

Loss of ‘homeostatic’ microglia and patterns of their activation in active multiple sclerosis

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Microglia and macrophages accumulate at the sites of active demyelination and neurodegeneration in the multiple sclerosis brain and are thought to play a central role in the disease process. We used recently described markers to characterize the origin and functional states of microglia/macrophages in acute, relapsing and progressive multiple sclerosis. We found microglia activation in normal white matter of controls and that the degree of activation increased with age. This microglia activation was more pronounced in the normal-appearing white matter of patients in comparison to controls and increased with disease duration. In contrast to controls, the normal-appearing white matter of patients with multiple sclerosis showed a significant reduction of P2RY12, a marker expressed in homeostatic microglia in rodents, which was completely lost in active and slowly expanding lesions. Early stages of demyelination and neurodegeneration in active lesions contained microglia with a pro-inflammatory phenotype, which expressed molecules involved in phagocytosis, oxidative injury, antigen presentation and T cell co-stimulation. In later stages, the microglia and macrophages in active lesions changed to a phenotype that was intermediate between pro- and anti-inflammatory activation. In inactive lesions, the density of microglia/macrophages was significantly reduced and microglia in part converted to a P2RY12⁺ phenotype. Analysis of TMEM119, which is expressed on microglia but not on recruited macrophages, demonstrated that on average 45% of the macrophage-like cells in active lesions were derived from the resident microglia pool. Our study demonstrates the loss of the homeostatic microglial signature in active multiple sclerosis with restoration associated with disease inactivity.

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Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS, which, with time, results in profound

neurodegeneration in the brain and spinal cord (Lassmann *et al.*, 2007). Active demyelination and axonal or neuronal injury are associated with the focal accumulation of microglia and macrophages (Babinski, 1885; Esiri and Reading,

1987; Ferguson *et al.*, 1997; Prineas *et al.*, 2001), but an understanding of the functional phenotype and origin of these cells in different stages of lesion formation is limited (Bogie *et al.*, 2014). Microglia and macrophages have been classified as pro-inflammatory (M1) or anti-inflammatory (M2) (Martinez and Gordon, 2014; Michell-Robinson *et al.*, 2015; Walker and Lue, 2015), though this strict classification is no longer considered valid (Ransohoff, 2016). Previous studies in multiple sclerosis demonstrated the expression of molecules involved in phagocytosis, antigen presentation and cytotoxicity in active lesions (Brück *et al.*, 1995; Höftberger *et al.*, 2004; Haider *et al.*, 2011; Fischer *et al.*, 2012). Macrophages and microglia have been shown to have an intermediate phenotype (Zhang *et al.*, 2011; Vogel *et al.*, 2013; Peferoen *et al.*, 2015) and no clear lesion stage-dependent polarization patterns have been identified, which may be due both to lack of well-defined multiple sclerosis lesion types included in these studies and the absence of unique markers that define microglia (Bogie *et al.*, 2014).

Microglia were originally defined as the resident phagocytic cells in the brain by their morphological appearance (small cell with elongated nucleus and slender, ramified cell processes), but it has already been noted in these earliest studies and confirmed later that these cells under pathological conditions can transform through an activated state into cells with classical round to oval macrophage phenotype (Kettenmann *et al.*, 2011). On the other hand, radiation bone marrow chimeric animals showed that myeloid cells recruited from the circulation can invade the brain and transform to a morphological phenotype resembling resident microglia (Lassmann *et al.*, 1993; Mildner *et al.*, 2007; Prinz *et al.*, 2014). Recently, it was shown that resident microglia populate the brain during early development from the yolk sac (Ginhoux *et al.*, 2010; Kierdorf *et al.*, 2013), but that under pathological conditions additional macrophages are recruited into the lesions from the blood (Hickey and Kimura, 1988). Thus, in descriptive studies on phagocytes within brain lesions, microglia and macrophages should be defined by a combination of their morphological and molecular phenotypes. However, when discussing their origin, the terms yolk sac-derived (resident) microglia versus recruited monocytes/macrophages should be used.

We performed a comprehensive investigation of microglia and macrophage phenotypes using new cell-specific markers in conjunction with a spectrum of functional markers (Walker and Lue, 2015) on carefully staged lesions in acute, relapsing and progressive forms of multiple sclerosis. We were able to determine the contribution of resident microglia versus recruited macrophages in multiple sclerosis lesions and the functional state of microglia and macrophages during the evolution of inflammatory demyelinating lesions at well-defined disease stages.

Materials and methods

Sample characterization

Our study was performed on an archival collection of brain autopsy tissue from 31 patients with multiple sclerosis and 18 age-matched controls collected during the past decades in the Center for Brain Research of the Medical University of Vienna. Patient demographics, clinical course and the spectrum of lesions included in this study are summarized in Table 1. The multiple sclerosis sample contained 12 cases of acute or relapsing multiple sclerosis, seven with primary and 12 with secondary progressive disease. The study was approved by the ethics committee of the Medical University of Vienna (EK. Nr.: 535/2004/2016).

We analysed pathological changes in the white matter of multiple sclerosis and focused on the following regions of interest (Figs 1 and 2): (i) normal-appearing white matter, at least 1 cm distant from any lesions (Fig. 1A and B); (ii) initial lesions, defined as ‘pre-phagocytic’ lesions by Barnett and Prineas (2004) (Fig. 1A and C), which are only present in cases with a pattern III of demyelination according to Lucchinetti *et al.* (2000); (iii) the early active lesion edge of classical active lesions following pattern I, II or III type of demyelination (Lucchinetti *et al.*, 2000), defined by the presence of macrophages or microglia with myelin degradation products reactive for myelin oligodendrocyte glycoprotein (MOG) (Fig. 1A, D, F, G and H) (Brück *et al.*, 1995); (iv) the late active lesion stage with macrophages containing degradation products reactive for major myelin proteins, such as proteolipid protein (PLP) or myelin basic protein (MBP); (v) the macrophage-rich inactive centre of classical active lesions, with foamy macrophages containing empty vacuoles as a footprint of neutral lipid degradation products (Fig. 1A, E and F); (vi) the active edge of slowly expanding (‘smouldering’) lesions present in the progressive stage of multiple sclerosis (Fig. 1J); (vii) the inactive centre of slowly expanding lesions (Fig. 1J); and (viii) the inactive centre of inactive lesions. The respective lesions were defined according to previously published criteria (Brück *et al.*, 1995; Lassmann, 2011; Frischer *et al.*, 2015). The respective cases and lesions were selected from a much larger sample of multiple sclerosis cases (Frischer *et al.*, 2015) based on the presence of the respective lesion types or lesion areas and their sufficient size for quantitative analysis of the respective markers. The normal white matter of age-matched controls was analysed as a reference background for microglia activation in multiple sclerosis.

Immunohistochemistry

To define microglia and macrophage phenotypes, we used Iba1 as a general marker for both microglia and macrophages. For microglia, we used TMEM119 and P2RY12. In the brain, TMEM119 is expressed on microglia-derived cells but not on recruited blood-derived macrophages (Butovsky *et al.*, 2014; Bennett *et al.*, 2016; Satoh *et al.*, 2016). P2RY12 is expressed in homeostatic microglia defined in experimental animals (Butovsky *et al.*, 2014). Other markers were used for phagocytosis (CD68), for antigen presentation, processing

Table 1 Clinical demographics

Case	Details	Sex	Age	Lesion type	Region of interest	Disease duration, months
C 1	Controls	F	30		NWM	
C 2	Controls	F	36		NWM	
C 3	Controls	M	37		NWM	
C 4	Controls	F	39		NWM	
C 5	Controls	F	42		NWM	
C 6	Controls	F	45		NWM	
C 7	Controls	M	46		NWM	
C 8	Controls	F	47		NWM	
C 9	Controls	M	65		NWM	
C 10	Controls	M	70		NWM	
C 11	Controls	F	71		NWM	
C 12	Controls	F	71		NWM	
C 13	Controls	M	72		NWM	
C 14	Controls	F	80		NWM	
C 15	Controls	M	83		NWM	
C 16	Controls	F	84		NWM	
C 17	Controls	F	88		NWM	
C 18	Controls	F	97		NWM	
MS 1	AMS	F	34	Act. lesion III	NAWM, initial, EA, act. centre	4
MS 2	AMS	M	35	Act. lesion III	NAWM, initial, EA, act. centre	1.5
MS 3	AMS	F	45	Act. lesion III, SEL	NAWM, initial, EA, LA, SEL:edge, SEL:core	0.2
MS 4	AMS	M	45	2 × Act. lesion III	NAWM, initial, EA, LA, act. centre	0.6
MS 5	AMS	F	46	Act. lesion II	NAWM, EA	0.5
MS 6	AMS	F	46	Act. lesion II	NAWM, EA, act. centre	7
MS 7	AMS	F	46	Act. lesion II	NAWM, EA, act. centre	3
MS 8	AMS	M	52	Act. lesion II	NAWM, EA, act. centre	1.5
MS 9	AMS	M	59	Act. lesion II	NAWM, EA, act. centre	5
MS 10	AMS	F	69	Act. lesion III	NAWM, initial, EA, LA,	2
MS 11	AMS	M	78	Act. lesion III	NAWM, EA, LA	2
MS 12	RRMS	F	40	Act. lesion III	NAWM, initial, EA, act. centre	120
MS 13	PPMS	F	34	3 × inactive lesion	NAWM, inactive core	204
MS 14	PPMS	M	36	Act. lesion II	NAWM, EA, act. centre	61
MS 15	PPMS	M	53	SEL	NAWM, SEL:edge, SEL:core	168
MS 16	PPMS	F	54	Act. lesion I	NAWM, EA, LA,	72
MS 17	PPMS	F	55	Act. lesion I	NAWM, EA, act. centre	168
MS 18	PPMS	M	67	SEL	NAWM, SEL:edge, SEL:core	87
MS 19	PPMS	F	77	SEL	NAWM, SEL:edge, SEL:core	168
MS 20	SPMS	M	34	SEL	NAWM, SEL:edge, SEL:core	120
MS 21	SPMS	M	41	SEL	NAWM, SEL:edge, SEL:core	137
MS 22	SPMS	F	42	Act. lesion I	NAWM, EA, LA,	216
MS 23	SPMS	F	46	SEL	NAWM, SEL:edge, SEL:core	444
MS 24	SPMS	F	48	Act. lesion I	NAWM, EA, act. centre	410
MS 25	SPMS	F	53	SEL	NAWM, SEL:edge, SEL:core	241
MS 26	SPMS	F	53	Inactive lesion	NAWM, inactive core	360
MS 27	SPMS	M	56	Act. lesion II	NAWM, EA, act. centre	372
MS 28	SPMS	F	59	SEL	NAWM, SEL:edge, SEL:core	492
MS 29	SPMS	F	61	Inactive lesion	NAWM, inactive core	288
MS 30	SPMS	F	62	SEL, inactive lesion	NAWM, SEL:edge, SEL:core	144
MS 31	SPMS	F	81	Inactive lesion	NAWM, inactive core	432

AMS = acute multiple sclerosis; RRMS = relapsing/remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis; NWM = normal white matter of controls; NAWM = normal-appearing white matter of multiple sclerosis patients. Act. Lesion = classical active lesions; SEL = slowly expanding lesions; EA = early active lesion; LA = late active lesion; M = male; F = female.

and co-stimulation (HLA-D, HC10/MHC I, and CD86), for oxygen and nitric oxide radical production (p22phox and iNOS), for microglia activation and iron uptake or storage (ferritin and ferritin light). The mannose receptor (CD206)

and the haptoglobin/haemoglobin receptor (CD163) were used as paradigmatic markers for an M2 anti-inflammatory phenotype. Immunohistochemistry for these markers was first standardized by analysis of lymph node tissue and normal

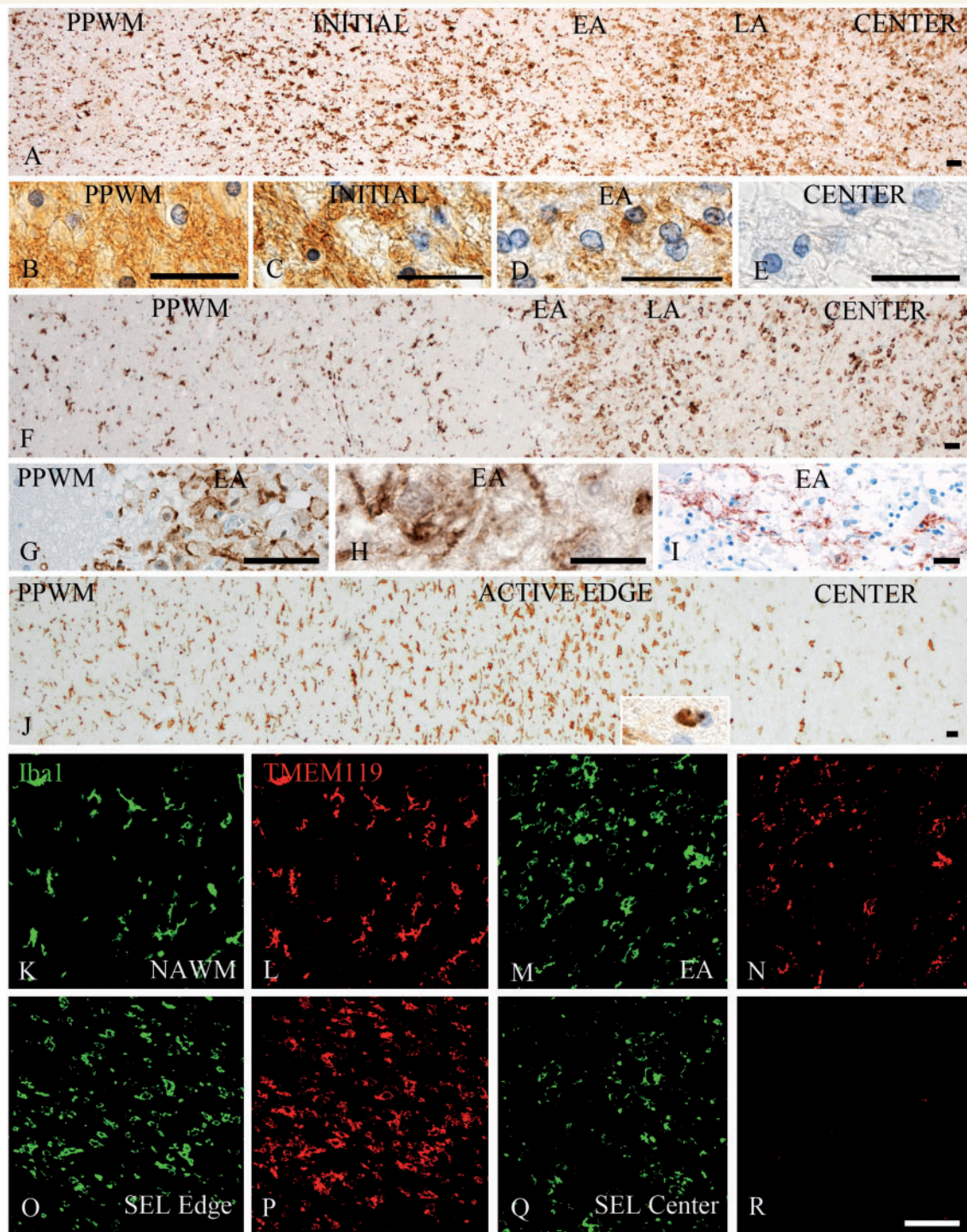


Figure 1 Patterns of microglia and macrophage reaction in different types of multiple sclerosis lesions. (A–E) Active lesion following pattern III demyelination as defined by Lucchinetti *et al.* (2000) in a patient with acute multiple sclerosis; (A) low magnification image depicting the distribution and morphology of Iba1-positive cells in different zones of the active lesions including the peri-plaque white matter (PPWM), the initial ‘pre-phagocytic’ lesion area (INITIAL), the early active (EA) and the late active (LA) lesion zones and the macrophage-containing inactive lesion centre (CENTER). There is already profound microglia activation in the initial lesion areas and these cells are transformed into or replaced by macrophage-like cells in the areas, where myelin has been destroyed (early active, late active and centre); the myelin pathology in these different lesion areas are shown in B–E; normal myelin and glia are seen in the PPWM (B). In the initial area myelin is still preserved, but there is some oedema and many oligodendrocytes show nuclear condensation and chromatin margination reflecting apoptosis (C). In the early active zone, myelin is lost, but there are many macrophages with intracytoplasmic myelin degradation products reactive for MOG (D). No myelin or MOG reactivity is seen in the demyelinated lesion centre, but there are still many macrophages with empty vacuoles reflecting the neutral lipid stage of myelin degradation (E). (F–I) Active lesion following pattern II demyelination as defined by Lucchinetti *et al.* (2000) in a patient with acute multiple sclerosis. (F) Low magnification image depicting the distribution and morphology of Iba1-positive cells in different

(continued)

brain samples before being used on multiple sclerosis brain tissue. Primary antibodies used for immunohistochemistry and specific staining conditions are summarized in Table 2.

We found that most of the markers used in this study were expressed on different macrophage subtypes in the lymph node (Supplementary Fig. 1). The only exception was P2RY12, which was not expressed at all in lymphatic tissue. TMEM119, which is unique to microglia in the brain and absent in recruited macrophages, was present on follicular dendritic cells (Satoh *et al.*, 2016). The macrophage populations stained with the other markers and their location within the lymph node tissue varied, apparently reflecting their functional polarization. In addition, other cells were stained with some of the markers; MHC II antigens being prominently expressed also on dendritic cells, MHC I antigen on a large variety of different cell types and p22phox on granulocytes. Only few macrophages within follicles expressed CD163 or CD206, while these antigens were mainly seen on medullary macrophages (Supplementary Fig. 1).

Immunohistochemistry was performed using a biotin-streptavidin technique (Fischer *et al.*, 2013). Sections were deparaffinized and antigen retrieval was done as outlined in Table 2. Non-specific protein binding was blocked by incubation with 10% foetal calf serum. Primary antibodies were applied overnight at the dilutions indicated in Table 2. Immunohistochemistry was completed by using species-specific biotinylated secondary antibodies against mouse, rabbit or goat immunoglobulins and incubation of sections with streptavidin/peroxidase complex and the reaction product was developed with diaminobenzidine. For control, immunohistochemistry was performed in the absence of the primary antibodies and by using normal rat and goat serum or isotype-matched monoclonal antibodies, directed against non-microglia/macrophage targets.

For double staining with primary antibodies derived from different species, the same antigen retrieval techniques and incubation with primary antibodies was used as described above. Antibody binding was visualized with either alkaline phosphatase-conjugated secondary antibodies or with biotinylated secondary antibodies and peroxidase-conjugated streptavidin. Alkaline phosphatase or peroxidase reaction products were visualized by development with fast blue BB salt (blue) or amino ethyl carbazole (AEC; red), respectively.

As both primary antibodies come from the same species (rabbit), double staining for P2RY12 or Iba1 and

TMEM119 was performed with a different protocol (Bauer and Lassmann, 2016) by using extensive heat-induced epitope retrieval between the subsequent immunohistochemical reactions. Details of the protocol and the respective control experiments are provided in Supplementary Fig. 3.

Quantitative evaluation

All sections were screened for white matter lesions after staining for myelin (Luxol[®] fast blue or immunohistochemistry for MOG or PLP). The selection of the proper areas for quantification was performed prior to immunohistochemistry for macrophage/microglia antigens on the basis of lesion staging as described above. Following immunohistochemistry with macrophage/microglia markers on respective serial sections the previously defined regions of interest were manually outlined. Each lesion type and randomly sampled areas of normal-appearing white matter were quantitatively analysed. In normal controls, randomly sampled areas of white matter were quantified in the same way. For the quantitative evaluation, sections stained by immunohistochemistry were overlaid by a morphometric grid (0.2256 mm²) placed within the ocular lens and two to three fields per region were quantified. For each case, average counts per square millimetre were calculated for each region of interest and compared by statistical analysis.

Digital optical densitometry was performed for markers, which were not exclusively expressed in macrophages and microglia, such as the MHC Class I marker HC10 and ferritin, according to a previously published protocol (Hametner *et al.*, 2013). Expression of these markers was quantified by calculating the positive DAB signal area fraction using ImageJ. One to two images per region of interest were taken with the 10× objective (0.43 mm²). Images were saved as TIFFs. For digitally removing haematoxylin counterstaining, a colour deconvolution plugin (freeware kindly provided by A.C. Ruifrok, NIH) was run. Further RGB images were converted into 8-bit grey-scale images and inverted. A threshold was set in resulting images and the area fraction was calculated.

Statistical analysis

Statistical analysis was performed with IBM SPSS and GraphPad Prism. Due to uneven distribution of our data,

Figure 1 Continued

zones of the active lesions, including the peri-plaque white matter, the early active and the late active lesion zones and the macrophage-containing inactive lesion centre. In contrast to pattern III lesions, there is no zone of initial demyelination with oligodendrocyte apoptosis; in contrast, microglia density is reduced in a small zone surrounding the actively demyelinating lesion area (**F** and **G**) possibly due to recruitment of peri-plaque microglia to the site of active demyelination (early active and late active zones), the actively demyelinating area is characterized by a high density of cells with macrophage phenotype (**F**), which contain early myelin degradation products (**H**). In addition, there is deposition of activated complement (C9neo antigen) at the sites of active demyelination in these lesions (**I**). (**J**) Slowly expanding lesion in a patient with secondary progressive multiple sclerosis; low magnification image depicting the distribution and morphology of Iba1-positive cells in different zones of the active lesions including the peri-plaque white matter, the active lesion edge and the inactive lesion centre. An increased density of Iba1-positive cells with a phenotype of activated microglia is seen at the active edge; in contrast, there are only very few Iba1-positive microglia-like cells in the inactive lesion centre; the *insert* shows a macrophage with early myelin degradation products. (**K–R**) Double staining for Iba1 (green) and TMEM119 (red) shows co-expression of these molecules in most cells in the normal-appearing white matter (**K** and **L**) and the active edge of slowly expanding lesions (**O** and **P**), while TMEM119 is expressed only in a subset of cells with macrophage or microglia phenotype in early active multiple sclerosis lesions (**M** and **N**). In the centre of classical active lesions and slowly expanding lesions (SEL) Iba1-positive macrophages can be present, which are negative for TMEM119 (**Q** and **R**). Scale bars = 100 µm.

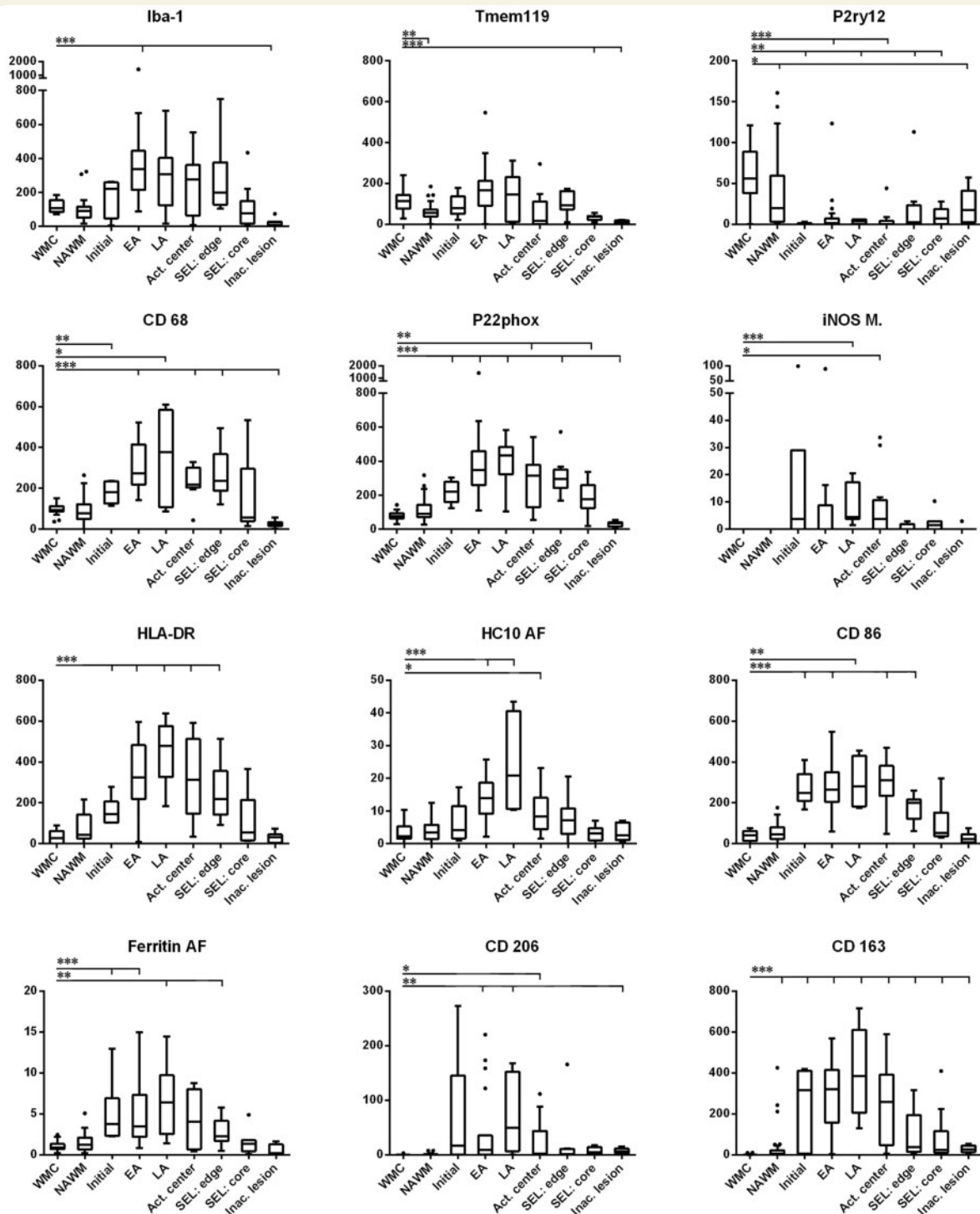


Figure 2 Quantitative evaluation of microglia/macrophages expressing different phenotypic markers in multiple sclerosis lesions. Following immunohistochemistry for the respective microglia/macrophage markers, the numbers of positive cells were quantified as described in the 'Materials and methods' section. Overall, Iba1-positive macrophages and microglia cells are similar in numbers in the normal white matter of controls and in the normal-appearing white matter of patients with multiple sclerosis. In active lesions, these cells increase already in initial lesion stages (when present in pattern III lesions) and reach their peak in early/late active lesion areas. Numerous macrophages are still present in the inactive lesion centre of these plaques. Slowly expanding lesions are surrounded by a rim of microglia cells with some intermingled macrophages and have only very few microglia or macrophages in the inactive lesion centre. A similar quantitative profile is seen for the pro-inflammatory markers CD68 (phagocytosis), p22phox (oxidative burst) and the molecules involved in antigen presentation and co-stimulation (HLA-D, HC10 and CD86). P2RY12, the marker associated with the homeostatic state of microglia in rodents is completely lost in active lesion stages, but reappears in microglia in the centre of inactive lesions. Putative M2 markers (CD163 and CD206) have their peak of expression in late active/ inactive lesions, although their expression patterns were highly variable from case to case. TMEM119 is expressed in about half of microglia

(continued)

Table 2 Antibodies used in immunohistochemistry

Antibody	Origin	Target	Dilution	Antigen retrieval	Source
PLP	Mouse (mAB, IgG2a)	Proteolipid protein	1:1000	St (E)	MCA839G; Serotec
MOG	Mouse (mAB, IgG1)	Myelin oligodendrocyte glycoprotein	1:1000	St (C)	Piddlesden <i>et al.</i> , 1993
IBA-1	Rabbit (pAB)	Ionized calcium binding adaptor molecule 1	1:3000	St (E)	019-19741; Wako
CD68	Mouse (mAB; IgG1)	CD68 110-kD transmembrane glycoprotein in macrophages	1:100	St (E)	M0814; Dako
HLA-DR	Mouse(mAB; IgG1)	MHC Class II antigen	1:100	St (C)	M0775; Dako
p22phox	Rabbit (pAB)	NADPH oxidase protein	1:100	St (C)	sc-20781; Santa Cruz
Tmem119	Rabbit (pAB)	Transmembrane protein 119	1:100	-	HPA051870; Sigma-Aldrich
P2ry12	Rabbit (pAB)	Purinergic receptor	1:2500	St (E)	Harvard, Dr. Butovsky
HC10	Mouse (mAB; IgG2a)	Heavy chain of MHC Class I	1:2000	St (E)	Stam <i>et al.</i> , 1990
Ferritin	Rabbit (pAB)	Iron storage protein	1:1000	St (E)	MO (F5012); Sigma-Aldrich
FTL	Rabbit (pAB)	Ferritin light chain	1:100	-	10727-1-AP; Proteintech
CD206	Mouse (mAB; IgG1)	Mannose receptor	1:100	St (E)	ab117644; Abcam
CD163	Mouse (mAB; IgG1)	Haemoglobin-haptoglobin scavenger receptor	1:1000	St (C)	NCL-CD163; Novocastra
iNOS	Rabbit (pAB)	Inducible nitric oxide synthase I	1:200	St (E)	PA1-37925; Thermo Scientific
CD86	Goat (pAB)	Co-stimulatory T cell signal	1:250	St (C)	AF-141-NA; R&D Systems

mAB = monoclonal antibody; pAB polyclonal antibody; ST (C) = antigen retrieval with citrate buffer; ST (E) = antigen retrieval with EDTA buffer; MHC = major histocompatibility complex.

statistical analysis was performed with non-parametric tests. Descriptive analysis included median value and range. Differences between two groups were assessed with Wilcoxon Mann-Whitney U-test. In case of multiple testing (comparison of more than two groups), significant values were corrected with the Bonferroni-Holm procedure. The reported *P*-values are results of two-sided tests. A *P*-value ≤ 0.05 was considered statistically significant.

Gene expression analysis

For the analysis of microglia/macrophage signature genes and genes related to phagocytosis, antigen processing/presentation, co-stimulation and oxidative stress, we re-evaluated whole-genome microarray data from multiple sclerosis white matter lesions and controls established previously in our laboratory (Fischer *et al.*, 2012). For this analysis we selected genes that were described to be microglia-specific in comparison to other brain cells (Chiu *et al.*, 2013; Hickman *et al.*, 2013) or in comparison to peripheral myeloid cells (Gautier *et al.*, 2012; Hickman *et al.*, 2013; Butovsky *et al.*, 2014) or to be involved in pro- or anti-inflammatory microglia/macrophage function such as phagocytosis, oxygen radical production, M1 or M2 phenotype or iron metabolism (Fig. 3). The data pool was derived from micro-dissected formalin-fixed paraffin-embedded tissue containing either normal-appearing white matter, the actively demyelinating lesion edge (containing parts of initial and early active lesions), or the inactive, macrophage-containing centre of highly active lesions from four patients with acute

multiple sclerosis (Marburg, 1906) or normal white matter of age-matched control subjects. Detailed information on sample selection/dissection, mRNA isolation and microarray technologies has been published (Fischer *et al.*, 2012). All microarray data have been deposited in NCBI's Gene Expression Omnibus (accession number GSE32915). Gene expression data derived from normal-appearing white matter, initial and active lesions were compared to normal white matter of controls and presented as fold-changes.

Results

Global dynamics of microglia/macrophage infiltration in multiple sclerosis lesions

Active lesion areas contained on average three times higher numbers of Iba1-positive cells in comparison to the number of microglia cells and perivascular macrophages in the normal white matter of controls and the normal-appearing white matter of multiple sclerosis patients (Figs 1A, F and 2). The highest numbers of these cells were seen in early active stages of classical active lesions and their density declined at later stages of lesion maturation. In slowly expanding lesions (Fig. 1J), high densities of microglia/macrophages were restricted to the active lesion edge, while in the inactive

Figure 2 Continued

and macrophages in active lesions and further decreases in the inactive lesion centres. The values for Iba1, TMEM119, P2RY12, CD68, p22phox, iNOS, HLADR, CD86, CD206 and CD163 are cells per mm² and thus the numbers in the y-axis are directly comparable. Due to the expression of the respective antigens in other cells in addition to microglia, the values for HC10 (MHC Class I) and ferritin were obtained by quantitative densitometry and are, thus, not directly comparable with the numerical counts for the other markers. WMC = white matter of controls; NAWM = normal-appearing white matter of multiple sclerosis patients; Initial = initial lesions ['pre-phagocytic' lesions (Barnett and Prineas, 2004)]; EA = early active lesions; LA = late active lesions; Act. center = macrophage-rich centre of active lesions; SEL: edge = active edge of smouldering lesions; SEL: core = lesion centre of smouldering lesions; Inac. lesions = inactive lesions.

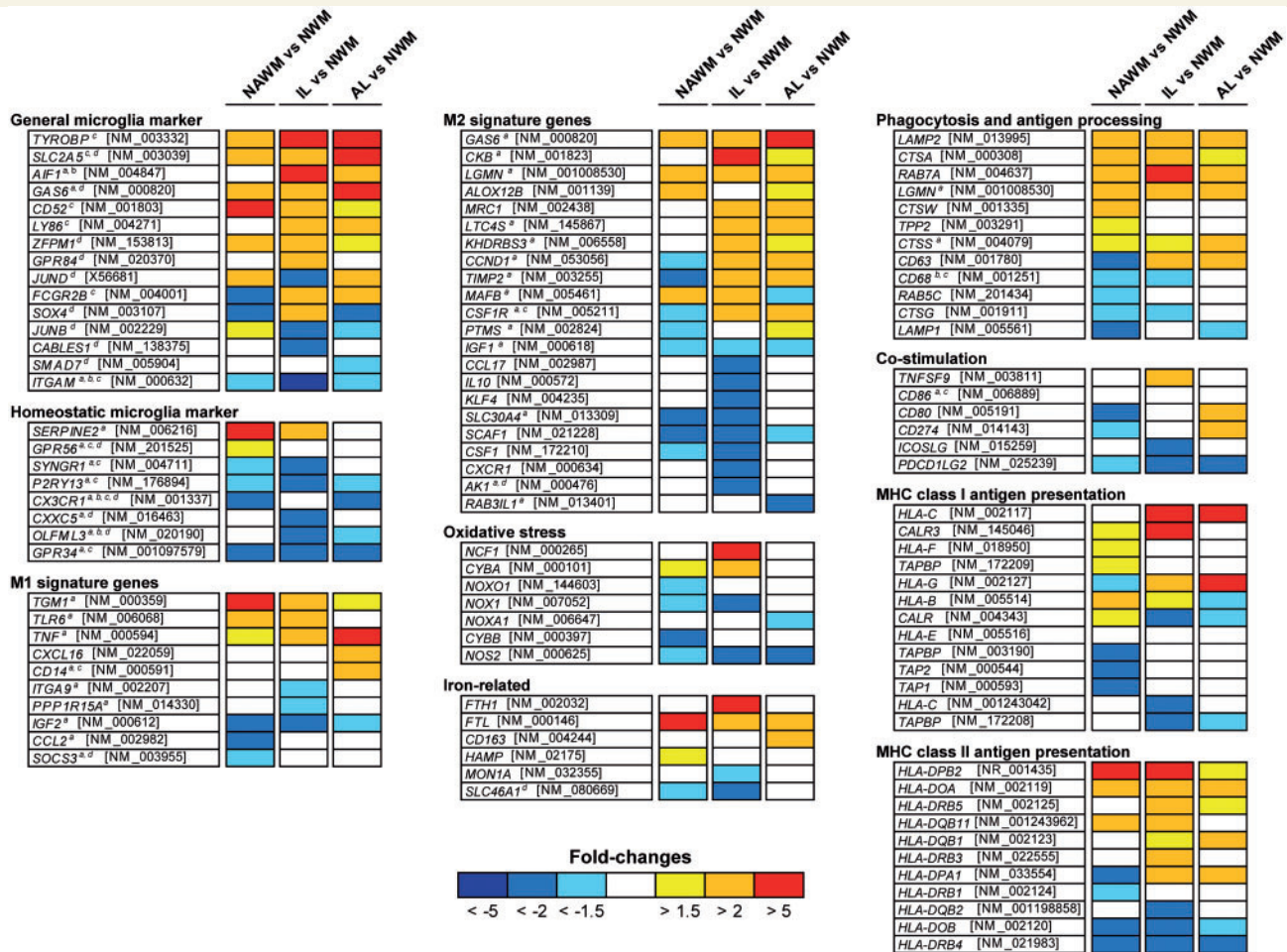


Figure 3 Expression of microglia genes in normal-appearing white matter, initial 'pre-phagocytic' lesion areas and the macrophage-rich centre of classical active multiple sclerosis lesions in comparison to normal white matter of controls. The respective lesion areas were microdissected from active lesions from four patients with acute multiple sclerosis following pattern III of demyelination according to Lucchinetti *et al.* (2000). Expression levels are provided as fold changes in comparison to those in normal white matter of controls. The microglia genes were grouped according to their functional designation provided by ^aButovsky *et al.* (2014); ^bChiu *et al.* (2013); ^cHickman *et al.* (2013); and ^dGautier *et al.* (2012). NAWM = normal appearing white matter of multiple sclerosis patients; IL = lesion edge containing initial (pre-phagocytic) and early active lesion areas; AL = centre of active lesions highly infiltrated by foamy macrophages.

lesion centre their numbers were low. Only few microglia cells or macrophages were seen in inactive lesions and their density in the tissue was significantly reduced in comparison to the microglia density in the normal or normal-appearing white matter of control and multiple sclerosis brains, respectively. The macrophage/microglia profile was related to the lesion type (classical active versus slowly expanding; Fig. 1A, F and J). No significant differences in microglia/macrophage numbers were seen when active lesions in acute and relapsing multiple sclerosis were compared with those that were formed in primary or secondary progressive multiple sclerosis. However, as described before (Frischer *et al.*, 2015), classical active plaques were mainly seen in early multiple sclerosis, while slowly expanding lesions were most prominent in patients with primary or secondary progressive multiple sclerosis. Thus, when active and slowly expanding lesions were combined, overall a lower degree of

microglia/macrophage infiltration was seen in progressive compared to acute/relapsing multiple sclerosis. No significant differences in microglia/macrophage density and activation status in multiple sclerosis lesions were seen in relation to age or gender of the patients.

The contribution of resident microglia versus recruited macrophages in multiple sclerosis brain

Overall, the markers used in our study did not discriminate between cells with a microglia (slender cell process bearing cells) or macrophage phenotype (round to oval cells without cell processes; Supplementary Fig. 2). An exception was P2RY12, which was never observed on cells with a macrophage phenotype in multiple sclerosis lesions and the

normal white matter of controls. The morphological phenotype of these cells does not allow conclusions whether they originated from the yolk sac-derived microglia pool or from recruited myeloid cells. Thus far, the only marker that may allow for such differentiation is TMEM119, which in experimental models and human brain tissue has been shown to be only expressed in microglia, but not on recruited myeloid cells (Bennett *et al.*, 2016; Satoh *et al.*, 2016). We found that the numbers of Iba1⁺ and TMEM119⁺ cells with microglia phenotype in the normal white matter of controls and the normal-appearing white matter of multiple sclerosis patients were similar and these cells were co-labelled with these markers (Figs 1K, L and 2). In contrast, in early stages of active lesions in multiple sclerosis, on average 43% of Iba1⁺ cells were TMEM119⁺ and there was a decreasing gradient of TMEM119⁺ cells from initial to advanced active lesions (Fig. 2). Similarly, the majority of activated microglia-like cells at the lesion edge of slowly expanding lesions were double-stained for Iba1 and TMEM119 (Fig. 1O and P), while the number of TMEM119⁺ cells decreased towards the inactive lesion centre (Fig. 1Q and R). In addition, we found a gradual transition of TMEM119⁺ cells with a morphological microglia phenotype into macrophages at the initial and active lesion edge (Figs 1 and 4). Double staining of TMEM119 and the microglia/macrophage markers CD68, p22phox and HLA-D showed co-expression of the markers in most microglia in the normal white matter of controls (Fig. 4) and the normal-appearing white matter of patients with multiple sclerosis. Our data indicate that in active multiple sclerosis lesions a major subset of cells, which ingest myelin debris and become phenotypically macrophages, are derived from the yolk sac-derived microglia pool, but that with maturation of the lesions yolk sac-derived microglia are lost in part and additional myeloid cells are recruited from peripheral monocytes.

Gene expression profile of macrophage/microglia markers in different multiple sclerosis lesion stages

To determine gene expression, microarray data from microdissected tissue areas containing normal-appearing white matter, the active lesion edge (containing parts of initial and early active lesions) and the macrophage-rich inactive lesion centre from active lesions of patients who died in the course of acute multiple sclerosis (Marburg, 1906) were compared to the normal white matter of age-matched controls.

In comparison to the normal white matter of controls, we found a profound downregulation of genes associated with homeostatic microglia function in active lesion areas with initial and active demyelination, whereas the changes were less pronounced in the normal-appearing white matter and in the lesion centre (Fig. 3). Genes dominantly expressed in active lesion areas were pro-inflammatory molecules related

to phagocytosis, antigen presentation and oxidative injury. Genes associated with anti-inflammatory (M2) molecules were up- and downregulated.

Immunohistochemical patterns of microglia/macrophage activation

Based on the differential gene expression profiles described above, we analysed key microglia/macrophage molecules by quantitative immunohistochemistry in relation to the activity of the demyelinating and neurodegenerative process in multiple sclerosis lesions.

Microglia in normal white matter of controls have a pre-activated phenotype

We found that in age-matched controls, similar numbers of Iba1-positive microglia expressed the microglia marker TMEM119, whereas P2RY12 was only present on average on 52% of microglia (Figs 2 and 4A). In addition, profound expression of the activation markers CD68 and p22phox was seen in 87% and 74% of Iba1⁺ microglia cells, respectively. Double staining with p22phox or CD68 revealed that the vast majority of P2RY12⁺ and TMEM119⁺ cells also expressed the activation markers (Fig. 4K and L). The expression of p22phox (NADPH oxidase) in microglia correlated significantly with the age of the patients ($P = 0.029$), but we did not find gender-dependent differences in microglia marker expression. In comparison, the expression of MHC II or CD86 was only seen in 26% and 38% of Iba1⁺ microglia-like cells, respectively (Fig. 2). The expression of iNOS and the M2 markers CD163 or CD206 was sparse and restricted to perivascular macrophages. Ferritin and ferritin light were found in oligodendrocytes and to a variable extent in microglia or perivascular cells (Hametner *et al.*, 2013). Overall, in the normal white matter of controls, microglia showed an intermediate phenotype between a homeostatic and pro-inflammatory state.

Loss of P2RY12⁺ microglia in the normal-appearing white matter of patients with multiple sclerosis

The expression profile of microglia markers in the normal-appearing white matter of patients with multiple sclerosis was similar to that seen in the normal white matter of controls, though the total numbers of microglia stained for P2RY12 and TMEM119 was reduced (Fig. 2; $P = 0.025$ for P2RY12 and $P = 0.001$ for TMEM119). In sections double-stained for P2RY12 a substantial number of cells were single positive for TMEM119 and some of these cells revealed a macrophage-like phenotype (Fig. 4B). Pro-inflammatory markers were moderately increased and this was significant for p22phox ($P < 0.01$), when calculated as percentage of total microglia. The expression of the phagocytosis-associated marker CD68 was significantly increased in the normal-appearing white matter of patients with progressive multiple sclerosis in comparison to the acute and relapsing multiple sclerosis ($P = 0.003$), but no

difference was seen between patients with primary or secondary progressive multiple sclerosis.

Classical active lesions

Classical active lesions contained high numbers of macrophage- and microglia-like cells; most of them in a state of activation and without expression of P2RY12 (Figs 2, 4D and F). When different stages of active lesions were compared, initial ‘pre-phagocytic’ lesions (according to Barnett and Prineas, 2004) primarily contained cells with a microglia phenotype and were in part positive for TMEM119, p22phox, CD68, CD86 and Class II MHC antigens (Figs 2, 4D and N). This pro-inflammatory activation state reached its peak in lesions containing macrophage-like cells with TMEM119 reactivity with early or late myelin degradation products (early or late active lesion stage; Figs 2 and 4F). In contrast, expression of iNOS was very low in macrophage- and microglia-like cells throughout all lesion stages. Expression of the M2 markers CD206 and CD163 was highly variable between initial and early active lesions of different cases. In some, these antigens were seen already on microglia-like cells in addition to perivascular and tissue macrophages, while in the majority of cases and lesions, their presence was restricted to perivascular cells and some tissue macrophages (Fig. 4O and P). In macrophage-containing inactive lesion sites TMEM119⁺ cells became rare despite the abundance of Iba1⁺ cells (Fig. 1Q and R). The expression of CD68 in macrophages remained, while the expression of pro-inflammatory molecules (p22phox and MHC antigen) declined. Expression of CD206, CD163 and ferritin peaked in the late active and inactive macrophage-containing core of active lesions (Figs 2 and 4O, P, R and S).

Comparison between active lesions following different patterns of demyelination

Classical active lesions with early active demyelination have previously been classified according to their patterns of demyelination (Lucchinetti *et al.*, 2000) (Fig. 1A and F). Besides the presence and absence of complement activation, they differ in their lesion architecture. Pattern III lesions have an ill-defined border and are surrounded by areas of variable size, which show the features of initial (‘pre-phagocytic’) lesions characterized by microglia activation, loss of distal oligodendrocyte processes and oligodendrocyte apoptosis (Lucchinetti *et al.*, 2000; Barnett and Prineas, 2004). In contrast, pattern I and II lesions are well demarcated from the surrounding normal-appearing white matter and initial stages of demyelination are seen within a dense rim of activated macrophages at the lesion edge (Lucchinetti *et al.*, 2000). When we compared the overall macrophage/microglia density and their activation phenotype in the active and inactive portions of these lesions, we found no differences between pattern I, II and III lesions. However, we did find that pattern II lesions had significantly lower expression of TMEM119 ($P = 0.038$) in comparison to pattern III lesions.

Slowly expanding (‘smouldering’) lesions

These lesions were characterized by a narrow rim of microglia with some intermingled macrophages containing early and late myelin degradation products (Figs 1J and 2). A large proportion of macrophages and microglia at the active lesion edge expressed TMEM119 (Fig. 1O and P) and were also stained by CD68, p22phox, MHC antigen and ferritin, while expression of M2 macrophage-related molecules was very low. Only few phagocytes were present in the inactive lesion core (Fig. 2).

Inactive lesions

Completely inactive lesions contained only very few macrophage-like or microglia-like cells (Figs 1J and 2). The density of microglial cells was significantly lower compared to the normal-appearing white matter. Such lesions were distinguished from other multiple sclerosis lesions by the reappearance of P2RY12 on the majority of the remaining microglia (Figs 2, 4H, I and J). Nonetheless, despite the reappearance of P2RY12, the majority of these cells also expressed pro-inflammatory activation markers. These results suggest that loss of the P2RY12 homeostatic marker is related to lesion activity.

Discussion

We investigated the innate immune response in the brain of subjects with multiple sclerosis using two unique markers: TMEM119, which differentiates microglia from recruited monocytes and P2RY12, which identifies a homeostatic microglia phenotype in rodent CNS tissue. We found that microglia cells in the control human brain have a pro-inflammatory activation state. Expression of some activation markers in microglia of the normal human brain has been described previously (Vogel *et al.*, 2013). This differs from rodents where microglia in normal brain display a homeostatic phenotype (Butovsky *et al.*, 2014), with little or no expression of pro-inflammatory markers (Schuh *et al.*, 2014). It is not clear whether co-morbidities, such as the systemic exposure to infections and environmental toxins or vascular and neurodegenerative events related to brain ageing (Conde and Streit, 2006; Perry *et al.*, 2010; Haider *et al.*, 2016) account for the pro-inflammatory microglia activation in the normal CNS or whether such background activation is an inherent property of microglia in the human CNS.

We found that P2RY12 reactive microglia are reduced in the normal-appearing white matter in multiple sclerosis in comparison to the normal white matter of controls and that this phenotype is completely lost in active lesions. Loss of the microglia homeostatic phenotype is seen in other neurodegenerative conditions of the CNS (Butovsky *et al.*, 2014, 2015). The expression of P2RY12 on microglia in the normal human brain has recently been confirmed and its loss on microglia has been shown in a single multiple sclerosis case and lesion of undefined activity stage

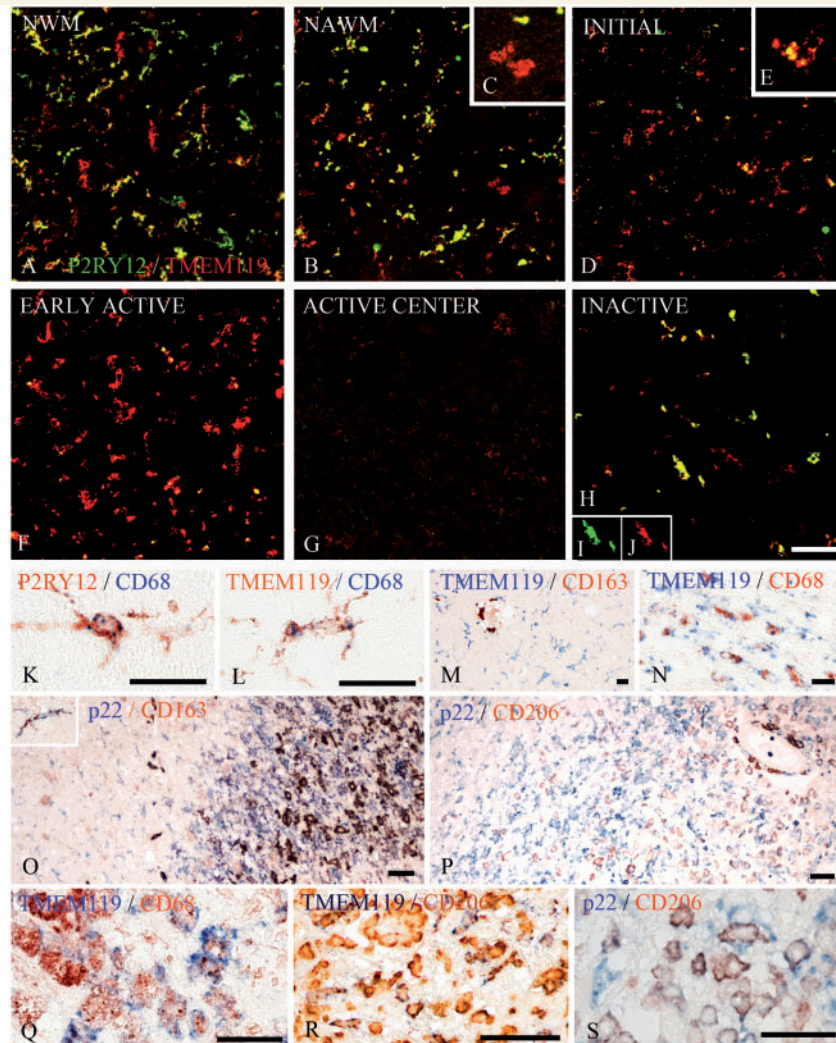


Figure 4 Co-expression of microglia/macrophage markers in multiple sclerosis lesions. (A–J) Double staining for P2RY12 (green) and TMEM119 (red) shows that in the normal white matter of controls most microglia co-express P2RY12, although there are some, which are single positive for TMEM119 (A). In the normal-appearing white matter of multiple sclerosis a similar pattern as in control normal white matter is seen, but some of the TMEM119 single positive cells have a macrophage-like phenotype (B and inset C). P2RY12 is massively reduced in TMEM119⁺ cells in initial lesions (D and inset E) and there is a further reduction of P2RY12 expression in early active lesions, which still contain high numbers of TMEM119⁺ cells (F) and in the active centre of active lesions, which contain only few TMEM119⁺ cells (G). In inactive lesions, only few microglia-like cells are seen, but the majority of these cells co-express P2RY12 and TMEM119 (H). The insets I and J show the expression of both antigens in a single double stained cell. (K–S) In the normal white matter of controls, the majority of microglia identified by the expression of P2RY12 (K) or TMEM119 (L) were activated and also expressed the phagocytosis-associated marker CD68. (M) An example of the normal-appearing white matter of an multiple sclerosis patient with numerous TMEM119⁺ microglia cells, while CD163 expression is restricted to perivascular macrophages. In N, activated microglia in initial lesion areas of a pattern III lesion are shown, which co-express TMEM119 and CD68. The edge of an active pattern III lesion is shown in O. In the initial lesion areas, most microglia cells express p22phox (NADPH oxidase) but are negative for CD163, although some microglia co-express both antigens (inset). In the early active lesion edge, there is still a dominant expression of p22phox, while co-expression of p22phox with CD163 increases towards the more advanced lesion parts. A similar profile is shown in P, which documents that CD206 appears in p22phox-positive macrophages predominately in advanced lesion stages. (Q–S) The expression profile of the markers in macrophages in the inactive lesion centre of active plaques. Some of the CD68⁺ (Q) or CD206⁺ macrophages (R) are co-labelled with TMEM119. In addition, some pro-inflammatory activated macrophages positive for p22phox co-express the anti-inflammatory M2 marker CD206 (S). Scale bars = 100 μm.

(Mildner *et al.*, 2016). In our study in multiple sclerosis, loss of P2RY12 reactivity on microglia was not restricted to the actual lesions, but also seen in the normal-appearing white matter, distant from focal plaques. This is true not only for chronic disease, but also in acute multiple sclerosis

with a disease duration of weeks to months. Loss of the homeostatic microglia phenotype has been reported in active EAE lesions (Butovsky *et al.*, 2014). The chronic inflammatory environment in the multiple sclerosis brain may contribute to its loss not only in the lesions, but also

in the normal-appearing white matter. This is supported by our observation that in completely inactive lesions, in which inflammation has subsided, P2RY12 is re-expressed on the remaining microglia cells.

Multiple sclerosis is a chronic inflammatory disease of the CNS and it would thus be expected that active demyelination and neurodegeneration are associated with a pro-inflammatory state of microglia or macrophages. In early lesions, the number of microglia and macrophages increase and the cells dominantly express markers, which are associated with phagocytosis, oxidative injury and antigen presentation or co-stimulation in the course of T cell activation. Several studies report active tissue injury with the various expression of M1 versus M2 markers (Zhang *et al.*, 2011; Vogel *et al.*, 2013; Peferoen *et al.*, 2015) and we also found that macrophage- and microglia-like cells in active multiple sclerosis lesions express both markers for M1 and M2 activation. This is not surprising, since M1 and M2 phenotypes are seen in macrophages *in vitro* after stimulation with defined mixtures of cytokines (Moore *et al.*, 2015; Healy *et al.*, 2017), while the situation may be more complicated in inflammatory brain lesions *in vivo*, where gene expression studies showed the simultaneous expression of pro- and anti-inflammatory cytokines within the same active lesion (Whitney *et al.*, 1999; Mycko *et al.*, 2003). However, we found a quantitative shift in microglia/macrophage activation from the initial stages of active lesions into established lesions, which contained macrophages in the process of myelin digestion. Thus, active demyelination and neurodegeneration was clearly associated with a dominant pro-inflammatory phenotype of phagocytes, while the peak expression of anti-inflammatory M2 markers was seen in the macrophage-containing inactive lesion centre. M2 polarization of microglia/macrophages follows myelin phagocytosis (Boven *et al.*, 2006; van Rossum *et al.*, 2008). It has been suggested to stimulate remyelination (Butovsky *et al.*, 2006; Miron *et al.*, 2013) and early stages of remyelination within multiple sclerosis lesions are mainly seen in the core of active lesions (Raine *et al.*, 1981; Prineas *et al.*, 1993; Raine, 2017).

A simple differentiation between M1 and M2 phenotype is complicated by the fact that some of the respective molecules also have other functions, which are relevant in the context of a multiple sclerosis plaque. For example, iNOS expression in multiple sclerosis lesions has been found mainly in macrophages at the lesion edge, which are loaded with iron and iron was mainly taken up by M1-polarized human macrophages *in vitro* (Mehta *et al.*, 2013). It is not only present in microglia/macrophages (Marik *et al.*, 2007), but also in astrocytes, as has been shown previously (Bö *et al.*, 1994). In a similar way, one of the classical M2 markers, CD163, is a haptoglobin/haemoglobin receptor, which is involved in iron uptake into macrophages (Andersen *et al.*, 2017) and the main function of ferritins is iron storage and sequestration in the cytoplasm (Lane *et al.*, 2015). Thus, the expression of certain microglia/macrophage molecules may also be in

part regulated by the iron metabolism, which is disturbed within the multiple sclerosis brain and lesions (Hametner *et al.*, 2013).

The phenotypic changes of microglia/macrophages we observed were similar in classical active lesions and in the active zone of slowly expanding ('smouldering') plaques, although in the latter the zone of active tissue injury was thin and did not allow an accurate separation of lesion stage-associated phenotype. In contrast to classical active lesions, macrophages were rare or absent in the demyelinated core of slowly expanding lesions and cells with an anti-inflammatory (M2) phenotype were extremely rare in these lesions, which also did not show signs of remyelination (Bramow *et al.*, 2010).

An important question that we were able to address is the degree to which macrophages in multiple sclerosis lesions are derived from the original microglia cell pool or from recruited monocytes. We found at the active lesion edge that there is a transition from classical microglia cells expressing P2RY12, to phagocytic cells phenotypically resembling process-bearing activated microglia that contain early myelin degradation products to typical round to oval debris-loaded macrophages. Many of these cells express TMEM119, which is expressed on microglia, but not on recruited peripheral myeloid cells (Bennett *et al.*, 2016). Using this marker, Satoh *et al.* (2016) described TMEM119-positive cells in the cortex of Alzheimer's disease, but not in multiple sclerosis lesions. On this basis, the authors concluded that phagocytes in Alzheimer's disease are derived from microglia, while in multiple sclerosis they come from recruited myeloid cells. In contrast, our study suggests that a major proportion of phagocytes present in early stages of multiple sclerosis lesions are indeed derived from the microglia pool, as suggested by Li *et al.* (1996). However, with the maturation of active lesions an increasing proportion of macrophages was TMEM119-negative and thus represents an additional pool of cells, which has entered the lesions from the blood. The proportion of recruited myeloid cells was higher in lesions following pattern II demyelination compared to pattern III lesions, as defined by Lucchinetti *et al.* (2000). Similar conclusions were reached through the analysis of the chemokine receptors CCR1 and CCR5 in multiple sclerosis lesions, which may allow differentiation between CCR5-positive resident microglia and CCR1/CCR5-double positive or MRP14-reactive recruited myeloid cells (Trebst *et al.*, 2001). Also with this approach, a similar difference between pattern II and III lesions has been observed (Mahad *et al.*, 2004).

In conclusion, our study provides evidence that during initial and early stages of plaque formation in multiple sclerosis a major fraction of the cells, which are associated with active demyelination and neurodegeneration, are derived from the original resident microglia pool, thus suggesting that therapeutic blockade of macrophage entry from the circulation into the lesions may be insufficient to halt the disease process. Active demyelination and neurodegeneration is associated with a pro-inflammatory activation of microglia

and macrophages, while macrophages polarized into an anti-inflammatory or neuroprotective phenotype are mainly seen in the lesion core of classical active lesions, where attempts of remyelination are frequent. They are sparse or absent in slowly expanding lesions of progressive multiple sclerosis, which fail to remyelinate. Thus, development of new PET markers, which distinguish these different functional states of microglia, could become useful para clinical markers for disease activity not only in relapsing but also in progressive multiple sclerosis. In contrast to experimental models of inflammatory demyelination, lesion formation in multiple sclerosis occurs on a background of pro-inflammatory microglia activation, which is seen already in the normal white matter of age-matched controls, and this may contribute to neurodegeneration in the disease.

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Supplementary material

Supplementary material is available at *Brain* online.

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