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Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection

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Multiple sclerosis is a chronic inflammatory disease of the CNS. Although progressive axonal injury and diffuse inflammatory damage has been shown in the chronic phase of the disease, little is known about the molecular mechanisms underlying these pathological processes. In order to identify these mechanisms, we have studied the gene expression profile in non-lesion containing tissue, the so-called normal-appearing white matter (NAWM). We performed differential gene expression analysis and quantitative RT-PCR on subcortical white matter from 11 multiple sclerosis and 8 control cases. Differentially expressed genes were further analysed in detail by *in situ* hybridization and immunofluorescence studies. We show that genes known to be involved in anti-inflammatory and protective mechanisms such as STAT6, JAK1, IL-4R, IL-10, Chromogranin C and Hif-1α are consistently upregulated in the multiple sclerosis NAWM. On the other hand, genes involved in pro-inflammatory mechanisms, such as STAT4, IL-1β and MCSF, were also upregulated but less regularly. Immunofluorescence colocalization analysis revealed expression of STAT6, JAK1, IL-4R and IL-13R mainly in oligodendrocytes, whereas STAT4 expression was detected predominantly in microglia. In line with these data, *in situ* hybridization analysis showed an increased expression in multiple sclerosis NAWM of HIF-1α in oligodendrocytes and HLA-DRα in microglia cells. The consistency of the expression levels of STAT6, JAK1, JAK3 and IL-4R between the multiple sclerosis cases suggests an overall activation of the STAT6-signalling pathway in oligodendrocytes, whereas the expression of STAT4 and HLA-DRα indicates the activation of pro-inflammatory pathways in microglia. The upregulation of genes involved in anti-inflammatory mechanisms driven by oligodendrocytes may protect the CNS environment and thus limit lesion formation, whereas the activation of pro-inflammatory mechanisms in microglia may favour disease progression. Altogether, our data suggests an endogenous inflammatory reaction throughout the whole white matter of multiple sclerosis brain, in which oligodendrocytes actively participate. This reaction might further influence and to some extent facilitate lesion formation.

Keywords: multiple sclerosis; inflammation; cytokines; chemokines; neuroprotection

Abbreviations: NAWM = normal-appearing white matter; EAE = experimental autoimmune encephalomyelitis; JAK = Janus tyrosine kinases; STAT = signal transducers and activators of transcription; MTI = magnetic transfer imaging

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS. Studies of the histopathology of the demyelinated lesions characteristic of this disease (Raine and Scheinberg, 1988; Lucchinetti *et al.*, 2000) have revealed a great deal of heterogeneity at the cellular and molecular level, which might partially reflect the diversity of the clinical disease course. A major effort in the study of the pathogenesis of MS has been to understand the molecular mechanisms of lesion formation (Lucchinetti *et al.*, 2000; Lock *et al.*, 2002), and in particular the role of various immune-modulating components in the different types of lesions (Lassmann and Ransohoff, 2004).

Pathological similarities to the animal model for Th1-mediated brain inflammation, experimental autoimmune encephalomyelitis (EAE), have suggested that MS is an autoimmune disorder caused by myelin-specific CD4+ T cells of the Th1 (Lassmann and Ransohoff, 2004; Sospedra and Martin, 2005) and/or Th17 type (Gutcher *et al.*, 2006; Weaver *et al.*, 2006). In contrast, other studies have highlighted a major role for CD8+ T cells in EAE and MS (Booss *et al.*, 1983; Babbe *et al.*, 2000; Sun *et al.*, 2001; Neumann *et al.*, 2002). Furthermore, Th2 helper-cell-mediated mechanisms have also been suggested to contribute to inflammation in a subset of patients with MS (Lafaille *et al.*, 1997). Cytokines are critical components in immune regulation and signalling, and thus, play a major role in determining the actions of different immune cells. Although still debated, anti-inflammatory cytokines of the Th2 type (e.g. Il-10, IL-4) are thought to be mostly beneficial and have been associated with remissions and recovery from disease (Cannella and Raine, 2004; Sospedra and Martin, 2005). In contrast, cytokines of the Th1 and Th17 type (e.g. Il-1, IL-6, IL-12, IL-17, IFN-γ and TNF-β) are thought to be predominantly pro-inflammatory and are likely to play a role in the pathogenesis of MS. In regard to CNS-Immune system communication cytokines are highly relevant as well. As an example CNS-targeted expression of IL-3 is sufficient to promote recruitment and activation of macrophage/microglial cells in white matter regions of the brain leading to subsequent demyelination (Chiang *et al.*, 1996), highlighting the crucial role of CNS specific modulation of the immune system.

There is accumulating evidence for the disregulation of various aspects of the immune response in the brain parenchyma as well as in the periphery in MS. An important signal transduction pathway involved in immune regulation is the JAK/STAT-signalling pathway, which consists of the protein families of Janus tyrosine kinases (JAK) and the signal transducers and activators of transcription (STATs). Other members of the JAK/STAT pathway are the protein tyrosine phosphatase (SH-PTP2) and the growth-factor-receptor-bound protein 2 (GRB2). GRB2 and SHP2 can form a complex together with the IL-6R (gp130) and are known to link the JAK/STAT pathway to the MAPK pathway (Schiemann *et al.*, 1997), which was shown recently to be upregulated in MS (Graumann *et al.*, 2003). Most interestingly in the context of MS, the JAK/STAT-signalling pathway plays a critical role in the regulation of the sensitivity to cytokines and in their expression. It is known that the JAK/STAT-signalling pathway is a sensitive switch playing a major role in the Th-polarization of the immune system. Members of the STAT family like STAT1, STAT3, STAT4 and STAT6 are thought to be critically involved in the polarization of Th1, Th17 or Th2 cells (Kaplan *et al.*, 1996; Takeda *et al.*, 1997; Harrington *et al.*, 2006). In EAE, it has been shown that STAT4-deficient mice are resistant to the induction of MOG-induced EAE, whereas STAT6-deficient mice show a more severe clinical course (Chitnis *et al.*, 2001). These findings suggest that STAT4 may be involved in the promotion of inflammatory mechanisms, whereas STAT6 may be important in their limitation. Therefore, we concentrated our work on STAT4, STAT6 and their particular downstream genes as markers for either pro- or anti-inflammatory mechanisms in resident cells of the brain.

In addition to the well-characterized inflammatory white matter lesions, studies by magnetic transfer imaging (MTI) have suggested that the normal appearing white matter (NAWM) of the majority of MS patients has significant abnormalities, such as blood–brain-barrier changes, axonal injury and to some extent astro- and microgliosis (Fu *et al.*, 1998; Silver *et al.*, 2001; Aboul-Enein *et al.*, 2003). In line with this, our recent microarray analysis of MS NAWM revealed the upregulation of a number of functionally related genes involved in endogenous neuroprotection, as well as in the maintenance of cellular homeostasis (Graumann *et al.*, 2003). In addition to these protective responses, the NAWM also mounts an immunomodulatory response, which is the subject of this study.

Material and Methods

Tissue collection

MS and control tissue samples were supplied by the UK Multiple Sclerosis Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland (registered charity 207495). Additional tissue samples were collected at the Department of Pathology, University Hospital Basel (Ethics Committee of the University Hospital Basel). Tissue samples representing normal control white matter and normal-appearing MS white matter were collected and analysed as described previously (Graumann *et al.*, 2003). All brains were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude other confounding pathologies. In particular, Bielschowsky silver staining and β-amyloid immunostaining were used to screen the tissues for age-related neurofibrillary tangles and amyloid plaques. A small number of diffuse amyloid plaques were observed in the entorhinal cortex in a number of cases, consistent with age-related changes, but no other pathological changes were seen. Nevertheless, additional age-related pathologies cannot be excluded. Therefore, we performed statistical analysis of the differentially expressed genes for age-dependent alterations. Furthermore, NAWM tissues showing extensive T-cell infiltration were also excluded from this study. From the 11 MS cases, five showed a secondary-progressive, four a primary-progressive, one a progressive-relapsing and one a silent disease course. The mean age of the control cases was 70.4 ± 10.7 years with a post-mortem delay time of 17.1 ± 5.9 h. The mean age of the MS cases was 66.5 ± 10.7 years with a post-mortem delay time of 11.3 ± 5.6 h (Table 1). No statistical significant difference was found in age of control and MS patients ($P = 0.387$). From these tissue blocks provided (Fig. 1A), about 20–40 mg pure subcortical white matter tissue was isolated and used for RNA isolation (hatched area).

Table 1 Summary of clinical data and characterization of MS and control cases

	Patient ID	Age (years)	Sex	MS type	Disease duration (years)	Post-mortem interval (h)	Cause of death	Array, LC
MS patients	MS1*	56	f	SP	31	12	Bilateral basal pneumonia	I, II, III, LC
	MS2	58	f	PP	22	16	Peritonitis	I, LC
	MS3	76	f	SP	>14	18	Myocardial infarction	I, II, III, LC
	MS5*	58	f	PP	21	6	Bronchopneumonia	I, II, III, LC
	MS10	69	f	PP	31	11	Acute pyelonephritis	I, II, LC
	MS12	78	f	PP	31	9	Lung infection	I, LC
	MS14	56	f	PR	34	6	Not known	LC
	MS18	78	f	SP	42	5	Lung cancer	I, LC
	MS20	66	f	SP	30	13	Aspiration pneumonia	I, LC
	MS25	54	f	SP	20	22	Bronchopneumonia	LC
	MS26	83	f	Silent	–	6	Myocardial infarction	I, II, III, LC
Controls	CLo3	85	f	–	–	9	Not known	LC
	CLo6*	73	m	–	–	21	Myocardial infarction	I, II, III; LC
	CLo7	77	m	–	–	26	Lung cancer	I, LC
	CLo8	64	f	–	–	18	Cardiac failure	I, II, LC
	CBS1	70	m	–	–	15	Myocardial infarction	I, II, III, LC
	CBS2	66	m	–	–	16	Lung cancer	I, II, III, LC
	CBS4	69	m	–	–	10	Myocardial infarction	I, LC
	CBS5	59	f	–	–	22	Acute pancreatitis	I, II, III, LC

Clinical and pathological information concerning the 11 MS and 8 control cases studied are shown. All MS and all control tissue samples except MS25 and CLo3 were used for microarray analysis. For MS1, MS5 and CLo6 (asterisk) two different tissue blocks were used for the microarray and qRT-PCR analysis. Abbreviations: SP = Secondary-progressive; PP = Primary-progressive; PR = Progressive-Relapsing; Silent = clinically silent disease course; I = BD Atlas™ Human 1.2 Array I; II = BD Atlas™ Human 1.2 Array II; III = BD Atlas™ Human 1.2 Array III; LC = Lightcycler.

Antibodies

For histochemical analysis the following antibodies were used: anti-STAT4 (R&D Systems, Cat.Nr. PA-ST4, 1:250), anti-STAT6 (R&D Systems, Cat.Nr. AF2167, 1:250), anti-Olig2 (Chemicon, Cat.Nr. AB9610, 1:250), anti-JAK1 (Chemicon, Cat.Nr. MAB3700, 1:250), anti-hIL-4R (R&D Systems, Cat.Nr. MAB230), anti-IL13R (Abnova, Cat.Nr. H00003598-MO1), anti-CD68 (DAKO, Cat.Nr.M0814, 1:500), anti-GFAP (SIGMA, Cat.Nr. G-3893), anti-PLP (kindly provided by K.-A. Nave, Göttingen), anti-MOG (kindly provided by Dr BP Morgan, Cardiff, UK), anti-CD3 (Novocastra Labs, Cat.Nr.NCL-CD3-PS1), anti-MBP (Chemicon, Cat.Nr. MAB386), pan neurofilaments (rabbit pAb cocktail; Biomol, Cat.Nr.NA1297). The secondary antibodies used were: donkey-anti-mouse-biotin (1:500), donkey-anti-rabbit-Cy2 (1:500), donkey-anti-rabbit-Cy3 (1:500), donkey-anti-rabbit-Cy5 (1:500), donkey-anti-mouse-Cy2 (1:500), donkey-anti-mouse-Cy3 (1:500), donkey-anti-rat-Cy5 (1:500), donkey-anti-goat-Cy2 (1:500) and donkey-anti-goat-Cy3 (1:500) (all from Jackson ImmunoResearch).

Immunohistochemistry

All tissue samples were analysed by immunohistochemistry. Cryostat sections (10 µm) used for tissue characterization using anti-CD68, –PLP, –MOG and –CD3 antibodies were fixed for 10 min in 10% formalin. Sections used for CD3 stainings were then boiled in 10 mM sodium citrate buffer, pH 6.0 for 30 min. For inactivation of endogenous peroxidase all sections were treated with 0.3% hydrogen peroxide and blocked with blocking buffer (1% normal donkey serum, 2% Fish skin gelatin, 0.15% Triton). After quenching, sections were incubated with primary antibodies overnight at 4°C. Secondary biotinylated antibodies were applied for 1 h at room temperature followed by the ABC complex reagent (Vector Labs) for 1 h. Colour reaction was performed with 3-amino-9-ethylcarbazole (Erne *et al.*, 2002). Counterstaining was performed in haematoxylin for 1 min followed by rinsing the slide in running tap water.

For fluorescent colocalization study using anti-STAT4, –STAT6, –MBP, –OLIG2, –JAK1, –Neurofilament, –GFAP and –CD68 antibodies, cryostat sections (10 µm) were air-dried for 20 min and fixed for 10 min in acetone at −20°C. For MBP/JAK1 colocalization immersed fixed tissue samples were used (fixed in 4% paraformaldehyde in PBS for 7 days). Sections were incubated with the primary antibodies at 4°C overnight. After washing with PBS, sections were incubated for 1 h with cupric sulfate in ammonium acetate buffer (10 mM $CuSO_4$, 50 mM CH_3COONH_4, pH 5.0) in order to reduce autofluorescence (Schnell *et al.*, 1999). Secondary antibodies were incubated for 1 h at room temperature. Slides were mounted with fluorosave and kept at 4°C.

Total RNA preparation

Total RNA isolation was performed by homogenization in guanidinium thiocyanate followed by a CsCl ultracentrifugation (Bothwell *et al.*, 1990). Freshly isolated RNA was tested for integrity by ethidium–bromide gel electrophoresis and GFAP Northern blot analysis as shown before (Graumann *et al.*, 2003).

Atlas™ cDNA expression array hybridization

The Clontech Atlas™ cDNA Expression Array 1.2 (I–III) contains 3'528 selected cDNA sequences arrayed on three different nylon membranes (1'176 per array). Array hybridization was

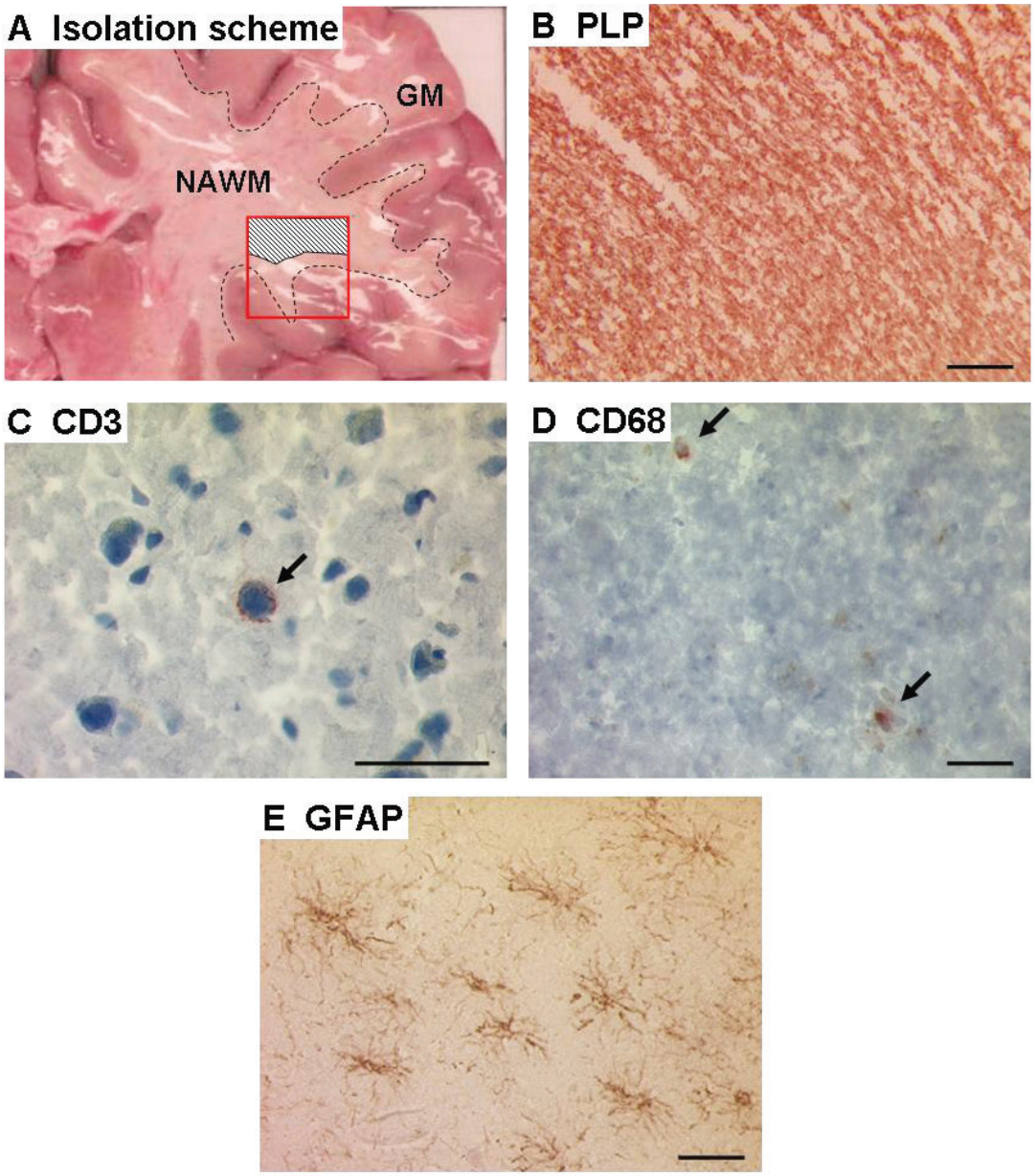

Fig. 1 Histopathological examination of subcortical MS NAWM tissue. To analyse gene expression changes in the NAWM we isolated pure subcortical white matter (hatched area) from tissue blocks (**A**, red box). Immunohistochemical analyses were made to characterize the NAWM and to exclude possible lesions. NAWM stained by PLP (**B**) showed normal myelin. CD3 positive cells were detected rarely in the NAWM (**C**, arrow). We could detect some CD68 positive cells in the NAWM (**D**, arrows). Furthermore, staining for GFAP revealed weakly activated astrocytes (**E**). Bars = 50 μm.

performed according to Graumann *et al.* (2003). Array I was hybridized with 11 MS and 8 control samples, Array II with 7 MS and 5 control samples and Array III with 6 MS and 4 control samples (Table 1). Quantification of differential hybridization signal intensities was achieved with the AtlasImage^{TM} 2.0 software program. Data analysis was performed according to Graumann *et al.* (2003). Median values showing fold changes above 1.5 were defined indicative to be upregulated and median values below 0.66 to be downregulated in a particular MS case. To verify gene expression data from the microarray study we performed quantitative RT-PCR. As we described previously, qRT-PCR results revealed an even greater fold difference, indicating that differential expression patterns are rather under- than overestimated from the microarray analysis (Graumann *et al.*, 2003).

Quantitative RT-PCR

Real-time RT-PCR was performed using the LightCycler system (Roche). Primer sequences were either provided by Clontech or designed from unique site over exon–intron junctions to prevent amplification of genomic DNA. Real-time RT-PCR was performed according to the manufacturer's protocol (Roche). RNA amounts were calculated with relative standard curves for all mRNAs of

Table 2 Primer sequences used for qRT-PCR

Primer name	Gene accession number	Sequence
5′ STAT4	NM.003151.2	5′-TCCGAAGTGATTCAACAGAGCC-3′
3′ STAT4		5′-TTCTTGGTGCGTCAGAGTTTATCC-3′
5′ STAT6	NM.003153.3	5′-CACTGGAAGCAGGAAGAACT-3′
3′ STAT6		5′-TCAAGCTGTGCAGAGACACT-3′
5′ SOCS3	NM.003955.3	5′-TGTGCCTCCTGACTATGTCT-3′
3′ SOCS3		5′-CCTGACTGGCCAATACTTAC-3′
5′ Aqp4	NM.001650.4	5′-TACTGGTGCCAGCATGAATC-3′
3′ Aqp4		5′-TTGTCCTCCACCTCCATGTA-3′
5′ HLA-DR	K01171	5′-ATGGCCATAAGTGGAGTCC-3′
3′ HLA-DR		5′-TTCACTGAGGTCAAGGATTG-3′
5′ nNOS	NM.000620.1	5′-TGGAGGTGCTGGAGGAGTTC-3′
3′ nNOS		5′-TGAGCCAGGAGGAGCATACG-3′
5′ MAG	NM.080600.1	5′-CCGCCGAAGACGGCGTCTATGC-3′
3′ MAG		5′-CTCTCGTAGATGACCGTGGACAGG-3′
5′ MOG	NM.002433.3	5′-CCTCCTCCTCCTCCAAGT-3′
3′ MOG		5′-CCATGCCTGTAGCGTTCTTC-3′
5′ PLP	NM.000533.3	5′-TTCTGTGGCTGTGGACATG-3′
3′ PLP		5′-GAGGCAGTTCCATAGATGAC-3′
5′ MBP	M30515	5′-ACCCGGCAAGAACTGCTCACTATGGCTC-3′
3′ MBP		5′-TGAGCCGATTTATAGTCGGACGCAC-3′
5′ GFAP	J04569	5′-GACGAGATGGCCCGCCACTTGC-3′
3′ GFAP		5′-CTCCACGGTCTTCACCACGATGTTC-3′
5′ NSE	NM.001975.2	5′-TGGTGTGCTGAGGTGTTAG-3′
3′ NSE		5′-CCTTATTAGCCAGGCGTGA-3′
5′ Snap25	XM.009497	5′-TAGTGGACGAACGGGAGCAGATGG-3′
3′ Snap25		5′-CGTTGGTTGGCCTCATCAATTCTGG-3′
5′ PDGFR-alpha	NM.006206.2	5′-CCACGCTACCAGTGAAGTCT-3′
3′ PDGFR-alpha		5′-CACAGCAGGATGGTCACTCT-3′
5′ 60s	X56932	5′-TCGTGCGTCTGAAGCCTAC-3′
3′ 60s		5′-TCTTCCGGTAGTGGATCTTG-3′
5′IL-4	NM.000589.2	5′-CCGAGTTGACCGTAACAGAC-3′
3′IL-4		5′-CTCTGGTTGGCTTCCTTCAC-3′
5′IL-4R	NM.000418.2	5′-CGTCAGCGTTTCCTGCATTG-3′
3′IL-4R		5′-CTCTTTGGCAGCCTTGTGAG-3′
5′IL-12p40	NM.002187.2	5′-AACCTGACCCACCCAAGAAC-3′
3′IL-12p40		5′-AGATGCCCATTCGCTCCAAG-3′
5′IL-13	NM.002188.2	5′-ATCCGCTCCTCAATCCTCTC-3′
3′IL-13		5′-AGCATCCTCTGGGTCTTCTC-3′

5′ and 3′ primers used for quantitative RT-PCR analysis together with the gene accession numbers are shown. The gene 60s was used as housekeeping gene for normalization.

interest and 60s ribosomal protein L13A was used for normalization. Normalization was further evaluated by the 40s ribosomal protein S9. Primer sequences used for qRT-PCR are shown in Table 2.

In situ hybridization

cDNA sequences of STAT6 (U16031, pos. 1248–2522) and HIF-1α (U22431, po. 1215–2394) were cloned from human cerebellum tissue into pBluescript KS II+. cDNA for HLA-DRα was kindly provided by Viktor Steimle (Department of Biology, University of Sherbrooke, Sherbrooke, Canada). Digoxigenin-labelled cRNAs were generated for Hif-1α and HLA-DRα. *In situ* hybridization was performed on cryosections (10 µm thick) of freshly frozen tissue as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Spiegel *et al.*, 2002). Hybridization was done overnight at 68°C and colour reaction time for the alkaline phosphatase reaction was 48 h for all probes.

Statistics

Statistical significance is expressed by *P*-values generated by the non-parametric Mann–Whitney U-Test. For each gene two *P*-values were calculated (Table 3). A hierarchical cluster analysis was performed using the program 'Cluster' and 'TreeView' from Michael Eisen (Eisen *et al.*, 1998). Hierarchical cluster analysis revealed two MS case groups based on similarities in their gene expression pattern. Therefore, two *P*-values were calculated. The first *P*-value (marked by an asterisk) was calculated using the signal intensities from the cases MS 1, 2, 3, 5, 12 and 26, whereas the second *P*-value (marked by double-asterisk) was calculated by using all MS cases. To evaluate differential gene expression data from the microarrays in more detail, a boxplot from each gene was generated and analysed (data are shown for particular genes in Fig. 5). For this, the hybridization signal intensities were normalized to one of the control samples according to Graumann *et al.* (2003). Statistical correlation was calculated using the non-parametric Spearman's test.

Table 3 Microarray analysis. List of differentially expressed genes in NAWM

		Median								*P*-value *	Median			*P*-value **
Gene bank accession	Gene name	MS26	MS1	MS1	MS12	MS5	MS5	MS3	MS2		MS20	MS10	MS18	
			A3B2	A3D1	P1A4	A3C2	A3C7	A4B4	P5C4		P3B3	A4D3	P3B4	
STAT signalling pathway														
U16031	STAT6	2.8	3.8	2.9	2.8	3	1.9	3.1	4.5	**0.005**	1.5	1.5	2	**0.005**
L29277	STAT3	1.4	2	2	1.7	2.4	1.5	1.5	1.5	**0.012**	1	1.2	1.2	**0.032**
M35203	JAK1	1.5	4.3	2.6	1.7	1.9	1.4	2.1	1.9	**0.003**	1.4	0.9	1.3	**0.005**
AF005216	JAK2	3	2.7	2.2	2	1.2	1.6	1	1.3	0.172	1.2	1.5	0.9	0.186
U09607	JAK3	3.3	3.4	2.3	1.1	1.5	1.5	2	8	0.074	3.5	0.6	1.3	0.137
X54637	TYK2	1.8	2.4	1.8	1.4	1.7	1.4	2.3	1	0.141	0.8	0.8	1	0.322
L08807	SH-PTP2	1.6	1.9	1.9	2.3	1.6	1	1.6	2	**0.012**	2.1	1.3	1.4	**0.008**
L29511	GRB2	2.2	2.1	2.5	2	2.7	2.2	2	1.4	**0.002**	1.4	1.1	0.9	**0.01**
M57230	IL-6Rb	2.1	3.2	2.1	1.6	2.7	1.9	1.5	1.1	**0.036**	1.5	0.9	1.8	0.069
L05624	MAPKK 1	3.7	6.3	4.1	2.3	2.1	2	1.9	1.6	**0.002**	0.7	1.3	0.7	**0.013**
X02751	N-ras	3.8	1.8	1.5	2.3	2.1	1.1	1	0.7	0.115	0.9	1.1	1.1	0.248
X03484	c-raf	3	1.7	1.9	2.1	1.3	1.1	1.4	1.8	**0.016**	1.3	1	0.6	0.083
U09579	WAF1	3.5	2.5	2.2	2.1	1.5	1.6	1.4	2	0.172	1.2	0.6	1.6	0.215
L34075	FRAP	2.8	4.1	1.4	2.5	1.7	1.3	2.7	0.4	**0.027**	1.1	2	0.5	0.075
Cytokines														
M57627	IL-10	2.4	2.7	1.3	1.8	2	1.7	1.1	1.3	**0.016**	1.1	0.8	0.8	**0.048**
M19154	TGF-β2	2.5	3.1	1.8	1.5	3	2.2	2.3	1.7	**0.006**	0.9	1	2.6	**0.01**
D49950	IL-18	2.3	2.2	1.5	1.2	1.6	1.6	1.1	1	0.093	0.6	1.5	0.5	0.248
K02770	IL-1β	2.6	2.6	1.7	1.5	2.2	1.9	1.6	1.2	**0.046**	0.9	0.8	1.5	0.117
Cytokine receptors														
X77722	INF-α/βR	1.2	2.4	1.2	2.3	2.6	1.5	1.1	0.9	**0.046**	1.5	0.9	0.8	0.099
M57230	IL-6Rβ	2.1	3.2	2.1	1.6	2.7	1.9	1.5	1.1	**0.036**	1.5	0.9	1.8	0.069
X01057	IL-2Rα/β	3.6	7.1	1	2.1	2.7	2.1	1.1	2.9	**0.021**	1.6	0.6	0.6	0.058
M33294	TNFR1	2.8	3	1.9	1.5	2.3	1.5	2.1	2.1	0.141	1.3	1.2	1.8	0.16
Chemokines/receptors														
M37435	MCSF	3.8	5.6	1.1	3.1	2.7	2.4	1.9	1.6	**0.009**	1.4	1.9	1	**0.01**
M24545	MCP1	3.7	4.6	2.1	1.6	1.7	1.9	2.2	6.1	0.172	1.3	0.5	1.4	0.215
U10117	EMAP II	3.1	3.1	1.3	2.9	1.9	2.2	1.4	1.9	**0.012**	1.9	1	1.1	**0.017**
M21121	RANTES	3.5	2.9	0.9	1.1	1.3	1.5	1	2	0.059	1.6	0.7	0.7	0.16
D10925	CCR1	1.1	4.5	1.3	1.7	2	1.8	1.1	1.2	0.115	0.9	0.6	1	0.283
M25756	Chromogranin C	2.4	2.5	4.1	2.3	2.7	3.6	5.1	1.9	**0.009**	0.9	1.8	0.9	**0.021**
Adhesion molecules														
J03132	ICAM1	2.2	3.5	2.6	1.8	1.9	1.1	1.2	1.9	**0.046**	1.2	0.7	2.5	**0.048**
X57766	MMP11	1.4	16.9	2.2	1.9	4	3.1	2.1	1.6	0.074	0.9	0.9	1.5	0.137
M15395	LFA-1	1.7	2.9	1.7	2	4.2	2.4	1.1	0.7	0.059	0.3	1.1	1.1	0.283
Hypoxia-related genes														
U22431	HIF-1α	1.5	6.5	3.6	2.3	2.2	1.8	2	1	**0.016**	2.3	0.5	1.4	**0.021**

Upregulation is shown in dark grey whereas downregulation is shown in yellow. Medians of ratios against all control samples are shown for each MS sample. Statistical significance is expressed as *P*-value (non-parametric Mann–Whitney U-test). *P*-values were calculated for the major group (*) and for all MS cases (**). *P*-values below 0.05 are printed in bold numbers, whereas *P*-values between 0.05 and 0.1 are shown in italic numbers.

Results

Histological and molecular examination of subcortical NAWM from MS and control brains

All brains used in this study were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude confounding pathologies, in particular neurofibrillary tangles and amyloid plaques. The clinical and pathological information on the MS and control cases was collected and is summarized in Table 1. To analyse gene expression changes in the NAWM, we dissected subcortical white matter from cortical grey matter to ensure that only RNA from white matter tissue was isolated (Fig. 1A, hatched area). Isolated RNA was tested for its integrity by ethidium–bromide gel electrophoresis and GFAP Northern blotting, and only samples with high-quality RNA were used. In order to confirm normal cellular integrity and composition, and to exclude any possible pathological changes such as demyelination and inflammation, we performed immunohistochemical analysis on the NAWM tissue. Staining for myelinated fibres (Fig. 1B, PLP) showed no signs of demyelination or shadow plaques. As tissues with extensive T-cell infiltration were excluded from this study, we detected almost no CD3-positive cells in subcortical NAWM (Fig. 1C). However, we detected CD68 positive cells distributed throughout the MS NAWM (Fig. 1D). There was no evidence of astrogliosis as revealed by an anti-GFAP staining (Fig. 1E, GFAP). As we have described before, some neurons were found in human subcortical white matter as well (Graumann *et al.*, 2003). In summary, all tissue included in this study showed no histological evidence of demyelinating lesions, remyelination or inflammatory infiltrates.

In a recent study, we performed a differential expression analysis of subcortical NAWM from MS cases and corresponding subcortical white matter from control cases (Graumann *et al.*, 2003). We performed a hierarchical cluster analysis to identify groups of MS cases based on similarities in their gene expression pattern. This analysis revealed a major group within the MS cases (MS1, 2, 3, 5, 12 and 26), which showed strong similarities in their expression patterns (Graumann *et al.*, 2003). A minor group of three MS cases (MS10, 18 and 20) showed an expression pattern with some similarities to the major group such as the upregulation of STAT6, but showed overall fewer alterations compared to the control cases.

Changed expression of genes of the JAK/STAT-signalling pathway

One of the major pathways involved in immune regulation is the JAK/STAT-signalling pathway (Dell'Albani *et al.*, 2003). By microarray and quantitative RT-PCR analysis of MS NAWM, we observed changes in the regulation of gene expression of several genes known to be involved in the JAK/STAT-signalling pathway (Table 3, Fig. 2A–D). One member of the STAT family, STAT6 was upregulated in all MS cases (Table 3, Fig. 2A, $P = 0.005$). Furthermore, we also found STAT3 to be significantly upregulated in these MS NAWM (Table 3). The other members, STAT1, 2, 4, 5a and 5b could not be detected by microarray analysis. However, using the more sensitive qRT-PCR, we revealed that STAT4 was also significantly upregulated in MS cases (Fig. 2D, $P = 0.002$). To identify the cell types expressing STAT4 and STAT6, we performed a immunofluorescent colocalization study (Figs 3 and 4). Most of the STAT4 expressing cells were CD68-positive, suggesting that the main cell population expressing STAT4 are activated microglia (Fig. 3A, arrow). Occasionally, we identified STAT4-positive astrocytes (Fig. 3B, arrow). We did not detect any STAT4-positive oligodendrocytes or neurons in MS NAWM. In contrast, immunofluorescent staining for STAT6 showed that STAT6 was mostly in cells expressing OLIG2 (Fig. 4A, arrows), demonstrating that cells of the oligodendrocyte lineage were the main cell population expressing this transcription factor. The arrangement of STAT6/OLIG2-positive cells in interfascicular rows indicates an expression by myelinating oligodendrocytes. However, STAT6 expression was not limited to oligodendrocytes, as occasionally GFAP/STAT6-positive astrocytes were also detected in the NAWM (Fig. 4B, arrow). Colocalization with neurofilament revealed STAT6/neurofilament-positive neurons in the NAWM as well (Fig. 4C, arrow). No colocalization of STAT6 together with CD68 could be detected. Triple immunofluorescence staining showed sporadically astrocytes positive for both STAT4 and STAT6 in the NAWM (Fig. 4D, arrow).

The expression of all four members of the JAK family could be detected by microarray analysis. Whereas JAK2 and TYK2 were not statistically significant changed, JAK1 was significantly upregulated in the NAWM of MS cases (Table 3, Fig. 2B). Another member of the JAK tyrosine kinase family, JAK3, was also upregulated in MS NAWM but with a weaker significance (Table 3). To identify the cell types expressing JAK1, we performed a colocalization immunofluorescent study, which revealed the expression of JAK1 in oligodendrocytes expressing MBP (Fig. 4E, arrow). Interestingly, we could show a positive correlation of JAK1 ($r = 0.505$, $P = 0.027$) and JAK3 ($r = 0.530$, $P = 0.020$) expression levels to the expression levels of STAT6 within the different MS cases. Although an overall increase in expression of JAK2 and TYK2 in MS could also be observed, the upregulation was not statistically significant. Other genes belonging to the JAK/STAT-signalling pathway, such as SH-PTP2 and GRB2 were also significantly upregulated in MS NAWM (Table 3). Furthermore, we found the upregulation of neuroblastoma RAS viral (v-ras) oncogene homolog (N-RAS), v-raf-1 murine leukaemia viral oncogene homolog 1 (c-raf) in some and mitogen-activated protein kinase kinase 1 (MAPKK1) in most MS cases. Also, cyclin-dependent kinase inhibitor 1A (p21, WAF1) and FK506-binding protein 12-rapamycin-associated

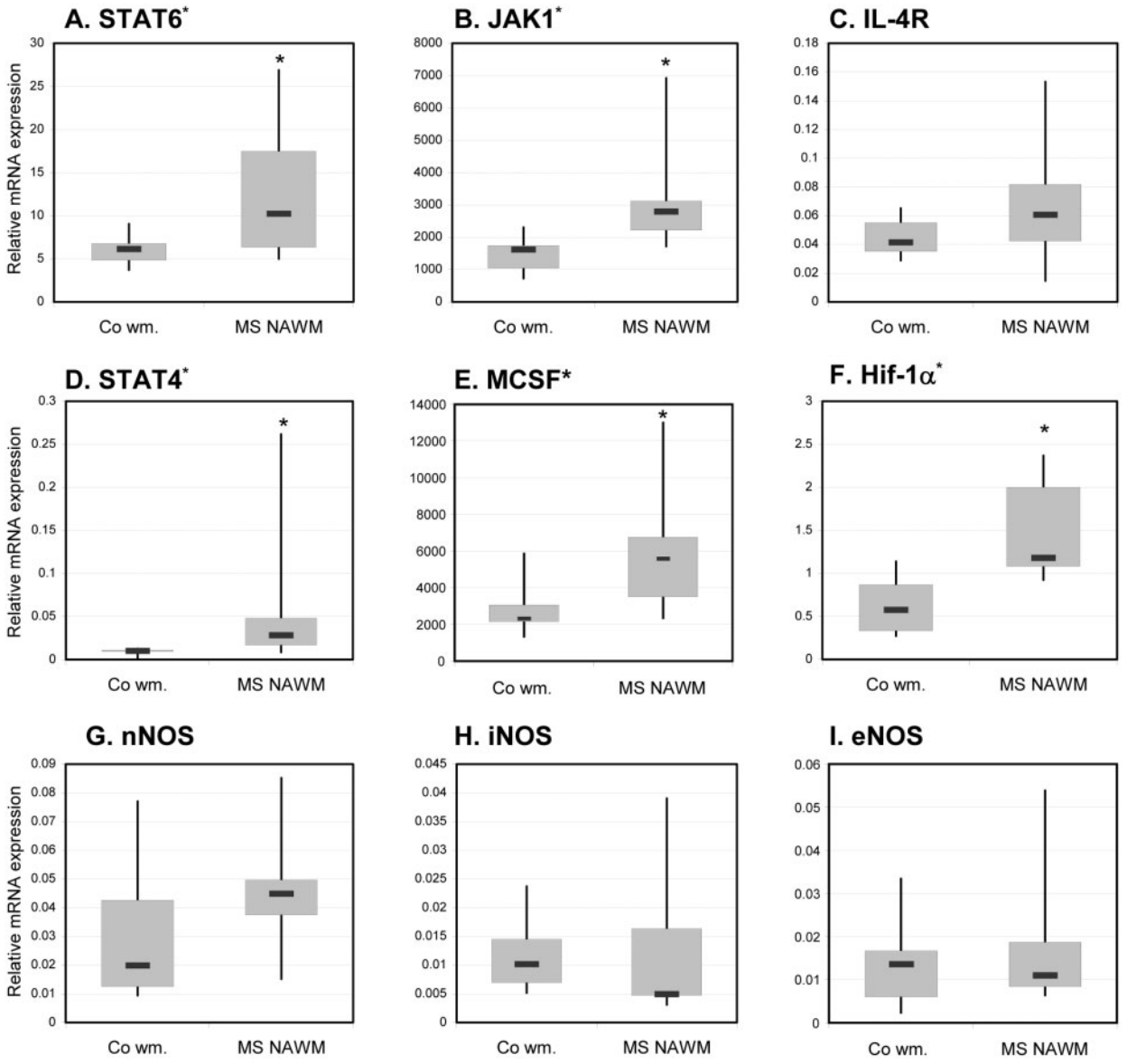

Fig. 2 Boxplots of selected differentially expressed genes analysed by quantitative RT-PCR and microarray. qRT-PCR was performed for STAT6 (**A**), IL-4R (**C**), STAT4 (**D**), HIF-1α (**F**), nNOS (**G**), iNOS (**H**) and eNOS (**I**). JAK1 (**B**) and MCSF (**E**) were analysed by microarray. Boxplots show differential gene expression in control white matter and MS NAWM. Genes significantly upregulated in MS NAWM are marked with an asterisk.

protein 1 (FRAP1) were upregulated in the majority of the MS cases. In summary, our results show the upregulation of genes from the Interleukin-4/Interleukin-13-signalling pathway expressed by oligodendrocytes and genes of other JAK/STAT-signalling pathways in resident brain cells in a majority of the MS cases studied.

Upregulated cytokine and cytokine-receptor expression in NAWM of MS patients

Changes in the balance between pro- and anti-inflammatory cytokines and their receptors have been implicated in the pathogenesis of MS. Therefore, we investigated the expression pattern of cytokines and their receptors in the NAWM of MS patients (Table 3). Most strikingly, we observed a significant upregulation of the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta 2 (TGF-β2) in the MS cases. On the other hand, IL-18 and IL-1β also showed elevated expression levels in the majority of MS cases. Other cytokines such as TGF-α and TGF-β3 showed only minor changes or were even decreased in some MS cases (data not shown). An endogenous expression of most of the cytokines, in particular IL-4, IL-6, IL-12, IL-13, IL-17, IL-23 and IFN-γ could not be detected by microarray analysis or by quantitative RT-PCR.

Microarray analysis of cytokine receptors revealed detectable expression of IL-2 receptor subunit alpha

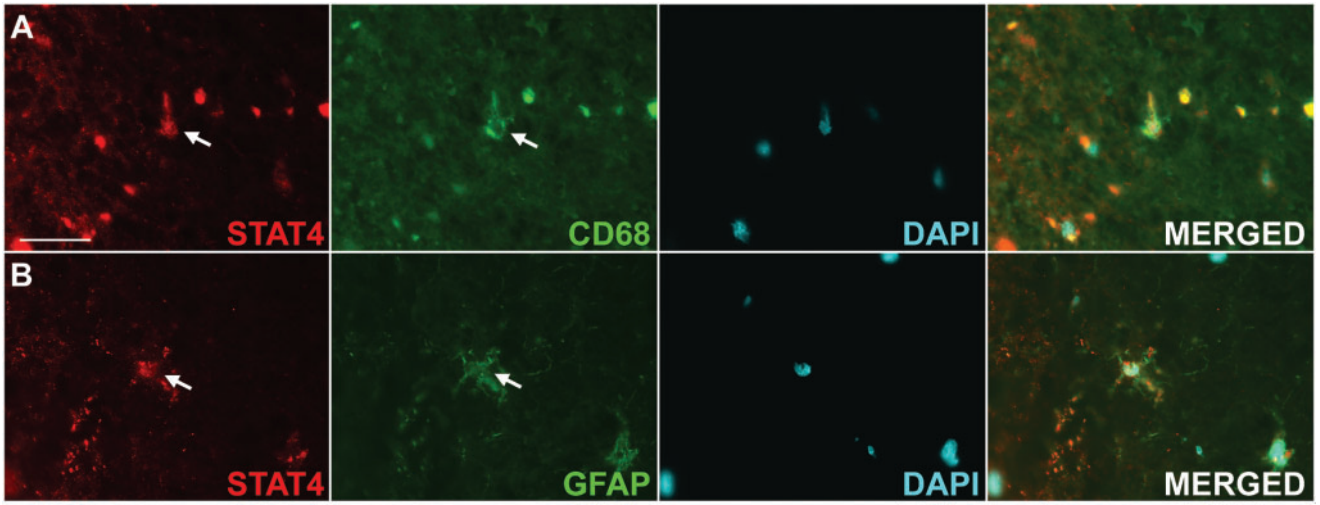

Fig. 3 Immunofluorescence localization of STAT4 in MS NAWM. Colocalization analysis showed STAT4 being expressed in CD68 positive cells (**A**). Furthermore, we could show some astrocytes positive for STAT4 (**B**). We did not detect any neurons or oligodendrocytes positive for STAT4 in the NAWM. Bar = 20 μm.

(IL-2Rα), IFN-γreceptor, IFN-alpha/beta receptor, IL-6 receptor (IL-6R) and tumour necrosis factor receptor 1 (TNFR1; Table 3). We detected an upregulation of the IL-6Rβ subunit (gp130) and TNFR1 in 9 out of 11 MS samples. In contrast to IL-6Rβ, which was significantly upregulated in MS NAWM, upregulation of TNFR1 was not statistically significant due to the fact that several control samples had also relative high TNFR1 expression levels (Table 3). One of the most consistently upregulated cytokine receptors was the interleukin-2-receptor alpha (IL-2Rα). Its co-receptors IL-2Rβ and IL-2Rγ, however, could not be detected by microarray (data not shown). Significant upregulation was also detected for the IFN-α/β receptor, whereas the IFN-γ receptor was only upregulated in 2 of 11 MS samples (Table 3).

To investigate the anti-inflammatory-signalling pathway of STAT6, JAK1 and JAK3 in more detail, we examined the expression pattern of one of its main receptors, the IL-4 receptor (IL-4R), by quantitative RT-PCR. We detected elevated expression levels of IL-4R in MS NAWM (Fig. 2C). Even though we could detect an overall higher expression of IL-4R in MS NAWM samples this was not statistically significant. The reason for this is a relative high expression of one of the controls (CLo6), and in addition, a moderate expression of IL-4R in the three cases of the minor group. However, IL-4R expression levels correlated well with the expression levels of STAT6 ($r = 0.657$, $P = 0.008$) within the analysed tissue samples. Immunofluorescent colocalization study with STAT6 revealed STAT6/IL-4R positive oligodendrocytes throughout the NAWM (Fig. 4F). Furthermore, immunofluorescent colocalization study for IL-13 receptor (IL-13R), which is another STAT6-activating receptor, revealed STAT6/IL-13R positive oligodendrocytes in the MS NAWM as well (Fig. 4G). In summary, our results show that oligodendrocytes in MS NAWM are expressing the necessary components for STAT6/JAK1 signalling.

Upregulated chemokines and chemokine-receptor expression in NAWM of MS patients

Chemokines and chemokine receptors play a critical role in the recruitment of lymphocytes and other inflammatory cells into the CNS (Sospedra and Martin, 2005). Microarray analysis revealed significant upregulation of several chemokines in MS NAWM (Table 3), in particular chromogranin C, monocyte colony-stimulating factor (MCSF) and endothelial-monocyte-activating polypeptide (EMAP-II). Boxplot analysis for MCSF shows an overall low-level expression in control cases and upregulation in almost all MS cases (Fig. 2E). Although monocyte chemotactic protein (MCP-1) showed an upregulation in all MS cases of the major group (Table 3), this was not statistically significant because MCP-1 expression among control cases was highly variable (data not shown). Other chemokines such as RANTES and its receptor CCR1 also showed elevated expression levels in particular MS cases, but for the same reason as for MCP-1 these were not statistically significant. Altogether, we could demonstrate that the expression levels of several chemokines involved in the attraction of peripheral immune cells into the CNS were increased.

Differential gene expression of cell adhesion molecules

Cell adhesion molecules play an essential role in many inflammatory processes, such as blood–brain-barrier changes, cell migration and differentiation. Therefore,

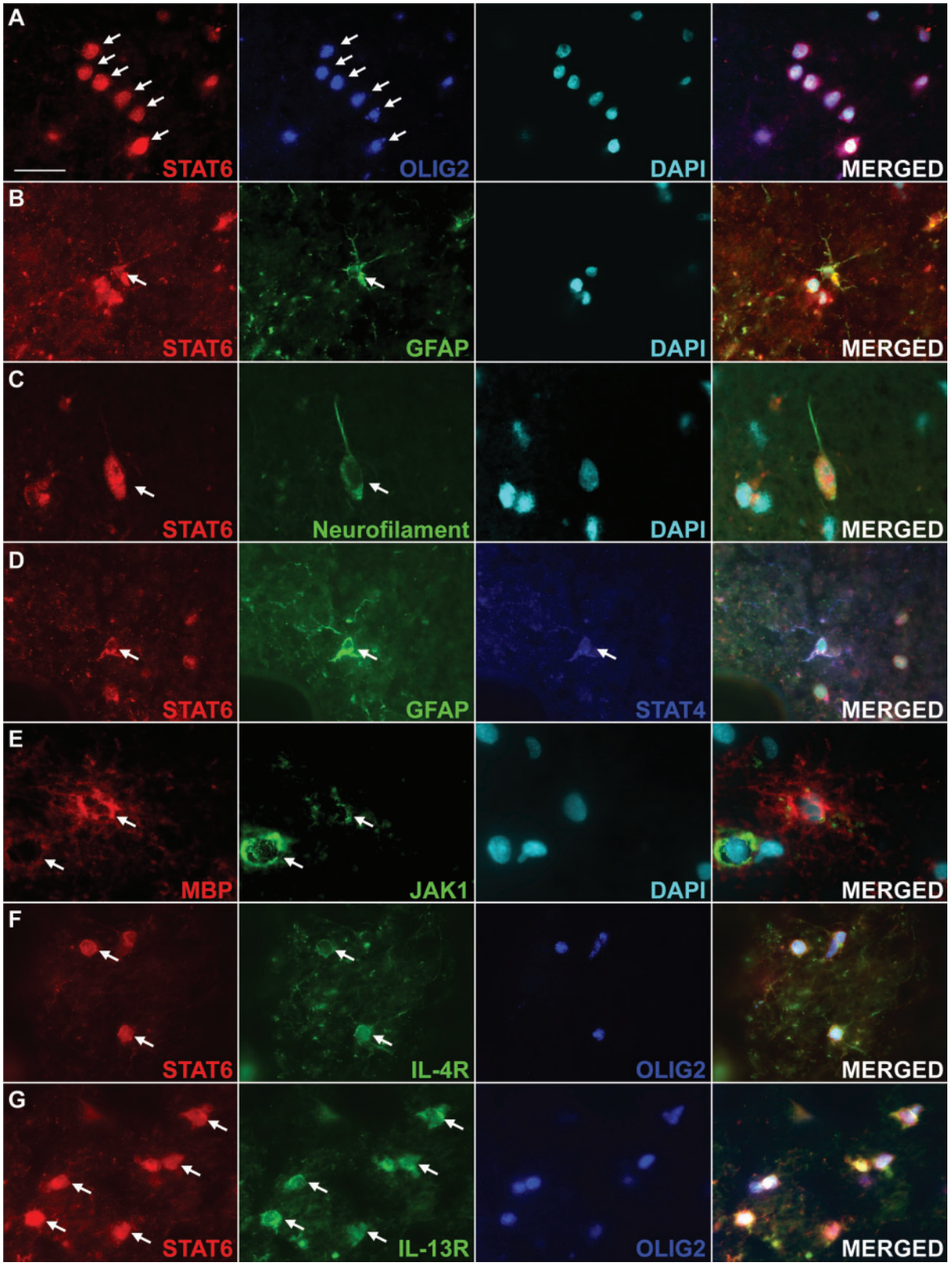

Fig. 4 Immunofluorescence localization of STAT6 and JAK1 in MS NAWM. STAT6 was mostly colocalized together with the oligodendrocytes marker OLIG2 (**A**). In few cases colocalization with astrocytes (**B**) and subcortical white matter neurons (**C**) could be observed. Sporadically, we could detect some astrocytes positive for both, STAT4 and STAT6 (**D**). Additionally, we could show that JAK1 mostly colocalized together with the myelin protein MBP (**E**). Furthermore, IL-4R (**F**) and Il-13R (**G**), the main receptors leading to STAT6 activation, were detected to colocalize with STAT6 and OLIG2. Bar = 20 μm.

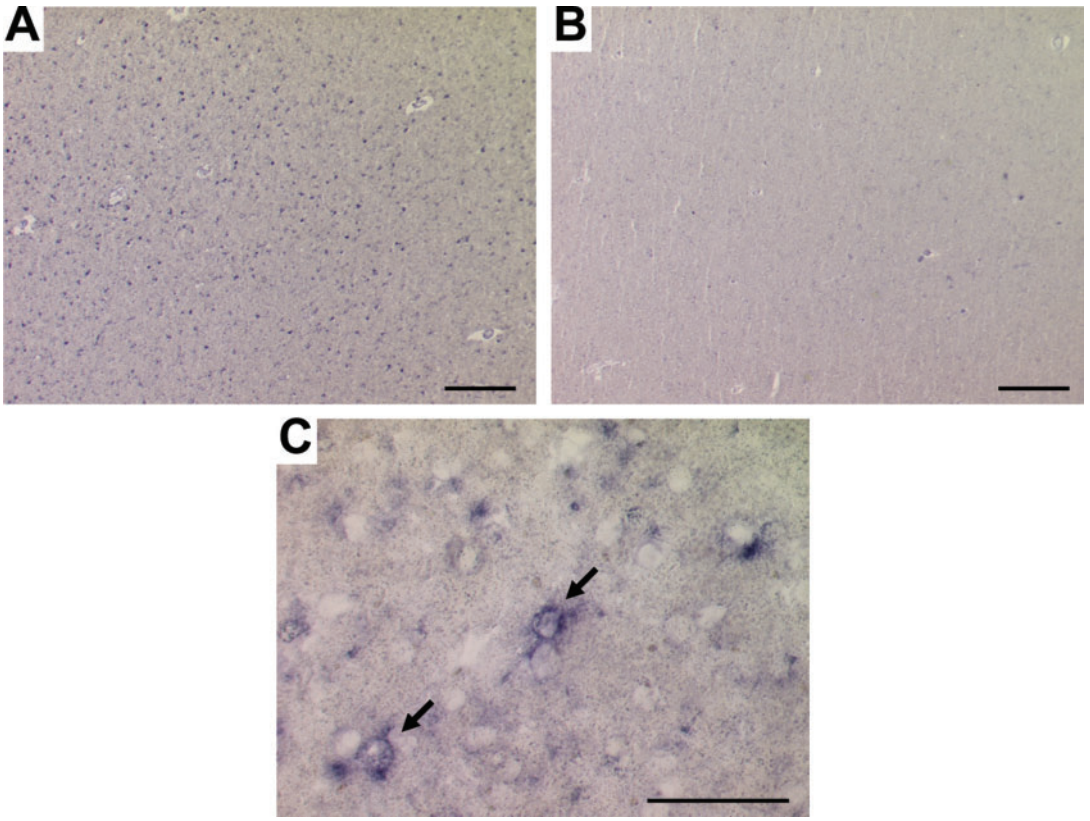

Fig. 5 *In situ* hybridization of HLA-DR mRNA in MS and control white matter. Many HLA-DRα positive cells were detected in the NAWM of MS cases (**A**) compared with white matter from control cases where the hybridization signal was below background levels (**B**). *In situ* hybridization shows that HLA-DRα mRNA expression in cells of the NAWM morphologically alike microglia cells (**C**, arrows). Bar = 50 μm.

we investigated the gene expression profile of different cell adhesion molecules (Table 3). Most of these genes did not show altered expression levels with the exception of ICAM-1, which was significantly upregulated in the majority of the MS cases (Table 3). Others such as LFA-1β had a more heterogeneous expression pattern (Table 3). Matrix metalloproteinases (MMP) are also implicated in the pathogenesis of the inflammatory process in MS, but our microarray study revealed that only MMP-11 (stromelysin-3) was upregulated in MS NAWM. MMP-7, MMP-9 and MMP-16 were found constitutively expressed in all control and MS cases, but their expression levels were not altered.

Upregulation of HLA-DRα in MS NAWM

The expression of the major histocompatibility complex HLA-DR α-chain, crucial for antigen presentation, was upregulated in the majority of MS cases, whereas upregulation of HLA II DP α-chain was less evident and only detected in a subset of MS cases (data not shown). To identify cells expressing HLA-DRα, we performed *in situ* hybridization analysis. This revealed the expression of HLA-DRα mRNA throughout the NAWM of the majority of MS cases (Fig. 5A), whereas in control white matter HLA-DRα was not detectable (Fig. 5B). Due to the morphology of the HLA-DRα expressing cells—small cells with thin processes—we identified most of the HLA-DRα expressing cells as microglia (Fig. 5C). Colocalization with anti-GFAP immunohistochemistry revealed that some astrocytes might also express HLA-DRα (data not shown). In summary, our data indicate that a part of the HLA Class II complex, especially HLA-DRα, is upregulated in MS NAWM.

Upregulation of the neuronal—but not of the inducible and endothelial nitric oxide synthase

Nitric oxide (NO), and thus nitric oxide synthases (NOS) play an important role under physiological as well as pathological conditions (Smith and Lassmann, 2002). Therefore, we performed qRT-PCR analysis and detected upregulation of the neuronal form of NOS (nNOS), in most of the MS NAWM tissue samples (Fig. 2G). In contrast, the inducible NOS (iNOS) showed comparable expression levels in MS NAWM as in controls cases (Fig. 2H). Also the expression of endothelial NOS (eNOS) in MS NAWM showed no difference to the control cases (Fig. 2H and I).

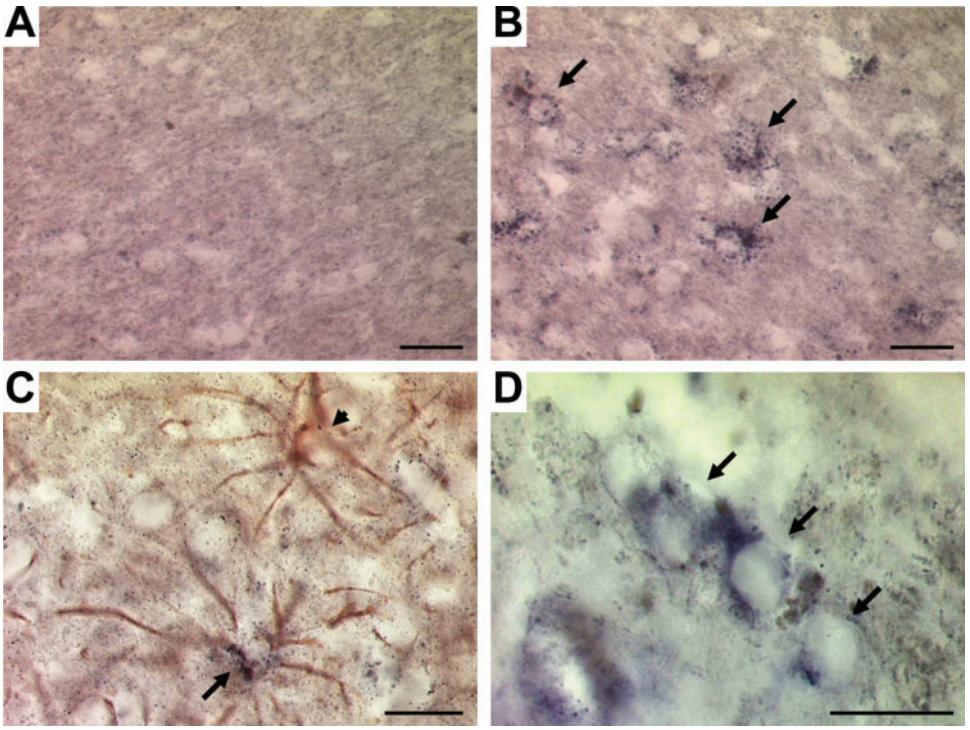

Fig. 6 *In situ* hybridization of HIF-1α mRNA in MS and control white matter. In subcortical white matter of control cases HIF-1α could not be detected (**A**). In MS NAWM, however, we could detect a hybridization signal for HIF-1α mRNA in a number of cells (**B**). Colocalization with anti-GFAP immunohistochemistry revealed a subpopulation of astrocytes expressing HIF-1α (**C**, arrow), whereas other astrocytes were negative for HIF-1α (**C**, arrowhead). Due to the chain-like arrangement and their morphology, we suggest that Hif-1α is also expressed by oligodendrocytes (**D**, arrows). Bars = 25 μm.

Upregulation of HIF-1α in NAWM

In our previous microarray study we showed that HIF-1α, a key regulator of hypoxia-induced gene regulation, and its downstream genes were significantly upregulated in MS NAWM (Graumann *et al.*, 2003). Quantitative RT-PCR analysis in 11 MS and 8 control cases for HIF-1αrevealed a consistent upregulation of HIF-1α in MS NAWM (Fig. 2F, $P = 0.002$), verifying our microarray data (Graumann *et al.*, 2003). In order to identify the cell types expressing HIF-1α, we performed *in situ* hybridization analysis (Fig. 6). We found that in subcortical white matter of control cases HIF-1α mRNA could not be detected (Fig. 6A), indicating an overall weak expression in white matter. However, in MS NAWM HIF-1α hybridization signals became detectable in a number of cells throughout the NAWM (Fig. 6B, arrows). Colocalization analysis for GFAP revealed that a small subpopulation of astrocytes expressed HIF-1α (Fig. 6C). Other HIF-1α positive cells were arranged in interfascicular rows, suggestive of myelinating oligodendrocytes (Fig. 6D, arrows).

Discussion

Very little is known about the earliest intrinsic changes to the CNS occurring in MS before the appearance of overt inflammatory and demyelinating plaques. The normal appearing non-lesional white matter looks normal from a morphological and cellular point of view. However, MRI and gene expression studies have shown significant changes in NAWM that might give indications concerning both, the early changes occurring before lesion formation and the attempts by the CNS to prevent the same (Fu *et al.*, 1998; Silver *et al.*, 2001; Aboul-Enein *et al.*, 2003; Graumann *et al.*, 2003). Furthermore, most recent data suggests a diffuse inflammatory damage spreading throughout the whole brain in the chronic phase of the disease associated with slow progressive axonal injury at sites without inflammation (Kutzelnigg *et al.*, 2005). In order to identify pre-lesional changes and immunological regulators, we analysed the gene expression profile in the NAWM of 11 MS cases. All tissues included in this study were routinely screened by a neuropathologist and showed no signs of additional neurological diseases. Tissues from MS cases with obvious confounding pathologies, in particular neurofibrillary tangles and amyloid plaques were excluded. Furthermore, to test whether the tissue used in this study show signs of age-related confounding pathologies, we correlated gene expression changes with age but found no statistical significant correlations; e.g. STAT6 ($r = -0.386$, $P = 0.103$), STAT4 ($r = -0.440$, $P = 0.116$), JAK1 ($r = -0.386$, $P = 0.103$), JAK3 ($r = -0.258$, $P = 0.286$) and IL-4R ($r = -0.121$, $P = 0.668$). Additionally, there is no statistical significant difference in age between the MS

and the control group, which further minimizes possible influence of age-related pathology. In particular, NAWM tissues with extensive T-cell infiltration were also excluded from this study. Thus, T-cell-specific transcripts were rarely detectable in single MS cases and no correlation of genes from the STAT-signalling pathways and T-cell-specific transcripts could be made. In addition, we could not detect any CD3/STAT6-expressing cells in MS NAWM. As gene expression changes were obtained by comparing MS cases with age-matched control cases, detection of differential gene expression due to age-related mechanisms was avoided. This so defined NAWM tissue is ideal to study the intrinsic changes of brain cells during the long-lasting disease course of MS.

Hierarchical cluster analysis of the MS cases based on the similarities of their expression pattern revealed two possible groups. But no grouping according to the MS type could be detected. Possibly, gene expression changes in the MS NAWM from different MS types might equalize during the long-lasting disease course. As gene expression changes taking place in the MS NAWM do not depend upon the MS type, this allowed us to analyse gene expression data from all MS types together.

Altogether, our analysis revealed that genes involved at different levels of the inflammatory response, such as signalling, transcription, cell adhesion and antigen presentation, were upregulated in the MS NAWM. Most interestingly, we detected upregulation of genes linked to both anti- as well as pro-inflammatory mechanisms. Two central players involved in these two mechanisms are the transcription factors STAT6 and STAT4 (Pfitzner *et al.*, 2004).

Diffuse damage of the CNS might be due to pro-inflammatory microglia in the MS NAWM

We found that in the NAWM, STAT4, a characteristic marker for pro-inflammatory mechanisms, was generally expressed by CD68-positive cells suggestive for activated microglia and occasionally astrocytes. In line, an upregulation of HLA-DRα, known to be generally expressed by microglia, was detected in the NAWM of MS cases. STAT4 is involved in IL-12 and IL-23-mediated signalling and as a consequence in Th1 cell differentiation (Watford *et al.*, 2004). The upregulation of STAT4 found in the NAWM tissue is strikingly prominent and might point to the development of a pro-inflammatory environment allowing or facilitating the infiltration of peripheral immune cells into the CNS. This is supported by a study investigating the role of STAT4 in the development of local allergic airway response. In this study, STAT4 knockout mice showed a significant decrease in airway hyperreactivity via local alteration of chemokine production, such as CCL5, CCL6, CCL11 and CCL17 (Raman *et al.*, 2003). Furthermore STAT4 is known to play a major role in IFN-γ regulation and is involved in the induction of IL-2R (Watford *et al.*, 2004). Also involved in IL-12 and IL-23 signalling are the Janus kinases JAK2 and TYK2, which were upregulated in some MS cases. Furthermore, IL-1β, which is described as a pro-inflammatory cytokine primarily secreted by activated microglia cells and infiltrating macrophages (Correale and Villa, 2004) was significantly upregulated in MS NAWM. An enhanced expression of IL-1β might also be one cause of the reported diffuse damage of the CNS (Kutzelnigg *et al.*, 2005), as intracerebral microinjections of IL-1β induced death of intrinsic CNS cells (Holmin and Mathiesen, 2000). The view of microglia playing a pro-inflammatory role in MS NAWM is further supported by a study showing that a blockage of microglial release of nitrite oxide, pro-inflammatory cytokines and chemokines, resulted in a strong reduction of CNS inflammation and an amelioration of the clinical signs in EAE (Heppner *et al.*, 2005). Furthermore, in MS NAWM, we could detect the upregulation of several chemokines, such as MCSF, EMAP II, MCP-1 (CCL2), ICAM-1 and RANTES (CCL5). This is inline with a study which showed an increased MCSF expression in the periplaque white matter of MS cases (Werner *et al.*, 2002). Altogether, we speculate that the alteration of the pro-inflammatory gene expression pattern in resident cells of the NAWM, most likely microglia, could prepare or later contribute to the enhancement and facilitation of the infiltration of active immune cells into the CNS. At this point, the production of these pro-inflammatory signals might still not be enough to promote T-cell recruitment but they might be already deleterious for the CNS, as the expression of one specific cytokine under certain circumstances can already lead to an infiltration of immune cells into the CNS (Chiang *et al.*, 1996).

Are oligodendrocytes playing an active role in immune regulation in the CNS?

A major finding of this study was the upregulation of genes involved in anti-inflammatory mechanisms in MS NAWM, illustrated by the upregulation of IL-10, a potent anti-inflammatory cytokine known to inhibit the activation of monocytes, dendritic cells and macrophages (Beebe *et al.*, 2002). Furthermore, TGF-β2, which was reported to reduce demyelination and macrophage recruitment in a viral model of MS (Drescher *et al.*, 2000), was strongly upregulated in NAWM of most of the MS cases. However, most interesting was the finding that STAT6 was upregulated in the NAWM of all MS cases. Although the function of STAT6 is not yet fully determined, this transcription factor is known to be involved in anti-inflammatory pathways. STAT6 is one of the major steps in IL-4 and IL-13 signalling (Takeda *et al.*, 1997) and this is further demonstrated by the exacerbation of EAE in STAT6-deficient mice (Chitnis *et al.*, 2001). The main cells expressing STAT6 in the MS NAWM were found to be oligodendrocytes. Furthermore, JAK1, IL-4R and IL-13R,

all belonging to the STAT6-signalling pathway (Hebenstreit *et al.*, 2006), were also expressed by oligodendrocytes. In the case of JAK1 and IL-4R, this is in agreement with the findings of Cannella and Raine (2004). Comparison of the expression pattern of STAT6, IL-4R, JAK1 and JAK3 showed a strong correlation within the different cases. This suggests an overall upregulation of the STAT6-signalling pathway in oligodendrocytes. Additionally, we show that HIF-1α, a transcription factor inducing hypoxic preconditioning (Bergeron *et al.*, 2000), is also expressed in oligodendrocytes in MS NAWM. Therefore, we hypothesize that in contrast to the pro-inflammatory response, the protective, anti-inflammatory response in the NAWM is mounted predominantly by oligodendrocytes. This conclusion is supported by a recent study showing that oligodendrocytes are capable of mounting protective mechanisms preventing demyelination (Lin *et al.*, 2007). Furthermore, it was recently reported that mice with suppressed oligodendrocyte responsiveness to IFN-γ developed EAE with an accelerated onset and markedly increased oligodendrocyte apoptosis (Balabanov *et al.*, 2007). In the same study, oligodendrocytes were shown to be capable of expressing several chemokines. This supports the view of oligodendrocytes participating in the regulation of CNS intrinsic immunity. Therefore, we speculate that the exacerbation of EAE in STAT6 knockout mice (Chitnis *et al.*, 2001) might also result from the deficiency in activating the STAT6-signalling pathway in oligodendrocytes. Because oligodendrocytes are highly susceptible to inflammation-mediated damage it may be crucial for them to compensate for the upregulated pro-inflammatory environment and to limit the inflammatory response and damage. The upregulation of an anti-inflammatory, and therefore also neuroprotective, environment in the MS NAWM is further supported by the activation of other neuroprotective pathways shown in our previous NAWM study (Graumann *et al.*, 2003).

Are signals from lesions leading to an imbalance of inflammatory mechanisms in NAWM or vice versa?

We hypothesize that there are two possible reasons for the differential regulation of immune modulating genes in the cells of the NAWM. One possibility could be that diffusing, soluble factors released by activated inflammatory cells found in active MS lesions might activate and modulate inflammatory gene expression in resident cells of the distant NAWM. This idea is supported by a study using quantitative magnetic resonance imaging (MRI) techniques, where the authors suggest that axonal damage and demyelination in NAWM mainly arise as a secondary result of visible lesions with the largest effect close to these lesions (Vrenken *et al.*, 2006). With the exception of IL-1β, IL-10, IL-18 and TGF-β2, most cytokines could not be detected by microarray in MS NAWM, which suggests that these cytokines are not, or very weakly, expressed in NAWM tissue. Because we observed STAT4 and STAT6 upregulation in MS NAWM, we performed quantitative RT-PCR to identify the possible expression of their main activating cytokines, IL-4, IL-13, IL12 and IL-23. The absence of IL-4, IL-12, IL-13 and IL-23 expression on one hand, and the simultaneous upregulation of genes from the STAT4 and STAT6 pathway in MS NAWM on the other hand, further imply that extrinsic signals might influence the expression of immune modulating genes in the resident cells of the CNS. Additionally, this might be supported by the fact that in different tissue samples from the same MS case some genes are differently regulated, such as MCSF or CCR1 in the case of MS1.

Another possibility could be that brain intrinsic events such as impairment of oligodendrocyte and/or neuronal function, and subsequent astrocyte and microglia activation may be the initial cause for the differential gene regulation observed in NAWM. This hypothesis is supported by the upregulation of the endogenous neuronal NOS (nNOS), but not of the inducible NOS (iNOS) and endothelial NOS (eNOS), suggesting a parenchymal disregulation. Immune modulating signals from the periphery would first activate microglia inducing iNOS or endothelial cells of the blood–brain-barrier activating eNOS. Moreover, the upregulation of HIF-1α in oligodendrocytes and neurons supports the view of oligodendrocyte and/or neuronal dysfunction in the NAWM as a possible primary cause. This idea is supported by a study of relapsing–remitting MS cases, showing widespread oligodendrocyte apoptosis as the earliest change in lesions in which other cells appeared normal (Barnett and Prineas, 2004). It could be that in certain regions of the NAWM activated endogenous neuroprotective mechanisms may gradually fail. This may lead to an imbalance between protective and pro-inflammatory mechanisms facilitating lesion formation.

Is MS NAWM in a subtle balance between inflammation and neuroprotection?

Overall our results suggest that although the NAWM of MS patients shows no visible signs of active inflammation, many different genes are expressed in the tissue, which are known to be involved in the regulation and activation of the immune response that are normally not expressed in the CNS. The expression of pro- as well as anti-inflammatory genes in the NAWM of the MS brain suggests that the CNS is in a state of low-level inflammation and an unsteady balance between protection and inflammation. This is further reflected by the heterogeneous regulation of cell adhesion molecules, matrix metalloproteinases and genes of the HLA complex. Yet, this combination of inflammatory factors seems not to be enough to promote T-cell recruitment. Possibly, these immune modulating genes are expressed below an active threshold or not in

the required combination. The expression of the different subunits of the interleukin 2 receptor may also suggest that some of these mechanisms are not yet functionally active. Furthermore, the presence of competing anti-inflammatory mechanisms may inhibit infiltration of peripheral immune cells. Nevertheless, the activation of pro-inflammatory components might contribute to the reported diffuse damage of the CNS in MS (Kutzelnigg *et al.*, 2005).

In summary, we show that a substantial set of genes involved in inflammation is expressed in resident cells of the NAWM. These genes are specifically regulated in the NAWM of MS patients compared with healthy controls, indicating an activation of the intrinsic immune regulation of the CNS, whereat oligodendrocytes actively participate. Therefore, the MS NAWM may be in a state of subtle balance between inflammation and neuroprotection, leading to an immune preconditioning of the non-infiltrated NAWM.

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