The stress-axis in multiple sclerosis: Clinical, cellular and molecular aspects

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Microglia in normal appearing white matter of multiple sclerosis are alerted but immunosuppressed

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ABSTRACT

Little is known about the functional phenotype of microglia in normal appearing white matter (NAWM) of multiple sclerosis (MS), although it may hold valuable clues about mechanisms for lesion development. Therefore, we studied microglia from NAWM obtained post-mortem from controls (n=25) and MS patients (n=21) for their phenotype *ex vivo* and their immune responsiveness *in vitro*, using a microglia isolation method that omits culture and adherence. By flow cytometry, microglia in MS NAWM displayed elevated CD45 levels and increased size and granularity, but were distinct from autologous choroid plexus macrophages by absent or low expression of additional markers, in particular CD206. Flow cytometric analysis of microglia from NAWM of 3 controls and 4 MS patients showed alterations in levels of Fc-gamma receptors in MS. In primary microglia from a bigger sample of subjects, analysis of Fc-gamma receptors by quantitative PCR indicated a significant increase in mRNA levels of the inhibitory CD32b isoform in MS NAWM. Despite their changed activation status, microglia from MS NAWM were unresponsive to LPS *in vitro*. Notably, culture with dexamethasone led to an impaired induction of the inflammation-limiting cytokine CCL18 in microglia from MS NAWM compared to those from control NAWM. Together, these data demonstrate that microglia in MS NAWM are in an alerted state, but display features of immunosuppression. Thus, the activation status of microglia in NAWM of MS patients likely reflects a response to ongoing neuroinflammation, which coincides with upregulation of immunoregulatory molecules to prevent full activation and damage to the vulnerable milieu.

INTRODUCTION

Microglia in NAWM of MS have been reported to show subtle changes in inflammatory and neuroprotective pathways and indicated changes in their activation status.[119,120] Moreover, elevated CD45 expression on microglia was found to precede the onset of autoimmune demyelination in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), supporting the idea that changes in microglial activation status represent a crucial step in the initiation of MS lesion pathology.[225] Therefore, detailed study of the phenotypic and functional properties of microglia in NAWM of MS patients may give insights into the events that lead to lesion development and reveal targets for modulation of microglial functioning in MS.

We recently developed a method for rapid isolation of pure microglia from post-mortem human brain tissue that omits effects of prolonged culture and adherence and therefore allows for observations on phenotypic and functional features of microglia that likely reflect their *in vivo* biology in a highly accurate way.[226] Using this approach, we found that microglia display CD45 expression levels and scatter characteristics that are significantly lower compared to those of autologous peripheral macrophages from the choroid plexus. Notably, the presence of peripheral inflammation at the time of dying coincided with a raise in CD45 protein levels on white matter (WM) microglia that went along with an increase in size and granularity, causing these parameters to overlap considerably with those of autologous choroid plexus macrophages. However, microglia were still negative for CD14, CD200 receptor (CD200R) and mannose receptor (CD206), in sharp contrast to choroid plexus macrophages. This showed that the two cell types display CD11b and CD45 expression and scatter characteristics with variable degrees of overlap, but can still be separated from each other by the use of additional markers. Importantly, we showed that absent CD14 protein expression on primary human microglia coincided with a lack of responsiveness to bacterial lipopolysaccharide (LPS), indicative of a profoundly immunosuppressed state, whereas distinct responses to IL-4 and dexamethasone were found.[226]

We here used this robust procedure for isolation and immediate *ex vivo*

characterization of human microglia to study the activation status and immune responsiveness of microglia in NAWM of MS. Knowing that CD45 is a sensitive indicator for microglial activation status in human and rodent brain, our first aim was to establish whether it may be used to assess phenotypic changes in microglia in NAWM of MS.[198,199,226,227] For these experiments, we excluded subjects with systemic inflammation, as it is associated with phenotypic changes of microglia in mouse and human.[226,228,229] To further define the activation status of microglia in NAWM of MS patients, we quantitatively studied expression of pattern recognition receptors, Fc-gamma receptors and molecules for T-cell co-stimulation, which were also selected on the basis of their putative role in MS pathogenesis.[230,231] Finally, we investigated *in vitro* responsiveness of primary microglia to LPS, IL-4 or dexamethasone, to determine their capacity for classical and alternative activation. We found that microglia in NAWM of MS patients are in a state of enhanced activation that concurs with suppression of pro-inflammatory immune responsiveness.

MATERIALS & METHODS

Subjects and tissue

Post-mortem brain tissue was provided by the Netherlands Brain Bank (www.brainbank.nl) of a total of 25 control and 21 MS brain donors (see table 1 for characteristics). Informed consent was obtained for brain autopsy and the use of tissue and clinical information for research purposes. At autopsy, WM from corpus callosum and choroid plexus were dissected and stored in Hibernate A medium (Brain Bits LLC, Springfield, IL, USA) at 4ºC. Dissected WM was normal appearing by macroscopic examination and for MS brain tissue also according to post-mortem magnetic resonance imaging (MRI) scanning of 1-cm slices.[232] For control subjects, mean age was 80 y (range 49–102 y), mean post-mortem delay (PMD) was 7:04 h (range 4:05–18:30 h) and mean pH value of cerebrospinal fluid (CSF) was 6.6 (range 6.0–7.7). For MS patients, mean age was 69 y (range 50–98 y), mean PMD was 9:26 h (range 7:05–10:50 h) and mean pH value of CSF was 6.4 (range 6.0–7.1). Due to

the post-mortem MRI and dissection of MS lesions, tissue from MS patients had a significantly longer PMD of about 2:20 h than that of control subjects ($p < 0.001$; see table 2 for further characteristics). In addition, MS patients had a significantly lower age ($p=0.02$) and pH of CSF ($p=0.03$).

Control subjects did not have neurological disorders, except for donors 2010-058 (leucoencephalopathy) and 2011-090 (cervical dystonia). At the time of death, three subjects had peripheral inflammatory conditions that are known to coincide with peripheral elevation of pro-inflammatory cytokines, namely donor 2011-120 and 2012-002 (both sepsis) and donor 2012-032 (pneumonia).[233–235]

Isolation procedure and experimental usage

Isolation procedures were started within 2 to 24 h after autopsy and were performed as described before.[226] Briefly, tissue was meshed through a stainless steel sieve in a glucose-potassium-sodium buffer (GKN-BSA; 8 g/l NaCl, 0.4 g/l KCl, 1.77 g/l $Na_2HPO_4.2H_2O$, 0.69 g/l $NaH_2PO_4.H_2O$, 2 g/l D-(+)-glucose, pH 7.4) with 0.3% bovine serum albumin (Roche Diagnostics GmbH, Mannheim, Germany), which was followed by enzymatic dissociation in 4 ml enzyme buffer (4 g/l $MgCl_2$, 2.55 g/l $CaCl_2$, 3.73 g/l KCl and 8.95 g/l NaCl, pH 6-7), with 300 units/ml collagenase type I (Worthington, Lakewood, NJ, USA) and 200 mg/ml DNase I (Roche Diagnostics GmbH) for 1 h at 37°C while shaking. After erythrocyte lysis, cells were resuspended in 20 ml Percoll of $\rho=1.03$, underlain with 10 ml Percoll of $\rho=1.095$, overlain with 5 ml GKN-BSA buffer and centrifuged for 35 min at 2500 rpm and 4ºC with slow acceleration and no break. The myelin layer on top of the $\rho=1.03$ Percoll phase was discarded, after which cells enriched for microglia were collected from the interface between $\rho=1.095$ and $\rho=1.03$ Percoll layer and transferred to a polypropylene coated 12-ml tube (Greiner Bio-One GmbH, Frickenhausen, Germany) for further use.

Flow cytometry to quantify CD11b, CD45 and scatter characteristics of microglia isolated from NAWM was performed for the majority of subjects included in the study (see table 1 for detailed information). This was done on cells obtained directly after Percoll density gradient separation. In a number of cases (see table 1), analysis was also done on autologous macrophages from the choroid plexus. This allowed

for confirmation of the microglial phenotype of myeloid cells isolated from NAWM and enabled us to rule out any contribution of perivascular and/or peripheral blood cells to our analyses by flow cytometric analysis for distinguishing markers such as CD14 and CD206. After density gradient separation, depletion of granulocytes and followed by positive selection of microglia was done by magnetic-activated cell sorting (MACS) according to the manufacturer's protocol with respectively anti-CD15 and anti-CD11b conjugated magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), leading to microglia isolates of around 99% purity. These cells were subsequently used for *in vitro* experiments or flow cytometric analysis for additional myeloid markers.

Flow cytometry

Quantification of CD11b and CD45 expression

In short, 1 x 10^5 cells in 100 µl GKN-BSA with 5% human pooled serum (HPS) were incubated in the dark for 30 min on ice with anti-human CD11b (clone ICRF44, labeled with PE; eBioscience, San Diego, CA, USA), CD45 (clone HI30, labeled with FITC; Dako, Glostrup, Denmark) and CD15 (clone HI98, labeled with APC; Biolegend, San Diego, CA, USA) antibodies. Aspecific staining was determined using isotype control antibodies (clone P3, labeled with FITC or PE; eBioscience). As a viability dye, 7-amino-actinomycin D (7AAD; BD Biosciences) was added to each sample in 1:40 about 15 min prior to flow cytometric analysis. Flow cytometry was performed with identical instrument settings on a FACSCalibur machine (BD Biosciences), which was calibrated at least once a week. Data were analyzed using FlowJo software version 8.7.1 (Treestar Inc. Ashland, OR, USA).

Phenotyping for additional surface markers

Antibodies with the following specificities were used: anti-human CD32 (clone 3D3, labeled with PE), CD45 (clone 2D1, labeled with PerCP), CD80 (clone L307.4, labeled with PE), CD86 (clone 2311 (FUN-1), labeled with FITC), CD163 (clone GHI/61, labeled with PE), CD206 (clone 19.2, labeled with APC; all from BD Biosci-

ences); CD16 (clone LNK16, labeled with AF647), CD200R (clone OX108, labeled with AF647; both AbD Serotec, Oxford, UK), CD64 (clone 10.1, labeled with AF488), HLA-DR (IgG2a, clone L243, labeled with AF488; both Biolegend), CD14 (clone 61D3, labeled with PerCP-Cy5.5; eBioscience). Mouse isotype control antibodies (IgG1, clone MOPC-21, labeled with PerCP-Cy5.5, PE or APC; BD Biosciences; IgG2a, clone MOPC-173, labeled with AF488; Biolegend) were used to determine background staining. Flow cytometric analysis was performed on a FACSCanto machine (BD Biosciences) using FlowJo software.

Cell culture and stimulation

In a 96-wells plate (Greiner Bio-One), 1.0 x 10^5 MACS-isolated microglia were cultured in a total volume of 200 μl Rosswell-Park-Memorial-Institute medium (RPMI) with 10% heat-inactivated HPS per well for 18 h with 100 ng/ml LPS (Salmonella minnesota R595; Alexis Biochemicals, Lausen, Switzerland) or for 72 h with 40 ng/ml IL-4 (Peprotech, London, UK) or 2 nM dexamethasone (Sigma Aldrich, Zwijndrecht, The Netherlands).

RNA isolation

Primary or cultured microglia were harvested in 800 μl Trizol reagent (Invitrogen, Carlsbad, CA, USA) and stored at -80°C for later use. For gene expression in cultured microglia cells, RNA was isolated using a modified protocol as described before, consisting of phase separation by addition of chloroform and centrifuging, after which the aqueous (top) phase was transferred to an RNeasy mini column (Qiagen, Hilden, Germany) followed by RNA extraction according to the manufacturer's instructions.[226]

For gene expression analysis in NAWM of control subjects and MS patients, corpus callosum tissue samples snap-frozen at the start of the microglia isolation procedure were homogenized in 1 ml Trizol reagent (Invitrogen) using a T 10 basic Ultra Turrax (IKA Werke GmbH, Staufen, Germany). For these samples, as well the samples of primary microglia, RNA was isolated by overnight precipitation in isopropanol at -20ºC, using glycogen as a carrier. Concentration of all extracted RNA was

Table 1 Overview of included subjects

NBB	Diag	Age	Sex	PMD	pH	Death cause	PI	GC
2008-100	MS	77	F	10:00	6.5	Euthanasia	No	No
2009-012	Con	78	M	<17:20	6.52	Cardiac failure	No	No
2009-102	Con	92	F	6:55	6.24	General deterioration and cachexia	No	No
2010-005	MS	68	F	10:40	6.4	Euthanasia	No	No
2010-038	Con	79	F	18:30	6.3	Cardiac failure	No	No
2010-045	MS	84	F	7:35	n/a	Euthanasia	No	No
2010-058	Con	98	M	5:50	6.54	Cachexia and decubitus after CVA	No	No
2010-062	Con	94	F	5:50	7.04	Cachexia	No	No
2010-105	MS	83	M	7:50	6.4	Lung cancer	No	No
2010-108	MS	74	F	10:15	6.43	Cardio-respiratory insufficiency	No	No
2010-117	MS	60	F	10:40	6.48	Euthanasia	No	No
2011-008	MS	54	M	8:15	6,39	Euthanasia	No	No
2011-021	Con	85	F	7:05	n/a	Renal insufficiency	No	No
2011-035	MS	50	F	7:35	6.45	Euthanasia	No	No
2011-039	Con	91	F	4:15	6.5	Cardiac failure	No	No
2011-044	Con	51	M	7:45	7.05	Suicide by withering	No	No
2011-046	Con	89	F	4:45	6.76	Euthanasia	No	No
2011-048	MS	53	M	10:00	6.38	Euthanasia	No	No
2011-049	Con	83	F	4:40	6.04	Metastasized pancreas carcinoma with ileus	No	n/y/a
2011-059	Con	84	F	6:05	7.65	Interstitial lung disease	No	Yes
2011-069	Con	49	M	6:15	6.23	Euthanasia	No	Yes
2011-070	Con	87	F	7:00	6.72	Cardiac failure	No	No
2011-072	Con	76	F	7:15	6.87	Cardiac arrest	No	Yes
2011-077	MS	66	F	9:35	6.45	Euthanasia	No	No
2011-081	Con	58	M	7:30	6.88	Euthanasia with metastasized esophagus carcinoma	No	Yes
2011-087	MS	62	F	12:35	n/a	Exhaustion and respiratory insufficiency	No	No
2011-089	MS	64	M	7:30	6.49	Euthanasia	No	No
2011-090	Con	85	F	8:25	6.51	Dehydration, cachexia	No	Yes
2011-091	Con	57	M	6:45	6.31	Lung cancer	No	No
2011-093	MS	67	M	10:10	7.1	Suicide by auto-intoxication	No	No
2011-100	MS	89	F	7:05	6.32	Cachexia	No	No
2011-105	Con	95	F	5:15	6.44	Euthanasia	No	No
2011-120	MS	73	M	8:45	6.4	Urosepsis and pneumonia	Yes	No
2012-002	Con	55	M	7:15	n.b.	Sepsis due to intestinal ischemia	Yes	No
2012-005	Con	83	F	5:35	6.68	Cardiac failure	No	No
2012-008	MS	66	F	10:45	6.73	Pulmonary hypertension	No	No
2012-027 ¶	MS	54	F	9:20	6.27	Cardiac failure	No	n/y/a
2012-032	MS	80	F	9:45	6.42	Pneumonia and cachexia	Yes	n/y/a
2012-043	MS	54	M	10:50	6.26	Euthanasia	No	n/y/a
2012-052	Con	64	F	5:40	6.35	Lung carcinoma and pneumothorax	No	n/y/a
2012-056	MS	78	M	8:45	6.41	Euthanasia	No	n/y/a
2012-062	Con	88	M	5:40	7.3	Intestinal ischemia	No	n/y/a
2012-064	MS	98	F	10:00	6.4	n/y/a	n/y/a	n/y/a
2012-067	Con	102	M	5:00	6.64	Ileus	No	n/y/a
2012-070	Con	79	M	5:45	6.38	Euthanasia	No	n/y/a
2012-071	Con	67	F	7:40	6.47	Euthanasia	No	n/y/a

NBB=NBB donor registration number; Diag=clinical diagnosis; PMD=post-mortem delay; pH=pH value of cerebrospinal fluid; PI=peripheral inflammation at time of death; GC=glucocorticoid treatment in 3 months prior to death; Figure=figure that depicts the data derived

determined using a nanodrop (ND-1000; NanoDrop Technologies, Rockland, DE, USA).

cDNA synthesis

For all gene expression analyses, synthesis of cDNA was done using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. For all samples, equal RNA inputs were used in a total volume of 12 μl RNAse free water. After addition of 2 μl gDNA wipeout buffer (7x), samples were incubated at 42°C for 2 min. After addition of the master mix with RT enzyme, buffer and RT primer mix, samples were incubated at for 15 min at 42°C and for 3 min at 95°C. Samples were subsequently stored at –20°C until further use.

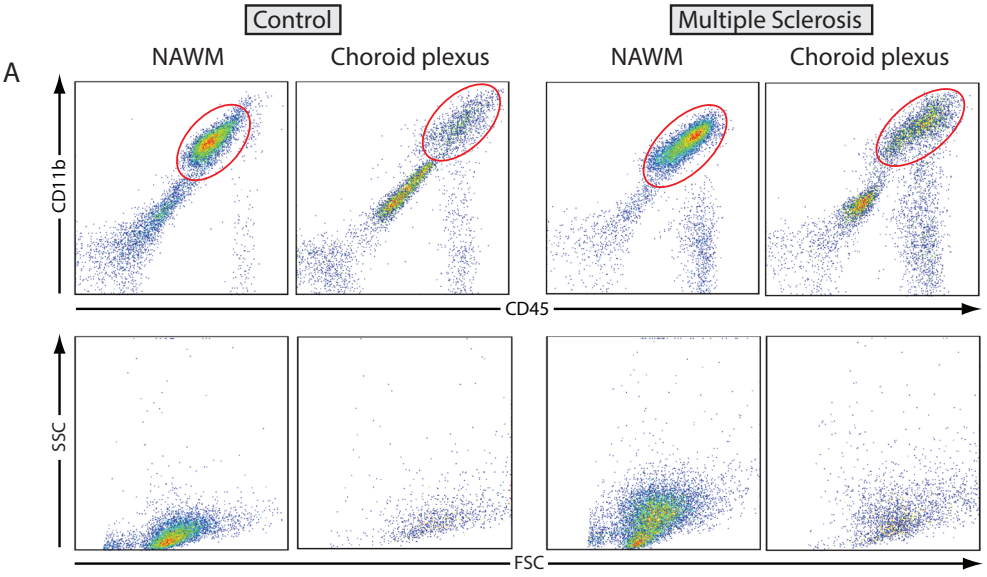
Quantitative real-time polymerase chain reaction

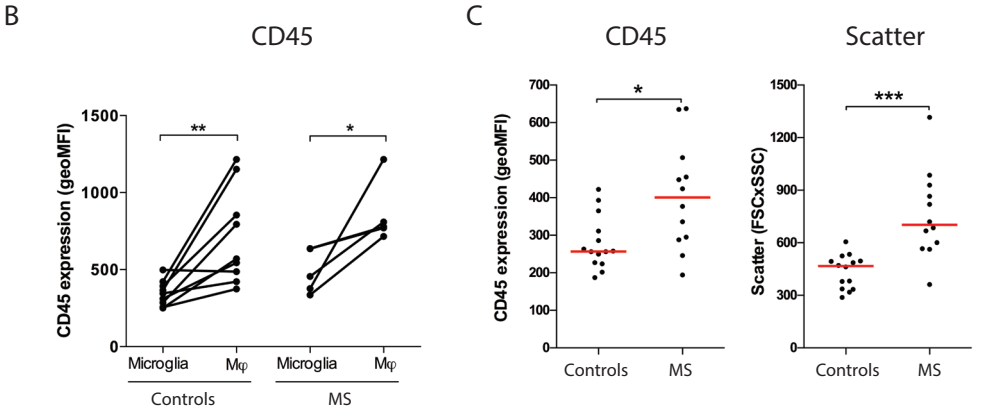
Quantitative real-time polymerase chain reaction (qPCR) was done with the 7300 Real Time PCR System (Applied Biosystems). The amount of cDNA used per reaction was based on an input of 4 to 6 ng original RNA in a final volume of 20 μl. Per reaction, 10 μl SYBRgreen PCR Master Mix (Applied Biosystems), and 2 μl primermix (2 pmol/μl) was added. Expression of selected genes was normalized to references genes 18S and GAPDH; efficiencies (E) of primerpairs were determined using LinRegPCR software. Absolute expression was calculated as: $E^{-CT\ target\ gene}$ / $E^{-CT\ reference\ gene}$.

An overview of the forward and reverse primers used for qPCR is present in the supplementary data. Primers were intron-spanning, except those for CCL18 and CCR7, and were designed with the primerS in-house software package for qPCR primer design.[236]

< Table 1 legend continued

from the donor; nd=not determined; n/a=not available; n/y/a=not yet available; ¶=white matter dissected from corpus callosum contained macroscopic MS lesion of unknown activity.


A
Control
Multiple Sclerosis
NAWM
Choroid plexus
NAWM
Choroid plexus
CD11b
CD45
SSC
FSC


B
CD45
CD45 expression (geoMFI)
1500
1000
500
0
**
*
Microglia
Mφ
Microglia
Mφ
Controls
MS
C
CD45
Scatter
CD45 expression (geoMFI)
700
600
500
400
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1500
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900
600
300
0
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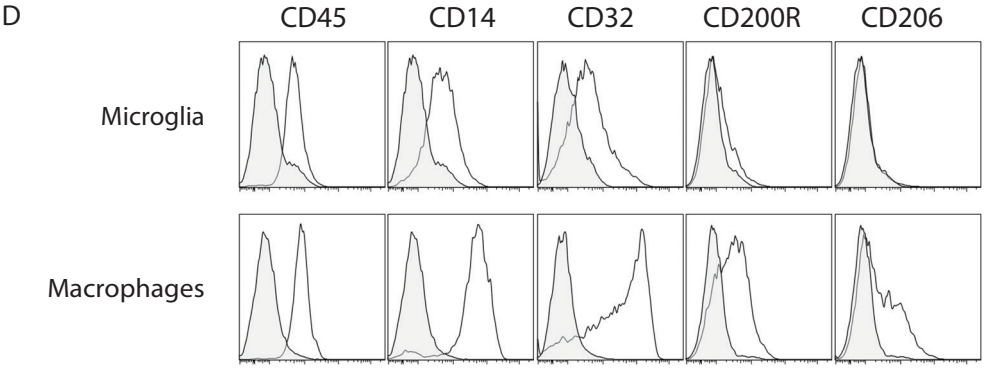
Controls
MS


D
CD45
CD14
CD32
CD200R
CD206
Microglia
Macrophages

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 5). Mann-Whitney U tests were performed to compare CD45 and CD11b geoMFI values between microglia from NAWM of control subjects and MS patients. The same test was used to compare mRNA expression levels between unstimulated microglia and microglia cultured with LPS, IL-4 or dexamethasone. Correlations were calculated using the Spearman's non-parametric correlation test. For all analyses, a p-value <0.05 was considered statistically significant. For analysis of gene expression profiles induced in microglia by LPS, IL-4 and dexamethasone, only changes of more than two-fold were considered significant.

RESULTS

Microglia in NAWM of MS patients display an enhanced activation status

Microglia from NAWM of both control subjects and MS patients could be discriminated from non-myeloid brain cells by CD11b expression and showed a 2-fold lower expression of CD45 and decreased forward and side scatter signals when compared to autologous macrophages from the choroid plexus (figure 1A and B). Compared to those from control subjects, microglia from MS patients displayed elevated CD45 expression levels ($p=0.015$) and exhibited markedly higher signals for forward and side

< Figure 1 Flow cytometric analysis of microglia from NAWM and macrophages from the choroid plexus in control subjects and MS patients. (A) Phenotypes of microglia from NAWM and choroid plexus macrophages, encircled in red, as indicated by ex vivo flow cytometric analysis for CD11b and CD45 expression in a control subject and an MS patient. The lower row depicts forward and side scatter profiles of red encircled populations in the upper panels and indicates the morphological changes of microglia in normal appearing white matter of MS patients. **(B)** Quantification of CD45 expression indicates microglia from NAWM consistently display a CD45low phenotype when compared to autologous macrophages isolated from the choroid plexus in control subjects as well as MS patients. **(C)** Quantification of CD45 expression and scatter signals shows that microglia from NAWM of MS patients display a highly significant elevation in CD45 levels and scatter signals. **(D)** Flow cytometric analysis for additional surface markers indicates that microglia in NAWM of MS patients can be distinguished from choroid plexus macrophages by lower levels of CD14, CD32 expression and absent expression of CD200R and CD206. FSC=forward scatter; SSC=side scatter; geoMFI=geometric mean fluoresence intensity; Mϕ=choroid plexus macrophages; *p <0.05, **p <0.01, ***p <0.001

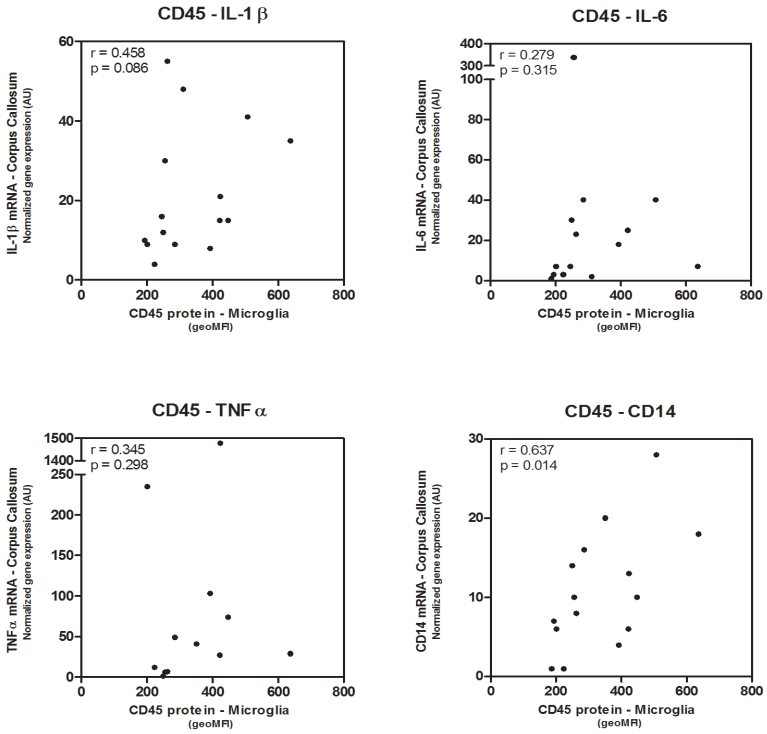
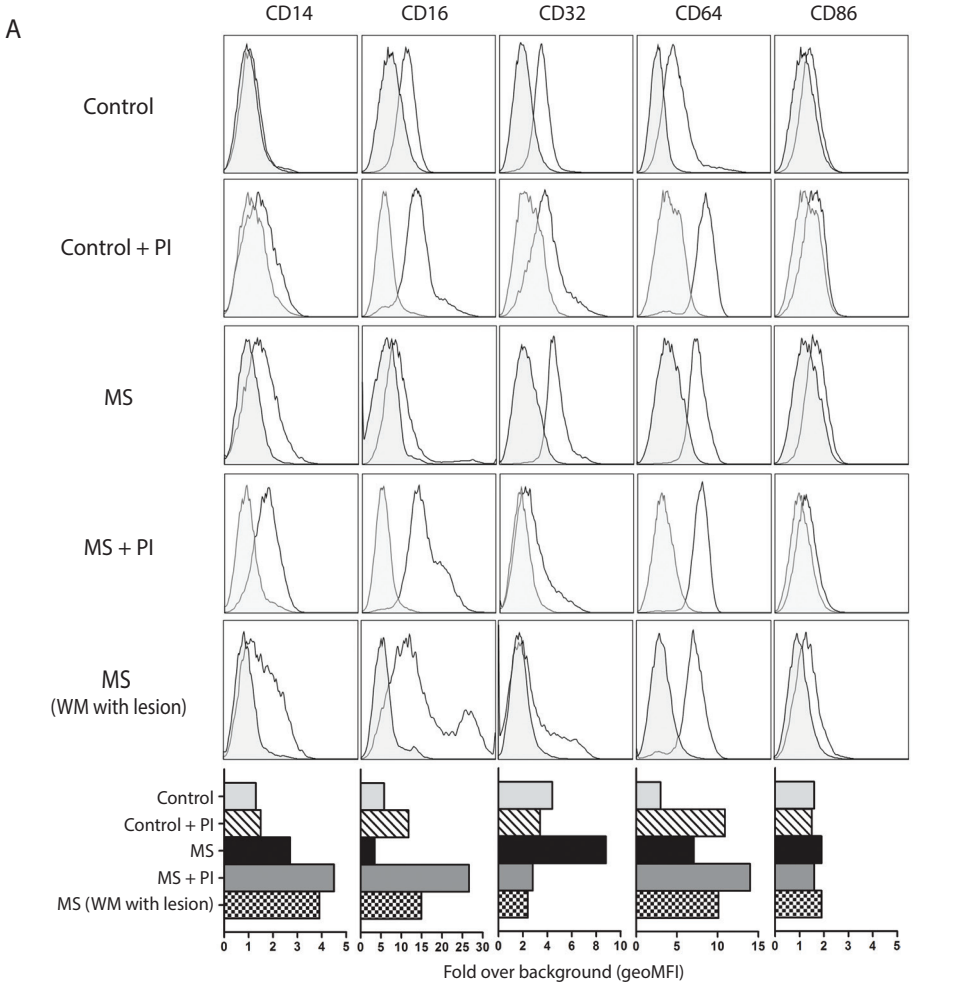


Figure 2 CD45 expression on ex vivo microglia from NAWM correlates to mRNA levels for innate inflammation markers in callosal parenchyma. Out of four markers known to be associated with levels of innate inflammation in the brain, mRNA levels of CD14 and IL-1β were correlated, in the latter case with a trend towards significance, to CD45 protein levels as determined by flow cytometry on ex vivo microglia from NAWM in control subjects and MS patients.

> Figure 3 Alterations in expression levels of Fc-gamma receptors on microglia in NAWM of MS patients. (A) Histogram overlays of CD14, CD16, CD32, CD64 and CD86 expression, as determined by ex vivo flow cytometry on microglia from NAWM of five donors with various clinical backgrounds. Open histograms represent stainings with the specified antibodies and shaded histograms represent stainings with appropriate isotype control antibodies. Graphs at the bottom of each column indicate quantified expression of the various surface markers. Expression of CD14 was detected in all MS cases and control subjects with peripheral inflammation. Notably, microglia from NAWM of the MS patient without PI displayed high CD32 expression and low CD16 expression, whereas the opposite was seen for microglia from NAWM of both subjects with PI, which was even more pronounced in cells isolated from WM with MS lesion pathology. **(B)** Scatter plots for quantified CD16, CD32 and CD64 expression, determined by flow cytometry in 3 control and 4 MS brain donors. Note the inverse correlation between CD16 and CD32, respectively the activating and inhibitory Fc-gamma receptor, and the positive correlation between CD16 and CD64, both of which are activating Fc-gamma receptors. PI=peripheral inflammation; WM=white matter; geoMFI=geometric mean fluorescence intensity


A
CD14
CD16
CD32
CD64
CD86
Control
Control + PI
MS
MS + PI
MS
(WM with lesion)
Control
Control + PI
MS
MS + PI
MS (WM with lesion)
Fold over background (geoMFI)
B
r = -0.786
p = 0.048
CD32
CD16
r = 0.893
p = 0.012
CD64
CD16
r = -0.571
p = 0.200
CD64
CD32

scatter characteristics ($p < 0.001$; figure 1C). No correlations were present between CD45 expression and PMD, pH or age. When analyzed for control subjects and MS donors together, scatter characteristics correlated with PMD ($r=0.537$, $p=0.006$), but not with pH or age. This suggests that PMD may have confounded the differences in scatter characteristics of microglia from NAWM of control subjects and MS patients, as PMD was longer in MS patients. However, microglial scatter signals and PMD did not correlate when analyzed for control subjects only ($r=-0.222$, $p=0.446$, $n=14$), indicating that PMD does not have a major impact on scatter features of microglia. Expression of CD45 correlated with scatter characteristics ($r=0.568$, $p=0.003$), revealing that elevated CD45 expression and morphological alterations coincide in microglia from NAMW of MS patients, which together demonstrate an enhanced activation status of these cells. Importantly, phenotyping for additional surface markers revealed that microglia from NAWM could be well distinguished from autologous choroid plexus macrophages, even in MS patients, by absent CD206 expression and much lower expression of CD14, CD32, and CD200R on microglia (figure 1D).

To find out to what extent microglial activation status is associated with the inflammatory state of the surrounding tissue, we investigated whether CD45 protein levels on isolated microglia related to mRNA levels of markers for innate inflammation in callosal parenchyma. We found that CD45 protein expression correlated to CD14 ($r=0.637$, $p=0.014$) and with a trend towards significance to IL-1β ($r=0.458$, $p=0.086$) mRNA levels (figure 2), suggesting that the enhanced microglial activation status goes along with elevated local synthesis of these pro-inflammatory molecules.

Altered expression levels of Fc-gamma receptors on microglia in NAWM of MS patients

To get a first indication whether the activation status of microglia in NAWM of MS patients is associated with altered expression of additional myeloid markers, we studied microglia from NAWM of control subjects and MS patients for expression of the pattern recognition receptors CD14, CD163 and CD206, the Fc-gamma receptors CD16, CD32 and CD64, the immunoregulatory CD200R and molecules for T-cell co-stimulation CD80 and CD86. This explorative analysis was performed in a

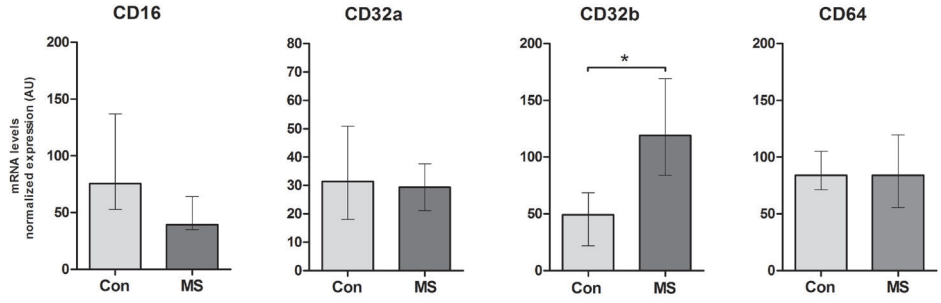
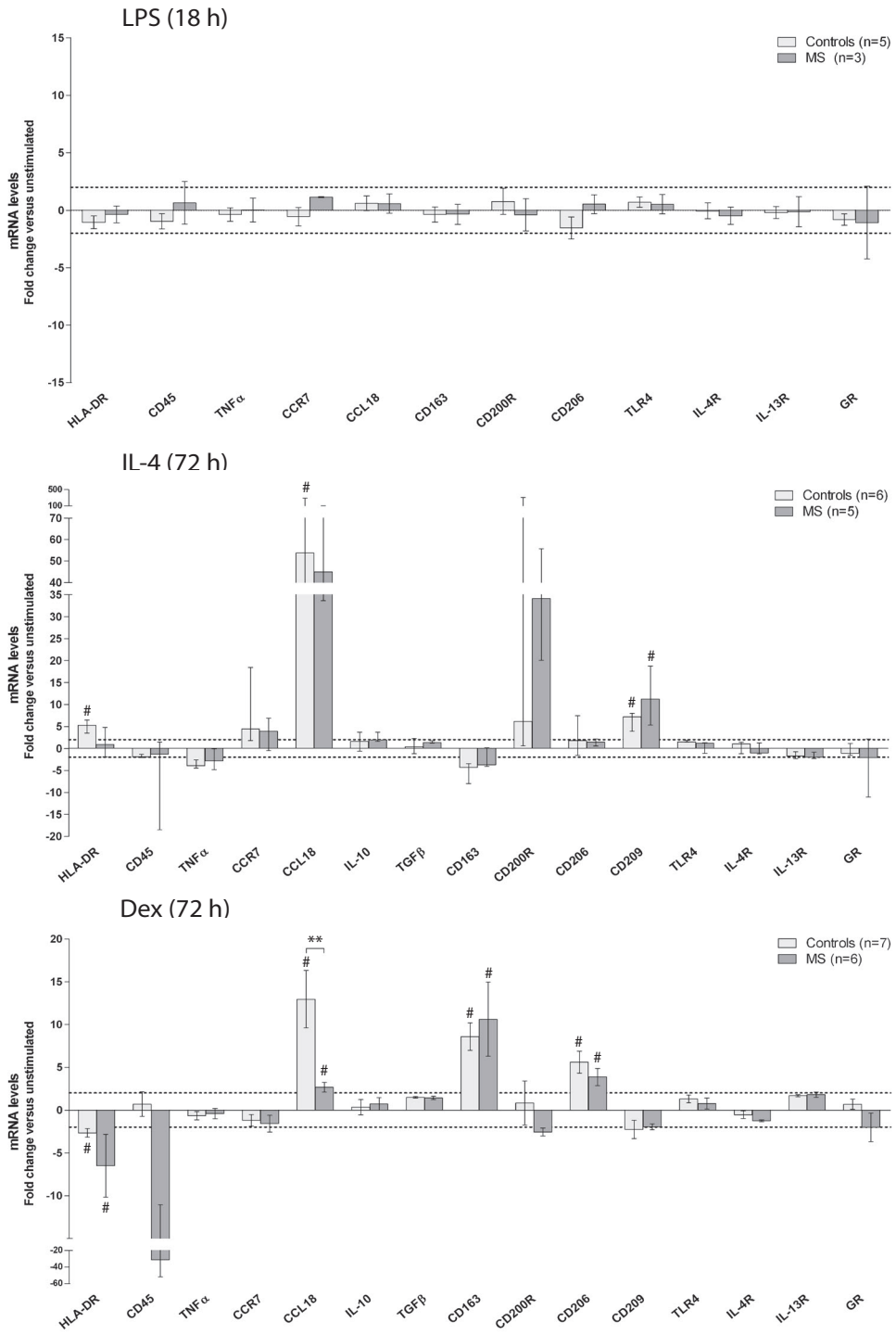


Figure 4 Significantly elevated expression of the inhibitory isoform of CD32 in microglia from NAWM of MS patients. Gene expression, as determined by qPCR analysis, in ex vivo microglia from NAWM of control subjects and MS patients indicates for CD16, the CD32 isoforms CD32a and CD32b and CD64. Interestingly, microglia from NAWM of MS patients had higher levels of CD32b, the isoform that specifically confers the inhibitory downstream effect of CD32 on cellular immune activation. Horizontal bars represent the median and interquartile range. * $p < 0.05$

limited number of subjects that differed with respect to the presence of peripheral inflammatory conditions while dying, which was previously demonstrated by our group to be associated with phenotypic alterations of human microglia.[226] Therefore, data are depicted separately for all included subjects in figure 3. Expression of CD14 was detected only in cases with MS and/or marked peripheral inflammation at the time of dying (figure 3A). Only in one MS case, low expression of CD163 was observed on microglia from NAWM, whereas expression of CD206 was totally absent on all microglia isolated from NAWM of control subjects and MS patients (data not shown). Levels of CD80 were undetectable in all cell isolates, while expression of CD86 was marginal and highly similar in all analyses. Expression of CD200R was found to be marginal at most (data not shown). In contrast, expression of Fc-gamma receptors was detected on all cell isolates and was modulated on microglia from NAWM of MS patients. Of note is that CD16 expression was high and CD32 expression low in microglia from NAWM of subjects with peripheral inflammation, which was even more pronounced in cells isolated from WM with MS lesion pathology (figure 3A). Expression levels of the activating Fc-gamma receptors CD16 and CD64 were correlated to each other ($r=0.893$, $p=0.012$). In contrast, expression of the inhibitory Fc-gamma receptor CD32 was inversely correlated to expression of CD16 ($r=-0.738$, $p=0.048$) and CD64 ($r=-0.571$, $p=0.200$), though in the latter case not significantly (f igure


LPS (18 h)
Controls (n=5)
MS (n=3)
mRNA levels
Fold change versus unstimulated
HLA-DR
CD45
TNFα
CCR7
CCL18
CD163
CD200R
CD206
TLR4
IL-4R
IL-13R
GR
IL-4 (72 h)
Controls (n=6)
MS (n=5)
mRNA levels
Fold change versus unstimulated
HLA-DR
CD45
TNFα
CCR7
CCL18
IL-10
TGFβ
CD163
CD200R
CD206
CD209
TLR4
IL-4R
IL-13R
GR
Dex (72 h)
Controls (n=7)
MS (n=6)
mRNA levels
Fold change versus unstimulated
HLA-DR
CD45
TNFα
CCR7
CCL18
IL-10
TGFβ
CD163
CD200R
CD206
CD209
TLR4
IL-4R
IL-13R
GR

3B). Apparently, expression of CD14 and the Fc-gamma receptors CD16, CD32 and CD64 is related to microglial activation status in NAWM, in contrast to that of CD80 and CD86.

Having found changes in expression levels of Fc-gamma receptors on microglia in NAWM of individual MS patients by flow cytometry, we studied expression of these molecules by qPCR in primary microglia isolates of 9 control subjects and 7 MS patients without any indications of peripheral inflammation to investigate whether consistent differences would be present in expression levels of Fc-gamma receptors. Interestingly, we observed a specific mRNA increase of the inhibitory CD32b isoform ($p=0.044$) in microglia from NAWM of MS patients compared to those from NAWM of control subjects, whereas mRNA levels of CD16, CD32 and CD64 were unaltered (figure 4). Apparently, the enhanced activation status of microglia in NAWM of MS patients coincides with a specific upregulation of the immunosuppressive Fc-gamma receptor CD32b.

Unresponsiveness to LPS and altered glucocorticoid responsiveness in microglia from normal appearing white matter of MS

The finding that NAWM in control subjects and MS patients harbors microglia with phenotypic changes associated with an altered activation status raised the question whether this is also reflected in their responses towards pro- and anti-inflammatory stimuli. Therefore, we investigated responses to LPS, IL-4 and dexamethasone of primary microglia from control subjects and MS patients without any peripheral inflammation. Intriguingly, a lack of responsiveness to culture for 18 h with LPS was seen in primary human microglia from NAWM of both control subjects as well as MS

< Figure 5 Lack of LPS-responsiveness in primary microglia from NAWM of control subjects and MS patients. Gene expression, as determined by qPCR analysis, in primary microglia cultured in the presence of LPS, IL-4 or dexamethasone. Note the absent upregulation of TNFα and CCR7 after culture with LPS and the specifically impaired induction of CCL18 after culture with dexamethasone. Bars represent median and interquartile range. #=$p<0.05$ for changes in gene expression in stimulated microglia compared to controls conditions (medium only); **=$p<0.01$ for differences in fold change of gene expression between stimulated microglia from NAWM of control subjects and MS patients.

patients, as induction of TNFα and CCR7 mRNA was absent (figure 5). Thus, the insensitivity of microglia to LPS, as recently described by us in NAWM of healthy subjects, was still conserved in MS NAWM.[226]

In contrast, microglia from control and MS NAWM displayed distinct responses to anti-inflammatory stimuli. Culture of microglia with IL-4 upregulated CCL18 in microglia from NAWM of control subjects and, with a trend towards significance (p=0.063) in those from NAWM of MS patients. CCL18 is a chemokine that is associated with alternative activation by IL-4 in macrophages.[207] In both control and MS microglia, IL-4 increased CD200R expression, yet not significantly, probably as a consequence of low power due to sometimes undetectable mRNA levels in microglia cultured in medium only.[222] Moreover, IL-4 induced the alternative activation marker dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209) in microglia from NAWM of control subjects and MS patients.[237] In contrast, culture of microglia from NAWM of control subjects and MS patients with IL-4 did not upregulate CD206, though it is strongly associated with IL-4 induced alternative macrophage activation.[238] Overall, no differences were observed for IL-4 induced immune responses between microglia from NAWM of control subjects and MS patients.

Culture of microglia with dexamethasone led to a significant upregulation of CD163, CD206 and CCL18. Interestingly, the latter was induced up to a 27-fold in microglia from control NAWM, but only about 3-fold in microglia from MS NAWM ($p < 0.05$). At the same time, basal expression levels of CD163, CCL18 and the glucocorticoid receptor (GR) after 72 h of culture did not differ from those of controls (data not shown). To find out whether basal or glucocorticoid-induced expression of CCL18 was affected by microglial activation status, we studied its relation with CD45 levels as determined by flow cytometry. We found that CD45 expression levels correlated negatively, with a trend towards significance, with fold induction of CCL18 in microglia cultured for 72 h with dexamethasone ($r=-0.635$, $p=0.096$) but not with basal CCL18 mRNA levels at the same time point ($r=0.151$, $p=0.957$).

DISCUSSION

This study identifies in microglia from NAWM of MS patients an enhanced activation status, indicated by increases in size, granularity and CD45 expression that coincides with elevated expression of CD32b and *in vitro* unresponsiveness to LPS. From this we conclude that microglia in NAWM of patients are in a state of alertness, defined by their activated appearance, that includes features of immunosuppression. Possibly, this alerted state represents a protective mechanism against ongoing neuroinflammation that concurs with enhanced expression of immunoregulatory molecules CD45 and CD32b to prevent unwanted damage to the highly vulnerable neuronal milieu. Our observations to some extent fit the concept of microglial priming.[239,240] This concept was originally described in a rodent model of prion disease in which microglia displayed an activated morphological appearance and expressed the anti-inflammatory cytokine transforming growth factor-beta (TGFβ) under basal conditions, which was followed by excessive microglial production of pro-inflammatory cytokines upon induction of systemic inflammation by peripheral LPS injection. Morphological changes and upregulation of immunoregulatory molecules also characterize the phenotype of microglia in NAWM of MS patients. However, we did not find any differences in TGFβ mRNA levels in microglia from NAWM of control subject and those of MS patients cultured under basal conditions for 72 h. Notably, we observed in these cells an absence of *in vitro* LPS responsiveness at 18 h. It therefore remains to be elucidated whether microglia alertness in NAWM of MS patients, as defined in this study, is indeed in line with the idea of priming. Importantly, microglia in NAWM of MS patients might still be more sensitive to superimposed immune challenges that coincide with ligation by other ligands than LPS, such as cytokines secreted by CNS-homing (autoreactive) T cells or perivascular macrophages during systemic inflammatory episodes. In line with this, clinical exacerbations of MS are associated with the occurrence of peripheral inflammation, which in its turn is known to induce phenotypic alterations of microglia in mouse and human.[226,228,229]

The phenotypic changes of microglia from NAWM of MS patients that we found by *ex vivo* flow cytometry are in line with studies reporting an enhanced microglial

activation status based on increased *in situ* HLA-DR and CD68 expression detected by semi-quantitative analysis.[16,241] Moreover, increased microglial CD45 expression, granularity and size has also been found to be an early event in EAE that precedes further cellular differentiation and onset of autoimmunity.[225] This is in line with our finding that quantified CD45 expression and scatter characteristics of microglia were correlated to each other, indicating that CD45 upregulation in microglia coincides with morphological alterations. In agreement with data from immunohistochemical studies, CD14 expression was absent on control microglia in the absence of peripheral inflammation.[242] However, CD14 was expressed by microglia isolated from NAWM of MS patients and control subjects with peripheral inflammation, as was also reported by *in situ* studies on NAWM of MS and virus-associated encephalitis patients.[198,243,226]

We carefully investigated our data for possible confounding effects of age, PMD and pH by correlation analysis. This was also done in control subjects only, to find out whether effects could be observed independent of MS pathology. None of the parameters described by us correlated to age or pH, which makes clear that findings reported in this study were not affected by pH or potential age-related alterations in microglial phenotypes. Moreover, potential confounding effects of systemically elevated pro-inflammatory cytokine levels on microglial activation status were eliminated from group-wise comparisons by exclusion of cases with peripheral or systemic inflammation at the time of dying.

The undetectable expression of CD80 and low levels of CD86 expression on all cell isolates, consistent with previous reports, indicate that microglial alertness in NAWM of MS patient is not associated with an enhanced potential for $CD4^+$ T-cell (co-)stimulation and activation.[215,244,245] Microglia were negative for CD206 in all phenotyping experiments, in contrast to autologous choroid plexus macrophages, which points out that the presence of perivascular and/or peripheral macrophages in microglia isolates used for our experiments can be ruled out.

Modulation of Fc-gamma receptor expression levels was observed on microglia from NAWM of MS patients. Earlier studies have shown that Fc-gamma receptors on cultured human microglia mediate cytotoxicity and phagocytosis of

antibody-coated targets and both myelin and axonal components have been identified as targets for autoantibodies in MS.[27,231,246,247] Therefore, triggering of pro-inflammatory and toxic microglial effector responses by binding of antigen–antibody complexes to Fc-gamma receptors may very well be an important mechanism underlying MS lesion pathogenesis and neuronal damage. Indeed, expression of IgG antibody-binding receptors CD16, CD32 and CD64 was found to be increased in active lesions.[246] Thus far, the relative expression levels of Fc-gamma receptors on microglia in NAWM of control subjects and MS patients was unknown, though they can be affected by the presence of neurodegenerative pathology and peripheral inflammation.[229,248] Flow-cytometric analysis gave a first indication of modulated Fc-gamma receptor expression on human microglia isolated from NAWM of control subjects and MS patients with and without peripheral inflammation. The correlation between the activating receptors CD16 and CD64 and the inverse correlation between CD16 and the inhibitory receptor CD32 suggested that modulation of Fc-gamma receptors is associated with microglial activation status. We therefore performed gene expression analysis in a well-powered sample of subjects, which allowed us to investigate for consistent differences between microglia from NAWM of controls subjects and MS patients, while excluding possible confounding effects of peripheral inflammation. These experiments revealed a specific increase in expression of CD32b, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail that confers a suppressive downstream effect on cellular immune activation.[249] The enhanced expression of the inhibitory CD32b in NAWM of MS patients may contribute to the absence of LPS responsiveness, as it has been found to suppress TLR4-mediated immune responses.[250]

Though commonly referred to as a marker for microglia activation, CD45 itself is an immunoregulatory molecule that transmits an immunosuppressive effect upon interaction with CD22 on neurons and has been reported to inhibit LPS-induced TNFα production by microglia *in vitro*.[251] Apparently, the phenotypic changes of microglia in NAWM of MS patients do not represent a type of pro-inflammatory activation, but rather a state of alertness that actually acts to oppose full-blown microglia activation and neurotoxicity.

Unresponsiveness to LPS could be a feature of microglia that is shared with other tissue-specific myeloid cells. A good example are intestinal macrophages, which exhibit very low expression levels for many pattern recognition receptors, lack CD14 expression and exhibit inflammatory anergy towards many pathogen-derived ligands.[252] Previous data from out group indicated that LPS-responsiveness is instigated in microglia isolated from control NAWM upon short-term culture that coincides with marked upregulation of CD14.[206,226] Responsiveness of microglia to pathogen-derived patterns other than LPS remains to be tested. Importantly, though pro-inflammatory cytokine production was absent upon LPS stimulation for 18 h, primary microglia from both control subjects and MS patients responded to IL-4 and dexamethasone at the same time point. This indicates that unresponsiveness of the cells to LPS cannot be attributed to an overall refractory state induced by the isolation procedure, suggesting that pro-inflammatory anergy might be a constitutive feature of microglia, similar to what is described for other tissue-specific myeloid cells.[252] IL-4 and dexamethasone are both well-known inducers of alternative activation in macrophages. Responsiveness of microglia to IL-4 was indicated by upregulation of CCL18 and CD209, whereas no induction of CD206 was observed, although the latter is an established marker for alternative macrophage activation.[84,253,222] Apparently, CD206 distinguishes microglia from macrophages *in situ, ex vivo* and after alternative activation by IL-4.[80,143] Importantly, culture of microglia with dexamethasone revealed a specific impairment of CCL18 induction in microglia from NAWM of MS patients, which may bear relevance for glucocorticoid therapy in MS. This was not due to reduced glucocorticoid responsiveness, as microglia from NAWM of control subjects and MS patients showed a similar upregulation of CD163, which is a well-known glucocorticoid-induced marker. Marked dexamethasone-induced CLL18 expression observed in microglia from NAWM of control subjects, has not been described for human macrophages before.[223] Indeed, CCL18 was only weakly induced by dexamethasone in human monocytes-derived macrophages in our hands.[226] Interestingly, CCL18 is one of the most abundant chemokines secreted by immature dendritic cells that preferentially attracts naïve and memory T cells and induces in these cells a $CD4^+CD25^+FoxP3^+$ regulatory T-cell phenotype.[254–256] As such, CCL18 might be a essential factor for suppression of local pro-inflammatory

immune responses in the CNS.

In conclusion, this study demonstrates that microglia in NAWM of MS patients are in an alerted but immunosuppressed state, characterized by elevated expression of the immunoregulatory molecules CD45 and CD32b, and apparent LPS-unresponsiveness. We postulate that the alerted but immunosuppressed phenotype of microglia in NAWM of MS patients represents a protective mechanism against ongoing neuroinflammation and neurodegeneration and that the relative expression of Fc-gamma receptors may be a determining factor in the transition of microglia from a resting phenotype to a state of alertness and ultimately full cellular activation. Secondary immune challenges, such as autoreactive T cells or systemic elevation of pro-inflammatory cytokines, may alter the phenotype of these alerted microglia in a way that makes them prone to full-blown activation and initiation of new MS lesions. Genome-wide profiling studies should be performed to define the exact nature of microglial alertness in NAWM of MS patients, which might produce valuable insight into the pathogenic mechanisms that underlie lesion development and may provide therapeutic opportunities for modulation of microglial functioning in MS.

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SUPPLEMENTAL DATA

Supplemental table 1 Characteristics of subjects included for flow cytometric analysis of CD11b and CD45 expression and scatter features.

	Controls (n=14)	MS patients (n=12)
Age (y)	84.0 (58 – 88)	65.0 (55 – 79)
PMD (h:m)	6:30 (5:10 – 7:10)	9:20 (7:25 – 10:25)
pH CSF	6.7 (6.2 – 6.9)	6.4 (6.4 – 6.5)

Values are provided as median with interquartile range; PMD = Post-mortem delay; pH = pH value of cerebrospinal fluid.

Supplemental table 2 Overview of primers used for gene expression analysis

Gene	NCBI database no.	Primer sequence forward	Primer sequence reverse
HLA-DR	NM_019111	CCCAGGGAAGACCACCTTT	CACCCTGCAGTCGTAAACGT
PTPRC	NM_002838.3	GCAGCTAGCAAGTGGTTTGTTC	AAACAGCATGCGTCCTTTCTC
CD14	NM_000591.3	ACAGGGCGTTCTTGGCTCGC	CGGGAAGGCGCGAACCTGTT
TNF	NM_000594	ATGTCCCAAGACAAGGGCAAGA	GGGCAGTCAATGGTCAGATATTGA
CCR7	NM_001838.3	TGAGGTCACGGACGATTACAT	GTAGGCCCACGAAACAAATGAT
CCL18	NM_0002988.2	CCCAGCTCACTCTGACCACT	GTGGAATCTGCCAGGAGGTA
MRC1	NM_002438	TGCAGAAGCAAACCAAACCTGTAA	CAGGCCTTAAGCCAACGAAACT
CD200R1	NM_138806	GAGCAATGGCACAGTGACTGTT	GTGGCAGGTCACGGTAGACA
CD163	NM_004244.5	AAGACGCTGCAGTGAATTGCA	GGATCCCGACTGCAATAAAGGAT
TLR4	NM_138554.4	TACAAAATCCCCGACAACCTC	AGCCACCAGCTTCTGTAAACT
IL4R	NM_000418.2	GCGTTTCCTGCATTGTCATCCT	GGCTGGGTTGGGAATCTGAT
IL13R	NM_001560.2	TGCTATGAGGATGACAAACTCTGGAGTA	CGACGATGACTGGAACAATGAGTAACAT
NR3C1	NM_001018077	GGATCCCGACTGCAATAAAGGAT	CAACCGATCCACCTCACCTT
CD16	NM_001127596.1	AGAATGGCAAAGGCAGGAAGT	AAAAGCCCCCTGCAGAAGTAG
CD32a	NM_001136219.1	GTGGTCATTGCGACTGCTGTA	AGCCTTCACAGGATCAGTGGAA
CD32b	NM_004001.4	CCACTAATCCTGATGAGGCTGACA	TCATCAGGCTCTTCCAGAGCAT
CD64	NM_000566.3	AGGTGTCATGCGTGGAAGGATA	TGGTGAGGTTAGAATTCCAGTGGAA
IL1B	NM_000576.2	CCAGCTACGAATCTCGGACCACC	TTAGGAAGACACAAATTGCATGGTGAAGTC
IL6	NM_000600.3	CAGCCACTCACCTCTTCAGAA	TGCCTCTTTGCTGCTTTCACA
TGFB2	NM_003238	GCTGGAGCATGCCCGTATTTAT	CGCAGCAAGGAGAAGCAGAT
CD209	NM_021155.3	GATTCCGACAGACTCGAGGA	CCTGACTTATGGAGCTGGGG
IL10	NM_000572.2	TGCCTTCAGCAGAGTGAAGACTT	TCCTCCAGCAAGGACTCCTTTA