumors, STAT3 has been found to exert dual effects including tumor suppression and promotion of oncogenesis (16). It is not only a potent negative regulator of helper T cell–mediated inflammation but also an important activator of many genes that are crucial for immunosuppression leading to tumor inflammation (18, 19). In CNS diseases with prominent neuroinflammation, such as multiple sclerosis (MS), a number of cytokines are upregulated and their effects are in part mediated by activation of STAT3 or other STAT members (8, 17). Signal transducer and activator of transcription 3 activates an anti-inflammatory program characterized by the upregulation of immunosuppressive cytokines such as Type 1 interferons that, in turn, inhibit proinflammatory gene expression (17, 20, 21). On the other hand, several studies have implicated phosphorylated STAT3 (pSTAT3) as a putative marker for inflammatory disease activity (22–24).

Genetic association studies have identified the *STAT3* gene as a potential MS susceptibility locus, suggesting some association between STAT3 and an increase in MS risk (25–27), although this association was not detected in another study (28). In animal studies, mice with targeted deletion of STAT3 in T cells did not develop experimental autoimmune encephalomyelitis, an animal model of MS (29). Significantly higher levels of pSTAT3 have been found in peripheral circulating monocytes from MS patients during relapses than in remission (30). Persistently high pSTAT3 levels in peripheral circulating CD4-positive T cells have been thought to favor the early conversion from a first event to clinically definite MS (31). Only one study has included examination of STAT3 expression and pSTAT3 immunoreactivity in CNS tissue of

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This study was supported in part by the Multiple Sclerosis Society of Canada and Canadian Institutes of Health Research to Christopher Power and V. Wee Yong.

The authors report no conflicts of interest regarding the contents of the article.

TABLE. Characteristics of MS and Control Patients and Samples Studied

Case	Sex	Age, years	Diagnosis	Cause of Death	No. MS Lesions Studied	Location of Lesions/Samples Studied
1	F	16	AID/MS*	N/A	1	Left frontal subcortical WM
2	F	27	AID/MS*	N/A	1	Left frontal subcortical WM
3	F	29	AID/RRMS*	N/A	1	Right posterior frontal subcortical WM
4	F	48	RRMS	Gastric adenocarcinoma	5	Right frontal, parietal, temporal WM
5	F	55	SPMS	Cardiorespiratory failure	9	Right frontal, parietal, temporal, occipital WM, basal ganglia, pons, and cerebellum
6	F	50	SPMS	Cardiac arrest	12	Right frontal, and left frontal, parietal, temporal, occipital WM, and midbrain
6	F	77	SPMS	Cardiorespiratory failure	7	Right occipital, and left frontal, parietal, occipital WM
8	M	63	SPMS	Pneumonia and pyelonephritis	10	Right frontal, and left parietal, occipital WM
9	M	60	SPMS	Cardiorespiratory failure	5	Right frontal, parietal, and occipital WM
10	F	58	PPMS	Cardiorespiratory failure	5	Left frontal, parietal, temporal WM, and midbrain
11	M	54	PPMS	Pneumonia and pancreatitis	10	Right parietal, temporal, occipital, and left frontal, parietal, temporal WM, and pons
12	M	52	AD	Cardiac arrest		Left temporal and right frontal subcortical WM
13	M	76	AD	Myocardial infarction		Right temporal subcortical WM
14	M	39	ALS	Multiorgan failure		Left temporal, right and left motor cortex and subcortical WM
15	F	75	ALS	Multiorgan failure		Right and left motor cortex and subcortical WM
16	F	63	Neuropathologically normal	Multiorgan failure		Right temporal subcortical WM
17	F	44	Neuropathologically normal	Multiorgan failure		Left frontal and temporal subcortical WM

*Biopsy, which MS diagnosis was confirmed by clinical and radiologic follow-up.

AD, Alzheimer disease; AID, active inflammatory demyelination; ALS, amyotrophic lateral sclerosis; F, female; M, male; MS, multiple sclerosis; PPMS, primary progressive MS; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; WM, white matter.

patients with MS (8). In that study, high levels of STAT3 and pSTAT3 immunoreactivity were found exclusively on astrocytes not only in the CNS tissues of MS but also those of normal subjects and other neurologic diseases. The present study examined pSTAT3 expression within MS lesions and in adjacent CNS tissues. Our results suggest that pSTAT3 was not correlated with inflammatory activity, but that it is more likely to be related to reactive changes around MS lesions.

MATERIALS AND METHODS

Subjects

This study was approved by local human research ethics boards. All brain tissues were obtained from the Departments of Laboratory Medicine and Pathology at the University of Alberta. We examined biopsy specimens from 3 patients with MS and postmortem tissues from 8 patients with MS, 2 patients with Alzheimer disease (AD), 2 patients with amyotrophic lateral sclerosis (ALS), and 2 patients with no CNS diagnostic abnormality (neuropathologically normal). The diagnosis of MS was clinically made and confirmed by neuropathologic examination. The patients with biopsies were diagnosed as having MS based on histopathologic findings and subsequent follow-up of clinical manifestations and magnetic resonance imaging scans. The MS lesions were histopathologically classified using a modification of the Bö/Trapp staging system (32, 33). None of the MS patients were found to have concomitant neurodegenerative or CNS inflammatory diseases. Clinical data for the MS patients (mean age ± SD, 48.8 ± 17.9 years) and other subjects are summarized in the Table.

Neuropathology and Immunohistochemistry

All postmortem brain tissues were fixed in 10% formalin for at least 2 weeks; biopsy tissues were fixed for more than 24 hours. Neuropathologic examination of the postmortem brains was performed on 1-cm-thick slices of the cerebral hemispheres and 0.5-cm-thick slices of the brainstem and cerebrum. The tissue blocks containing MS lesions and control areas were sampled from various brain regions and were embedded in paraffin. The samples were cut into 5-μm-thick tissue sections, deparaffinized, and stained with hematoxylin and eosin and Luxol fast blue (LFB) to identify lesions. The nature of the plaques was morphologically assessed and confirmed by immunohistochemistry. Immunohistochemistry was performed on tissue sections using the EnVision FLEX Mini Kit, High pH (Autostainer/Autostainer Plus; Dako, Carpinteria, CA)

detection system after the tissue was deparaffinized and rehydrated according to standard protocol. Primary antibodies to the following antigens were used: mouse monoclonal pSTAT3 antibody (B-7, SC-8059; Santa Cruz Biotechnology, Santa Cruz, CA), glial fibrillary acidic protein ([GFAP] 6F2; Dako), CD3 (A0452; Dako), and CD68K (KP1; Dako).

For double-immunofluorescence microscopy to localize pSTAT3 within specific cell types, deparaffinized sections were first subjected to antigen retrieval by steaming in Epitope Retrieval Solution (pH 9.0) for 10 minutes, then 3% H_2O_2/methanol for 10 minutes. Triton X-100 (0.25%) in PBS was added for 20 minutes, and sections were blocked for nonspecific binding (using HHG^+ skimmed milk blocking solution [1:1] with 5% normal human serum). The primary antibodies were then applied simultaneously overnight, followed by appropriate Alexa Fluor 488 or 546 secondary antibodies (1:500 dilutions; Jackson Laboratories, West Grove, PA). Sections were analyzed using an Olympus Fluoview FV10i confocal microscope. The primary antibodies used for these analyses were rabbit anti-GFAP (Dako Z0334, 1:200 dilution), rabbit anti-Iba1 (Dako 019–19741, 1:500 dilution), rabbit anti–Nogo-A (AB5664P, 1:200 dilution; Millipore, Billerica, MA), rabbit anti–myelin basic protein ([MBP] AB40390, 1:300 dilution; Abcam, Cambridge, MA), and mouse anti-pSTAT3 (B-7, SC-8059, 1:100 dilution; Santa Cruz Biotechnology).

We characterized the MS lesions by defining active lesions as actively demyelinating with inflammatory activity throughout the lesion with considerable infiltration of CD68-positive macrophages and CD3-positive T cells (34). Chronic active lesions were hypocellular in the center and hypercellular at the leading edge that contained ongoing demyelination and inflammatory activity. Chronic inactive lesions were

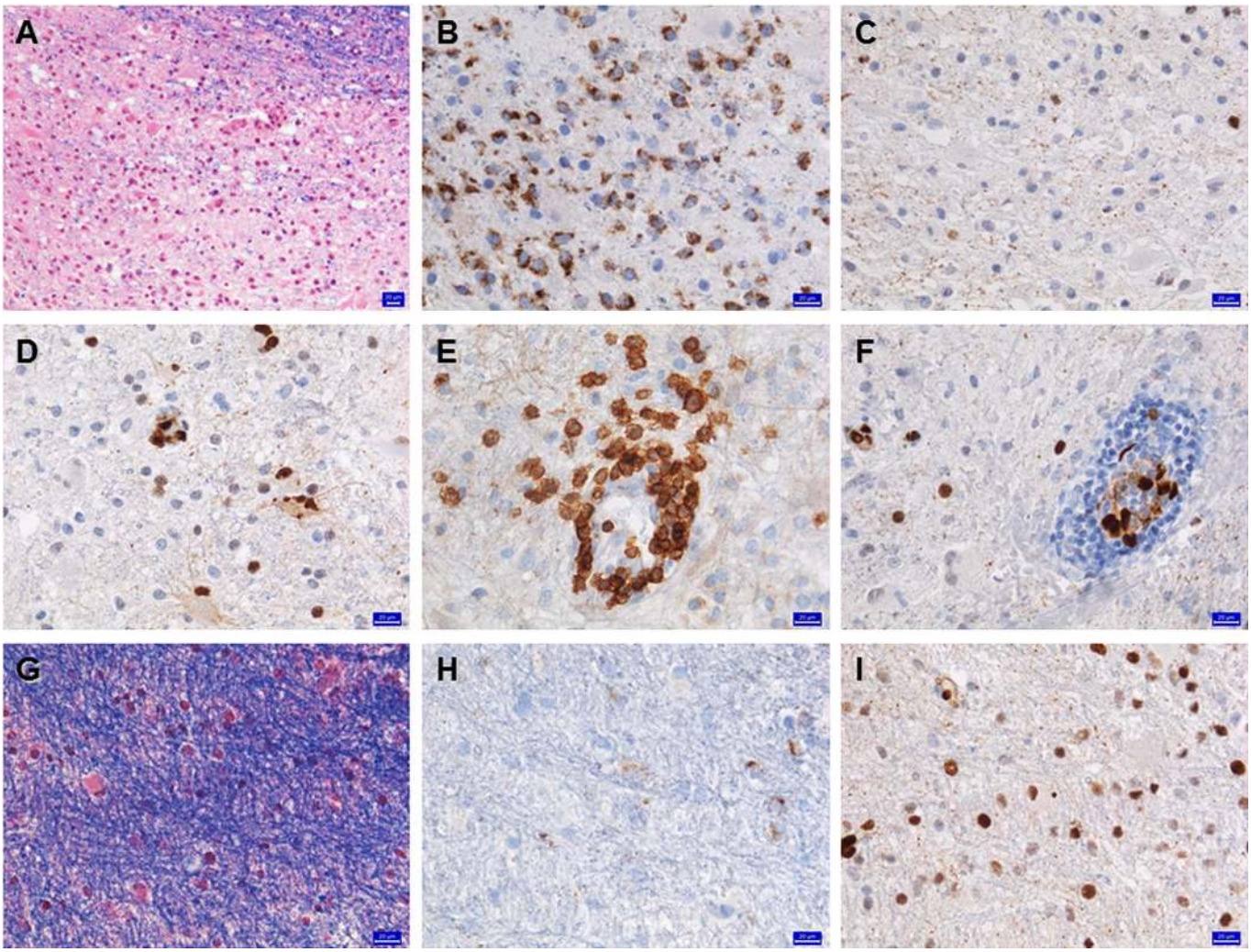

FIGURE 1. Examination of pSTAT3 immunoreactivity in a biopsy of a multiple sclerosis (MS) lesion. The specimen is an active lesion from the left frontal lobe in a patient with MS whose diagnosis was confirmed by follow-up (Table, case 1). The lesion shows decreased LFB staining for myelin and increased cellularity **(A)** with abundant CD68-positive macrophages/microglia **(B)**. Within the lesion, the immunoreactivity for pSTAT3 is scattered **(C)** and identified in some reactive astrocytes **(D)**. CD3-positive T cells are numerous and are preferentially perivascular **(E)** but mostly negative for pSTAT3 **(F)**. The WM adjacent to this active lesion exhibits preserved LFB staining with scattered reactive astrocytes **(G)** and infrequent CD68-positive macrophages/microglia **(H)**, but frequent pSTAT3-positive cells **(I)**. Scale bar = 20 μm.

hypocellular in both the center and edge, with no significant ongoing demyelination and infrequent CD68-positive macrophages/microglia. Astrocytic gliosis was associated with inflammatory demyelination in the lesion and also prominent in the adjacent brain tissue. ''Normal-appearing white matter'' (NAWM) was defined as the areas away from lesions and containing minimal astrogliosis and infiltration of inflammatory cells.

Semiquantitative Analysis of pSTAT3 Immunostaining

Phosphorylated STAT3 immunoreactivity was assessed semiquantitatively by scoring the frequency of pSTAT3-positive cells per high-power field (original magnification: 200×, defined as 0.79 mm^2) as follows: 0, none; 1, rare (1%–4%); 2, scattered (5%–24%); 3, frequent (≥25%). Immunoreactive cells were identified as those with visible positivity within the nucleus and/or cytoplasm. The scores of 10 consecutive high-power fields were then summed for analysis. The WM adjacent to MS lesions was defined as the area within 0.1 cm (1 high-power field outward) along the boundary of distinct LFB staining.

Western Blotting

Brain specimens were collected and frozen on dry ice at autopsy from 3 control subjects (C1, a 57-year-old man with normal neuropathologic examination; C2, a 60-year-old man with cerebral atherosclerosis; C3, a 63-year-old woman with normal neuropathologic examination) and 3 patients with MS (44-year-old and two 54-year-old men). Nonlesional WM

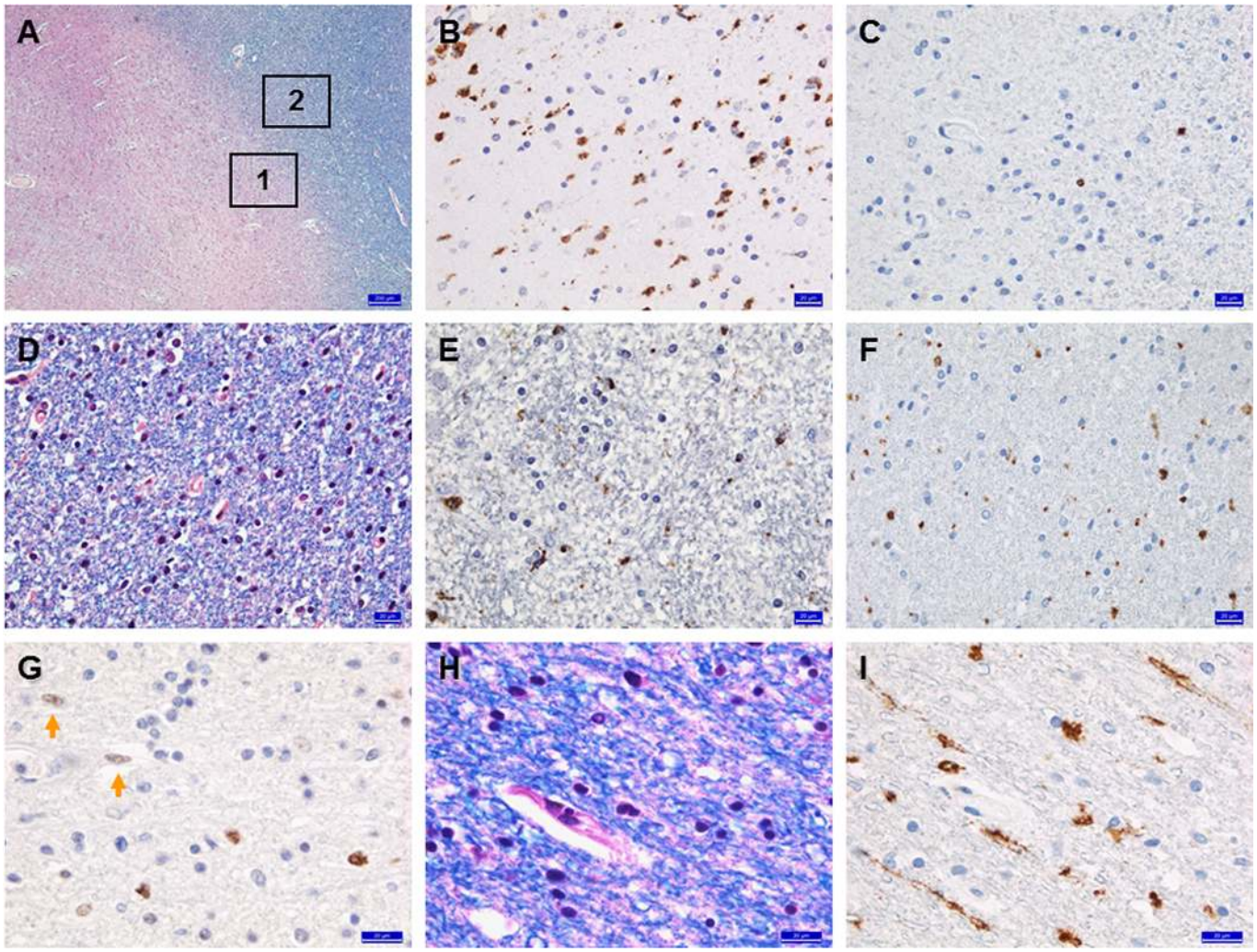

FIGURE 2. Phosphorylated STAT3 immunoreactivity is localized preferentially around chronic active multiple sclerosis (MS) lesions. A chronic active lesion of the right temporal periventricular region (Table, case 4) shows a hypocellular center with diminished LFB staining for myelin and a hypercellular edge (**[A]** rectangle 1 indicates the area of **B** and **C**; rectangle 2 indicates the area of **D**–**F**), with many CD68-positive macrophages/microglia **(B)**. Immunoreactivity for pSTAT3 is sparse within the lesion **(C)**. The WM adjacent to this chronic active lesion exhibits preserved LFB staining **(D)** and scattered CD68-positive macrophages/microglia **(E)**; there are numerous pSTAT3-positive cells **(F)**. **(G–I)** Some cells with macrophage/microglial morphology are positive for pSTAT3 (**[G]** arrows). Focally in the WM (**[H]** LFB staining), there are scattered pSTAT3-positive linear structures **(I)**. Scale bars = **(A)** 200 μm; **(B–I)** 20 μm.

tissues were sampled and homogenized in Laemmli buffer with 0.1% β-mercaptoethanol in lysing matrix tubes on a Fast Prep-24 tissue homogenizer (MP Biomedicals, Santa Ana, CA). The samples were boiled at 95°C for 10 minutes and loaded on mini protein TGX 12% polyacrylamide gels (456–1044 Bio-Rad, Hercules, CA). The protein fractions were transferred to Hybond ECL membranes (RPN303D; GE Health Care), probed with 1:250 anti-pSTAT3 antibody (B-7, SC-8059; Santa Cruz Biotechnology) overnight at 4°C and detected using horseradish peroxidase–conjugated goat anti-mouse antibody (105-035-003; Jackson ImmunoResearch). Beta-actin–horseradish peroxidase was used as a loading control (1:1000; Santa Cruz Biotechnology). Membranes were developed with Pierce ECL Western blotting substrate (32106; Thermo Scientific) and exposed on film (Canon) (35).

Statistical Analysis

The one-way analysis of variance test was used to assess the difference in scores of pSTAT3 immunostaining among groups, followed by Tukey post hoc test for further comparisons between groups. GraphPad Prism 6 software was used for this statistical analysis. Values of $p < 0.05$ were regarded as significant.

RESULTS

Identification of MS Lesions

The Table summarizes the clinical features of MS patients and controls, as well as the location and number of lesions examined. Multiple sclerosis lesions were identified by discrete decreased intensity of LFB staining for myelin in the WM (Figs. 1–3). Active (both active and chronic active) demyelinating lesions had considerable presence of CD68-positive macrophages/microglia and CD3-positive T lymphocytes, as well as associated astrocytic gliosis within the lesion and adjacent brain tissue (Figs. 1, 2). Ongoing demyelination was characterized by the presence of macrophages with LFB-positive myelin debris within their cytoplasm (Fig. 1A). Chronic inactive lesions were sharply demarcated and hypocellular and lacked inflammatory and demyelinating activities (Fig. 3). Three biopsied lesions (Table, cases 1–3) were assessed separately for comparison.

Phosphorylated STAT3 Immunoreactivity Preferentially Surrounding MS Lesions

By Western blot, pSTAT3 in the nonlesional MS WM of MS patients was found to be elevated compared with that of non-MS control specimens (Fig. 4). Immunohistochemistry for pSTAT3 was then performed to localize the protein. Phosphorylated STAT3 immunoreactivity was identified in the nucleus and/or in cytoplasm of cells within or nearby MS lesions from both biopsy (Fig. 1) and postmortem (Figs. 2, 3) specimens. Remarkably, pSTAT3 immunoreactivity was sparse within MS lesions. There was no significant difference in pSTAT3 (nuclear and cytoplasmic) immunoreactivity between active and inactive MS lesions ($p > 0.05$; Fig. 5). In contrast, the WM adjacent to MS lesions exhibited much more frequently

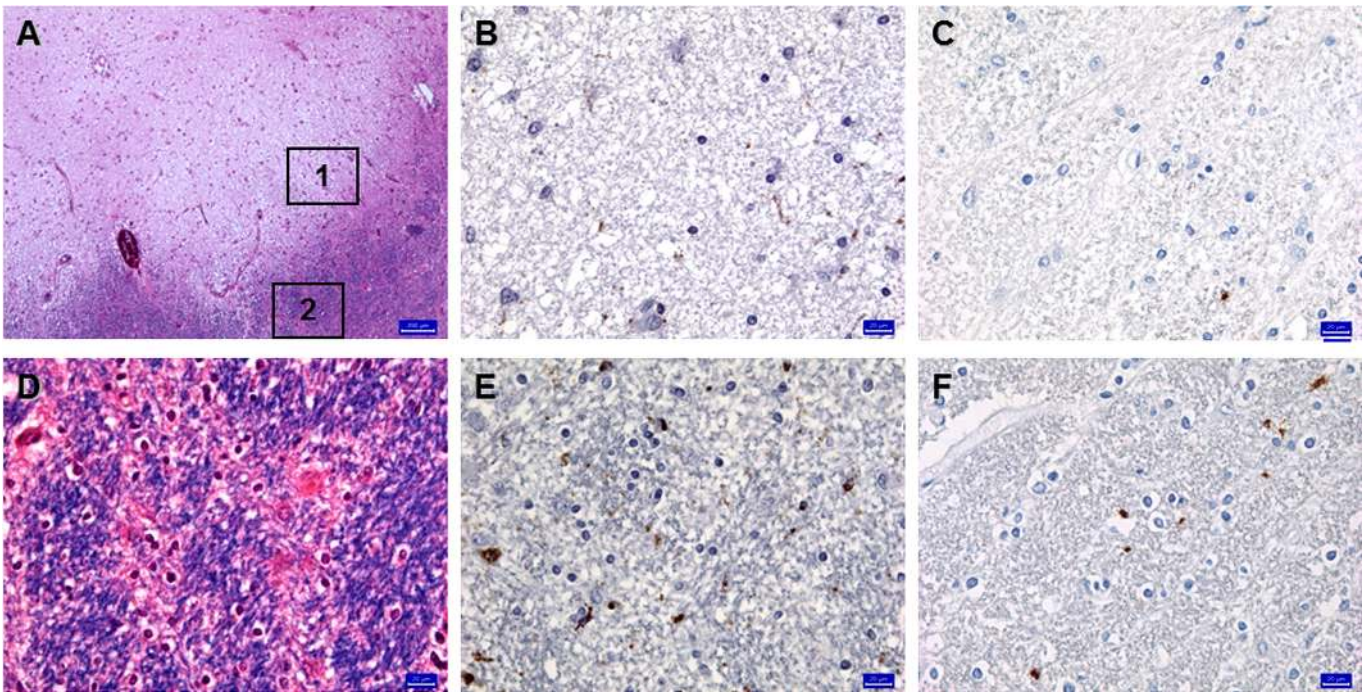

FIGURE 3. Phosphorylated STAT3 immunoreactivity in the WM adjacent to a chronic inactive multiple sclerosis (MS) lesion. A chronic inactive lesion of the left frontal periventricular region (Table, case 6) shows sharply diminished LFB staining (**[A]** rectangle 1 indicates the area of **B** and **C**; rectangle 2 indicates the area of **D**–**F**). There is minimal infiltration of CD68-positive macrophages/microglia **(B)** and rare immunoreactivity for pSTAT3 **(C)**. The WM adjacent to this chronic inactive lesion exhibits preserved LFB staining **(D)**, sparse CD68-positive macrophages/microglia **(E)**, and infrequent pSTAT3-positive cells **(F)**. Scale bars = **(A)** 200 μm; **(B–I)** 20 μm.

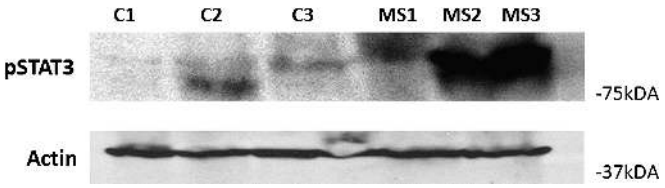

FIGURE 4. Western blot analysis of pSTAT3 expression in nonlesional WM tissue lysates from 3 control and 3 MS brains. Actin is used as an internal loading control. Phosphorylated STAT3 protein levels are enhanced in MS nonlesional WM tissues but minimal in the control tissues.

detected pSTAT3 immunoreactivity (Figs. 1–3). The scores of pSTAT3 immunoreactivity in WM adjacent to active and inactive lesions were significantly greater than those within active (Fig. 2) and inactive lesions (Fig. 3), respectively ($p < 0.001$; Fig. 5). The WM adjacent to active lesions had significantly greater scores of pSTAT3 immunoreactivity than that adjacent to inactive lesions ($p < 0.001$) and NAWM ($p < 0.001$), but there was no significant difference in the scores of pSTAT3 immunoreactivity between the WM adjacent to inactive lesions and NAWM ($p > 0.05$; Fig. 5). Normal-appearing WM remote from lesions displayed minimal pSTAT3 immunoreactivity (not shown).

Phosphorylated STAT3 Immunoreactivity in Subsets of Cells

We sought to determine the cell types expressing pSTAT3. Reactive astrocytes were frequently pSTAT3 immunopositive (Fig. 1D), whereas T cells were largely negative for pSTAT3 (Fig. 1F). Macrophages/microglia that were abundant in active lesions (Figs. 1B, 2B) rarely exhibited pSTAT3 immunoreactivity (Figs. 1C, 2C), although some pSTAT3-positive cells detected in the WM adjacent to active lesions had the appearance of macrophages or microglia (Fig. 2G). In the areas proximal to chronic active lesions there were focally sparse to scattered pSTAT3-positive linear structures that resembled myelin sheaths (Fig. 2I).

We next identified pSTAT3-positive cells by double-immunofluorescence microscopy. Using antibodies directed against cell-type specific markers, we found that pSTAT3-positive cells were mostly GFAP-labeled astrocytes (Fig. 6A–C) or Iba1-labeled macrophages/microglia (Fig. 6D–F), but not Nogo-A labeled oligodendrocytes (Fig. 6G–I). Some MBP-labeled oligodendrocyte soma appeared positive for pSTAT3 (Fig. 6J–L).

Phosphorylated STAT3 Immunoreactivity in Non-MS Brain Samples

To compare the pSTAT3 immunoreactivity in MS with that in other neurologic diseases, we examined postmortem tissues of 2 patients with AD, 2 patients with ALS, and 2 neuropathologically normal subjects (Table). The cerebral WM of patients with AD showed a normal appearance (Fig. 7A), with infrequent CD68-positive microphages/microglia (Fig. 7B) and rare pSTAT3-positive cells (Fig. 7C). The temporal subcortical WM of patients with ALS was normal appearing (Fig. 7D) and contained sparse CD68-positive macrophages/microglia (Fig. 7E), as well as occasional pSTAT3-positive cells (Fig. 7F). In contrast, the WM adjacent to the motor cortex of patients with ALS exhibited a normal myelinated appearance (Fig. 7G), frequent CD68-positive macrophages/microglia consistent with neurodegenerative changes (Fig. 7H), and focally scattered to frequent pSTAT3-positive cells (Fig. 7I).

DISCUSSION

In this study, we observed that immunoreactivity for pSTAT3 was greater in the WM adjacent to MS lesions than within lesions; this is the first to describe the distribution of pSTAT3 activity relative to MS lesions. Our findings suggest that pSTAT3 is more involved in reactive astrocytic and microglial gliosis, rather than demyelinating or inflammatory activity, in MS.

Activated STAT3 is present in several cell types after CNS injury. Astrocytes have been found consistently to show upregulated pSTAT3 after CNS injury (7–9, 12–14), which suggests important roles for STAT3-mediated astrocytic gliosis, possibly in restricting inflammation (8) and protecting myelin development in neonatal brain injury (36). Activation of the STAT3 pathway has been observed in astrocytes after neonatal WM injury; pSTAT3-containing astrocytes have been identified focally in neonatal postmortem brains with WM injury but not in age-matched controls (36). The colocalization of pSTAT3 and GFAP immunostaining has been seen in specimens of the temporal neocortex surgical resection in patients with drug-resistant epilepsy (14). In rodent studies, a subset of astrocytes exhibit activation of STAT3 focally after various injuries, including transient focal cerebral ischemia (13, 36), *N*-methyl-D-aspartate–induced excitotoxic cell death (12), epileptic process (14), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–induced neurodegeneration (9), and spinal cord injury (7). A few rodent studies have observed that

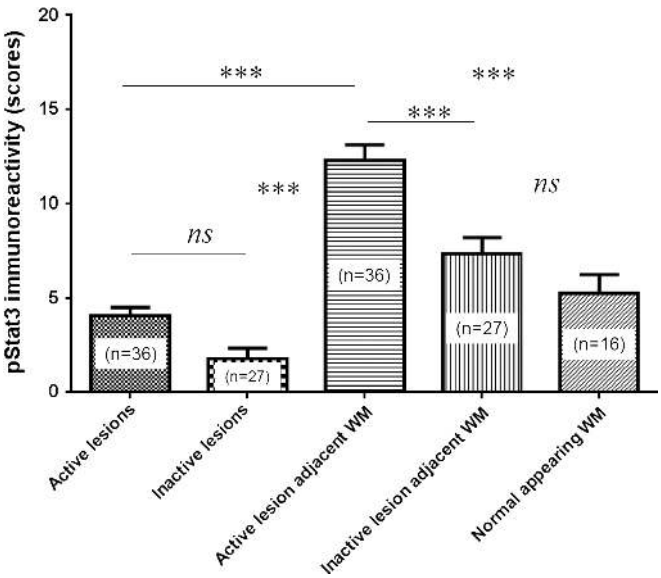

FIGURE 5. Semiquantitative scores of pSTAT3 immunoreactivity in lesion and nonlesional WM of MS patients. The groups were compared using one-way analysis of variance followed by Tukey post hoc test. *** $p < 0.001$; ns, not significant. The number of lesions sampled is indicated in the bars.

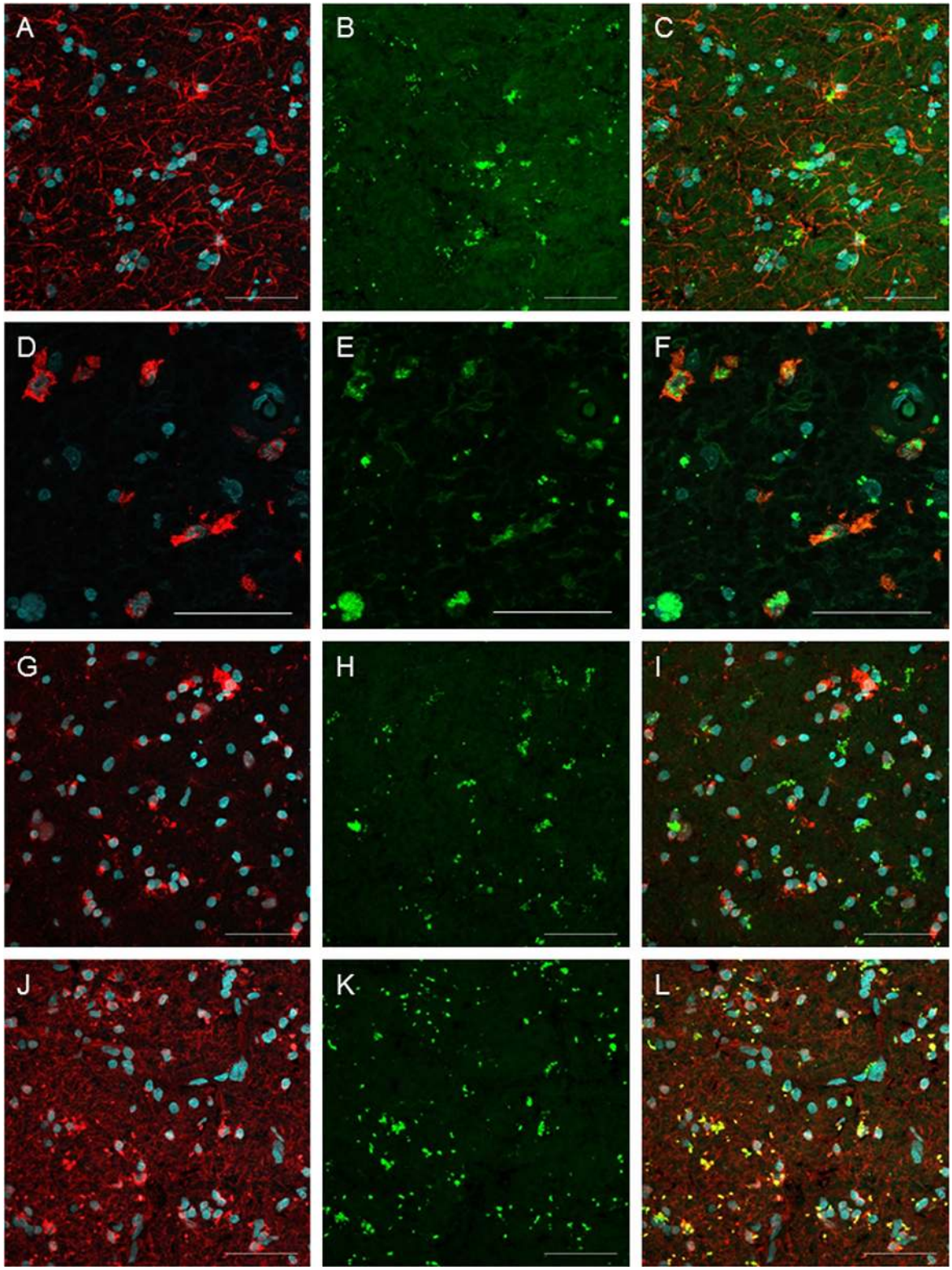

FIGURE 6. Cellular immunoreactivity for pSTAT3 surrounding MS lesions. **(A–L)** Sections were immunostained for glial fibrillary acidic protein **(A, C)**, Iba1 **(D, F)**, Nogo-A **(G, I)**, myelin basic protein **(J, L)**, and pSTAT3 **(B, E, H, K)**. Nuclei were counterstained with DAPI (blue color in **A**, **D, G, J** and **C, F, I, L**). The immunoreactivity for pSTAT3 in soma is merged (orange-yellow color in the merged images **C, F, L**) in some cells that are immunopositive for the markers of astrocytes **(C)**, macrophages/microglia **(F)**, and oligodendrocytes **(L)**. Phosphorylated STAT3 was found in some MBP-positive soma **(L)** but not detected in Nogo-A–positive soma **(I)**, both of which are markers of oligodendrocytes; the reason for this is unknown. Scale bar = 50 μm.

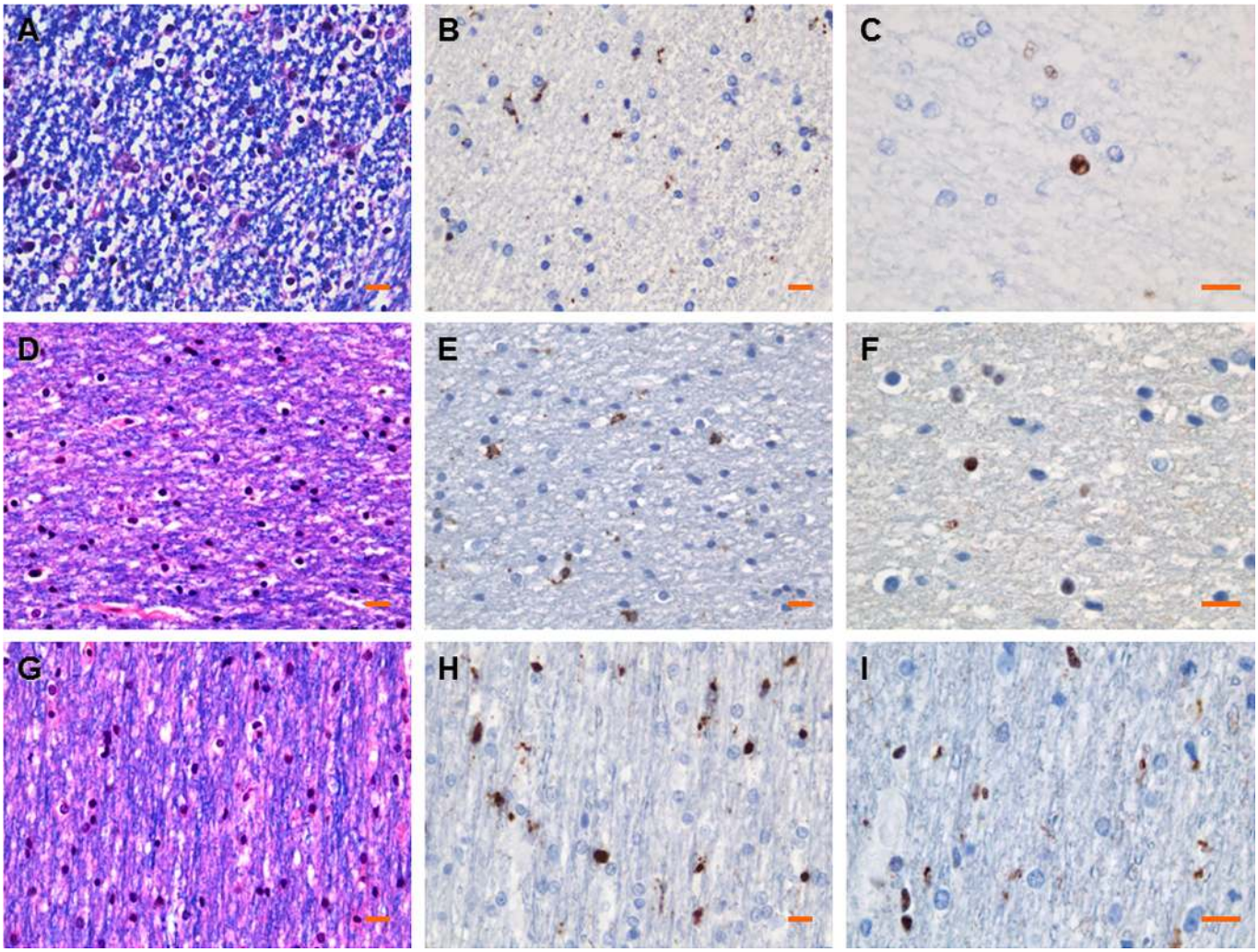

FIGURE 7. Phosphorylated STAT3 immunoreactivity in control cases. The temporal WM of a patient with AD (Table, case 12) shows normal-appearing LFB staining for myelin **(A)**, with sparse CD68-positive macrophages/microglia **(B)** and rare pSTAT3-positive cells **(C)**. **(D–I)** In a patient with amyotrophic lateral sclerosis (Table, case 14), the temporal subcortical WM shows unremarkable LFB staining **(D)**, with sparse CD68-positive macrophages/microglia **(E)** and occasional pSTAT3-positive cells **(F)**. The WM adjacent to the motor cortex demonstrates relatively preserved LFB staining **(G)** and scattered CD68-positive macrophages/microglia **(H)** and focally more numerous pSTAT3-positive cells **(I)**. Scale bar = 20 μm.

activation of STAT3 precedes cell hypertrophy and GFAP upregulation and follows a time course in response to the injury (9, 10, 12, 13). These results have suggested that STAT3 signaling plays a critical role in proliferation of astrocytes and that pSTAT3 regulates reactive astrogliosis. Activation of STAT3 has also been noted in macrophages/microglia (12) and neurons (10, 13) in rodents after CNS injury, which is confined to specific times and locations. One study in postmortem spinal cord tissue of patients with ALS found persistent activation and nuclear translocation of STAT3 and pSTAT3 immunoreactivity in both CD68-immunoreactive ramified microglia and amoeboid microglia (37). In the mouse model of neonatal WM injury, conditional deletion of *STAT3* gene induced hypertrophic reactive astrogliosis and resulted in exacerbated WM injury, which was associated with delayed maturation of oligodendrocytes (38). Another mouse study found that STAT3 has antiapoptotic effects in Olig2-positive oligodendrocytes (39). Activation of STAT3 has been reported in peripheral circulating T cells in human inflammatory diseases, including MS (22, 23) and experimental autoimmune encephalomyelitis (29). However, it is unknown how those pSTAT3-containing T cells traffic into the CNS and where they disperse within the CNS.

In the present study, pSTAT3 immunoreactivity was identified in a subset of astrocytes, microglia/macrophages, and in the soma of MBP-labeled cells despite the absence in Nogo-A–labeled cells, and preferentially in the WM adjacent to MS lesions (more in those adjacent to active lesions than inactive lesions). Within the active MS lesions, T cells and macrophages were predominantly negative for pSTAT3. Our observations are compatible with the findings of those previous studies.

Phosphorylated STAT3 immunoreactivity has thus far been noted in the brain tissue of patients with MS in only 1

study (8). Therein, pSTAT3-positive cells were observed in astrocytes but not in microglia or oligodendrocytes. The location of pSTAT3-positive cells was not specifically mentioned, however, although their examination was performed in frozen sections from tissue blocks containing MS lesions. The absence of pSTAT3 immunoreactivity in macrophages and oligodendrocytes may be caused by the location of sampling in their study, that is, their Figure 2H exhibiting pSTAT3 immunoreactivity in an active lesion appears less cellular with a few astrocytes but infrequent inflammatory cells. Their findings may be compatible with those of the present study in which pSTAT3-positive cells, including astrocytes and macrophages/microglia, are predominantly found in the WM adjacent to active lesions but rarely seen within lesions.

The activation of STAT3 and its phosphorylation are not specific to MS lesions. As previously mentioned, the activation of STAT3 and pSTAT3 immunoreactivity are observed focally in the CNS after various injuries. Phosphorylated STAT3 immunoreactivity has been found in patients with other neurologic diseases, including ALS (37), stroke, and olivopontocerebellar degeneration, and in normal subjects (8). Immunoreactivity for pSTAT3 has also been noted in surgical resections of patients with epilepsy (14). In the present study, the finding of pSTAT3 immunoreactivity in the brains of non-MS patients further suggests its nonspecific nature. Although the major focus of our present study is MS, 1 limitation of this study is the small group of non-MS cases. Several studies by the same group (22, 23, 30, 31, 40) have noted increased pSTAT3 in peripheral circulating T cells and monocytes collected from patients with MS, but it has been unclear whether this occurred within the CNS. The present study has revealed that pSTAT3 immunoreactivity is preferentially in the WM adjacent to MS lesions (more surrounding active than inactive lesions), but there was no difference within the lesions of active from inactive lesions, and inflammatory cells in active lesions are predominantly negative for pSTAT3. These results indicate that pSTAT3 immunoreactivity does not correlate with ongoing demyelination and inflammatory activity at least in this study. Instead, as suggested by many animal studies, activation of STAT3 may be a critical regulator of reactive astrogliosis particularly to restrict inflammation. Because our analysis of pSTAT3 immunoreactivity is performed at 1 time point in a limited series of tissues samples of MS, we cannot exclude that STAT3 signaling occurs in immune cells in other lesions or at a different time point during the disease process.

In summary, we report that pSTAT3-containing cells are preferentially in the areas adjacent to MS lesions but significantly less within lesions. Reactive astrocytes and some macrophages/microglia express pSTAT3, whereas lymphocytes are predominantly negative for this protein. Our findings show that pSTAT3 does not correlate with inflammatory or demyelinating activity in MS, but that it may play an important role in regulating the reactive changes adjacent to MS lesions.

ACKNOWLEDGMENTS

The authors thank Drs. Kenneth G. Warren, Edward Johnson, and Lothar Resch for contributing several cases to this study; Mr. William G. Branton for technical assistance; Ms. Yan Fan for performing double label immunofluorescence. This study is supported in part by the Multiple Sclerosis Society of Canada and Canadian Institutes of Health Research (Dr. Christopher Power and Dr. V. Wee Yong).

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