Imbalanced immunity in multiple sclerosis

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Publication date
2009

Immune suppression in MS

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Down-regulation of macrophage inhibitory molecules in multiple sclerosis lesions

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Ann. Neurol. 2007, 62 (5):504-514

Abstract

Inflammatory and demyelinating activity of activated resident macrophages (microglia) and recruited blood-borne macrophages are considered crucial in multiple sclerosis (MS) lesion development. The membrane glycoproteins CD200 and CD47, highly expressed on neurons, are mediators of macrophage inhibition via their receptors CD200R and SIRPa, respectively, on myeloid cells. We determined the expression pattern of immune inhibitory molecules in relation to genes involved in macrophage activation and MS lesion pathology. Laser dissection microscopy was combined with real time-PCR to quantitatively study these gene expression patterns in specific sub-areas (i.e. rim, centre and normal appearing white matter) of chronic active and inactive MS lesions. Hallmarks of MS pathology were confirmed by mRNA expression patterns of GFAP, NF, MBP, growth factors, chemokines and receptors, and macrophage activation markers, although expression of osteopontin and αB-crystallin was decreased. CD200 and CD47 were down-regulated in the centre of chronic active and inactive MS lesions. CD47 expression was also decreased in the rim of chronic active lesions, where complement expression was increased. This expression profile was also found in normal appearing white matter surrounding these lesions, but not surrounding inactive lesions. Expression of CD200R and SIRPa was not altered. These data suggest that diminished immune inhibition via decreased CD200 and CD47 expression contributes to a disturbed equilibrium in macrophage and microglia activation in MS lesions. Furthermore, this may result in a pro-inflammatory predisposition in the area surrounding chronic active lesions, thereby contributing to axonal injury, demyelination and possible lesion expansion.

Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) with unknown etiology. Inflammation is a possible primary feature of demyelination[133] and has been shown to be correlated with axonal damage that may reflect the progressive decline in MS.[134] Inflammatory macrophages are likely to play a crucial role in lesion development, also demonstrated in animal models of MS.[42,43,108] The extent and distribution of macrophage infiltration can be used to categorize the inflammatory activity of MS lesions.[34,135] The highest level of inflammation is found in the hypercellular active lesion, with perivascular infiltration and myelin laden macrophages. This is followed by a chronic active stage, defined as having a hypocellular and demyelinated gliotic centre, surrounded by a hypercellular rim containing foamy macrophages. When the inflammation resolves, few macrophages remain present throughout the lesion which is now considered inactive. At present, it is not understood which factors promote inflammation or the transition from chronic active into inactive lesions. Understanding how the inflammatory and possible demyelinating activity of macrophages and microglia increases during lesion formation and why progression at some point stops would help to comprehend MS pathology and will possibly contribute to development of new therapeutic strategies.

Macrophage behavior is tightly regulated by the integration of activating and inhibitory signals. The membrane glycoprotein CD200 is such an inhibitory ligand which is broadly expressed and abundantly present in the CNS and provides an inhibitory signal to myeloid cells, including macrophages and microglia, via the CD200 receptor (CD200R).[121,125,136] Inhibition through CD200R signalling occurs via phosphorylation of its cytoplasmic NPXY motif that recruits DOK1, which subsequently inhibits the Ras/MAPK pathways.[123,124] Mice lacking CD200 have an expanded and activated myeloid compartment and show a significantly accelerated development of experimental autoimmune encephalomyelitis (EAE), an animal model for MS.[125] A second immune inhibitory receptor is signal-regulatory protein α (SIRPα or SHPS-1).[137,138] SIRPα is expressed on myeloid cells and neurons and is triggered by its ligand CD47, which, like CD200, is also highly expressed in the CNS.[139-141] CD47-SIRPα interaction causes inhibition of macrophage activity via activation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs), present in the cytoplasmic part of SIRPα.[113,142,143] Furthermore, this interaction has been shown to be important in the inhibition of phagocytosis by macrophages.[144]

The abundant expression of CD200 and CD47 suggests an important role for immune inhibitory molecules in the CNS, a site that typified as being immunologically

privileged. However, it is unknown whether expression of inhibitory molecules in the CNS of MS patients is altered in a way that it might contribute to an environment with an inflammatory predisposition. Since the activation of macrophages and microglia is pivotal in MS, research has so far focused on factors stimulating this activation. However, the number of studies on immune inhibitory molecules, that may in principle be equally important in MS lesions, is limited.

In the present study we analyzed gene expression profiles of CD200, CD200R, CD47, SIRPα, and other macrophage regulatory molecules in relation to a set of genes thought to be involved in the development of MS lesions. We show that in and around lesions in MS brains, immune suppression may be hampered relative to non-MS controls, since expression of CD200 and CD47 in MS lesions is decreased.

Methods

Subjects

Snap-frozen tissue specimens of 19 MS patients containing white matter lesions and white matter tissue of 10 controls obtained at autopsy were provided by the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. Permission was obtained for brain autopsy and the use of the tissue and clinical information for research purposes. All MS patients were clinically diagnosed by a neurologist (Prof. C.H. Polman, VUMC, Amsterdam), and this diagnosis was confirmed post-mortem by a neuropathologist. Exclusion criteria for MS patients and controls were treatment with immune suppressive agents in the last three months before death, death from bacterial sepsis and having neurological disorders other than MS. At the time of death, fifteen MS patients had a secondary progressive form of the disease. One patient had a primary progressive disease course, one had a relapsing-remitting course and for two patients, the disease course could not be determined. Control subjects were age and gender matched. Mean post-mortem delay (PMD) was 8.28 h (range 4.15 – 17 h) for MS patients and 8.43 h (range 4.50 – 22.15) for control subjects. Mean age of MS patients was 59.9 years and 63.4 years for control subjects. Differences were not significant. Detailed patient characteristics are shown in Table 1.

Immunohistochemistry

Lesion inflammatory activity was characterized as described previously,[34,135] in cryostat sections (8 µm) stained for proteolipid protein (PLP) (Serotec, Oxford, UK) and HLA-DP, -DQ, -DR (DakoCytomation, Glostrup, Denmark). In brief, acetone-

fixed sections were incubated in 10% human serum in PBS for 20 minutes followed by incubation with primary antibody, diluted in PBS containing 1% bovine serum albumine (BSA), o/n at 4°C. Then, the sections were incubated with secondary antibody dilutions of biotinylated horse anti-mouse IgG, containing 10% horse serum, for 45 minutes. Staining was completed by incubation with horseradish peroxidase-conjugated avidin-biotin-complex (VectaStain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), which was visualized with DAB (Sigma Laboratories, St. Louis, MO, USA). Sections were counterstained with haematoxylin for 30 seconds, dehydrated and mounted in entallan. Nine MS lesions were characterized as chronic active, by presence of HLA-positive foamy macrophages in the rim surrounding the gliotic centre of the lesion, and 10 lesions were chronic inactive, as determined by absence of foamy macrophages. MS lesions and sections (8 µm) of cortical grey matter and spinal cord derived from healthy donors were also stained for CD200 (clone OX104, hybridoma was kindly provided by Prof. A.N. Barclay) and CD47 (Serotec).

Laser dissection microscopy

To dissect immunohistochemically defined sub-regions of MS lesions, two series of 10 sections (20 µm each) were mounted on PALM MembraneSlides (P.A.L.M. Microlaser Technologies AG, Germany) and dried 48 h at RT, in a sealed box containing silica gel. Sections preceding and following these series were stained for HLA and PLP to visualize possible alterations in the topography of the lesion (Fig. 1A, B).

Using a laser dissection microscope (LDM; Zeiss, Oberkochen, Germany), elements that represented (parts of) the centre or rim of both chronic active and inactive lesions, as well as normal appearing white matter (NAWM) surrounding the lesions were cut and isolated from the dried, unstained sections. The same elements were cut in each of the twenty sections, with slight adjustment to the elements to match alterations in lesion topography. Afterwards, sections were stained in thionine to confirm that isolated areas were properly dissected (Fig. 1C). In control white matter, comparable elements were cut as those taken from NAWM from MS patients. The isolated tissue fragments were placed directly into TRIZOL (Invitrogen Life Technologies, Carlsbad, CA, USA) such that from each control subject one sample was obtained containing white matter, and from each MS patient three samples were obtained representing precisely isolated material from centre and rim of the lesion as well as NAWM.

Table 1. Clinical and neuropathological data of donors

Patient	Sex	Age	PM time	Duration	MS type	Lesion type	Cause of death
1	m	46	3:45	23	SP	chronic active	pneumonia
2	f	40	7:00	11	SP	chronic active	aspiration pneumonia
3	f	71	8:20	24	ND	chronic active	pneumonia
4	f	69	13:20	26	RR	chronic active	probable viral infection
5	f	48	8:10	8	SP	chronic active	euthanasia
6	f	53	10:45	27	SP	chronic active	euthanasia
7	f	66	6:20	15	SP	chronic active	pneumonia
8	f	50	7:45	17	SP	chronic active	euthanasia
9	f	43	10:45	23	SP	chronic active	subdural heamatoma, pneumonia
10	f	71	10:25	24	SP	Inactive	respiratory insufficiency
11	f	52	8:25	22	SP	Inactive	respiratory failure/pneumonia
12	f	66	6:20	43	SP	Inactive	metastases and liver failure
13	f	75	8:00	42	SP	Inactive	pneumonia
14	m	77	4:15	26	PP-SP	Inactive	CVA
15	f	71	10:15	23	SP	Inactive	post-surgery respiration problems
16	f	48	4:50	25	SP	Inactive	euthanasia
17	f	55	17:00	19	SP	Inactive	possible CVA
18	f	68	7:30	39	SP	Inactive	bronchitis/ aspiration pneumonia
19	m	70	7:45	46	ND	Inactive	cardiac arrest
20	m	75	7:15	-	-	-	heart failure
21	f	80	6:15	-	-	-	dehydration
22	f	81	22:15	-	-	-	coronary shock
23	f	54	8:00	-	-	-	acute renal failure
24	f	71	4:50	-	-	-	cardiac arrest
25	f	65	12:50	-	-	-	cardiac arrest
26	f	55	5:35	-	-	-	intracerebral bleeding
27	m	48	5:30	-	-	-	euthanasia
28	f	64	6:00	-	-	-	cachexia
29	f	41	13:30	-	-	-	massive lung bleeding

PM time = post-mortem delay until end of autopsy expressed in hours:minutes; Duration = disease duration in years; MS type = type of MS at time of death; SP = secondary progressive; RR = relapsing-remitting; PP = primary progressive; ND = not determined; CVA = cerebrovascular accident.

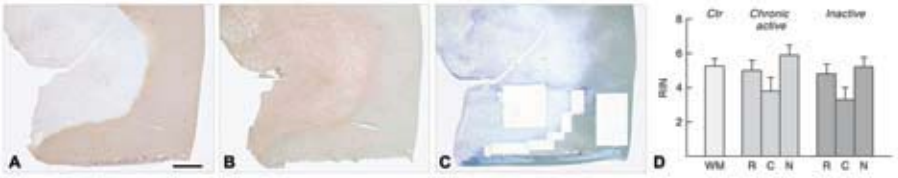


Fig. 1 Dissection of specific MS lesion areas using laser dissection microscopy. To determine immune activity and topography of the lesions, staining was performed for PLP (A) and MHC class II (B). Per patient, 2 series of 10 adjacent unstained sections (20 μm) were used to laser-dissect parts of the rim, centre and normal appearing matter of the lesion. After laser dissection microscopy, the lesion was stained with thionine (C). Quality of RNA, isolated from control white matter (WM) and from the rim (R), centre (C) and NAWM (N) of chronic active and inactive lesions, was indicated by the RNA integrity number (RIN) (D). Results are expressed as mean ± SEM, bar is 2 mm.

RNA isolation and reverse transcription

Following removal of the PALM membrane, chloroform was added to the TRIZOL lysate. An equal volume of ethanol was added to the collected aqueous phase, and this mixture was loaded onto an RNeasy Mini Kit column (Qiagen, Hilden, Germany). Further RNA isolation was performed according to the RNeasy protocol (RNeasy Mini Handbook 06/2001) and samples were stored at -80°C. RNA quantity and quality control was performed using a NanoDrop (ND-1000, NanoDrop Technologies, Rockland, DE, USA) and Bioanalyzer (2100, Agilent Technologies, Palo Alto, CA, USA), which allowed RNA quality assessment using the RNA integrity number (RIN), ranging from 1 (worst quality) to 10 (best quality).

On average 9 mm^2 brain tissue per lesion sub-area per section was isolated. The amount of total RNA isolated per sub-area from 20 sections ranged from 350 ng to 2630 ng. The pH of the cerebrospinal fluid (CSF), that can reflect post-mortem processes that might affect the quality of RNA, was significantly different ($p < 0.05$) between control subjects (pH = 6.75) and MS patients from which inactive lesions were derived (pH = 6.28). However, average RIN was 5.3 in control white matter and did not differ significantly from RIN values from rim, centre or NAWM of chronic active and inactive lesions (Fig. 1D). No correlations were found between these RIN values and qPCR results (see below for statistics).

Aliquots of 100 ng total RNA per area of nine chronic active lesions were pooled, and similarly from ten chronic inactive lesions and ten white matter controls, resulting in 0.9 μg total RNA per rim, centre and NAWM from chronic active lesions and 1.0 μg total RNA per area from chronic inactive lesions and from controls. Reverse transcription was performed in a reaction mixture of 25 μl containing 900-1000 ng total RNA, 500 μg/μl oligo(dT) and random hexamer primers (40:1) for 10 min at 80°C and 200 U/μl SuperScript II RT (Invitrogen), first strand buffer, 100 mM

dithiothreitol (DTT) and 10 mM dNTP, incubated for 1h at 42°C. All cDNA samples were diluted to a concentration of 2 ng/µl of initial total RNA and stored at -20°C, until used for further analysis.

Real-time quantitative PCR

Primer pairs for real-time quantitative PCR (qPCR) (Suppl. Table 1) were designed using the PrimerS software package for qPCR primer design (developed by R.M.H.) and specificity was tested on cDNA from brain and tonsil by assessment of the dissociation curve and PCR product as determined by size fractionation on an 8% SDS-PAGE gel. The qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with samples containing equal cDNA concentrations of 10 ng initial total RNA per reaction. Analysis was performed according to manufacturer's protocol and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Target genes were normalized to 18S ribosomal RNA. For each primer pair, the primer efficiency (Effpr) was calculated using LinRegPCR software.[145] Fold differences were calculated by $(Effpr)^{-\Delta\Delta CT}$ method.[146]

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney U test and the Spearman's correlation test. Based on the resolution of the qPCR technique, differences in gene expression ≥ 2.0 fold were considered reliable (see discussion). P-values ≤ 0.05 were considered significant.

Results

Gene expression in chronic active lesions

We measured the expression levels of 41 genes involved in macrophage regulation or in other processes expected to be important for lesion formation, on specific micro-locations within chronic active lesions. All gene expression data are presented relative to non-MS control white matter and details are listed in Supplemental Table 2.

Centre

As indicators of demyelination, axonal damage and gliosis, the hallmarks of MS, we measured the mRNA expression of myelin basic protein (MBP), neurofilament (NF) and glial fibrillary acidic protein (GFAP), respectively. MBP and NF expression was decreased in the centre of the lesion, and GFAP levels were increased (Suppl. Table

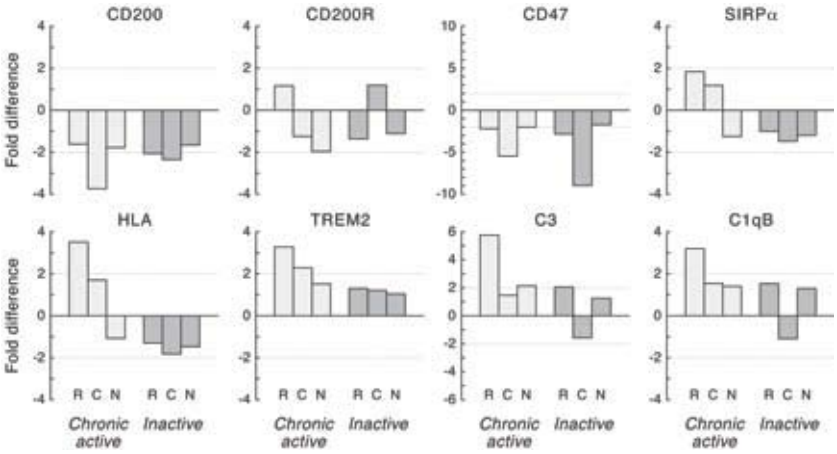


Fig. 2 Gene expression of macrophage inhibitory and activating molecules in micro-locations of MS lesions. The expression of CD200, CD200R, CD47, SIRPα, MHC class II, TREM2, C3 and C1qB was measured in pooled mRNA from the rim (R), centre (C) and NAWM (N) from chronic active and inactive MS lesions and compared to control white matter. Dotted lines indicate a two-fold difference (see Methods and Discussion sections).

2). Of macrophage activation markers, only triggering receptor expressed on myeloid cells 2 (TREM2) expression was found to be increased (Fig. 2). Further activity of the immune system was indicated by the increased expression of the chemo-kines CCL3 and CCL5 and the chemokine receptor CCR5. However, the cytokine profile was anti-inflammatory, as IL-10 expression was high and that of IL-12 and IL-23 was low compared to controls (Fig. 3). Expression of both immune suppressive molecules CD47 and CD200 was decreased, whereas expression of their receptors SIRPα and CD200R was not altered (Fig. 2). Remarkably, the expression of two molecules that are suggested to play a role in lesion development, αB-crystallin and osteopontin, were decreased. Furthermore, expression of the glucocorticoid receptor (GR) was decreased and expression of the estrogen receptor α (ERα) was highly increased.

Rim

In the rim of chronic active lesions, NF and MBP expression was decreased and that of GFAP was increased, like in the centre of the lesions. The hallmark of chronic active lesions is that the rim contains highly activated macrophages. Indeed, we found increased levels of HLA, CD11b, DAP12 and TREM2 (Fig. 2). Also, expression of the complement factors C3 and C1qB was increased (Fig. 2). The expression of CD47 was decreased, whereas expression of CD200 was not different from controls and expression of their receptors was unaltered as well (Fig. 2). The higher immune activity was further reflected by the expression of chemokines (CCL3, CCL5 and

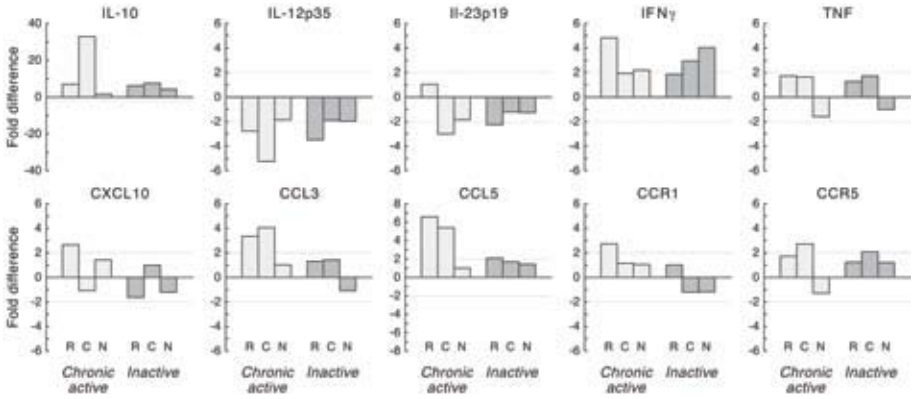


Fig. 3 Gene expression of cytokines, chemokines and chemokine receptors in micro-locations of MS lesions. Expression of IL-10, IL-12p35, IL-23p19, IFN-γ, TNF, CXCL10, CCL3, CCL5, CCR1 and CCR5 was measured in pooled mRNA from the rim (R), centre (C) and NAWM (N) from chronic active and inactive MS lesions and compared to control white matter. Dotted lines indicate a two-fold difference (see Methods and Discussion sections).

CXCL10), chemokine receptor (CCR1) and of the cytokine IFN-γ, although, similar to the centre of the lesions, IL-10 expression was increased and IL-12 expression decreased (Fig. 3).

NAWM

Only few molecules were found to be differentially expressed in the NAWM surrounding chronic active lesions. Remarkably, expression of NF and MBP were again decreased relative to controls. However, GFAP expression was not altered. Complement factor C3 expression was increased and expression of CD47 was decreased (Fig. 2). Furthermore, IFN-γ expression was increased and that of IL-17 was decreased.

Gene expression in inactive lesions

Gene expression profiles were also determined on micro-locations of inactive lesions, where macrophages have ceased their (phagocytic) activity. Therefore, the rim of an inactive lesion is a sharp boundary between the gliotic tissue and surrounding normal appearing white matter. Results of the gene expression profile analysis in inactive lesions are listed in Supplemental Table 2.

Centre

In the gliotic centre of inactive lesions, NF and MBP expression was decreased and GFAP was increased. The expression levels of CD47 and CD200 were decreased. Expression of their receptors was not different from controls, neither was the expres-

sion of molecules involved in macrophage activation (Fig. 2). Low immune activity was found as only CCR5 and IFN-γ expression was increased. Like in chronic active lesions, IL-10 expression was highly increased (Fig. 3). Also comparable to the centre of chronic active lesions was the decreased expression of GR, αB-crystallin and osteopontin and the increased expression of ERα.

Rim

In the rim of inactive lesions, NF and MBP expression was decreased and GFAP expression was increased. Also, the expression of both CD47 and CD200 was decreased and complement factor C3 expression was increased (Fig. 2). Further indication of activity of the immune system was restricted to increased CCL5 expression. Similar to the centre of chronic active and inactive lesions, IL-10 expression was high, whereas that of IL-12 and IL-23 was lower compared to controls (Fig. 3). We detected decreased expression of αB-crystallin, osteopontin and GR, and an increased expression of ERα.

NAWM

In contrast to the NAWM adjacent to chronic active lesions, NF expression was not altered. Also GFAP expression was comparable to controls, but MBP expression was decreased. No change in expression was found for CD200, CD47, CD200R and SIRPα (Fig. 2). In this area, expression of IL-10 and IFN-γ was increased (Fig 3.). Like other sub-areas of inactive lesions, expression of GR and osteopontin was decreased and that of ERα was increased.

T cell transcripts are hardly detectable in chronic active and inactive MS lesions

To determine the contribution of T cells in these lesions, we included measurement of the T cell marker CD3. In controls, rim of inactive lesions or NAWM of both types of lesions, CD3 was not detected (Ct>40). Only very low abundant transcript levels were detected in the rim of chronic active lesions (Ct = 36.23), and in the centre of both chronic active and inactive lesions (Ct = 34.86 and 38.03, respectively).

Individual gene expression closely resembles pooled mRNA data

The amount of RNA obtained from LDM-dissected lesion areas was insufficient to 1) repeatedly measure each gene, and 2) to determine the expression of every gene on each individual patient. To address these issues, the expression of a few selected genes (CD200, CD200R, CD47, SIRPα and osteopontin) was repeatedly measured in the sub-areas of 4 chronic active and 4 inactive individual lesions, and were compared with 4 controls. Expression of these genes was also determined on

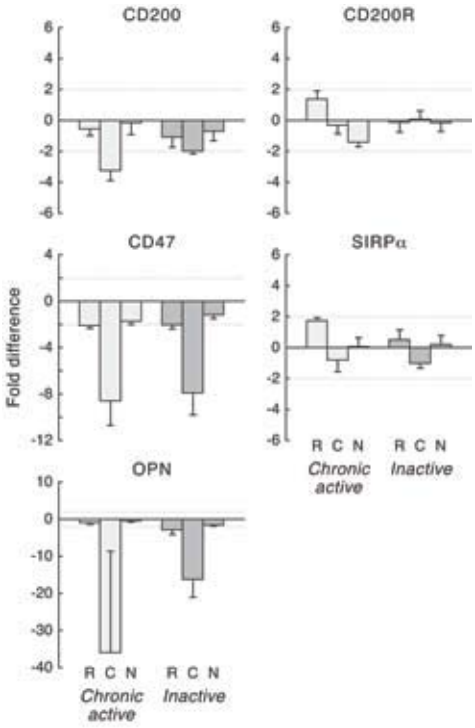
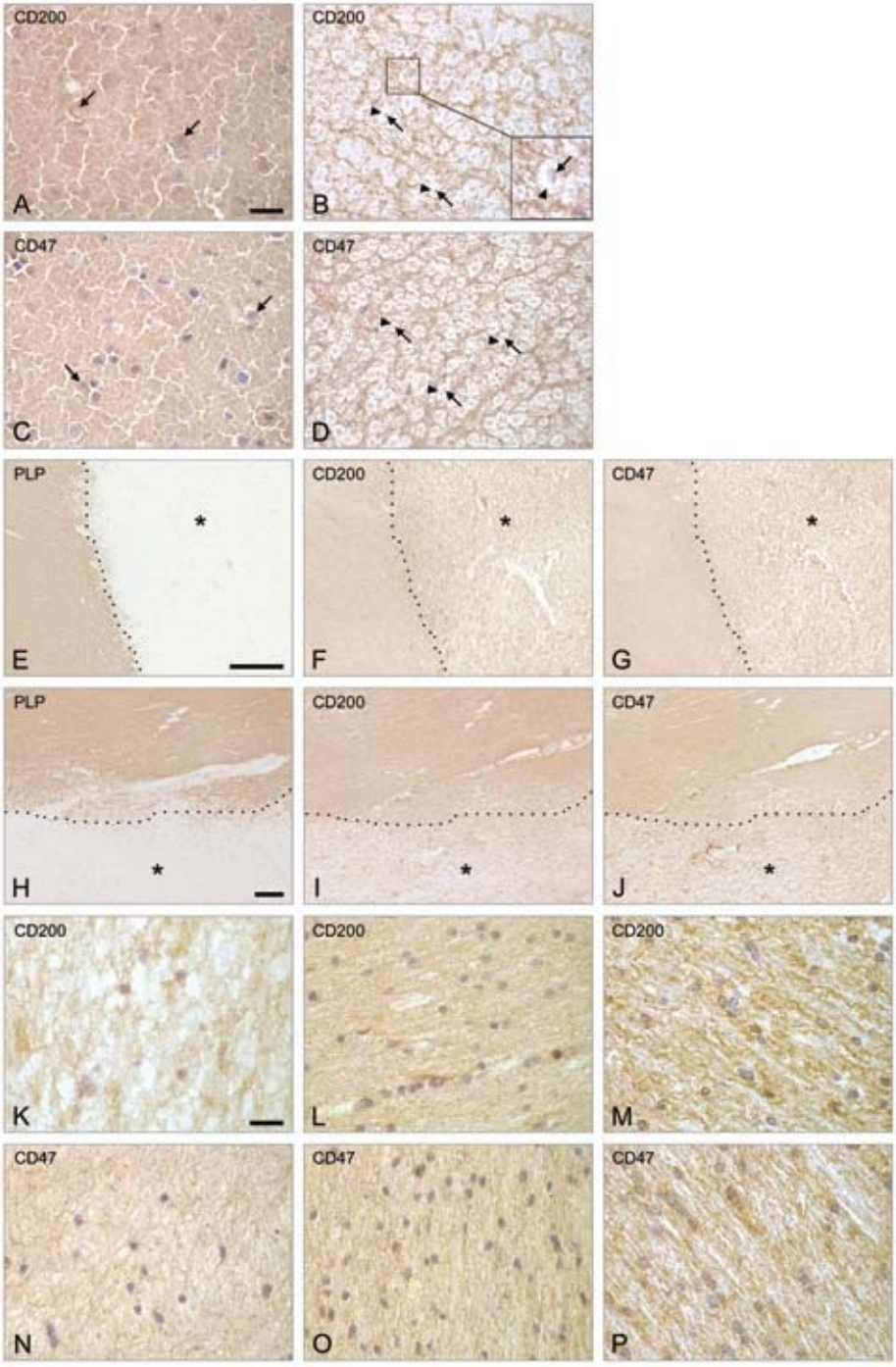


Fig. 4 Gene expression of CD200, CD200R, CD47, SIRPα and osteopontin (OPN) in MS lesions. Gene expression was determined in three experiments. In the first experiment gene expression was measured in pooled RNA per sub-area of all chronic active and all inactive lesions (see Fig. 1). In the second experiment expression was determined in 4 individual chronic active and 4 inactive lesion areas (mean of 3 measurements) and in the third experiment expression was determined in pooled cDNA of lesion sub-areas of these 4 individual chronic active and inactive lesions (mean of 2 measurements). Data are expressed as mean of the three experiments ± SEM.

pooled cDNA from each sub-area from these individuals. Low variability between the experiments was found, as shown by the combined data of gene expression in individual patients, in the pool of individual patients and in the pool of all patients (Fig. 4). Furthermore, individual values confirmed the data of these genes obtained from the pool of all patients, with no significant change in expression of CD200R and SIRPα, and a significant decrease in expression of CD200 ($p<0.05$), CD47 ($p<0.05$) in the centre of chronic active and inactive lesions (Suppl. Fig. 1). The expression of CD47 tended to be decreased in the rim of chronic active lesions and the NAWM surrounding it, but in these 4 individuals, the difference was not significant. Osteopontin expression was decreased in the centre of chronic active and inactive lesions ($p<0.01$) and also in the rim of inactive lesions ($p<0.05$). As these data are contradicting previously published data on osteopontin expression,[147,148]


CD200
A
CD200
B
CD47
C
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D
PLP
E
CD200
F
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PLP
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we measured osteopontin expression with a second primer set that was used in the study of Tajouri *et al*. Again, in our tissue samples, osteopontin expression was decreased to a comparable extent as determined with the first primer set (data not shown).

Protein expression of CD200 and CD47 is down-regulated in MS lesions

We stained sections of 6 chronic active and 6 inactive lesions with CD200 and CD47 mAb to investigate protein expression in MS lesions. Immunoreactivity to these molecules was high in the CNS, with the most intense expression mainly found in grey matter, confirming previous findings.[120] Neuronal cell bodies were found positive in cortical grey matter from controls (Fig. 5A, C). Transverse sections of spinal cord from controls show immune-reactivity for both CD200 and CD47 on axons. No reactivity was present on compact myelin sheaths, but structures surrounding them are positive (Fig. 5B, D). On low magnification a chronic active and inactive lesion, respectively, was visualized by PLP staining (Fig 5E, H). Expression of both CD200 and CD47 was diminished within the centre of chronic active (Fig. 5F, G) and inactive MS lesions (Fig. 5I, J) when compared to the surrounding white matter. Note that RNA expression of CD200 and CD47 was compared to white matter from controls. CD200 and CD47 staining is also shown on higher magnification in the centre of a lesion (Fig. 5K, N), NAWM surrounding the lesion (Fig. 5L, O) and in white matter from control subjects (Fig. 5M, P).

Discussion

In the present study, we analyzed sub-areas of MS lesions, isolated by immunohistochemically-guided laser dissection microscopy. This technique is very precise, but the amount of RNA that can be isolated from these small tissue samples is

< Fig. 5 Protein expression of immune suppressive molecules. Sections of cortical grey matter derived from controls show CD200 (A) and CD47 (C) positive neurons (arrows). Furthermore, the surrounding tissue is also intensely stained. Transverse sections of the spinal cord from control subjects were stained for CD200 (B) and CD47 (D). Axons (arrows) are weakly positive for CD200 (enlarged in the inset), and are more intensely stained for CD47. The compact myelin sheaths surrounding the axons are unstained (arrowheads), but structures surrounding the myelin are positive. Bar is 25 µm. Low magnification of sections of a chronic active MS lesion, stained for PLP, (E) CD200 (F) and CD47 (G) shows absence of PLP intensity in the lesion center (asterisk), and presence of PLP in the NAWM at the other side of the border (dotted line) The same was done for sections of inactive lesions, stained for PLP (H), CD200 (I) and CD47 (J). In both types of lesions, the expression of CD200 and CD47 is less intense in the lesion centre than in the adjacent NAWM. Bars are 300 µm. High magnifications are shown for CD200 and CD47 respectively in the center of a lesion (K, N), the NAWM (L, O) and in white matter from controls (M, P). Bar is 25 µm. Data are representative for 6 chronic active and 6 inactive lesions.

low. Therefore it was necessary to perform the main part of this study on pooled RNA samples as this had the most important advantage that as many as 41 genes could be measured. To compensate for the lack of information on individual variability, a few genes that were considered important in this study were chosen and analyzed, using samples from individual patients and controls, on RNA that was retained after pooling the RNA samples. On the results from these experiments, statistical analysis was performed, which confirmed the findings from the data set derived from pooled RNA (Suppl. Fig. 1). Furthermore, we considered the data from real-time quantitative PCR experiments to show a reliable difference when the difference in transcript levels was at least two-fold. We chose this cut-off value because the resolution of real-time PCR is theoretically limited to a difference in cycle threshold of 1, which corresponds to a 2-fold difference, since this technique is based on doubling the target template each cycle. However, it should be kept in mind that differential expression of a molecule by a factor less than 2 could still have a biological effect.

Figure 6 gives an overview of pathological events in well defined sub-locations in MS lesions based on the present gene expression analysis. We used chronic active lesions, defined by having a hypercellular rim consisting of foamy macrophages, and inactive lesions, in which foamy macrophages are absent. From Figures 2 and 6 it is clear that the expression levels of the macrophage specific molecules HLA, CD11b, DAP12 and TREM2 were increased almost exclusively in the rim of chronic active lesions. The expression of the astrocyte marker GFAP appeared to be increased in all areas where gliosis can be found, i.e. in the rim and centre of both chronic active and inactive lesions, as shown in Fig. 6. The growth factors BDNF and NGF were decreased or not altered, indicative of a lack of repair mechanisms.

The expression levels of pro-inflammatory chemokines and chemokine receptors confirm a prominent degree of inflammation especially in chronic active lesion areas.[1] However, the unchanged expression levels of IL-1β and TNF, the decreased expression of IL-23p19, and the high level of IL-10 suggest that the chronic active lesions used in the present study probably have gone past the peak level of inflammation. In addition, based on the CD3 expression levels, there is little evidence for the presence of T cells in these stages of MS lesions, although the expression level of IFN-γ was increased. The source for this might be either NK cells or even macrophages that have previously been reported to produce IFN-γ under inflammatory conditions.[149,150]

Although the expression patterns of MBP, NF, GFAP and growth factors, summarized in Figure 6, fit the general model on pathological events in MS lesions like demyelination, axonal damage, gliosis and lack of repair mechanisms, some

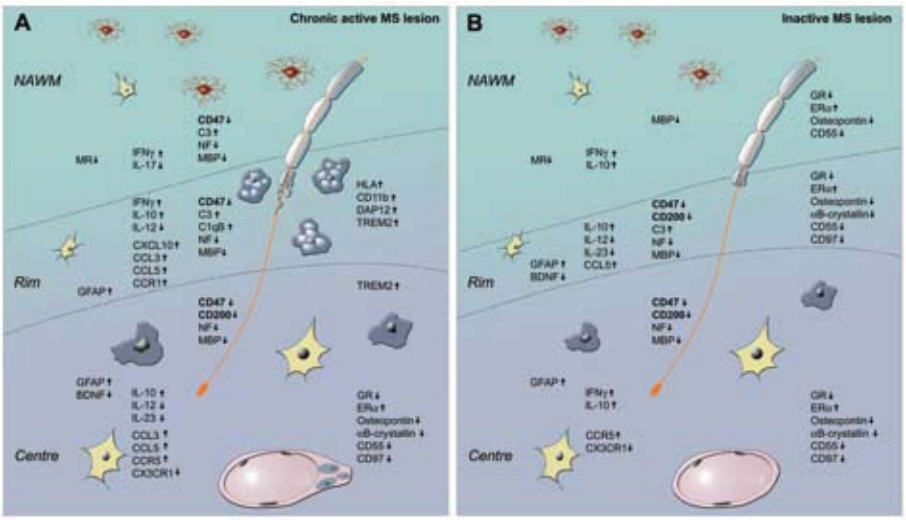

Fig. 6 Overview of gene expression profiles of genes involved in macrophage regulation, inflammation and lesion development in specific areas of chronic active (A) and inactive MS lesions (B). Expression of the immune-inhibitory molecules CD200 and/or CD47 was lower than controls in both chronic active and inactive lesions and in NAWM surrounding chronic active lesions. Most inflammatory genes were found to be expressed in chronic active lesions. Elevated expression of complement was found in the rim of both lesion types and also in NAWM around chronic active lesions. Furthermore, neuronal and oligodendrocyte-specific genes (NF and MBP, respectively) were expressed at highly reduced levels in lesions and the latter also in surrounding tissue. The astroglial gene GFAP was expressed at high levels in the lesions. Of the steroid hormones, GR expression was decreased in the centre of chronic active lesions and in all isolated regions of inactive lesions, whereas expression of ERα in these areas was increased. In the centre of both lesion types and in the inactive rim, αB-crystallin and osteopontin expression was decreased, the latter also in NAWM around inactive lesions. See text for details on the specific molecules.

remarkable differences with other studies were found.[147,148] For example, Chabas and colleagues detected in a high-throughput screening of expressed sequence tags abundant transcripts of αB-crystallin and osteopontin in two non-normalized cDNA libraries of MS tissue, which were not detected in the cDNA library of a control brain. Tajouri and colleagues detected an increase of osteopontin expression of over 50-fold in acute plaques, but a much smaller increase of up to 5-fold in chronic active plaques.[148] Acute lesions were not included in the present study, but in chronic active and inactive lesions, we detected osteopontin levels that were strongly reduced, especially in the centre of both lesion types, compared to white matter from non-MS controls (Fig. 6). These data were confirmed by a second primer pair for osteopontin that was previously used by Tajouri *et al*. The inconsistency with the former studies may be explained by the difference in techniques that were used in the study by Chabas *et al*., the smaller sample size of the other

studies, or the possibility that the control brain tissue was taken from different anatomical locations.

Steroid hormone receptors are described to have immune modulating properties in the CNS. A role for estrogens in neuroprotection has been proposed for a variety of neurological disorders.[151] Studies using MRI showed that treatment with estrogens or derivates resulted in decreased lesion numbers and volumes in MS patients[152] and decreased inflammation and demyelination in EAE.[153] Therefore it is interesting that the present study shows high expression levels of the estrogen receptor in MS lesions (Fig. 6), as these may, upon stimulation, exert beneficial effects in MS. Interestingly, GR expression was diminished in the centre of both lesion types and also in the rim and surrounding NAWM of inactive lesions. This may be a relevant finding as corticosteroids are one of the most commonly applied treatments of MS relapses.[154]

To our knowledge, this is the first study to reveal the decreased expression pattern of CD200 and CD47 in MS lesions. Importantly, expression of their receptors CD200R and SIRPα was unaltered. Biological importance of altered expression levels of molecules may be determined by several factors, for example the function of the molecule, the distribution pattern of the molecule and the possibility of the ligand to interact with its receptor. CD200 and CD47 are immune suppressive molecules that are highly abundant in the CNS. The CNS is protected by several mechanisms against harmful effects from the immune system and is thus called 'immune-privileged'. It is very likely that the presence of the normally high levels of CD200 and CD47 contribute to the immune-privileged state of the CNS. CD200 and CD47 can specifically inhibit macrophage/microglia activity. Absence of either CD47 or SIRPα in the presence of activating signals via opsonization increases phagocytic activity of macrophages.[115,144] It is thus interesting that in areas where lesions can expand, like the rim of chronic active lesions and NAWM surrounding it, expression of CD47 was diminished and expression of C3, and in the rim also C1qB, was increased suggesting a shift in balance that promotes macrophage phagocytic and inflammatory activity in and around expanding chronic active lesions and thereby likely contributes to lesion expansion and axonal injury. In the rim of inactive lesions, CD47 and CD200 expression was also reduced and expression of C3 was increased. However, the finding that in the NAWM surrounding inactive lesions, expression of these molecules was restored to levels comparable to controls (Fig. 6), suggests re-establishment of an environment that suppresses macrophages in their activity and may therefore comprise a mechanism to cause lesion progression to halt. CD47 and CD200 are expressed on neurons and their receptors on microglia. This implicates that neuron-microglia interaction is important to regulate

microglia. In the absence of CD200, microglia are spontaneously activated and EAE develops more rapidly.[125] On the other hand, up-regulated CD200 expression due to altered ubiquitination in mice with a spontaneous mutation in the *Wld*^{s} gene mediates axonal protection during EAE.[129] Blocking CD200 interactions in these mice significantly worsened the disease. Not only neurons, but also glial cells may express CD47 and CD200. These cells may therefore also serve to protect neurons as they may also contribute to maintain immune suppression via these molecules. In the present study we found that in all areas where CD200 or CD47 expression was decreased, also expression of NF and MBP was decreased (Fig. 6). Loosing 2- to 9-fold of the expression of CD200 and CD47 is thus a strong indication that immune inhibition and thereby also the protection of neurons is hampered.

As mentioned before, it is highly unusual for the CNS to get inflamed due to its immune-privileged status. MS is one of the very few diseases in which CNS inflammation in the parenchyma does occur. CD200 and CD47 bind to receptors on myeloid cells, so a decreased expression of CD200 and CD47 mainly affects macrophages and microglia. As macrophages are thought to be crucial effector cells in MS, it is therefore likely that decreased myeloid cell inhibition has specific implications for this disease. Although it is unknown whether CD200 and CD47 expression is altered in other human CNS disorders, studies in *Wld*^{s} mice, that have increased levels of CD200, showed that neurons are protected against several forms of axonal injury other than induced by EAE, such as Wallerian degeneration after peripheral and CNS nerve transections, and after apoptosis of the neuronal cell bodies.[155-157]

In conclusion, in this study the expression levels of multiple genes were characterized in specific MS lesion areas. The expression of the immune inhibitory molecules CD200 and CD47 is normally high in the immune-privileged CNS, but expression is decreased in MS lesions. This suggests that the balance in macrophage regulation has shifted towards activation by a hampered inhibitory input via CD200 and CD47 in areas where myelin and axonal damage are found. Combined with presence of immune activating molecules, this microenvironment may promote ongoing inflammation around chronic active lesions with irreversible axonal damage as a consequence and a possible enhancement of phagocytic activity that can lead to expansion of the lesion. In terms of therapeutic interventions we show that expression of the receptors SIRPα and CD200R was not changed, making them a potential target for artificial ligand ligation to maintain or restore the balance in macrophage inhibition and to halt inflammation and possibly phagocytosis early in lesion development, thereby preventing the extension of axonal injury.

Acknowledgments

We thank Dr. Jörg Hamann, Prof. René van Lier and Prof. Dick Swaab for helpful discussions. This study was supported by a grant of the Dutch Foundation MS Research (MS02-496).

Supplemental data

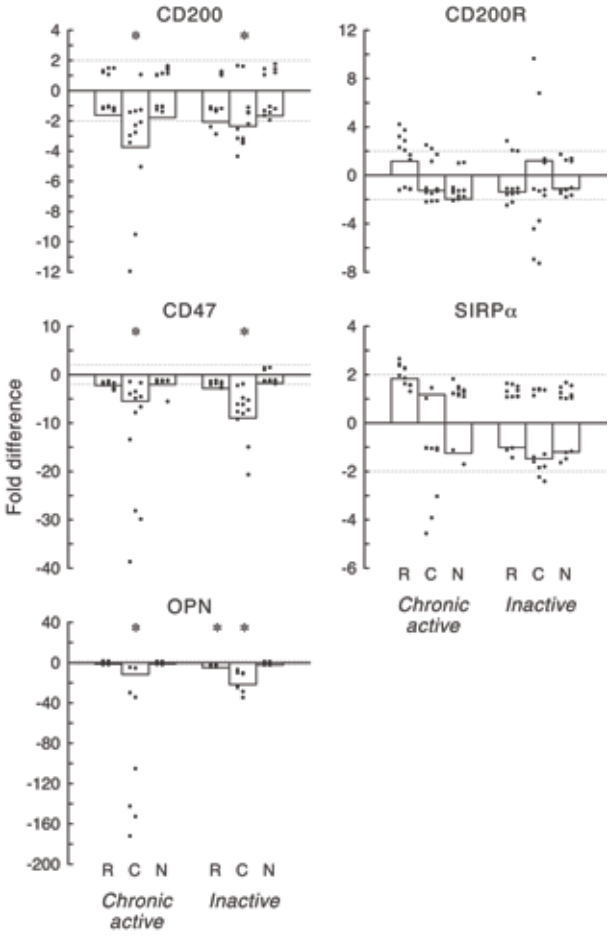

Fig. 1 Gene expression in 4 chronic active and 4 inactive MS lesions. Individual gene expression data are shown for CD200, CD200R, CD47 and SIRPα (3 experiments) and for osteopontin (OPN) (2 experiments). The bar represents the value obtained from the pooled sub-areas of chronic active and inactive lesions. Significant changes are indicated with *.

Table 1. Primer sequences

Protein	Database no	Primer sequence forward	Primer sequence reverse
HLA-DR	NM_019111	CCCAGGGAAGACCACCTTT	CACCCTGCAGTCGTAAACGT
CD11b	NM_000632	TGCTTCCTGTTTGGATCCAACCTA	AGAAGGCAATGTCACTATCCTCTTGA
DAP12	NM_003332	GGGTCAGAGGTCGGATGTCTA	AGGAATGGCTGGATCCAGGTAT
TREM2	NM_018965	CCACCCACTTCCATCCTTCT	GTCCCTGGCTTCTGTCCAT
CD55	NM_00574	GGGCAGTCAATGGTCAGATATTGA	GGCTGTTTGAGGGATGCAGAAT
CD97	NM_078481	GGTGCTGACCTATGTGTTTACCA	AGGCCCACTTCCGGTATTCTT
CD200	NM_005944	CCAGGAAGCCCTCATTGTGA	TCTCGCTGAAGGTGACCATGT
CD47	NM_001777	ATGGAGCTCTAAACAAGTCCACTGT	TGTGAGACAGCATCACTCTTATCCAT
CD200R	NM_138806	GAGCAATGGCACAGTGACTGTT	GTGGCAGGTCACGGTAGACA
SIRPα	NM_080792	GTCTGGAGCAGGCACTGA	GGACTCGCAGGTGAAGCT
TGFβ2	NM_003238	GCTGGAGCATGCCCGTATTTAT	CGCAGCAAGGAGAAGCAGAT
IL-1β	NM_000576	CCGACCACCACTACAGCAA	GGCAGGGAACCAGCATCT
IL-17	NM_002190	CAACCGATCCACCTCACCTT	GCACTTTGCCTCCCAGATCA
TNF	NM_000594	GGCGTGGAGCTGAGAGATA	CAGCCTTGGCCCTTGAAGA
IFN-γ	NM_000619	GCAAGATCCCATGGGTTGTGT	CTGGCTCAGATTGCAGGCATA
IL-10	NM_000572	TGCCTTCAGCAGAGTGAAGACTT	TCCTCCAGCAAGGACTCCTTTA
IL-12 p35	NM_000882	GGAGGCCTGTTTACCATTGGA	GGCCAGGCAACTCCCATTA
IL-23 p19	NM_016584	GGATCCACCAGGGTCTGATT	CCCAGTAGGGAGGCATGA
IP-10	NM_001565	TACGCTGTACCTGCATCAGCATT	GCAATGATCTCAACACGTGGACAAA
CCL3	NM_002983	GCAGCAGACAGTGGTCAGT	GTGCAGAGGAGGACAGCAA
CCL5	NM_002985	TCTGTGACCAGGAAGGAAGTCA	GAGACGGCGGAAGCTTAAGA
CCR1	NM_001295	ACGGAGGTGATCGCCTACA	AACAACTGCCGCAGGTACTT
CCR5	NM_000579	TCTTCTGGGCTCCCTACAACATT	CTGCATAGCTTGGTCCAACCT
CX3CR1	NM_001337	TTGGCCTGGTGGGAAATTTGT	AGGAGGTAAATGTCGGTGACACT
Fractalkine	NM_002996	CAGGCCACAAGACCCTTGT	GCCCAGCCTCAGGAAAGAAT
SEMA 3A	NM_006080	CCTTACTGTGCTTGGGATGGT	AGTGAGTCAGTGGGTCTCCAT
MR	NM_002438	TGCAGAAGCAAACCAAACCT	CAGGCCTTAAGCCAACGAAACT
GM-CSF	NM_000758	CCCGGAAACTTCCTGTGCAA	CTGGCTCCCAGCAGTCAA
NGF	NM_002506	TGTACCACGACTCACACCTTTGT	CACAGGCCGTATCTATCCGGATAA
BDNF	NM_170735	GGGACGGTCACAGTCCTT	CCCATGGGATTGCACTTGGT
c-KIT	NM_000222	TGCACTGATCCGGGCTTT	TGTTGGTGGCTTCTGCCTTT
NF	NM_021076	CCCAGCTGCGAGAATACCA	CCAAAGCCAATCCGACACTCT
MBP	NM_001025081	GGGTCTTCCTGGAGATTTGGT	GCTGTGGTTTGGAAACGAGGTT
αB-crystallin	NM_001885	GTCCTCACTGTGAATGGACCAA	GGTGACAGCAGGCTTCTCTT
Osteopontin	NM_001040058	CGAGGACATCACCTCACACA	CCACGGCTGTCCCAATCA
GFAP	NM_002055	CCGCCACTTGCAGGAGTA	GGGAATGGTGATCCGGTTCT
BZRP	AY998017	TACCGTGGCCTGGTACCA	TCCCGCCATACGCAGTAGTT
GR	NM_001018077	GCCAAGGATCTGGAGATGACAA	GAGGAGAGCTTACATCTGGTCTCA
ERα	NM_000125	ACGGTTCAGATAATCCCTGCTG	CCATTGGTGTTGGATGCATG
C3	NM_000064	CGGACGGTCATGGTCAACAT	ATGTCCCAAGACAAGGGCAAGA
C1qB	NM_000491	CCGCTTCGACCACGTGAT	AGTAGAGACCGGGCACCTT

Table 2. Fold change in gene expression in MS lesions relative to control white matter

	Chronic active lesion			Inactive lesion		
	Rim	**Centre**	**NAWM**	**Rim**	**Centre**	**NAWM**
HLA	3.51	1.70	-1.07	-1.30	-1.82	-1.47
CD11b	2.25	1.55	-1.11	1.00	-1.11	-1.40
DAP12	3.34	1.26	-1.04	-1.08	-1.81	-1.20
TREM2	3.28	2.29	1.52	1.30	1.21	1.04
CD55	-1.94	-5.82	-1.70	-3.14	-11.34	-2.14
CD97	-1.03	-2.58	-1.55	-2.81	-8.26	-1.95
CD200	-1.62	-3.74	-1.78	-2.06	-2.35	-1.66
CD47	-2.21	-5.48	-2.02	-2.83	-8.97	-1.73
CD200R	1.16	-1.25	-1.96	-1.37	1.19	-1.11
SIRPα	1.84	1.18	-1.25	-1.01	-1.48	-1.20
TGFβ2	1.65	-1.11	-1.04	1.09	-1.19	-1.14
IL-1β	1.04	-1.33	-1.35	-1.14	1.18	1.07
IL-17	-1.09	-1.18	-2.03	-1.51	1.35	-1.43
TNF	1.73	1.66	-1.58	1.28	1.73	-1.01
IFN-γ	4.82	1.93	2.19	1.85	2.94	4.04
IL-10	7.03	32.93	1.71	6.18	7.59	4.31
IL-12	-2.77	-5.23	-1.84	-3.48	-1.87	-1.96
IL-23	1.05	-3.00	-1.84	-2.24	-1.20	-1.24
IP-10	2.66	-1.07	1.43	-1.62	1.00	-1.19
CCL3	3.36	4.06	1.03	1.32	1.43	-1.08
CCL5	6.58	5.39	1.00	2.10	1.70	1.43
CCR1	2.74	1.16	1.05	1.02	-1.19	-1.16
CCR5	1.74	2.73	-1.28	1.25	2.08	1.21
CX3CR1	-1.43	-2.01	-1.40	-1.77	-2.76	-1.68
Fractalkine	1.66	1.07	-1.14	1.34	1.38	1.20
SEMA 3A	1.25	1.13	1.24	1.29	1.43	1.30
MR	-1.05	-1.25	-2.77	-1.81	1.38	-3.63
GM-CSF	1.49	-1.49	-1.19	-1.46	1.34	1.02
NGF	1.08	-1.63	-1.19	-1.44	1.23	-1.21
BDNF	1.00	-3.63	-1.52	-2.65	-1.58	-1.63
c-KIT	-2.42	-2.25	-2.85	-2.72	-1.82	-1.67
NF	-2.33	-2.54	-4.69	-7.27	-5.05	-1.96
MBP	-3.94	-59.53	-2.04	-3.56	-142.33	-2.15
αBcrystallin	-1.89	-5.13	-1.98	-2.03	-4.52	-1.50
Osteopontin	-1.22	-11.64	-1.11	-4.88	-21.57	-2.10
GFAP	3.08	3.60	1.48	2.74	3.16	1.86

	Chronic active lesion			Inactive lesion		
	Rim	**Centre**	**NAWM**	**Rim**	**Centre**	**NAWM**
BZRP	1.24	1.24	-1.24	-1.09	-1.40	-1.12
GR	-1.79	-4.23	-1.74	-4.23	-7.11	-2.08
ERα	1.89	18.57	1.40	9.02	7.40	6.54
C3	5.77	1.47	2.14	2.06	-1.57	1.25
C1qB	3.20	1.54	1.40	1.53	-1.09	1.30