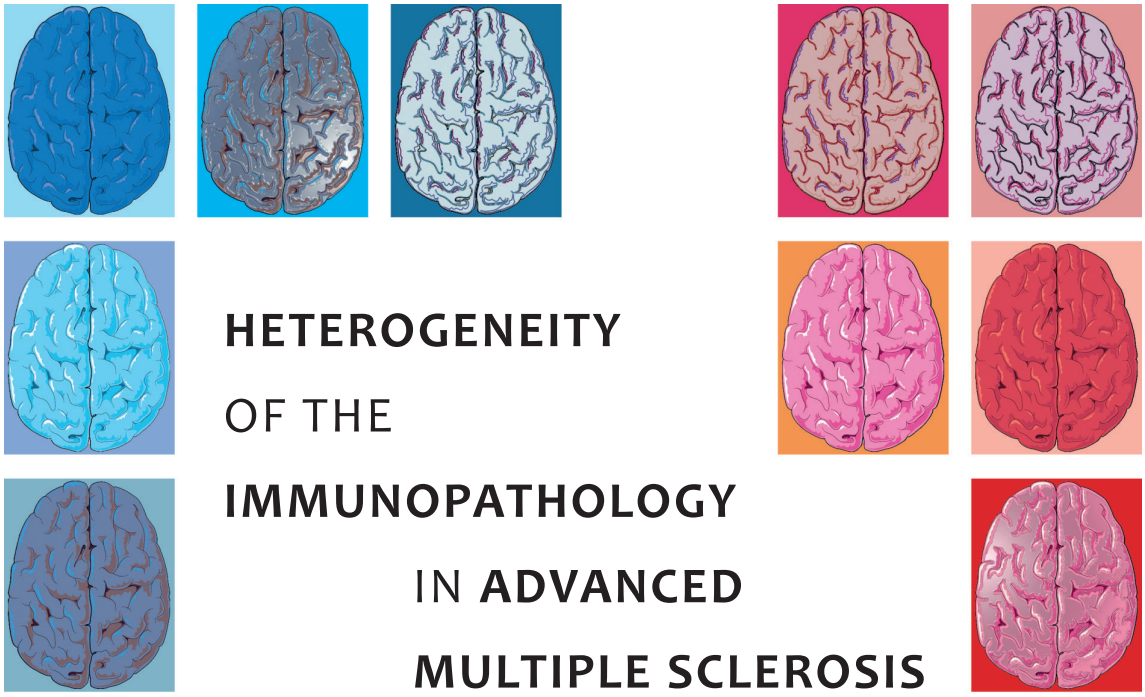
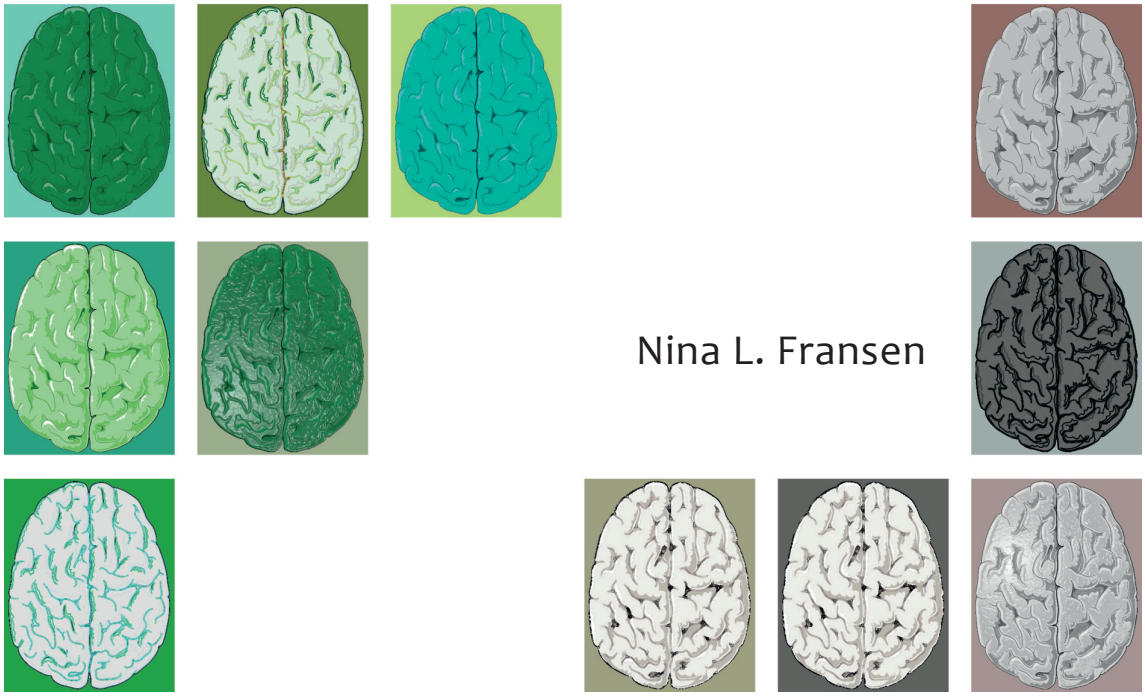


HETEROGENEITY OF THE IMMUNOPATHOLOGY IN ADVANCED MULTIPLE SCLEROSIS

An autopsy cohort analysis



Nina L. Fransen

Heterogeneity of the immunopathology in advanced multiple sclerosis

An autopsy cohort analysis

N.L. Fransen

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Heterogeneity of the immunopathology in advanced multiple sclerosis

An autopsy cohort analysis

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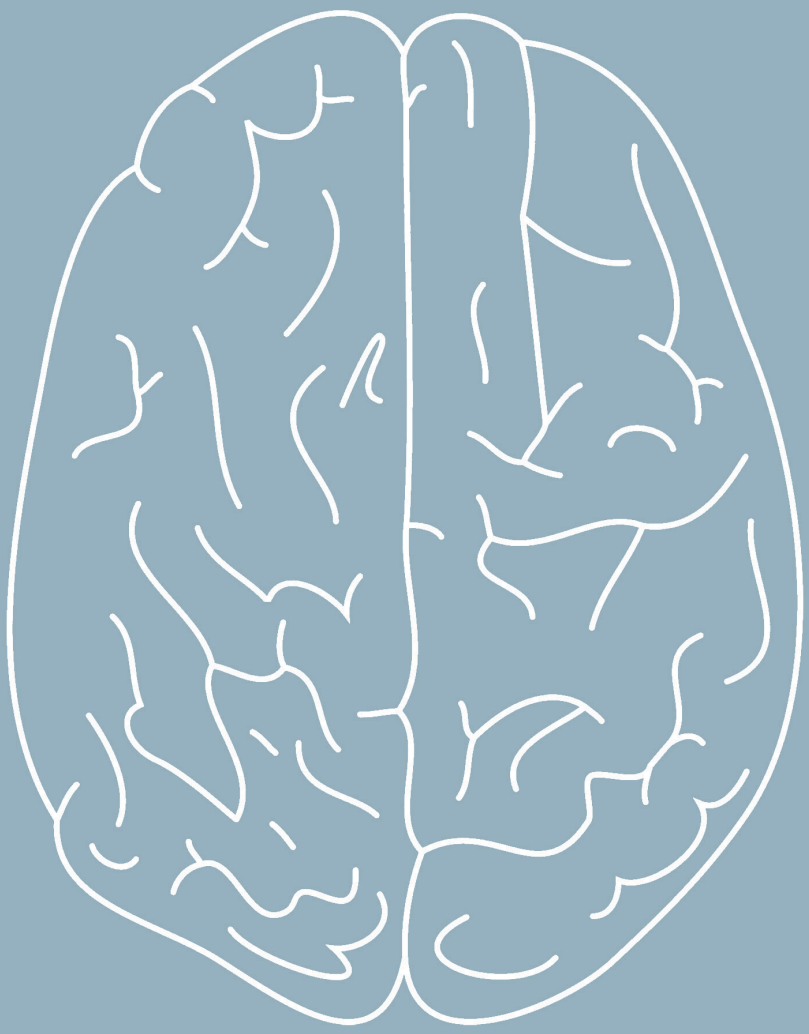
THESIS CONTENT

CHAPTER 1

Introduction and aim of thesis



MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is the most prevalent chronic inflammatory disease of the central nervous system. It presents mostly in young adults and the mean age at diagnosis is around 30 years.[1,2] Its world-wide prevalence is 30 patients per 100.000 people and between 1990 and 2016 the world-wide prevalence increased with 10.4%. There is a strong latitudinal gradient for prevalence, with a 3% relative increase for each degree increase of latitude.[3] The geographical location before the onset of MS is a risk factor for susceptibility.[4] These observations illustrate the importance of environmental risk factors in the development of MS.[5] It has been proposed that the strong latitudinal gradient in MS prevalence can be explained by regional UVB exposure and vitamin D deficiency.[5–9] The strongest environmental risk factor is infection with Epstein-Barr virus, however the mechanisms underlying this association still remain to be determined.[5,10,11]

MS is a typical example of a common complex disease that results from a combination of environmental and genetic risk factors. The cause of MS and whether this varies from one patient to the other still remains elusive. The disease arises in genetically susceptible individuals, where genetic risk factors influence the penetrance of environmental factors.[12] GWAS studies have identified over two hundred genetic risk loci for MS with the MHC region being the main genetic determinant for the susceptibility of MS.[13,14] Most of the susceptibility associated genes are related to immunological pathways and there is notable overlap with other auto-immune diseases, suggesting common predisposing immunological processes.[15,16] Pathway analysis and few functional studies have been performed for genetically associated genes to identify the related disease relevant pathways. These studies indicate central tolerance mechanisms (the negative selection of developing T and B cells in the primary lymphoid organs), peripheral differences in effector T cell function due to altered cytokine responsiveness, cytokine production and homeostatic proliferation and alterations in microglial function in the predisposition for multiple sclerosis.[14,17,18] Overall there are strikingly few genetic associations shared with neurodegenerative conditions indicating that non-immunological, from a genetic perspective, primary neurodegenerative processes are less likely to contribute to the susceptibility of MS.[17]

Heterogeneity in MS clinical disease course

The clinical presentation of MS is dependent on the location of the lesions. Patients typically present with monocular visual loss due to unilateral optic neuritis or facial sensory loss due to brainstem dysfunction, ataxia and nystagmus due to cerebellar lesions, limb weakness or sensory loss due to partial myelopathy. Clinical features that are suggestive for demyelination as a cause of these symptoms are age younger than 40, acute or subacute onset over hours or days, maximal neurological deficit within 4 weeks after onset and spontaneous remission. The diagnosis MS requires the dissemination of clinical symptoms and/or MRI lesions in time and space.[19]

In general practice, three clinical disease phenotypes are distinguished; relapsing remitting, secondary progressive and primary progressive MS,[20] these are illustrated in **Figure 1.** In 78% of the MS patients the disease starts with a relapsing onset, while in 22% of the MS patients the disease starts with a progressive onset.[2] MS patients that show a progressive onset of the disease are older (38.5 years vs 29.5 years) compared to the MS patients with a relapsing onset. MS patients with a relapsing onset mostly show conversion to a secondary progressive phase, generally this conversion occurs after a median time of 16 years from onset and at a median age of 40 years. In the more recent definitions of MS, the clinical course of primary progressive MS is not considered pathophysiological distinct from relapsing forms of MS, that have entered a progressive course (SPMS).[21,22] Therefore in recent years secondary and primary progressive MS subgroups are often combined and described as progressive and/or advanced MS.

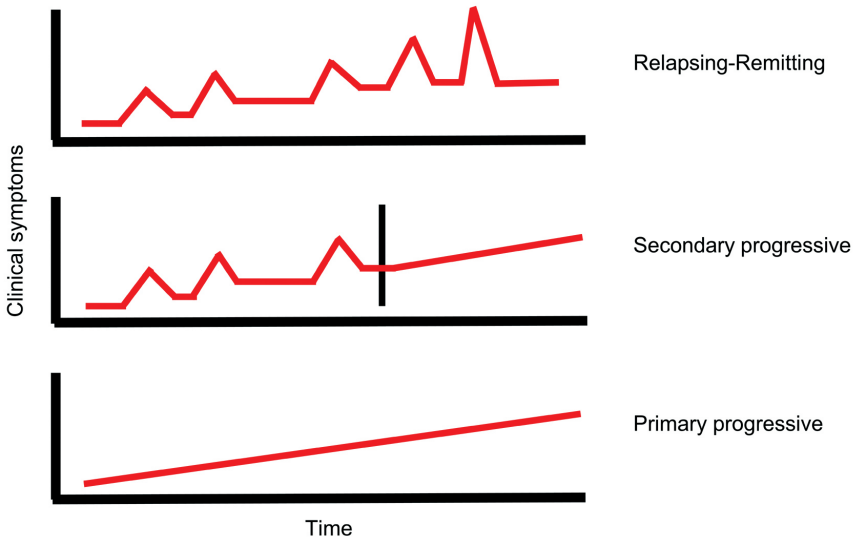


Figure 1. Different clinical disease courses in multiple sclerosis.
Illustrating the development of clinical symptoms over time in the relapsing-remitting, secondary progressive and primary progressive disease course.

Most MS patients accumulate disability over time, however the rate of disability progression is variable between patients. There is limited information regarding the predictors of the disease course. The time from first symptoms until the time a patient needed a walking aid (Expanded Disability Status Score 6) was longer in patients with a relapsing onset compared to a primary progressive onset.[23–25] Differences in the rate of disability progression between MS cases have been described as either benign or malignant MS, although such a clinical classification and its definitions are controversial since MRI lesion load and cognitive decline are neglected. Benign MS has been defined as EDSS 2 or lower, which means minimal disability in only one of the functional systems, after a disease duration of more than 10 years. Benign MS cases have a higher chance to

stay stable compared to non-benign MS patients.[26] Malignant MS has been used when the need to walk continuously with a walking aid is reached within 5 years. Over 10% of the MS patients develop a malignant form of MS. Patients with an older age at onset, motor symptoms at onset and a progressive disease course, male gender and positive smoking history are more likely to develop malignant MS.[27] Furthermore, patients with a motor pathway deficit at onset were less likely to have a benign MS disease course.[26]

Sex differences in MS susceptibility and clinical course

In MS both the susceptibility and the clinical disease course shows sex differences. The global prevalence of MS in adults differs significantly by sex, with overall a higher prevalence in females compared to males, with a sex ratio of 2:1. Interestingly, in a progressive onset of MS, the male-female ratio is significantly lower compared to the relapsing onset MS patients.[2] In MS patients with a relapsing onset males show a faster disability progression compared to females, while the disability progression is comparable between males and females in patients that show a progressive onset.[2,25,28] Imaging studies showed that males show more destructive white matter lesions and more often cortical grey matter lesions compared to females.[29,30] Altogether these observations demonstrate that males are more susceptible to develop a severe and often progressive disease course. Interestingly the incidence of multiple sclerosis in females doubled between 1950 and 2009, whereas an increase among males has been modest.[31] This may be attributed to environmental changes affecting the behavior of females more compared to men, which include rise in obesity, increased cigarette smoking in females and changes in the frequency of breastfeeding.[32] The hypothesis that sex steroids are also contributing to the differences between males and females is supported by the observation that MS relapses decrease during pregnancy and increase post-partum, when estrogen and progesterone levels rapidly decrease. Both sex steroids produced outside the CNS as well as "neurosteroids" produced within the CNS are potentially impacting on the MS disease activity.[33] Especially progestogens and androgens show inhibition of demyelination and promotion of remyelination, anti-inflammatory and neuroprotective effects in models for MS.[33–35] In human MS white matter lesions it has been demonstrated that there is altered expression of progesterone synthetic enzymes and their receptors; showing in females there is an increased progesterone signaling while this was not increased in males.[36] This all suggests that sex steroids are contributing to the differences in susceptibility and clinical disease course between males and females.

Imaging and biomarkers monitoring MS disease activity

Several imaging and biomarkers have improved the monitoring of disease activity, the prediction of the clinical disease course and response to immunomodulatory therapies in MS patients. Furthermore they provide insight in disease mechanisms that contribute to the development of relapses and disability progression in MS. A role of blood brain barrier damage in the development of relapses is suggested by two meta-analysis.[37,38] showing that the best predictor of the effect of immunomodulatory therapies on relapses is the number of new or enlarging T2 lesions or

gadolinium enhancing lesions in the white matter. In a study of 3635 RRMS patients disability progression could be predicted by brain volume loss which is a marker for brain atrophy.[39] Furthermore, imaging measures that reflect the extend of brain tissue damage in lesions and also outside lesions improve the prediction of disability progression.[40,41] These observations indicate that neurodegenerative changes in the MS lesions correlate with the accumulation of disability in MS patients. Interestingly, disability progression was inversely correlated with remyelination of white matter lesions as shown by combining PET and MRI in longitudinal imaging studies.[42] Therefore, besides the occurrence of focal inflammation and neurodegeneration, differences in remyelination capacity between MS patients appears to impact on disability progression.

Recently, mixed active/inactive lesions (also referred to as chronic active or smoldering lesions) were defined on MRI as non-gadolinium enhancing lesions with rims. These lesions are suspected to feature ongoing demyelination, remyelination failure and axonal degeneration. A longitudinal imaging study showed that lesions without a rim shrank over time, while the lesions with a rim stayed stable or expanded over time. Rim lesions had a longer T1 time suggesting more tissue destruction compared to lesions without rim. Finally, the presence of rim lesions associated with a more aggressive disease course with faster disability progression.[43] Altogether, these observation suggest that ongoing demyelination, remyelination failure and axonal degeneration are contributing to disability progression.

Biomarkers in CSF and circulation improve the monitoring of disease activity and the prediction of disability progression. The presence of CSF-unique oligoclonal bands is a well-known feature of MS at diagnosis, with only 10% of patients lacking this biomarker. The latter small proportion of MS patients develop more often a benign disease course.[44] Recently, it was confirmed that the presence of oligoclonal bands at first symptoms is an independent predictor of disability progression.[45] In radiologically isolated syndrome, where there are incidental radiological findings highly suggestive for MS in patients who are asymptomatic, the presence of oligoclonal bands and also high neurofilament light chain levels are associated with a faster progression to the clinical diagnosis MS.[46] In large studies in MS patients the serum neurofilament light chain level is associated with new and enlarging lesions, contrast enhancement of lesions and brain and spinal cord volume loss on MRI, illustrating that this is an objective surrogate of ongoing disease activity.[47,48] These observations illustrate that biomarkers of intrathecal immune activation and axonal loss reflect biological processes impacting on the disease course of people with MS.

The clinical disease course and disability progression is variable between MS cases. Longitudinal radiological studies show that MS patients with more inflammatory disease activity, less remyelination and more neurodegenerative changes show a faster disability progression in follow-up. However, the molecular and cellular mechanisms that underly these differences remain poorly understood. Several GWAS studies have identified genes associated with clinical disability progression and/or MRI outcome measures, but the functional pathways implicated in disability progression still remain to be determined.[49–51] Since current immunomodulatory therapies only

show modest effect on disability progression a better understanding of pathological mechanisms that contribute to disability progression is warranted and the aim of this thesis.

Multiple sclerosis lesion pathology

Histologically MS is characterized by sharply demarcated focal inflammatory lesions with demyelination, variable axonal loss and gliosis in the white and grey matter.[52–54] The lesions can be present in any location in the brain, including the white matter, the deep grey matter and the cortical grey matter. In the cortex the lesions are classified based on their location as subpial, intracortical and leukocortical lesions. Subpial cortical demyelination is spatially associated with meningeal T cell infiltrates,[55–57] however the general inflammatory and demyelinating activity in cortical MS lesions is lower compared to the adjacent white matter.[52] Inflammatory lesions in the white matter are dominated by T cells and activated microglia and/or macrophages.[58] Blood brain barrier damage is classically seen in inflammatory active white matter lesions.[59]

The formation of MS lesions is a dynamic process and the neuropathological appearance of lesions is determined by the development of myelin phagocytosis at the time of biopsy or autopsy.[52] Since microglia/macrophages migrate slowly from the lesions, the presence or absence of microglia/macrophages provides an identification for the age of a lesion. In MS autopsy tissues different types of MS lesions can be distinguished and over the past decades a number of classification systems have been introduced. Recently, a consensus characterization of MS lesions has been described by Kuhlmann et al 2017[52] based on Luxol Fast Blue (LFB) histological staining and immunohistochemistry for MBP or PLP for myelin proteins, CD68 for microglia/macrophage activity, and CD3 for the presence of T cells. In this classification system active, mixed active/inactive (mixed) and inactive lesions are distinguished in the white matter. Active lesions are hypercellular, characterized by infiltration of $CD68^+$ cells most of them with a foamy morphology and a loss of myelin. T cells are localized perivascular, but they are also encountered throughout the lesion in the brain parenchyma. It has been suggested that the presence of foamy microglia/macrophages in MS lesions indicates that they are formed within days or weeks before pathological analysis, however their presence is not a measure of ongoing myelin destruction since they do not always show myelin degradation products inside. Demyelinating and post-demyelinating lesions can be distinguished based on the MBP, PLP or Luxol fast blue positive myelin degradation products inside the microglia/macrophages. Active demyelinating lesions show microglia/macrophages that show myelin degradation products inside while the active and post-demyelinating lesions are infiltrated with foamy lipid containing microglia/macrophages lacking LFB or other myelin degradation products.[52]

In contrast to the active lesions, mixed lesions are characterized by a hypocellular lesion center and a rim of activated microglia/macrophages at the lesion border. The center of the lesion is depleted of microglia/macrophages. To classify it as a mixed lesion, the rim of active microglia/macrophages does not need to surround the entire lesion. In these active and mixed lesions

moderate T cell infiltrates are present perivascular and also in the brain parenchyma. In the mixed lesions the same subdivision into demyelinating and post-demyelinating is made as in active lesions, based on myelin degradation products inside the microglia/macrophages. Frequently the mixed lesions show a narrow rim of microglia/macrophages containing MBP or PLP degradation products, reflecting ongoing demyelination, these lesions have been called slowly-expanding or smoldering lesions.[52]

Inactive lesions are sharply demarcated, gliotic and hypocellular, only few T cells and microglia/macrophages are present and axonal loss is evident in these lesions.

Remyelination in MS lesions has been proven at the ultra-structural level.[60,61] MS lesions can be partly or completely remyelinated and using immunohistochemical myelin staining remyelinated axons can be identified having thinner myelin sheaths resulting in a paler myelin staining intensity. Remyelination is present in all lesion types and also the active and demyelinating lesions contain remyelinated lesion areas. Inactive shadow plaques can be distinguished that show extensive remyelination with only few microglia/macrophages. The extend of remyelinated shadow plaques has been shown to be heterogenous between MS patients, where the presence of more shadow plaques is related to a longer disease duration, suggesting that potentially genetic and environmental factors may influence the ability to remyelinate in MS patients.[52,62] A schematic overview of the stages of MS lesions that are distinguished in the Kuhlmann et al. 2017 consensus are shown in **Figure 2** together with the staging system used by the NBB.

Characterization of MS lesions in the NHB MS cohort

At the Netherlands Brain Bank (NBB) all MS tissue samples have been histologically characterized over de past few decades. From MS autopsy cases at NBB tissue is dissected from standardized locations from the brainstem and the spinal cord, and macroscopically visible MS plaques (PLA) from white and grey matter are dissected. In addition, since 2001, MS lesions are dissected on post-mortem MRI guidance (MRI) on 1 cm thick coronal brain slices.[63] The characterization of MS lesions at NBB uses PLP immunohistochemistry for myelin and HLA-DR immunohistochemistry for microglia/macrophages. The characterization of MS lesions is comparable to the Kuhlmann consensus, except that in active and mixed lesions the NBB does not distinguish demyelinating and post-demyelinating but uses the morphological appearance of microglia/macrophages to characterize the microglial/macrophage activity. Active lesions are characterized by a demarcated area of partial or paler PLP staining and HLA-DR$^+$ microglia/macrophages that are identified throughout the lesion area. The mixed lesions contain a completely demyelinated and hypocellular center, with a hypercellular rim of HLA-DR$^+$ cells. The morphology of the microglia/macrophages in both active and mixed lesions were classified as either ramified (suggestive for a resting, trophic state), rounded (suggestive for activation, or infiltration of peripheral macrophages) or foamy (suggestive for demyelination).[64] Inactive lesions were sharply demarcated demyelinated areas that are hypocellular, with little HLA-DR$^+$ cells. The inactive shadow plaques show paler PLP

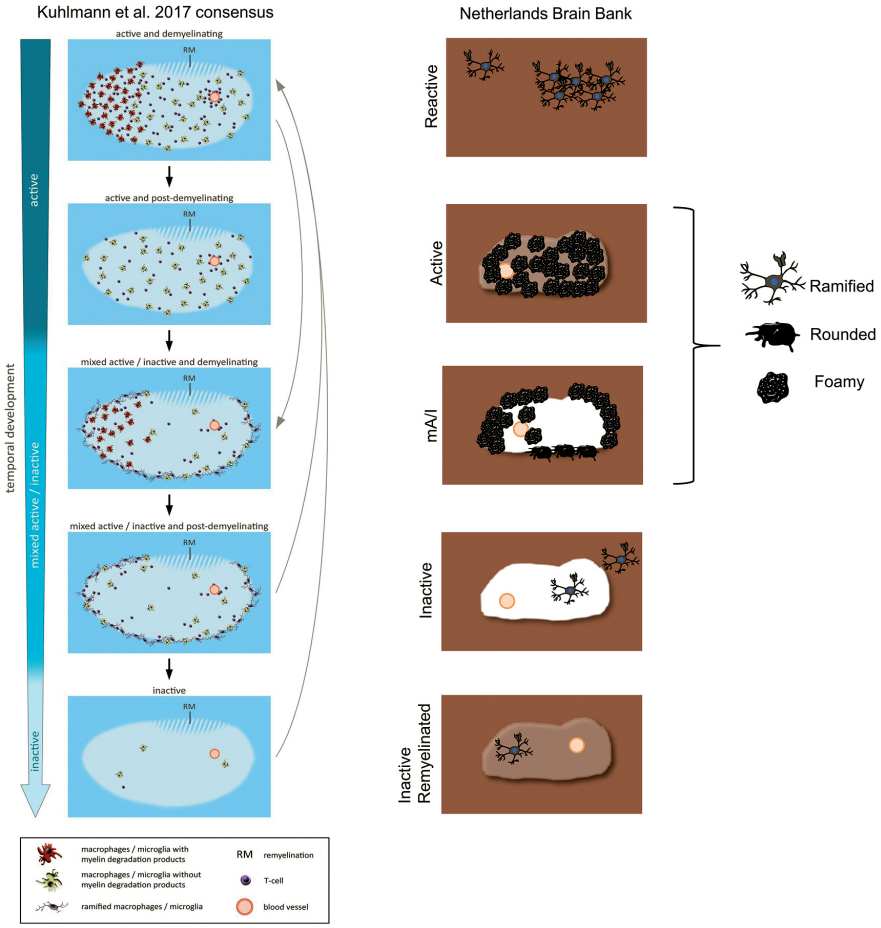


Figure 2. Histopathological staging of multiple sclerosis lesions.
In active and mixed lesions the Kuhlmann et al. consensus distinguishes demyelinating and post-demyelinating based on the myelin degradation products inside microglia/macrophages, while the NBB uses microglia/macrophage morphology to characterize the microglia/macrophage activity. Image from Kuhlmann et al. consensus derived with permission of Kuhlmann et al. (2017) *Acta Neuropathologica*.

staining compared to the normal appearing white matter with little HLA-DR$^+$ cells, suggestive for a remyelinated area. Demyelinating and post-demyelinating lesions were not distinguished based on myelin degradation products inside microglia/macrophages, however the presence of foamy microglia/macrophages is suggestive for relatively recent demyelination. The mixed lesions, the inactive lesions and most of the active lesions are comparable in the NBB characterization and the Kuhlmann consensus characterization. However, the subset of active lesions with a ramified microglia/macrophage morphology at the NBB would not be considered as active lesions in the Kuhlmann consensus since the microglia throughout these lesions are not considered activated. These lesions are rather considered as (active) remyelinated areas since they show a paler myelin

staining and lack evident ongoing demyelination. The stages of MS lesions that are distinguished by the NBB are included in **Figure 2**.

At the NBB it has since long been appreciated that the characteristics of MS lesions in the post-mortem MS brain are relatively consistent within a brain donor, while there are clear differences in for example the number of active lesions or the number of inactive remyelinated shadow plaques between MS brain donors.[65,66] These differences between donors may represent the dynamic process of MS lesion formation and is potentially related to the clinical disease stage at time of autopsy. However, they might also reflect differences in genetic or environmental factors that influence the inflammatory lesion activity or the capacity to remyelinate in subsets of MS patients.

The immunopathology of early MS

In biopsy samples of early MS or in autopsy cases of acute MS with a very rapid disease course (died within 1 years), the dominant MS lesion type is the active and demyelinating white matter lesion.[58] In these early MS biopsy lesions heterogeneity in immunoglobulin and complement depositions or oligodendrocyte apoptosis have been described.[67] Therefore, it has been considered that these early MS lesions may arise from different etiologies. Initially four, but more recently three different immunopathological patterns of MS biopsy lesions have been proposed.[1,67] Pattern I lesions show demyelination and the presence of activated microglia/macrophages and T cells. Pattern II lesions show next to activated microglia/macrophages and T cells also complement activation and antibody depositions, suggesting more involvement of the humoral immune response. Finally, pattern III lesions are characterized by the presence of oligodendrocytes with nuclear condensation and fragmentation, resembling apoptotic cell death. This is associated with a selective loss of MAG, a myelin antigen that is located in the most distal (peri-axonal) oligodendrocyte processes. Since in each patient only 1 pattern of MS lesions was identified, this suggests that different pathological mechanisms may lead to demyelination.[68] However, whether these different patterns represent different etiologies or simply reflect temporal stages of MS lesion development remains to be determined. Furthermore, it needs to be considered that the cases used for the identification of the lesion patterns do not have a typical clinical presentation of MS, otherwise a brain biopsy would not have been performed. Finally, some of the antibodies required to identify these different patterns are not commercially available, therefore the identification of patterns is not considered within the scope of neuropathological analysis at the NBB and in the Kuhlmann et al. consensus.[52] The characterization of the different patterns is illustrated in **Figure 3**, derived from Reich et al.[1]

Interestingly, it has been shown that cases with pattern II MS lesions did show a better effect on plasma-exchange compared to the patients with pattern I and pattern III MS lesions.[69] Which illustrates that the potential differences in the involvement of the humoral immune response between MS patients, has potential clinical and therapeutic implications. Also in post-mortem autopsy lesions differences in the presence of IgG deposits have been described, where both the presence and absence of IgG deposits in MS lesions has been described.[70,71] Whether this

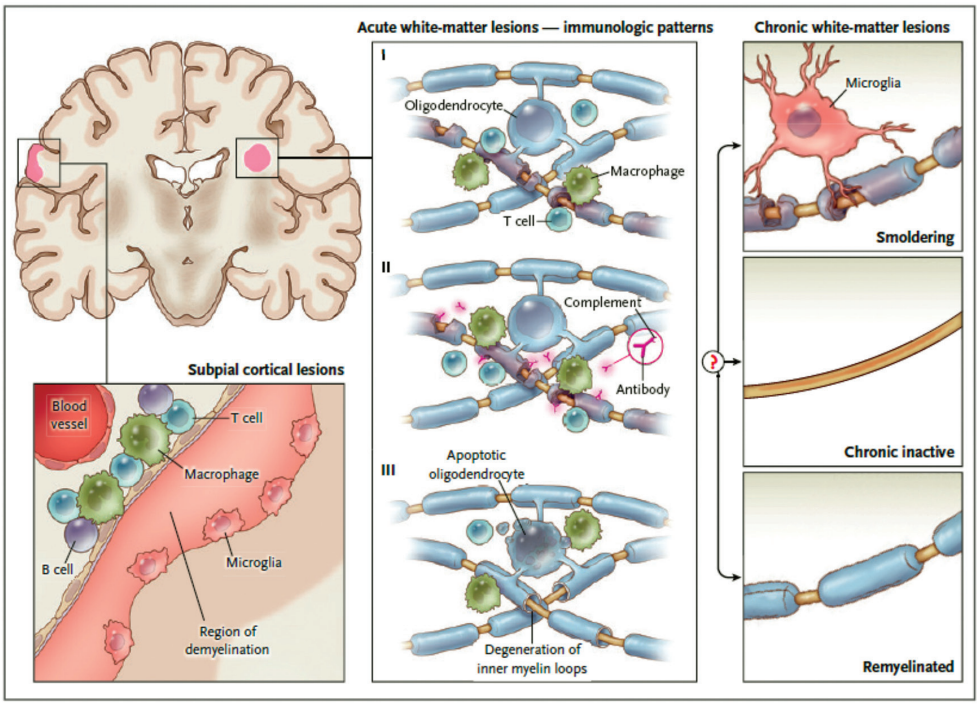


Figure 3. Heterogeneity in the immunopathology of MS in early biopsy lesions.
Early active white matter demyelination falls into three major categories. The most common types (patterns I and II) show a background of mononuclear phagocytes with perivascular and parenchymal T cell infiltration. Pattern II is further distinguished by immunoglobulin and complement deposition. In approximately 25% of biopsied active lesions (pattern III), oligodendrocyte apoptosis is accompanied by a "dyingback" oligodendrogliopathy, starting at the portion of myelin closest to the axon. Image derived with permission of Reich et al. (2018) *NEJM*.

represents different etiologies or donor specific differences in immune response due to genetic or environmental factors remains to be determined.

The immunopathology of advanced MS

The pathology of secondary progressive and primary progressive MS in autopsy cases is different compared to the early MS lesion pathology in biopsy lesions, since active demyelinating lesions with disruption of the blood brain barrier are less frequent.[58] However in advanced MS substantial numbers of the lesions show inflammatory activity at their margin composed of T cells and microglial cells.[58,71] As described previously the microglial cells in the lesion margins contain myelin degradation products and are therefore suggestive for a slow rate of ongoing demyelination in the progressive stages of the disease. It has been shown that these mixed active/inactive lesions occur more often in the progressive cases compared to the early and acute MS cases.[72] In progressive MS also the normal appearing white matter is abnormal, where there is diffuse microglial activation which is suspected to be associated with diffuse axonal injury and destruction.[73]

There are several pathological studies that describe the infiltration of T-cells, B cells and plasma cells in autopsy tissue derived from advanced MS patients.[74–79] Which suggests these cells potentially contribute to MS lesion progression, also in the advanced stages of the disease. Lymphocytes in advanced MS lesions are mostly found in the perivascular space and meninges,[52,57,80] which are since decades two compartments that are considered connected with each other and since a few years also to the meningeal lymphatic vessels.[81–86] These compartments are considered relevant for the immune cell reactivation potentially driven by CNS derived antigens.[86] This is illustrated in **Figure 4** derived from Esiri et al. 1990.[86]

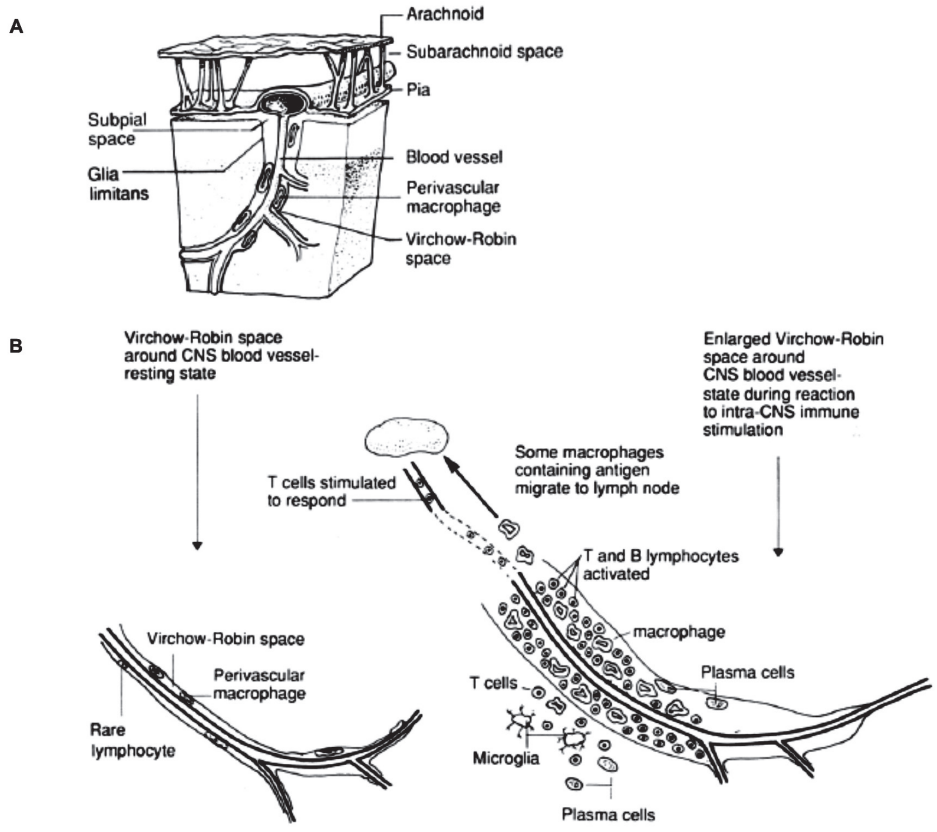


Figure 4. Neuroinflammation in the peri-vascular Virchow Robin space and meninges.
A. Illustration of the relationship of the Virchow-Robin space to the subpial and subarachnoid space.
B. Diagram illustrating the view put forward that the Virchow-Robin space is an immunological space that can become expanded and filled with immune competent cells interacting together under conditions of immune stimulation in the brain. Image derived with permission of Esiri et al. (1990) *Journal of Neurological Sciences*.

Interestingly, due to the ineffectiveness of current immunomodulatory therapies which target lymphocytes outside the CNS to reduce progression of disease, a large role for neurodegenerative mechanisms has been advocated in progressive MS. Although cortical demyelination has been

repeatedly related to the presence of meningeal infiltrates by several research groups,[55,57,87] the inflammatory activity in cortical grey matter lesions is limited compared to the adjacent white matter.[52,88] This raised the hypothesis that neurodegeneration in progressive MS is potentially independent of the inflammatory response.[89–91]

However, pathological studies in progressive MS show that in line with the early phases of the disease, neurodegeneration occurs on the background of inflammation consisting of microglia, T cells and B cells.[71,73,76] And both primary and secondary progressive MS shows substantial lymphocytic infiltrates.[79] **Figure 5** shows the hypercellular rims and the perivascular infiltrates that are considered characteristic for secondary progressive MS, derived from Revesz et al 1994.[79]

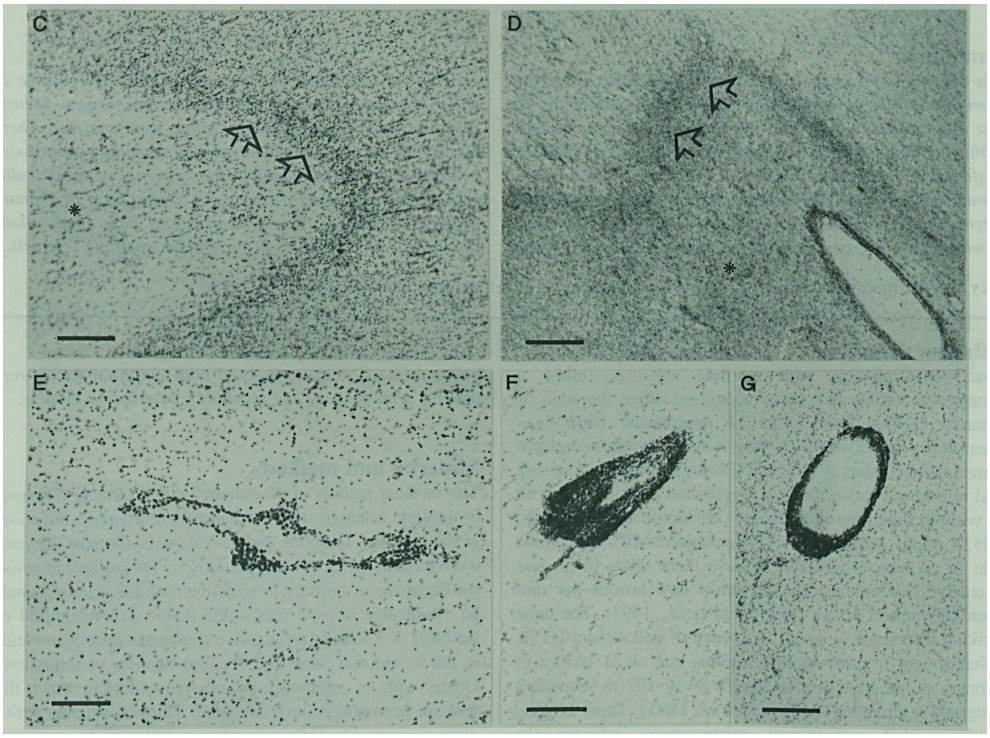


Figure 5. Lymfocyte involvement in progressive MS pathology.
H&E staining showing hypercellular lesion rim (C,D) and perivascular lymfocytic infiltrates (E-G). Image derived with permission of Revesz et al. (1994) *Brain*.

All together these observations illustrate that neuroinflammation in advanced MS lesions, comprising both the innate and adaptive immune response, is different from 'neuroinflammation' as described in classical neurodegenerative diseases as Alzheimer's or Parkinson's disease. Neuroinflammation has been classically defined as immune-mediated pathology in the central nervous system (CNS).[92] Classically neuroinflammation in the CNS is similar to that seen in

other organs and it shows the same tissue characteristics;[92] the elevation in pro-inflammatory cytokines and chemokines, activation of macrophages, recruitment of leukocytes and local tissue damage.[93] In the CNS this is seen in MS and also acute and chronic infections, stroke and trauma. However the changes observed in Alzheimer's and Parkinson's disease comprise complement and microglial activation without the recruitment of leukocytes and therefore do not equal the classical definition of neuroinflammation.[94] Because it has been shown that microglial cells can detect, process and respond to signals also in a non-inflammatory way.[92,95] This non-inflammatory type of microglia activation seen in neurodegenerative diseases is different from the microglial activation in advanced MS lesions, where they show a foamy morphology and contribute to the ongoing demyelination. Furthermore the activated microglial cells in the advanced MS lesions are accompanied by pronounced activation of T cells and to lesser extend B cells.[52,94] The pathological phenomena of immune cell infiltrates in the meninges in the advanced stages of MS also mimic the pathology of chronic auto-immune diseases in other organs, for example Sjogren's disease and Rheumatoid Arthritis.[96] This all corroborates the idea that advanced MS lesion pathology is immune mediated and differs principally from 'neuroinflammation' as it occurs in classical neurodegenerative diseases.

Although advanced MS can be considered an immune mediated disease with involvement of T and B cells there is surprisingly limited data available on T and B cells in the MS white matter lesions from MS autopsy cases. Quantifications of T cells in limited numbers of MS autopsy cases showed that they are slightly increased in active and mixed lesions compared to the inactive lesions,[74–77] suggesting that the inflammatory and demyelinating activity in the MS lesions in the progressive phase is related to the presence of T cells. However, the characteristics of these T cells and to what extend also B cells and plasma cells contribute to the immune response in MS lesions remains to be further characterized in the advanced stage of MS. Furthermore, the heterogeneity in inflammatory activity and lymphocytes infiltration between MS autopsy cases and how this correlates with the clinical disease severity remains to be analyzed in a clinically and pathologically well-characterized MS autopsy cohort.

Clinically and pathologically well-characterized MS autopsy cohorts with substantial numbers of advanced MS cases with a progressive disease course are very limited available world-wide.[72,97] Possibly the fact that progressive MS patients often die at home or in nursery homes, explains why relatively few progressive MS cases are presented to the neuropathologists in the hospital. At the NBB MS patients register as brain donor during life. Over the past 30 years almost 200 MS brain donors with a neuropathologically confirmed diagnosis of MS came to autopsy at the NBB. Extensive clinical information of the donors was collected retrospectively. Over the past ten years the MS lesions of the MS tissue collection at the NBB have been systematically characterized. This allows the study of the heterogeneity of the immunopathology of MS in relation to clinical characteristics in an autopsy cohort of advanced MS cases.

AIM OF THESIS

The aim of the thesis is to characterize the heterogeneity of the immunopathology in advanced MS in an autopsy cohort and identify mechanisms that contribute to the heterogeneity and sex differences in the clinical course of MS.

In **Part 1** – entitled *substantial inflammatory lesion activity in advanced MS* – we aim to characterize the immune cells that are involved in advanced MS lesion pathology in autopsy tissue and their correlation with the clinical disease course and sex.

In **Chapter 2** we ask the question *whether neurodegenerative changes in progressive MS brainstem lesions relate to the innate and adaptive immune response*. We used post-mortem MS brainstem tissue where we first staged lesions based on demyelination – detected with the proteolipid protein (PLP) marker of myelin – and microglia/macrophage accumulation, distribution and morphology – detected with the human leukocyte antigen (HLA-DR) marker of myeloid cells. Across all lesion stages in the MS brainstem we analyzed neurodegenerative changes including metabolic stress (mtHSP70), axonal transection/impaired transport (SMI312, APP) and synaptic alterations (synaptophysin) as well as the localization and extent of deposits of early (C1q, C3d) and terminal (MAC) complement factors, and the localization and density of T ($CD3^+$, $CD4^+$, $CD8^+$) and B ($CD20^+$, $CD138^+$) lymphocytes. Findings in the MS brainstem were compared to brainstem tissue of non-neurological controls and controls with other neurodegenerative diseases.

In **Chapter 3** we ask the question *what are the pathological correlates of MS clinical disease course and sex* in the autopsy cohort of the Netherlands Brian Bank, containing 182 MS brain donors. Using the standardized autopsy procedures from the Netherlands Brain Bank including systematic dissection from standard location, 3188 tissue blocks containing 7562 MS lesions were dissected. Based on previously proposed criteria, MS lesions in white matter, cortical grey matter and deep grey matter were categorized. Lesion demyelinating and innate inflammatory activity were visualized by immunohistochemistry for proteolipid protein (PLP) and human leukocyte antigen (HLA-DR). Lesions in the white matter and deep grey matter were classified into active, mixed active/inactive, inactive or remyelinated, while microglia/macrophage morphology was classified as ramified, amoeboid or foamy. Lesions in the cortical grey matter were characterized as leukocortical, intracortical or subpial based on their location. Lesion load, lesion type prevalence and microglia/macrophage morphology were analyzed in relation to clinical course, disease severity and sex, and in relation to each other.

In **Chapter 4** we ask the question *whether T cells that were previously identified in brain autopsy tissue represent a brain specific population of tissue resident memory T cells (T_{RM})*. We performed flow-cytometric phenotyping of human T cells isolated from the post-mortem brain tissue. We analyze the expression profiles of molecules associated with cellular differentiation, migration,

effector functions, and transcriptional control in these cells as well as cytokine profiles after stimulation. We analyzed the existence and characteristics of two $CD69^+$ subsets distinguished by the surface presence of CD103. Furthermore we explore the characteristics of the lesser abundant brain $CD4^+$ T cell fraction and analyze whether these also are enriched for T_{RM} cell associated surface markers.

In **Chapter 5** we ask the question *whether T cells are involved in the ongoing inflammatory lesion activity in advanced MS autopsy cases and whether they show a T_{RM} cell phenotype*. We used a combination of immunohistochemistry and flow cytometry to study localization, quantity, and phenotypic profile of T cells in control white matter, MS normal-appearing white matter and MS white matter lesions. We quantified T cells and perivascular T-cell cuffing at a standardly dissected location in 146 MS, 10 neurodegenerative control and 20 non-neurological control brain donors. In addition, we quantified $CD3^+$, $CD4^+$, and $CD8^+$ T cells in 141 subcortical white matter lesions. The location of $CD8^+$ cells, either in the perivascular space or in the brain parenchyma was determined using CD8/laminin staining and confocal imaging. We compared early MS biopsy and late MS autopsy lesions for the presence of $CD103^+$ and $S1P1^+$ T cells. Finally, we analyzed $CD8^+$ T cells, isolated from fresh autopsy tissues from subcortical MS white matter lesions (n=8), MS normal-appearing white matter (n=7), and control white matter (n=10), by flow cytometry. The $CD8^+$ T cells were phenotyped for CD69, CD103, CD44, CD49a, CXCR6, PD-1, GPR56, Ki67, and granzyme B.

In **Part 2** – entitled *heterogeneity of the immunopathology in advanced multiple sclerosis* – we aim to identify pathophysiological mechanisms that contribute to the heterogeneity and sex differences in the immunopathology and clinical disease course of MS.

In **Chapter 6** we ask the question *whether differences in the presence of B cells and plasma cells in MS are correlated with clinical and pathological characteristics, the IgG ratio and presence of OCBs in the cerebrospinal fluid (CSF)*. Autopsy tissue from 140 MS and 24 non-neurological controls and early MS biopsy lesions from 24 MS patients were stained for $CD20^+$ and $CD138^+$ to detect B cells and $CD138^+$ plasma cells, respectively. The presence of B cells and $CD138^+$ plasma cells in white matter lesions and in the standardly dissected brainstem was correlated with pathological and clinical donor characteristics. In corresponding CSF and plasma, immunoglobulin (Ig)G ratio and oligoclonal band patterns were determined. Additionally, to determine whether oligoclonal bands could disappear over time in advanced MS the presence of oligoclonal bands was determined in a clinical cohort of 73 patients, at diagnosis and during follow-up.

In **Chapter 7** we ask the question *whether altered progesterone and androgen synthesis in the normal appearing cortical grey matter of males and females contributes to the increased susceptibility of males for the development of cortical grey matter lesions*. In the standardly dissected superior temporal gyrus from 40 MS (20F/20M) and 35 non-neurological controls (20F/15M) cortical grey matter lesions were characterized. We analyzed if there were sex differences in gene expression of the progestogen and androgen synthetic enzymes and the progesterone receptor in normal

appearing cortical grey matter. Secondly this was correlated with neuroprotective and anti-inflammatory effects by measuring gene expression of GABA/Glutamate synthesis and re-uptake, the neuroprotective protein BDNF, the anti-inflammatory cytokines and the cytotoxic T cell response.

In **Chapter 8** we ask the question *whether genotype correlates with MS lesion characteristics in autopsy tissue*. We genotyped 179 MS brain donors from the Netherlands Brain Bank MS autopsy cohort for 102 SNPs, selected based on their reported associations with clinical outcome or their associations with genes that show differential gene expression in MS lesions. In order to link genotype to pathological parameters we analyzed the correlation of allelic distributions for each SNP with the proportion of lesion subtypes that was scored for each donor. Total lesion load, reactive site load, presence of cortical grey matter lesions and the proportions of lesion subtypes, either active, mixed active/inactive, inactive or remyelinated were tested for correlation with genotype for each SNP. For the SNPs that showed significant association after multiple-testing correction the effect on gene expression levels in brain autopsy tissue was analyzed using eQTL database validated by qPCR from MS brain autopsy tissue. An immunohistochemical examination of MS lesions and flow-cytometric analysis of T cells derived from blood and brain was performed for the related proteins.

FIGURE REFERENCES

Fig 2. Reprinted from: Kuhlmann T, et al. (2017) An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol* 133:13–24, with permission of Springer Nature Customer Service Centre GmbH: Springer Nature.

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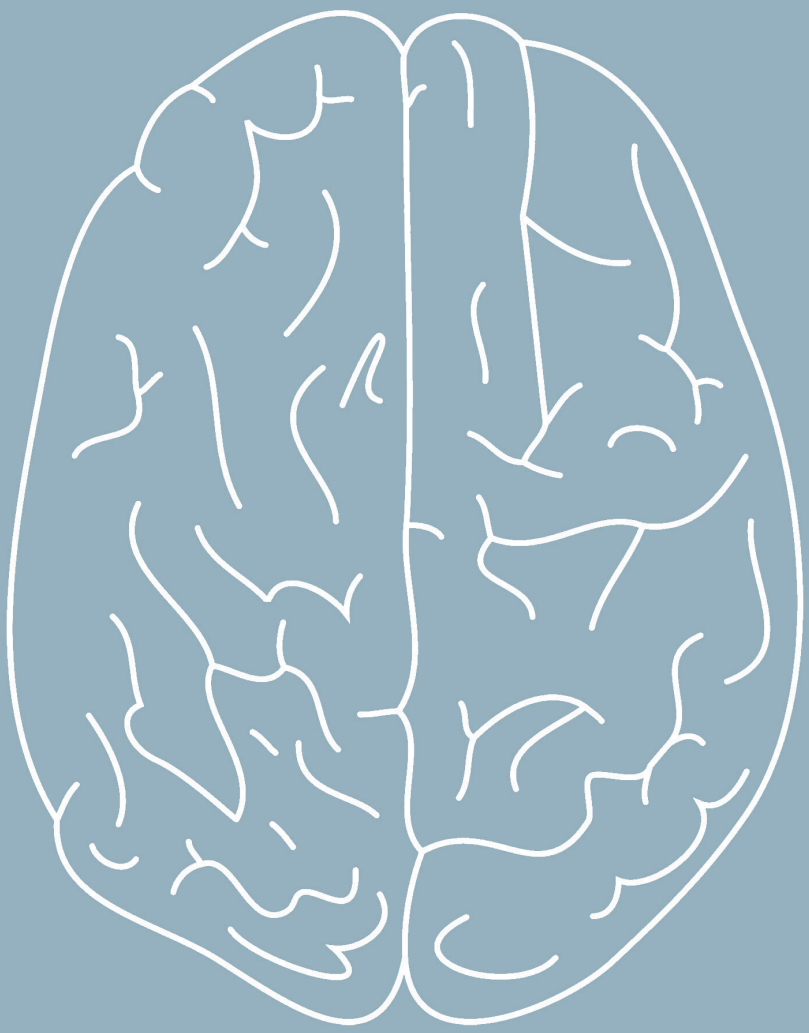
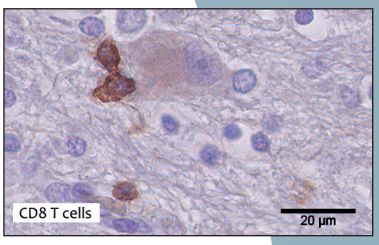
PART 1

SUBSTANTIAL INFLAMMATORY LESION ACTIVITY IN ADVANCED MULTIPLE SCLEROSIS



CHAPTER 2

Inflammation is associated with demyelination and neurodegeneration in the brainstem of patients with progressive multiple sclerosis



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In prep

ABSTRACT

In progressive multiple sclerosis (MS), demyelinated lesion load in the brainstem strongly correlates with disease severity, but we know surprisingly little about the immunopathology of these lesions. Here we investigated the lesional activity, the extent of inflammation and neurodegenerative changes in 38 staged brainstem lesions from 9 progressive MS cases, compared to normal-appearing MS brainstem, 6 donors with other neurodegenerative diseases and 3 non-neurological controls. We found that 74% of MS brainstem lesions analyzed are active or mixed active/inactive (mixed). In mixed lesions, neurons and axons showed increased metabolic stress, disturbed axonal transport, reduced axonal density and loss of synapses. C1q deposits were detected at synapses while C3d was found on neuronal perikarya and focally on axons, a feature shared with other neurodegenerative diseases. MAC immunoreactivity, identified within phagocytic microglia/macrophages and on astrocytes, was a specific feature of MS. A dominance of $CD8^+$ T cells was detected in mixed lesions but $CD4^+$ T cells were also seen (CD8:CD4 ratio, ~3:1), and both infiltrated the parenchyma with some $CD8^+$ T cells found in close association to neurons. $CD20^+$ B cells were detected in mixed lesions but were more rare compared to T cells (CD3:CD20 ratio, ~7:1) and unlike T cells they localized perivascularly. $CD138^+$ plasma cells, although scarse, infiltrated the tissue. Importantly, inflammation associated significantly with injury and loss of axons. Altogether, we report substantial inflammation in the brainstem of progressive MS patients which closely associates with demyelination and neurodegeneration. While deposits of early complement components in both neurodegenerative diseases and MS point to shared mechanisms of complement-mediated injury, MAC deposition may constitute a specific feature of the inflammatory response with pathogenic significance in MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS) with a neurodegenerative component.[16,17,30,66] Although MS has long been considered a disease of the white matter, several studies have now highlighted the involvement of grey matter demyelination in the supratentorial cortex[10,38,41,59] and in the deep grey matter nuclei including the hippocampus,[29] the hypothalamus[35] and the brainstem.[48] While the immunopathology of cortical demyelination has received widespread attention in recent years,[6,33,34,47,51,52,67,68] we know surprisingly little about lesions in the deep grey matter nuclei, yet they are of interest since they appear to contribute considerably to the neurological disabilities of MS patients.[14,19,36,46,53] The brainstem is a deep grey matter structure particularly relevant in MS because brainstem atrophy, a measure of neurodegeneration, occurs from the earliest stages of the disease and is an important predictor of clinical disability.[19] Brainstem involvement in MS can be manifested with different symptoms, among which diplopia is the most common (68%) followed by facial sensory symptoms (32%), unstable gait (30.7%) and vertigo (18.7%).[31] Notably, our recent study using the autopsy MS cohort of the Netherlands Brain Bank (NBB), has shown that the brainstem grey and white matter are heavily demyelinated, and the lesion load strongly correlates with disease severity.[48] However, the lesional activity, the extent of inflammation and changes to neurons, axons and synapses in these lesions are currently unknown. It is also unknown whether demyelination and neurodegeneration in the MS brainstem progress independently from inflammation or whether these processes are linked.

Using post-mortem MS brainstem tissue collected at rapid autopsy, we first staged lesions based on demyelination – detected with the proteolipid protein (PLP) marker of myelin – and microglia/macrophage accumulation, distribution and morphology – detected with the human leukocyte antigen (HLA) marker of myeloid cells. Across all lesional stages of the MS brainstem, we analyzed neurodegenerative changes including metabolic stress (mtHSP70), axonal transection/impaired transport (SMI312, APP) and synaptic alterations (synaptophysin) as well as the localization and extent of deposits of early (C1q, C3d) and terminal (MAC) complement factors, and the localization and density of T ($CD3^+$, $CD4^+$, $CD8^+$) and B ($CD20^+$, $CD138^+$) lymphocytes. Findings in the MS brainstem were compared to brainstem tissue of non-neurological controls and controls with neurodegenerative diseases. Finally, we correlated the extent of inflammation with the extent of neurodegenerative changes at neurons, axons and synapses. Understanding the type of lesional activity, the extent of inflammation and neurodegeneration, and the relationship between these processes in the brainstem of progressive MS patients, will inform on the mechanisms leading to brainstem injury in progressive disease and could guide development of therapies aimed at slowing deterioration of clinical symptoms related to this region.

METHODS

Post-mortem brainstem tissue and inclusion criteria

All paraffin-embedded or fresh frozen brainstem tissue blocks were collected at rapid (average PMD 7h:19m) autopsy by the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands) from 4 standardized fronto-caudal locations in the brainstem (nigra, pons/locus coeruleus, pons/colliculus inferior, medulla oblongata). Brainstem tissue was collected from 9 MS donors, 3 non-neurological control (NNCo) donors and 6 control donors with other neurodegenerative diseases (referred to as neurodegenerative controls, NDCo). The diagnoses for MS or neurodegenerative diseases were confirmed by a certified neuropathologist. Donors diagnosed with neuromyelitis optica, known to trigger complement activation and preferentially affect the brainstem, were excluded. NNCo included 3 cases without neurological diseases. NDCo included 3 cases of Alzheimer's disease (AD, Braak 6, female:male 3:0; age 54–60 years), 2 cases of frontotemporal dementia (FTD, female:male 2:0; age 61–63 years) and 1 case of amyotrophic lateral sclerosis (ALS, female; age 64 years). The brainstem of donors with other neurological disease showed disease-associated pathological changes such as amyloid β plaque, neurofibrillary tangles (not shown). Multiple sclerosis samples comprised 6 cases with SPMS and 3 cases with PPMS. Since patients with PPMS and SPMS share very similar pathological changes in the brain and spinal cord, for the purpose of this study they were considered as one clinical group of progressive MS donors. Of note, the MS cases analyzed included a higher number of males compared to NDCo cases in which females predominated. Detailed clinical and demographic data of all donors are provided in **Table 1** and **Suppl. Table 1**. NBB obtained permission from donors for brain autopsy and the use of tissue and clinical information for research purposes as part of a program approved by the Ethical Committee VU University Medical Center (Amsterdam, The Netherlands).

Table 1. Overall clinical and demographic data of MS donors and controls.

	Cases (n)	**Age** (years)	**Sex** (M/F)	**pH value**	**Brain weight** (g)	**Disease duration** (years)	**Disease course**
Multiple sclerosis	9	58 ± 11,5	8/1	6,5 ± 0,3	1260 ± 112,6	27,1 ± 13,2	6 SP 3 PP
Neurodegenerative controls	6	59,8 ± 3,8	0/6	6,25 ± 0,2	1008,8 ± 100,4	–	–
Non-neurological controls	3	51,7 ± 17,0	2/1	6,43 ± 0,1	1390,7 ± 192,5	–	–

Values are expressed as mean ± SD (standard deviation).
F, female; M, male; PMD, post-mortem delay; PP, primary progressive; SP, secondary progressive.

Neuropathology techniques

Paraffin or frozen sections from each tissue block were stained by standard histological methods with hematoxylin and eosin (HE) and luxol fast blue (LFB) myelin stain and by immunohistochemistry

for proteolipid protein (PLP) as a marker of myelin and human leukocyte antigen (HLA-DP-DQ-DR) as a marker of microglia/macrophages. Since brainstem lesions are almost always mixed grey/white matter lesions, the lesional activity was inferred from the white matter portion of the lesion and staged as reactive, active, mixed active/inactive and inactive, according to the classification routinely used at the NBB and the latest international consensus as previously published.[24,40,48] Normal-appearing brainstem was defined as a region of the brainstem located at least 1 cm away from demyelinated plaques and reactive lesions. Lesions were staged by two independent observers (NLF and VR). All lesions and randomly selected areas of normal-appearing brainstem underwent immunohistochemical and quantitative analysis (see below).

Selection of markers for inflammation and neurodegeneration

To evaluate the involvement of innate immunity in the brainstem, we determined the accumulation, distribution and morphology of microglia/macrophages across the lesions and normal-appearing tissue using an antibody against the human leukocyte antigen (HLA). In addition, we evaluated the deposition of key components of the complement system, using antibodies against the recognition protein and initiator of the classical complement pathway (C1q), the cell-bound product of activation of the common pathway complement component C3 (C3d) and the neo epitope of C9 that is formed when a polymer of C9 is incorporated into the membrane attack complex (MAC) as a result of terminal complement activation.[64] To determine the basic composition of adaptive immune cellular infiltrates we used antibodies against all T cells (CD3), against MHC class I restricted T cells (CD8α), MHC class II restricted T cells (CD4) and B cells (CD20). Plasma cells were identified by their enlarged cytoplasm and expression of CD138. To determine evidence of neurodegeneration, we used an antibody against a mitochondrial stress response molecule (mitochondrial heat shock protein 70, mtHSP70) because impaired energy metabolism has been detected in axons within active and inactive white matter lesions and has been proposed as a possible cause of neuronal injury in MS.[78] In addition, we used an antibody against a fast axonal transport protein (amyloid precurson protein, APP) that accumulates focally in swollen axons and transected axons forming axonal spheroids or end-bulbs[8,20,26,39] was used as measure of axonal injury since these features may persist at sites of damage for an extended period.[45] An antibodies against a neurofilament (SMI312) was used to visualize axons, including axonal swelling. An antibody against a pre-synaptic element (synaptophysin) was used to visualize synapses as a measure of structural dis/connection of brainstem networks, since loss of synapses has been previously detected in the MS grey matter.[17]

Immunohistochemistry

Seven micron thick paraffin or cryo sections were mounted on Superfrost Plus glass slides (Knittel Glass, Germany). Cryostat sections were fixed in formalin for 10 minutes and washed in phosphate-buffer saline (PBS, pH 7.4, Life technologies, Bleiswijk, The Netherlands). Paraffin sections were deparaffinated and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol or incubation medium for 20 minutes as previously described.[23,24] For some antigens,

epitopes were exposed by heat-induced antigen retrieval in either appropriate buffer solutions, depending on the primary antibody used (see **Table 2**). For both paraffin and cryostat sections, non-specific binding of antibodies was blocked using 10% Normal Goat or Horse Serum (DAKO, Glostrup, Denmark) in PBS for 20 minutes at room temperature. Primary antibodies (**Table 2**) were diluted in Normal Antibody Diluent (Immunologic, Duiven, The Netherlands) or incubation medium (SUMI) as previously described[24] and incubated for 1 hour at room temperature. Detection was performed by incubating the sections in the secondary Poly-HRP-Goat anti Mouse/Rabbit/Rat IgG (Immunologic, Duiven, The Netherlands) antibodies diluted 1:1 in PBS for 30 minutes at room temperature followed by incubation in 3,3- diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA) as chromogen and counterstaining with hematoxylin (Histolab, Gothenburg, Sweden) for 5 minutes. Sections stained with secondary antibody alone were included as negative controls with each test. After dehydration, slides were mounted in Pertex (Histolab). Images were captured with a light microscope (Olympus BX41TF, Zoeterwoude, The Netherlands) using the Cell D software (Olympus, Zoeterwoude, The Netherlands).

Immunofluorescence

For the immunofluorescence staining, primary antibodies were detected with FITC-conjugated goat anti-rabbit IgG or Cy3-conjugated goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, MI, USA) diluted 1:200 in PBS and incubated for 1 hour at RT. After washing, sections were counterstained with 4.6-diamidine-2-phenylindole dihydrochloride (DAPI) (blue, 280nm) (Vector Laboratories), air dried and mounted in Vectashield (Vector Laboratories). Images were captured digitally with a fluorescence microscope (DM LB2; Leica Microsystems, Rijswijk, The Netherlands).

Quantification of immunohistochemistry

All sections were digitally scanned using a Philips scanner 3.1.1.2. Lesions and normal-appearing tissue were identified based on HLA and PLP staining and manually outlined using QuPath (Version: 0.2.0, University of Edinburgh, Scotland).[4] $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, $CD20^+$ cells, $CD138^+$ cells, APP^+ axonal swellings and $C1q^+$ neurons were manually counted using the points tool in QuPath. Values are expressed as counts per square millimeter. $C3d^+$ and MAC^+ deposits were manually counted on digitally photographed section using a light microscope (Olympus BX41TF, Zoeterwoude, The Netherlands) and the Cell D software (Olympus, Zoeterwoude, The Netherlands). Lesions and normal-appearing tissue were outlined and the size of the areas was measured using the "measurement" function of the Image Pro Plus 7.0 imaging software (MediaCybernetics, Rockville, MD, USA). Five to 20 fields per lesion (0.1-30mm^2) and 20 to 100 fields per normal-appearing tissue (130-230mm^2) were quantified. Values are expressed as counts per square millimeter. SMI312 and mtHSP70 staining were quantified as the percentage of immunopositive surface area using the positive pixel count tool in QuPath and dividing the positive pixel area in um^2 by the total area in um^2. Down sample factor was set at 5, gaussian sigma was set at 3, positive DAB threshold was set at 0.2 for SMI312 and 0.3 for mtHSP70. Values are expressed as percentage of stained area per area analyzed.

Table 2. Antibodies for immunohistochemistry.

Antigen	Target	Host	Clone	Conc.	Tissue fixation	Product code, company
PLP (Proteolipid protein)	Myelin	Mouse	Plpc1	1:1000	FFPE [b]	Bio-Rad Cat# MCA839G, RRID:AB_2237198
HLA-DP, DQ, DR (Human leukocyte antigen)	Microglia/ macrophages	Mouse	CR3/43	1:100	FFPE [b]	Agilent Cat# M0775, RRID:AB_2313661
GFAP (Glial fibrillary acidic protein)	Astrocytes	Rabbit	Poly	1:4000	FF	Agilent Cat# Z0334, RRID:AB_10013382
CD3	All T cells	Rabbit	Poly	1:100	FFPE [a]	Agilent Cat# A0452, RRID:AB_2335677
CD4	MHC class II restricted T cells	Rabbit	EPR6855	1:500	FFPE [a]	Abcam Cat# ab133616, RRID: AB_2750883
CD8	MHC class I restricted T cells	Rabbit	Poly	1:500	FFPE [a]	Abcam Cat# ab4055, RRID:AB_304247
CD20cy	B cells	Mouse	L26	1:100	FFPE [a]	Agilent Cat# M0755, RRID:AB_2282030
CD138	Plasma cells	Mouse	B-A38	1:500	FFPE [a]	Bio-Rad Cat# MCA2459, RRID:AB_566507
IgM (Immunoglobulin M)	Immunoglobulins	Rabbit	Poly	1:16000	FFPE [c]	Agilent Cat# A042602, RRID:AB578520
C1q	Classical pathway of complement	Mouse	34E2	1:100	FFPE [c]	Abcam, ab235454
C3d	Common pathway of complement	Rabbit	Poly	1:2000	FFPE [a]	Agilent Cat# A006302, RRID: AB_578478
C9neo (MAC, membrane attack complex)	Terminal pathway of complement	Mouse	aE11	1:100	FF	Agilent Cat# M0777, RRID:AB_2067162
mtHSP70 (Mitochondrial heat shock protein 70)	Mitochondrial stress	Mouse	30A5	1:50	FFPE [c]	Enzo Life Sciences Cat# SPS-825E, RRID:AB_916897
APP (Amyloid precursor protein)	Impaired axonal transport	Mouse	22C11	1:300	FFPE [c]	Millipore Cat# MAB348, RRID:AB_94882
Synaptophysin	Synapses	Chicken	Poly	1:100	FFPE [c]	Synaptic Systems Cat# 101 006, RRID:AB_2622239
SMI312 (Pan-neurofilament)	Axons	Mouse	SMI 312	1:1000	FFPE [d]	BioLegend Cat# 837904, RRID:AB_2566782

[a] 10mM Sodium Citrate, pH 6.0; [b] 50 mM Tris base, 150 mM NaCl, pH 7.6; [c] 50mM Tris base, 150 mM NaCl, 1mM EDTA, pH 9.0; [d] 50 mM Tris base, 150 mM NaCl, pH 9.0; FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; poly, polyclonal.

Statistical analysis

Comparison between MS brainstem lesions or normal-appearing tissue and brainstem from NNCo and NDCo were analyzed using Kruskal-Wallis test and Dunn's post-hoc tests in GraphPad Prism 8.1.1. (©1992-2019 GraphPad Software, Inc.). Correlation analysis between measures of inflammation and measures of neurodegeneration in the MS brainstem was performed in R studio (Version 1.2.5033) using the psych and ggcorrplot package and using Spearman correlation coefficient. FDR multiple testing correction was applied on all correlation tests.

RESULTS

Staging of MS brainstem lesions

The collection of MS brainstem tissue blocks used in this study included a total of 38 lesions, comprising of 2 reactive, 5 active, 24 mixed active/inactive lesions (referred to in the text and figures as mixed lesions) and 7 inactive lesions, classified based on immunostaining for myelin (PLP) (**Figure 1A**) and microglia/macrophages (HLA) (**Figure 1B**). Compared to brainstem tissue of non-neurological controls, which showed normal PLP staining and HLA^+ cells of resting, thinly-ramified appearance (**Figure 1C-E**), the normal-appearing MS brainstem showed PLP^+ myelin but enhanced HLA staining (**Figure 1F, G**), suggesting increased microglia/macrophages reactivity in non-lesional brainstem compared to controls, as previously shown for normal-appearing white matter.[75] In addition, we observed clusters of HLA^+ microglia/macrophages throughout the normal-appearing MS brainstem, which we refer to as nodules.[55] The nodules localized along segments of nerve fibers (**Figure 1H**) which, in transverse view, appear as clusters of ramified microglia/macrophages around a central core (**Figure 1H'**), similarly to profiles we and others previously observed in normal-appearing MS white matter.[5,62] Reactive lesions were positive for PLP but showed obvious accumulation of HLA^+ microglia/macrophages (**Figure 1I-K**). Active lesions showed profound loss of PLP staining, with microglia/macrophages infiltrating across the lesion (**Figure 1L-N**). In these lesions the morphology of microglia/macrophages varied from thickly ramified to foamy, indicating myelin phagocytosis (**Figure 1N**). Mixed lesions in the brainstem were characterized by a border of activated myeloid cells, whose morphology ranged from thickly ramified to amoeboid and foamy. The lesion core of mA/I brainstem lesions was usually hypocellular (**Figure 1O-Q**). Inactive lesions comprised a sharp PLP positive lesion border lacking activated microglia/macrophages and a gliotic acellular core (**Figure 1R-T**). Notably, in this cohort of progressive multiple sclerosis brainstem donors, we found predominantly active (n=4; 11% of all detected lesions) and mixed (n=24; 63% of all detected lesions) lesions, demonstrating that the disease remains active in the progressive stage, in line with previous reports.[27,35,48]

Neurodegeneration in the MS brainstem

We first determined neurodegenerative changes in the MS brainstem compared to brainstem of NNCo and NDCo cases.

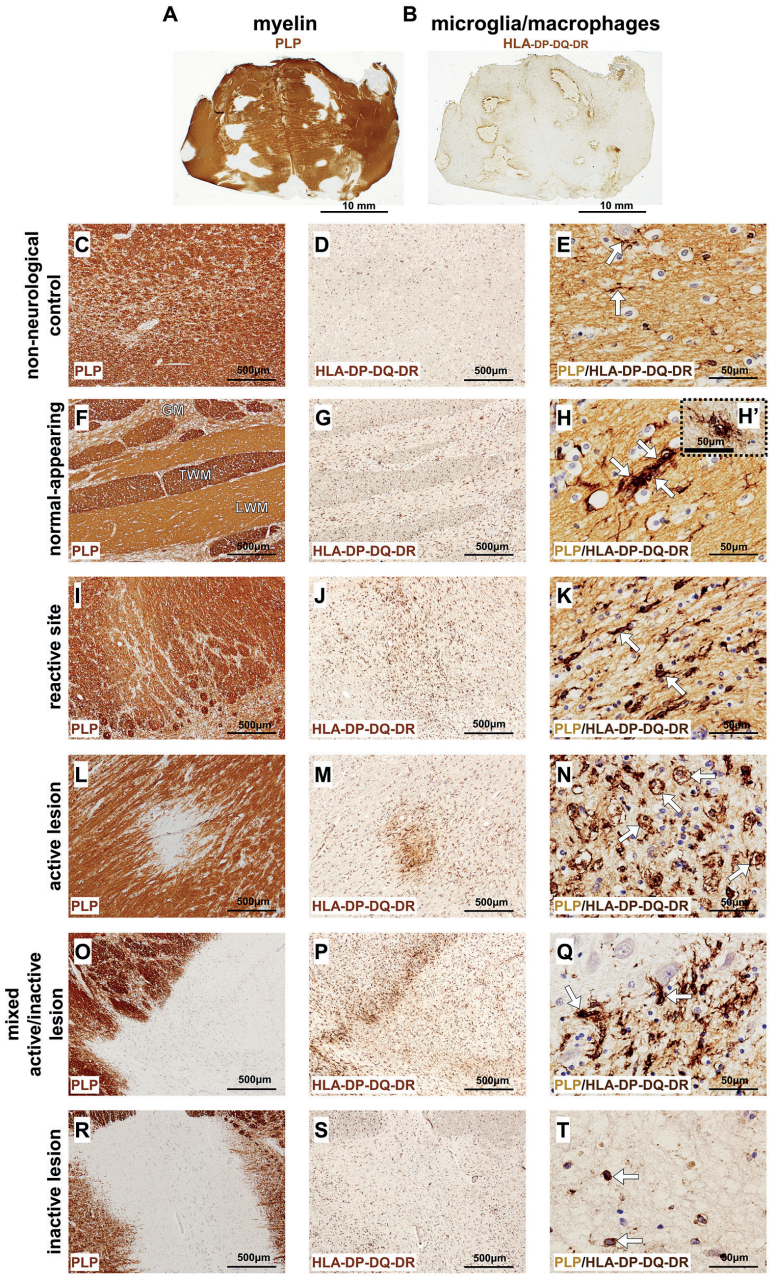


Figure 1. Multiple sclerosis brainstem lesions.
Representative sections of post-mortem brainstem from a donor with progressive multiple sclerosis, stained for (A) proteolipid protein (PLP) to visualize myelin, and (B) human leukocyte antigen (HLA-DP-DQ-DR) to visualize microglia. Examples of demyelinating lesions (lack of PLP staining, in A) with or without a rim of microglia (HLA-DP-DQ-DR staining, in B) are shown. (C-T) High magnification images of PLP, HLA-DP-DQ-DR or double PLP/ HLA-DP-DQ-DR immunostaining of (C-E) brainstem sections from a non-neurological control and (F-T) multiple sclerosis donors. Examples of various stages of MS brainstem lesions, classified based on PLP and HLA-DP-DQ-DR staining as described in the text, are shown: (F-H) lesion free MS brainstem, (I-K) reactive site, (L-N) active lesion, (O-Q) mixed active/inactive lesion or (R-T) inactive lesion. In F, GM indicates grey matter; LWM indicates longitudinal white matter tract; TWM indicates transverse white matter tract. Scale bars in A and B, 10mm. Scale bars in the single staining PLP or HLA-DP-DQ-DR, 500µm. Scale bars in the double staining PLP/ HLA-DP-DQ-DR, 50µm.

Mitochondrial heat shock protein 70 (mtHSP70)

While NNCo showed no mtHSP70$^+$ signal (not shown), and normal-appearing brainstem showed only a few immunopositivity neurons and axons (not shown), mtHSP70$^+$ neurons and axons were detected in NDCo (**Figure 2A**) and in mixed MS brainstem lesions (**Figure 2B, zoom 2B**), indicating metabolic stress in this cell type (PMID: 19591199).

Amyloid precursor protein (APP)

APP signal was detected in neurons of NNCo brainstem (not shown), NDCo brainstem (**Figure 2C**) and MS brainstem lesions (**Figure 2D**). In addition, mixed MS lesions showed staining of axonal swellings and axonal bulbs especially at the lesion rim (**Figure 2D, zoom 2D**), demonstrating impaired fast axonal transport and transected axons.[8,20,26,39]

SMI312

While we did not detect obvious changes of axonal density in the brainstem of NNCo (not shown) and NDCo (**Figure 2E, zoom 2E**), axonal density appeared reduced in most MS brainstem lesions (**Figure 2F, zoom 2F**) compared to brainstem of NDCo, NNCo and the normal-appearing brainstem. In addition, mixed MS lesions often showed SMI312$^+$ axonal swelling and end-bulbs, demonstrating transected axons in the lesion rim (**zoom 2F**).

Synaptophysin

Compared to NNCo (not shown) and the NDCo, synaptophysin staining was evidently reduced in MS brainstem lesions (**Figure 2G and H, zoom 2G and zoom 2H**), indicating a loss of pre-synaptic elements.[17]

Quantitative analysis of mtHSP70$^+$ staining, APP$^+$ end-bulbs and SMI312$^+$ axons across all lesion types compared to NNCo and NDCo demonstrated a robust axonal degenerative component to the pathology of the MS brainstem, which is most evident in the active rim of mixed lesions (normal-appearing vs mixed mean rank: mtHSP70: 16,43 vs 17,82 p=n.s.; APP: 15,40 vs 32,61, p=0.01.; SMI312: 35,40 vs 19,40, p=0.02; by Kruskal-Wallis and Dunn's post-hoc test) (**Figure 2I-K**).

The extent of complement deposits is highest in active and mixed MS brainstem lesions

Next, we analyzed the localization and extent of complement deposits in the MS brainstem in relation to lesional activity and in comparison to NNCo and NDCo brainstem.

C1q

C1q is a pattern recognition molecule that together with the proteases C1r and C1s (the C1 complex) can trigger the classical pathway of complement activation by binding to immunoglobulins[13,37] In addition, antibody-independent binding of C1q to myelin[76] and to synapses[56] have been described. In line with previous reports which described C1q deposits at synapses during normal ageing[72] and neurodegeneration,[32] we found staining for C1q (although weak) around neurons in the brainstem of NNCo (not shown) and NDCo (**Figure 3A**). In the MS brainstem, C1q immunoreactivity was marked around neurons in mixed lesions and showed a punctate staining pattern that is consistent with a synaptic localization (**Figure 3B**). To verify whether C1q deposits in mixed brainstem lesions

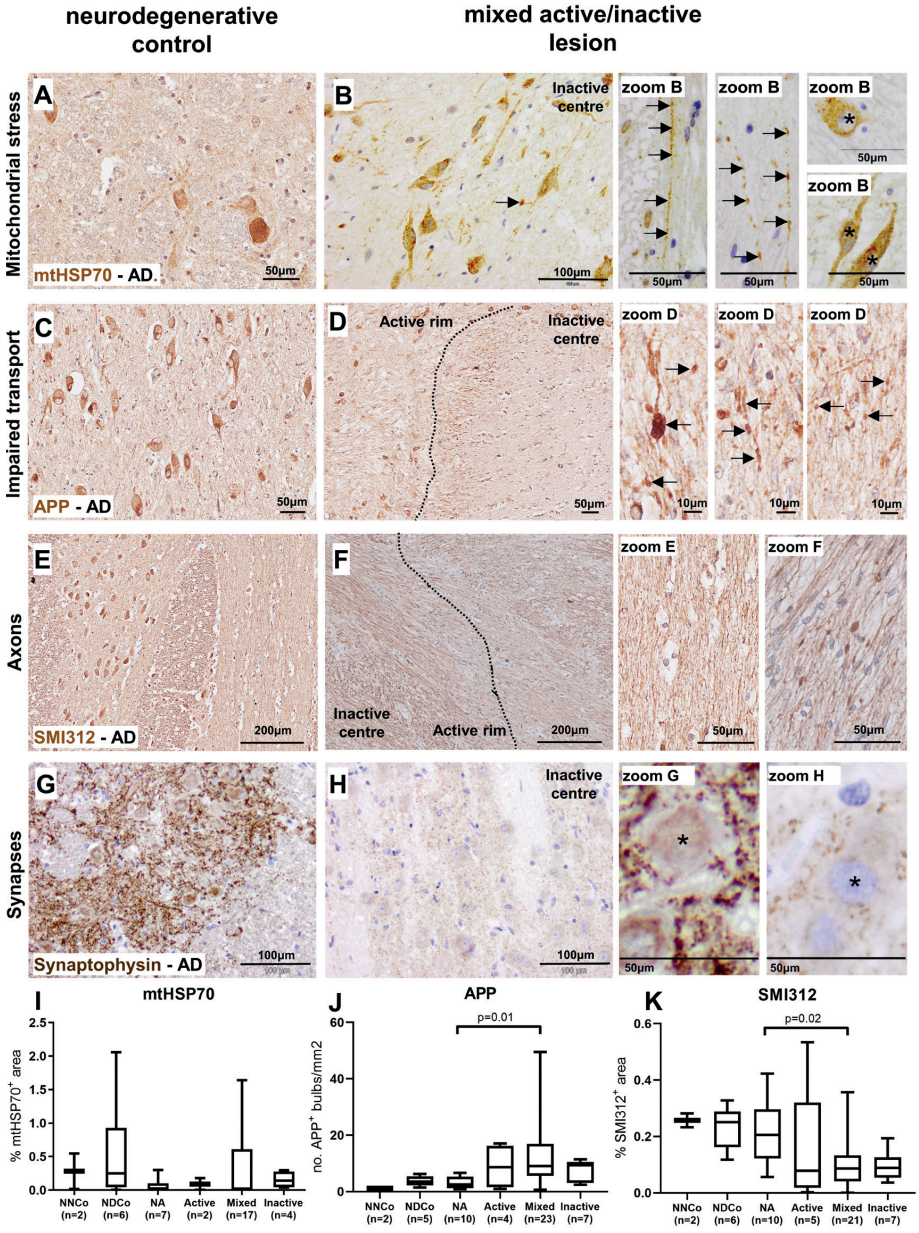


Figure 2. Neurodegeneration in multiple sclerosis brainstem.
Representative brainstem sections from progressive multiple sclerosis cases and neurodegenerative disease. (A and B and zoom B) Mitochondrial heat shock protein 70 (mtHSP70) visualizes metabolic stress in neurons and axons in both MS and neurodegenerative disease. (C and D and zoom D) Amyloid precursor protein (APP) visualizes disturbed axonal transport, specifically in the active rim of mixed active/inactive lesions where they show axonal end-bulbs. (E and F and zooms) Pan-neurofilament marker of axons (SMI312) visualizes axons demonstrating variable axonal loss in mixed active/inactive lesions, demonstrating also axonal end-bulbs in the active rim of the mixed active/inactive lesion. (G and H and zooms) Synaptophysin visualizes synapses, in neurodegenerative disease and mixed active/inactive lesions, showing synapse loss in MS lesions. Asterisks indicate neurons. Hematoxylin stains nuclei in blue. Staining patterns are representative of those seen in the brainstem lesions from 9 donors with progressive multiple sclerosis. Isotype-matched antibodies were used as negative controls. (I-K) Quantification of mtHSP70, APP$^+$ axons and bulbs and SMI312 in MS, neurodegenerative disease and non-neurological controls.

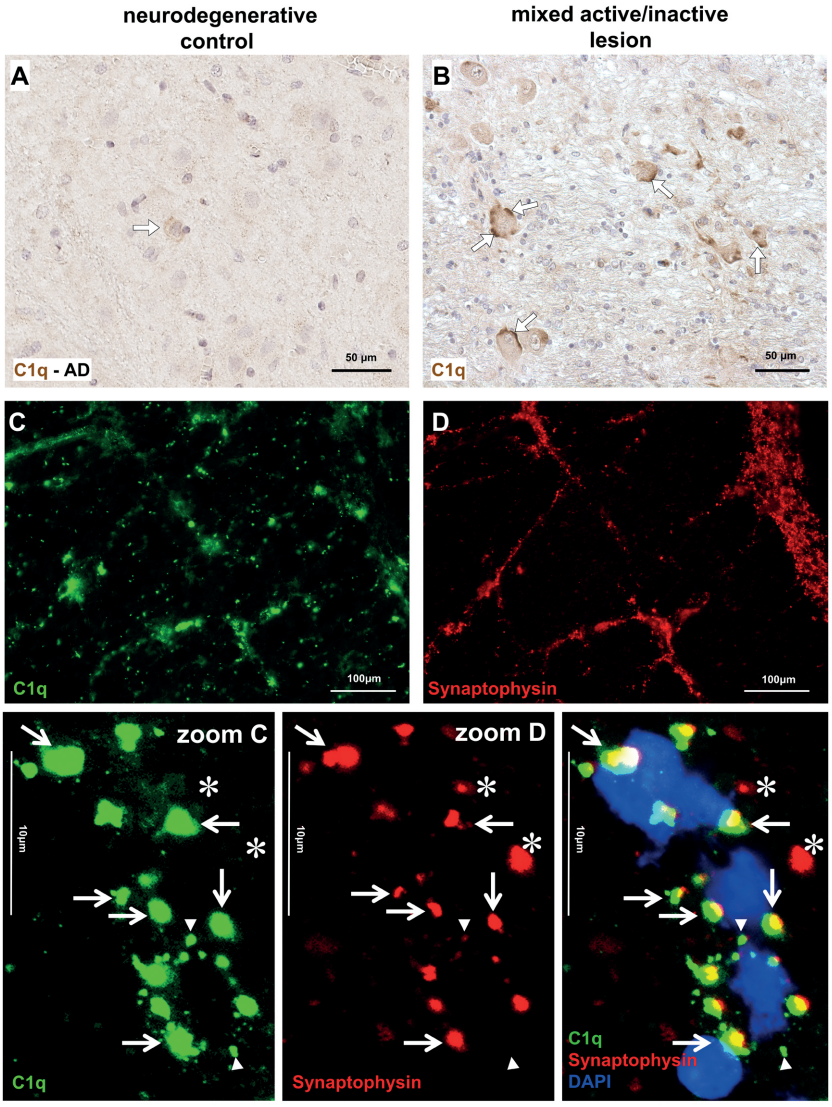
localized at synapses, we performed double immunofluorescent studies using the anti-C1q antibody together with an antibody against synaptophysin to label synapses (**Figure 3C and 3D**). We found punctate C1q deposits that localized at synaptophysin^{+} synapses (**Figure 3 arrows in zoom C and zoom D**). Although we also detected synaptophysin^{+} synapses that where negative for C1q (**Figure 3 asterisk in zoom C and zoom D**). Additional C1q deposits that did not co-localize with synaptophysin^{+} synapses was also noted (**Figure 3 arrows head in zoom C and zoom D**), possibly indicating local production of C1q by neurons or microglia as we previously reported.[56] These data suggest that C1q tags a subset of pre-synaptic terminals in the mixed lesions of the MS brainstem. Since antibodies are known to recruit C1q to the targeted tissue,[13,37] we tested whether immunoglobulins (Ig) deposition could be detected in the MS brainstem. Staining for IgM and IgG (not shown) was negative in all cases except for one MS case where IgM deposits were found in the vicinity of a few neurons at the edge of a mixed lesions (**Suppl. Figure 1A and zoom A**). These data suggest that immunoglobulins are likely not involved in the tagging of synapses by C1q.

Quantitative analysis of C1q deposition on the perikarya of neurons across all lesion types compared to NA brainstem, NNCo and NDCo demonstrated increased deposition of C1q on neurons in the mixed MS brainstem lesions compared to the normal appearing brainstem in MS (normal-appearing vs mixed mean rank: C1q: 11,43 vs 23,46 p=0,04.; by Kruskal-Wallis and Dunn's post-hoc test). (**Figure 3E**)

C3d

C3d is the end-product of the activation of the complement component C3, which is a common factor to all (classical, alternative and lectin) complement pathways. Deposition of C3d in tissue indicates C3 activation with release of opsonins (molecules that enhance the ability of macrophages and neutrophils with complement receptors to phagocytose material – C3b, iC3b, C4b etc.) and anaphylatoxins (peptides that induce local and systemic inflammatory responses, increasing the permeability of blood vessels and attracting neutrophils through their chemotactic properties – C3a, C4a and C5a).[64] While NNCo showed little or no evidence of C3d deposits in the brainstem (not shown), we found C3d deposits on short stretches of nerve fibers in NDCo brainstem (**Figure 4A**) and normal-appearing MS brainstem (**Figure 4B, C**). In some cases the C3d deposits also appeared as enlarged punctate profiles (**Figure 4D**). Double staining for C3d and PLP showed that C3d immunoreactivity localized on axons with disrupted PLP^{+} myelin profiles (**Figure 4E, F**). Double staining for C3d and SMI312 showed that C3d immunoreactivity localized on swollen axons and end-bulbs (**Figure 4G, H**). These data are in line with earlier findings from our group showing that C3 focally tags injured axons in normal-appearing white matter, possibly as part of a physiological mechanism to remove irreversibly damaged axons in chronic disease.[55]

In mixed MS brainstem lesions and brainstem of NDCo, we found in addition C3d deposits on the perikarya of neurons (**Figure 4I**), which in some cases showed morphological evidence of injury (i.e. decentrated nucleus) (**Figure 4J**), and on demyelinated axons (**Figure 4K and L**) with signs of impaired energy metabolism, as shown by the colocalization with mtHSP70 (**Figure 4M and N**). NDCo also showed C3d staining associated with specific neuropathological features of the neurodegenerative disease, including amyloid δ deposits or hyperphosphorylated tau (not



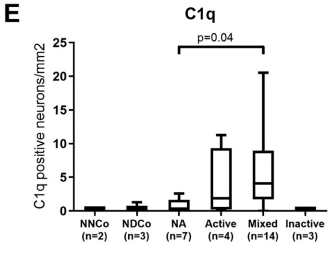


Figure 3. Complement activation products C1q are deposited on synapses in both neurodegenerative disease and multiple sclerosis brainstem lesions.
(A) Representative immunohistochemical staining for the initiator of the classical pathway C1q in neurodegenerative disease, showing positive but faint staining on a neuronal perikarya. (B) Representative C1q immunohistochemical staining in mixed active/inactive lesions showing a dark punctuated staining pattern on neurons, indicated with arrows. (C and D) Representative C1q and synaptophysin confocal images of mixed active/inactive brainstem lesions from progressive multiple sclerosis donors. (Zoom C and D) Staining of C1q localizes at synaptophysin-positive synapses. In zoom C and zoom D, open arrows show synaptophysin-positive synapses also positive for C1q; arrow heads indicate C1q staining on synapses with reduced synaptophysin immunoreactivity; asterisks indicate synaptophysin-positive synapses negative for C1q. DAPI stains nuclei in blue. Isotype-matched antibodies were used as negative controls. E. Quantification of number of C1q positive deposits in non-neurological controls, neurodegenerative disease and MS lesions. Results are expressed as median and interquartile range. Kruskal-Wallis and Dunn's post-hoc test *p*-values are shown.

shown). Quantification analysis showed that the most pronounced density of C3d deposition occurred in NDCo and mixed MS lesions which was significantly increased compared to the normal appearing MS brainstem. (normal-appearing vs mixed mean rank: C3d: 9,80 vs 41,63 p=0.0002.; normal-appearing vs NDCo mean rank: 9,80 vs 50,5 p<0.0001 by Kruskal-Wallis and Dunn's post-hoc test) (**Figure 4O**).

MAC

The membrane attack complex (MAC) is the terminal effector complex of the complement system. It occurs downstream of C3 and C5 convertase activity and forms from the binding of C5b to C6, C7, C8 and a polymer of C9. In lytic levels it forms pores in the phospholipid bilayer of the target cell[64] whereas sublytic levels can activate the NLRP1 inflammasome.[74] While MAC deposits were absent in NNCo (not shown) and were almost undetectable in NDCo (**Figure 5A and zoom A**), active and mixed MS brainstem lesions showed substantial MAC immunoreactivity (**Figure 5B**). In the active white matter part of the MS lesions, MAC positive inclusions were seen inside myelin laden microglia/macrophages, suggesting phagocytosis of MAC-coated myelin debris (**Figure 5 zoom B, white matter**) in line with earlier observations of MAC deposits in myeloid cells within active demyelinating white matter lesions.[12] Notably, in the grey matter part of active or mixed MS brainstem lesions MAC immunoreactivity appeared predominantly on astrocytes, particularly those located adjacent to neurons (**Figure 5 zoom B grey matter, and C-D**). These astrocytes showed no signs of necrosis, indicating resistance to MAC-induced lysis. These observations point to the possibility that MAC is deposited at sub-lytic amount on astrocytes in the grey matter portion of MS active brainstem lesions, suggesting a potential role in activating the inflammasome on the targeted astrocyte. Quantification analysis showed that the most pronounced density of MAC deposits was seen in both active and mixed lesions and this was significantly higher compared to the NDCo. (NDco vs active mean rank: C9neo: 2,50 vs 20,11 p=0.0087.; NDCo vs mixed mean rank: 2,50 vs 22,56 p=0,0015 by Kruskal-Wallis and Dunn's post-hoc test) (**Figure 5E**).

Altogether these findings indicate that in the MS brainstem the extent of complement deposits correlates with lesion activity. In addition, the MS brainstem shares features of early complement activation in common with classic neurodegenerative diseases, suggesting a convergence of neurodegenerative processes, whereas MAC deposition may constitute a specific feature of the inflammatory response with pathogenic significance in MS.

The density of inflammatory infiltrates is highest in active and mixed MS brainstem lesions

Next, we analyzed the localization and density of inflammatory infiltrates in the MS brainstem in relation to lesional activity, and in comparison to NNCo and NDCo brainstem.

T cells

In NNCo and NDCo $CD3^+$ T cells were present and localized around blood-vessels, as expected in virtue of their role in immune surveillance at steady state.[70,71] In the MS brainstem $CD3^+$ T cells were more frequently seen in active and mixed lesions. They infiltrated the parenchyma and were found

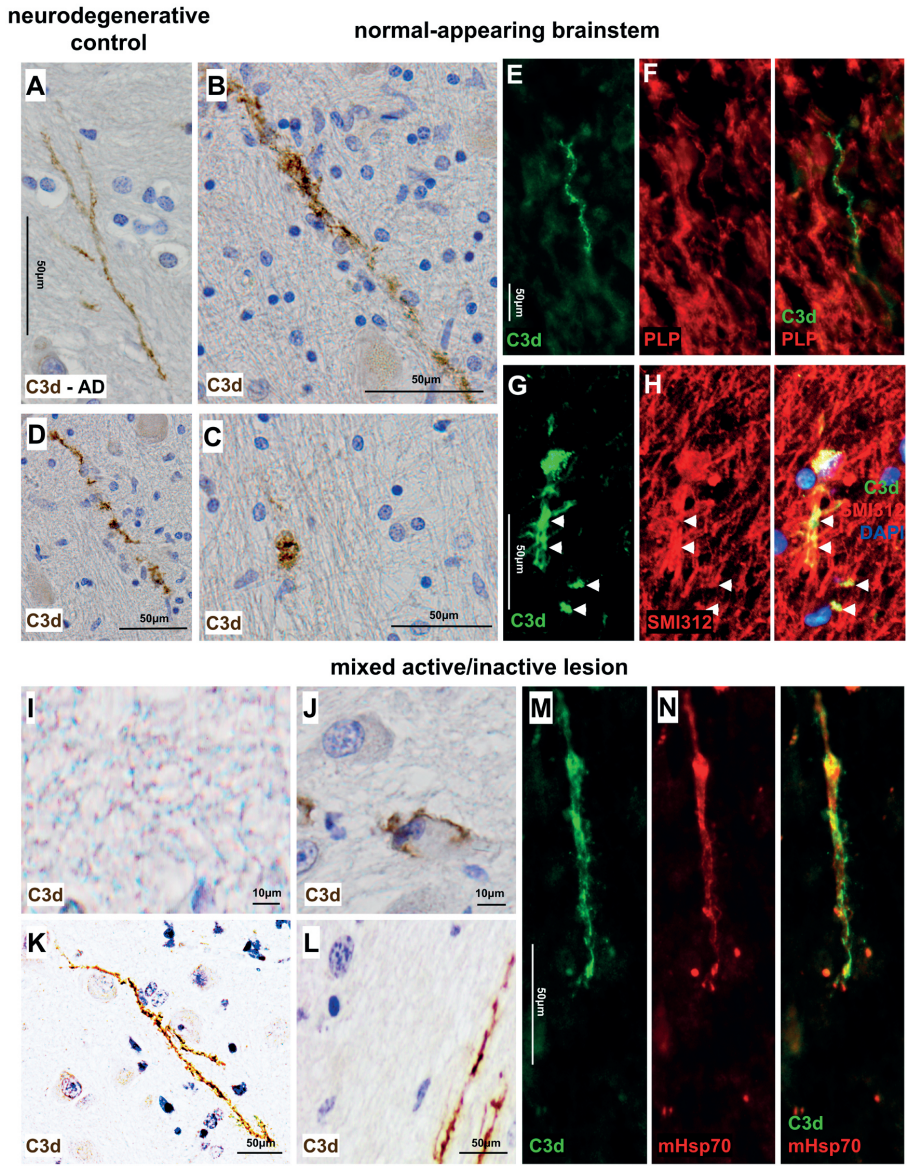


Figure 4. C3d deposits are localized and neuronal perikaryal and axons in both neurodegenerative disease and multiple sclerosis brainstem lesions.

(A-D) In neurodegenerative controls and in normal appearing brainstem C3d, the final activation product of the central component of the complement pathways, C3, localizes stretches of axons. (E-F) C3d localized on myelined axons showing degenerating myelin. (G-H) C3d localized on SMI312 positive axonal bulbs in the normal appearing white matter. In mixed active/inactive lesions C3d is localized on the neuronal perikarya (I-J) and on stretches of axons (K-L). (M-N) The axons deposited with C3d in mixed active/inactive lesions showed impaired energy metabolism since they were positive for mtHSP70. (O) Quantification of C3d in non-neurological controls, neurodegenerative disease and MS brainstem lesions demonstrated both increased C3d deposits in MS lesions and neurodegenerative brainstem. Results are expressed as median and interquartile range. Kruskal-Wallis and Dunn's post-hoc test *p*-values are shown.

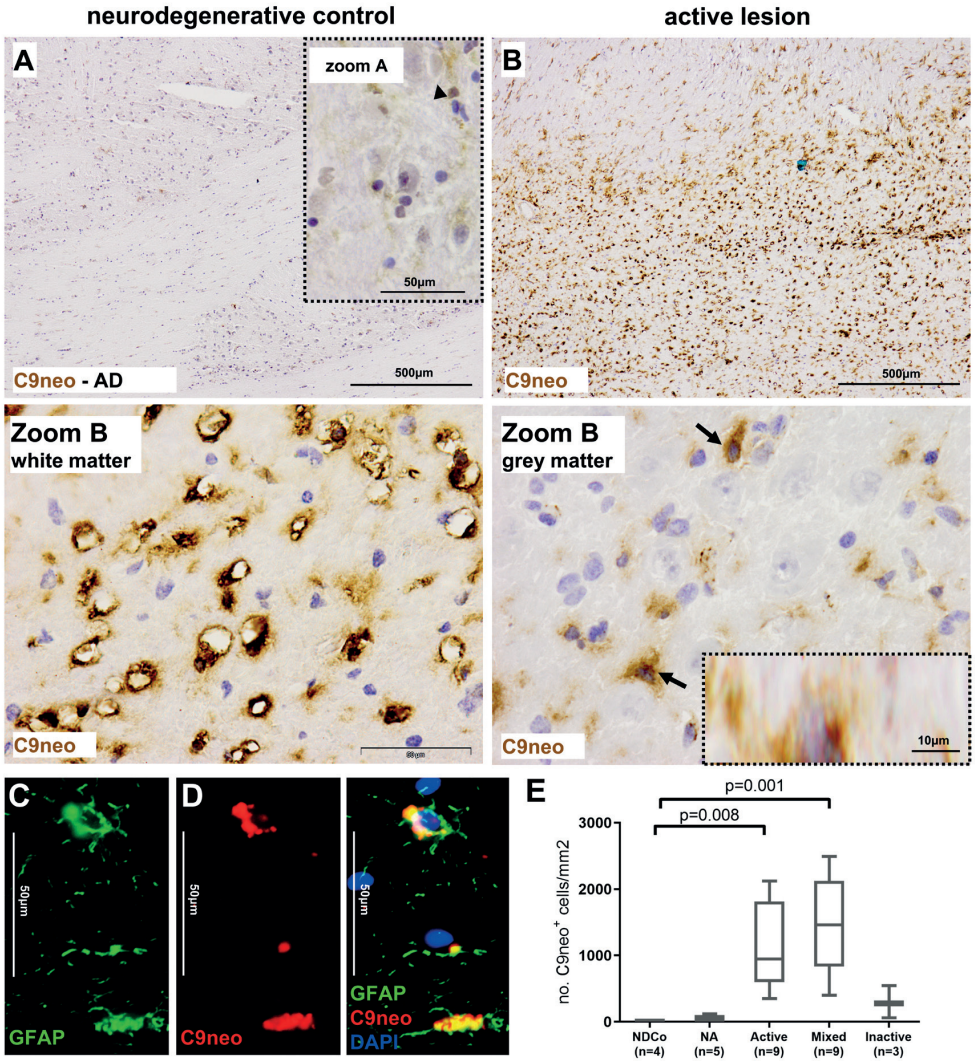


Figure 5. Membrane attack complex, MAC, is specifically deposited in multiple sclerosis brainstem lesions in myelin laden microglia/macrophages and peri-neuronal astrocytes.
(A) Staining for the terminal effector of the complement system, membrane attack complex (MAC, C9neo epitope), shows in neurodegenerative controls a light staining of potentially blood vessels. (B and zoom B white matter) In active MS lesions C9neo deposits are shown inside microglia/macrophages throughout the lesion, indicating phagocytosis of MAC opsonized myelin debris. (Zoom B grey matter) MAC deposits are also localized on astrocytes in close proximity to neurons in the grey-matter part of the lesions (arrows indicate MAC-positive astrocytes). (C and D) Confocal images of MS lesions shows colocalization of GFAP and C9neo. Isotype-matched antibodies were used as negative controls. (E) Quantification of MAC in neurodegenerative disease and MS brainstem lesions shows it is specifically deposited in MS lesions. Results are expressed as median and interquartile range. Kruskal-Wallis and Dunn's post-hoc test *p*-values are shown.

to localize along the active rim and in close proximity to neuronal cell bodies (**Figure 6A-C**) (NDco vs active mean rank: 8,30 vs 38,0 *p*=0.043.; NDco vs mixed mean rank: 8,3 vs 35,5 *p*=0,002; NA vs mixed mean rank: 15,6 vs 35,5 *p*=0,010 by Kruskal-Wallis and Dunn's post-hoc test). Consistent with

prior studies,[3,25,28] further analysis of the $CD4^+$ and $CD8^+$ T cell subtypes revealed that the majority of T cell infiltration in the MS brainstem lesions consisted of $CD8^+$ T cells, with a median CD8:CD4 ratio of ~3:1 in mixed lesions. This ratio was in line with findings in NNCo and NDCo (**Suppl. Figure 2**). The density of $CD8^+$ T cells was highest in active and mixed lesions where they localized at the active rim and in close proximity to neuronal cell bodies (**Figure 6D-F**) (NDco vs mixed mean rank: 15,0 vs 34,1 *p*=0,03; by Kruskal-Wallis and Dunn's post-hoc test). Although in lower numbers, $CD4^+$ T cells were also detected in the MS brainstem, with the highest density in active and mixed lesions. Unlike $CD8^+$ cells, $CD4^+$ cells rarely infiltrated the brainstem parenchyma and localized mostly in close proximity to blood vessels (**Figure 6G-I**) (NDco vs active mean rank: 7,83 vs 37,50 *p*=0.030.; NDco vs mixed mean rank: 7,83 vs 34,32 *p*=0,002; normal-appearing vs mixed mean rank: 14,20 vs 34,32 *p*=0,010 by Kruskal-Wallis and Dunn's post-hoc test).

B cells

$CD20^+$ B cells were not found in the brainstem of NNCo and NDCo, as expected. In the MS brainstem they were found in clusters localized in enlarged perivascular spaces around blood vessels, with the highest density in active and mixed lesions (**Figure 6J-L**) (NDco vs mixed mean rank: 6,0 vs 32,2 *p*=0,001; by Kruskal-Wallis and Dunn's post-hoc test). Of note, the density of $CD20^+$ B cells was highly variable between MS donors, and only 44% (4/9 cases) of the MS cases showed >4/mm^2 $CD20^+$ B cells in the lesions, reflecting the heterogeneity of MS lesion pathology.

Plasma cells

$CD138^+$ plasma cells were not observed in the brainstem of NNCo and NDCo. They were identified only in 22% (2/9) of MS cases analyzed but, when detected, they were found to infiltrate the parenchyma, localizing at the rim of both active and inactive lesions (**Figure 6M-O**).

Altogether these findings indicate that in the MS brainstem the extent of inflammatory infiltrates correlates with lesional activity. In line with previous studies in active and mixed white matter lesions,[26,50] the relative higher abundance of $CD8^+$ T cells and B cells may underline the potential importance of these immune cells in the inflammatory response in established multiple sclerosis lesions.

Inflammation in the MS brainstem correlates with neurodegeneration

To analyze the potential link between inflammation and neurodegeneration in brainstem lesions from progressive MS patients, we performed correlation analyses between the density of inflammatory infiltrate or complement deposits and neurodegenerative changes detected on serial sections from each MS brainstem tissue analyzed.

Spearman correlation and FDR multiple testing correction was performed on the measures for SMI312, APP, mtHSP70, C3d, C1q, CD4, CD8, CD20, CD138, and a correlation plot is shown in **Suppl. Figure 3**. Reduced fast axonal transport as shown by APP immunoreactivity, which was specifically seen in the inflammatory active rim of mixed lesions, positively correlated with the number of

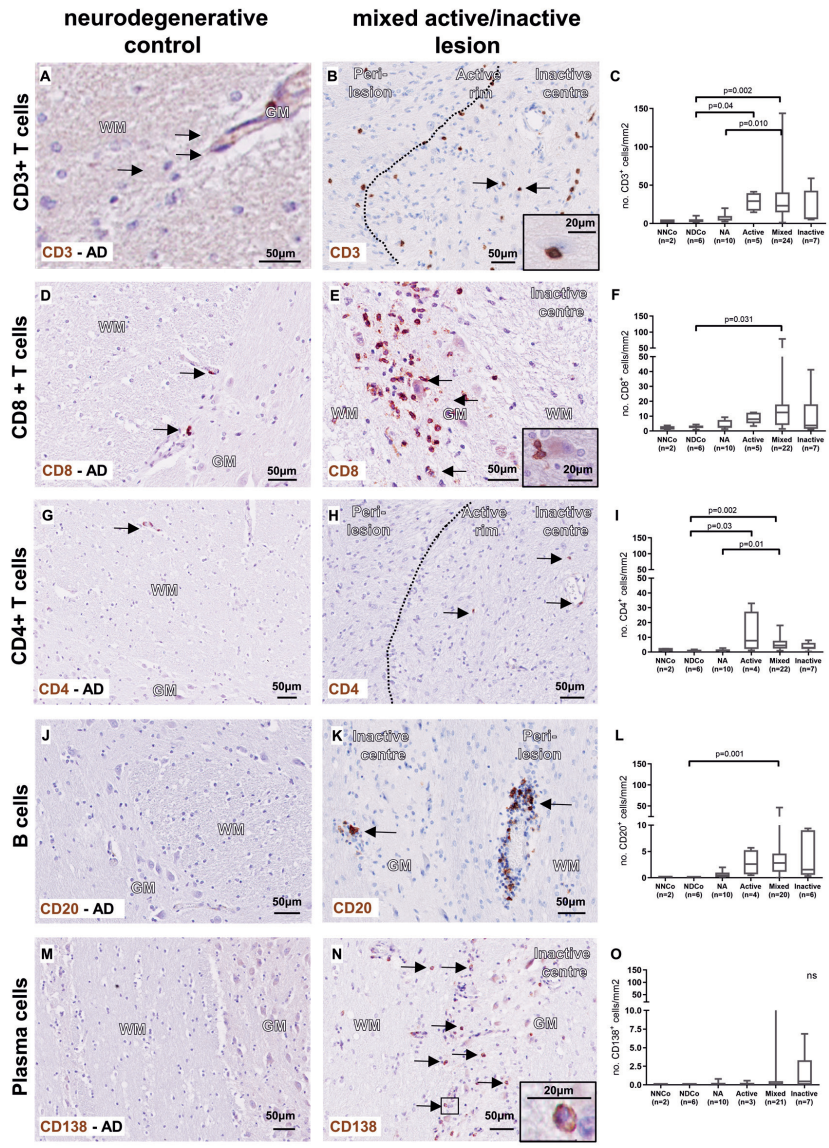


Figure 6. In mixed active/inactive lesions CD8 T cells are found in the brain parenchyma in close contact with neurons and B cells are present in the perivascular space.

(A-C) In active and mixed active/inactive lesions the number of T cells is increased compared to neurodegenerative brainstem and they are located in the brain parenchyma in the inflammatory active lesion rim. (D-F) T cells are mostly $CD8^+$, in both the control, neurodegenerative and MS brainstem. In mixed active/inactive lesions $CD8^+$ T cell number is increased, and in the GM part of MS lesions $CD8^+$ T cells are found in close proximity to neuronal cell bodies. (G-I) The number of $CD4^+$ T cells in the brainstem is lower compared to $CD8^+$, however in both active and mixed active/inactive brainstem lesions the number is increased. $CD4^+$ T cells are less often encountered in the brain parenchyma. (J-L) The number of $CD20^+$ B cells is low and highly variable between MS brain donors, B cell number is increased in mixed active/inactive lesions and they are most often encountered in clusters around blood vessels. (M-O) The number of plasma cells was low and only in 2/9 MS cases they were encountered in the brain parenchyma, the number of plasma cells was not associated with the inflammatory activity of the lesions. Scale bars 50 µm, in the zoom scale bars are 20um.

$CD3^+$ T cells (Spearman's Rho = 0,40, FDR p=0,04) and $CD8^+$ T cells (Spearman's Rho = 0,37 and FDR p=0,05) (**Figure 7A**). Axonal loss as measured by the surface area of SMI312 negatively correlated with the number of C1q deposited neurons (Spearman's Rho = -0,49, FDR p=0,04) and negatively correlated with the number of $CD20^+$ B cells (Spearman's Rho = -0,49, FDR p=0,01). There was a trend for a positive correlation with the number of $CD138^+$ plasma cells (Spearman's Rho = 0,36 and FDR p=0,056) (**Figure 7B**). The extent of C3d deposits positively correlated with the number of $CD8^+$ T cells (Spearman's Rho = 0,36 and FDR p=0,05) and $CD4^+$ T cells (Spearman's Rho = 0,42 and FDR p=0,03) (**Figure7C**).

These data show that in patients with progressive MS injury to neurons, axons and synapses is associated with inflammation. This is different from what we found in the brainstem of NDCo, where signs of metabolic stress in neurons shown by the immunoreactivity for myHSP70 was not associated with the density of T cells, B cells and plasma cells and no deposits of the terminal complement complex MAC were identified within the tissue.

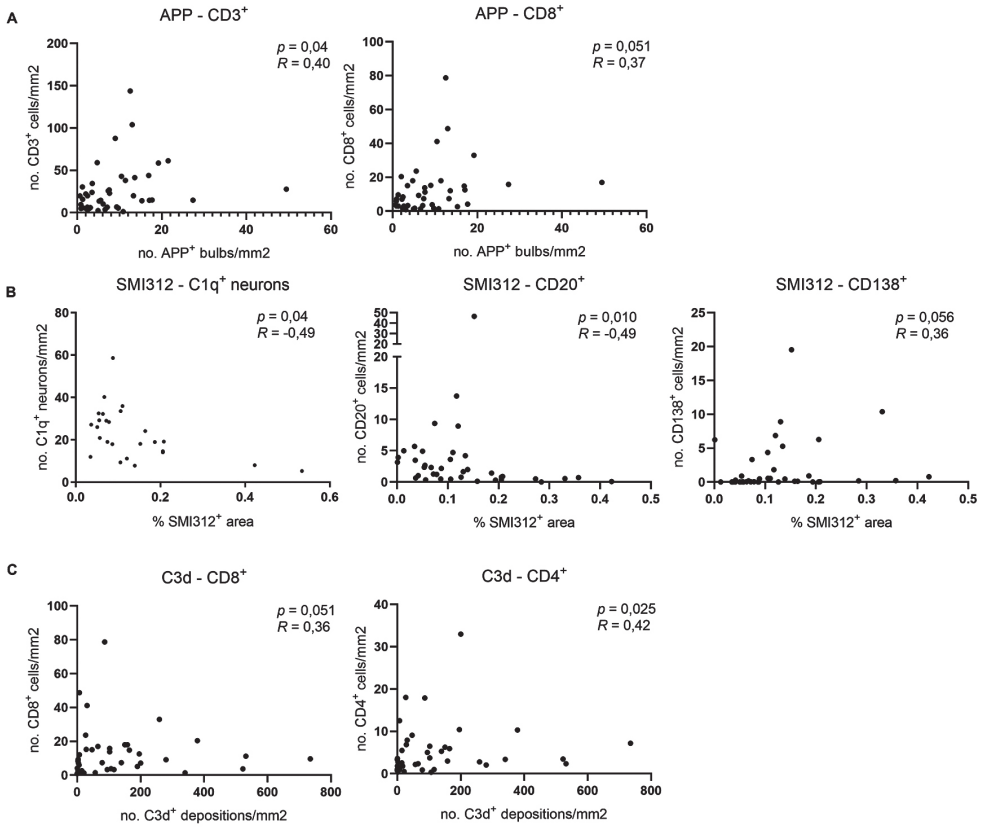


Figure 7. Correlation of reduced fast axonal transport, axonal loss and C3d depositions with lymphocytes in MS brainstem lesions.
(A) The number of $CD3^+$ and $CD8^+$ T cells positively correlated with the number of APP^+ bulbs. (B) The number of $CD20^+$ B cells and $C1q^+$ neurons negatively correlated with the axonal density, while the number of $CD138^+$ plasma cells positively correlated with the axonal density. (C) The number of $CD8^+$ and $CD4^+$ T cells positively correlated with the number of C3d deposits.

DISCUSSION

Here we show that in the brainstem of progressive MS patients neurodegeneration and inflammation are extensive. In addition, we show that the extent of microglia/macrophages activation, complement deposition and lymphocyte infiltration are linked to demyelination and neurodegeneration in this tissue, suggesting a role for inflammation in the pathology of the progressive MS brainstem.

A possible link between inflammation and neurodegeneration in progressive MS has been largely debated, since magnetic resonance imaging (MRI) data show only rare Gadolinium (Gd)-enhancing lesions in patients with advanced and progressive MS, dissociating BBB-leakage (which is the entry venue for serum proteins and peripheral immune cells) from progressive damage of the grey and white matter.[2,7,21,79] In addition, clinical findings have shown that current anti-inflammatory treatments, although effective in modulating peripheral immunity, do not prevent or resolve neurodegenerative changes in the brain (reviewed in[15]). One theory supporting a key role of inflammation in neurodegeneration, while reconciling imaging and clinical findings of progressive MS, proposes that this injury may be driven by a compartmentalized immune response within brains that have a relatively intact BBB. In support, several studies have identified aggregates of lymphoid cells of variable size and organization, referred to as Tertiary Lymphoid Tissues (TLT), in the leptomeninges lining the cortex.[6,67] Importantly, these leptomeningeal TLT are associated with underlying neuronal loss and rapid disease progression,[33,51,52] suggesting that these structures can shape pathological processes into the CNS tissue. Recent studies reported that in active white matter lesions of progressive MS patients a subset of the infiltrating $CD8^+$ T cells with features of tissue-resident memory cells show loss of the surface molecules S1P1 or CCR7, which are involved in the egress of leucocytes from inflamed tissue, possibly reflecting the compartmentalization of the inflammatory response also in the subcortical white matter.[25,50,58,69,70]

Here we show that in the deep grey matter of progressive MS patients, parenchymal inflammation is extensive, further supporting a role for inflammation in the pathology of MS lesions during the progressive phase of the disease. The brainstem of patients with progressive MS displayed a substantial number of inflammatory active and mixed lesions (74% of total lesions analyzed) determined by the abundance, distribution and morphology of microglia/macrophages, classified according to criteria previously described for classical MS white matter lesions.[40] Importantly, foamy macrophages, thought to be involved in ongoing demyelination, were also detected. Our data is in line with results from a larger cohort previously reported by our group,[48] showing that 70% of all brainstem lesions in 182 MS cases analyzed have either active or mixed demyelination. Our data is also in line with reports of active and mixed lesions in another deep grey matter structure, the hypothalamus.[35] Interestingly, the abundance of microglia/macrophages in and around brainstem lesions of progressive MS patients is unlike what is often observed in cortical grey matter lesions of patients with a similar advanced stage of MS. While foamy macrophages

can be found in cortical grey matter lesions of patients with early MS,[47] cortical grey matter demyelination and neuronal injury in patients with progressive MS do not typically associate with microglia/macrophage activation.[9,33]

We also report substantial lymphocytes infiltration within the brainstem parenchyma of progressive MS patients. Similar to what we observed for microglia/macrophages, while T cells have been observed at early MS biopsy in intracortical lesions and to a lesser extent in subpial lesions,[47] lymphocytes are rarely found to infiltrate the subpial parenchyma in cortical grey matter lesions of progressive MS patients.[11,59] Instead, lymphocytes aggregate in the meningeal compartment from where they are thought to contribute to pathology by producing cytotoxic mediators that diffuse across the injured glial limitans into the underlying subpial cortex (reviewed in[1]). The difference in the localization of leukocytes and extent of microglia/macrophage activation in cortical versus deep grey matter lesions of progressive MS patients may reflect tissue-specific differences in the inflammatory response between these two sites. For example, perivascular fibroblastic cells, which are known to remodel during inflammation participating in the scaffolds that supports immune cells in local niches,[60] may exhibit inherent differences in their immune-stimulating potential at postcapillary venules compared to perivascular fibroblastic cells in the meninges.

Specifically, we found an abundance of $CD8^+$ T cells in active lesions and at the rim of mixed lesions suggesting a role in demyelination. We also found few $CD8^+$ T cells in close contact with neurons, which may reflect immunosurveillance or a cytotoxic $CD8^+$ T cell response against neuronal antigens.[57] $CD4^+$ T cells although less abundant were also present at sites of demyelination but localized more perivascularly, in line with previous reports in MS lesions of the white matter.[50] In terms of B cells, the density of $CD20^+$ B cells although variable across donors was generally higher in enlarged perivascular spaces within active and mixed lesions, suggesting that B cells could be involved in antigen presentation[42,49] and reactivation of $CD4^+$ and $CD8^+$ T cells in the brainstem of progressive MS patients.[25,50,58] The density of $CD20^+$ B cells in the brainstem was inversely correlated with the axonal density, suggesting that increased involvement of B cells is related to a decreased number of axons in this tissue. Interestingly, we found a positive correlation between the plasma cell density and the axonal density, suggesting that an increased number of plasma cells relates to more preserved axonal density in progressive MS brainstem lesions. Furthermore, the number of $CD138^+$ plasma cells appeared to be enriched (although not significantly likely due to low power) in inactive lesions compared to normal-appearing tissue or active and mixed lesions. This accumulation of $CD138^+$ plasma cells in the brainstem at a later lesional stage (i.e. inactive lesions) and concomitant with a decrease in $CD20^+$ B cells number compared to more active lesional stages is in line with findings in MS white matter lesions[61] and suggests the existence of a population of long-lived plasma cells, which may accumulate in the CNS during chronic inflammation. The significance of these potentially long-lived plasma cells is currently unknown, but recent studies have shown that they may exert regulatory roles during neuroinflammation.[65]

In addition to lymphocytes, we also report substantial complement activation within the brainstem of progressive MS patients. While classical complement activation is generally linked to antibody-mediated demyelination which occurs in cases of myelin oligodendrocyte glycoprotein (MOG) antibody-associated inflammatory demyelinating disease or neuromyelitis optica,[43] over the past decade several studies have identified early proteins of the complement system as key components of synaptic "pruning" during normal ageing,[72] development,[73] viral infection[77] and neurodegeneration.[32] In line with previous studies from our team showing that in the hippocampus of progressive MS patients C1q/C3 tags synapses for elimination by microglia in the absence of antibodies and terminal MAC activation,[56] we show that C1q (but not antibodies or MAC) deposits at synapses also in the MS brainstem suggesting a C1q-dependent mechanism of synaptic pruning. What recruits C1q at synapses is unknown but a recent study proved that local pruning of dendritic spines is initiated by the mitochondrial production of reactive oxygen species and/or by the activation of the N-methyl-D-aspartate receptor (NMDA) receptors locally in dendrites.[18] Both, mitochondrial oxidative stress[22,54,78] and changes in the glutamate neurotransmitter system[17] have been reported in progressive MS. Therefore it is possible that similar pathways of synapse elimination are at play in the MS brainstem. C1q deposition on neuronal perikarya was significantly increased in the mixed MS brainstem lesions compared to the normal appearing MS brainstem. Furthermore the extend of C1q deposition negatively correlated with the extend of axonal loss suggesting that the deposition of C1q is related to axonal degeneration in chronic MS lesions.

In line with our previous work which identified C3d deposits on short stretches of axons with impaired transport in the periplaque white matter,[55] we found C3d on axons with disrupted myelin profiles, swollen axons and axonal end-bulbs also within the normal-appearing brainstem and brainstem from NDCo. These profiles are also found in advanced stroke lesions,[55] therefore they likely represent a shared mechanisms aimed at removal of irreversibly damaged axons in chronic disease.

The terminal complement pathway, culminating in the formation of the MAC, is undoubtably the most inflammatory of complement axes.[63] Lytic levels of MAC can directly disrupt the membrane integrity of a target cells, which is then engulfed by phagocytes recruited to the site of inflammation by anaphylatoxins. In addition, sublethal amounts of MAC can induce NLRP3 inflammasome activation on the target cell, resulting in the secretion of pro-inflammatory cytokines.[44,74] Our finding of MAC deposits within foamy macrophages in the white matter portion of active brainstem lesions and on the surface of astrocyte on the grey matter portion of the active lesions is in line with these functions. In summary, while we show that early complement activation components (C1q and C3d) are a common feature between MS and classic neurodegenerative diseases, suggesting a convergence of neurodegenerative processes, deposition of the terminal complement component MAC may constitute a specific feature of the inflammatory response with pathogenic significance in progressive MS.

Altogether, while our work does not answer the question of causality or direction of causality, it does establish a link between an innate and adaptive immune responses, demyelination and neurodegeneration in the brainstem of progressive MS patients. Understanding the relation between inflammation, demyelination and neurodegeneration is critical to guide future therapeutic strategies aimed at slowing deterioration of clinical symptoms related to this region in progressive MS patients.

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Disclosures

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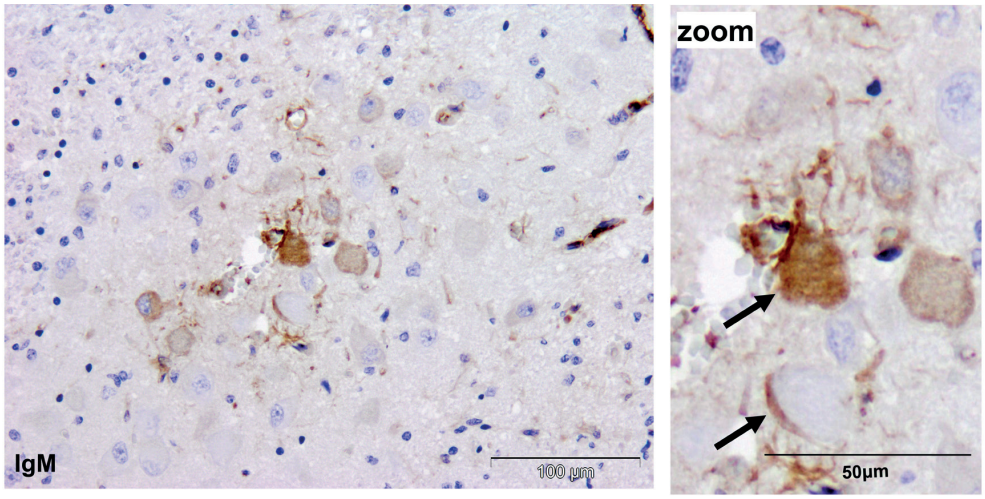
SUPPLEMENTARY MATERIAL

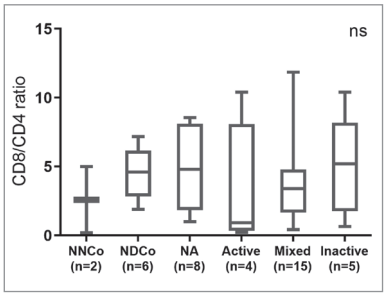
Supplementary Table 1. Donor information per autopsy case.

Donor	Tissue block	Fixation	Diagnosis	Age (years)	Sex	Disease duration (years)	Disease course	Brain weight (g)	pH	Pmd (h:m)	Cause of death
1	BRS-E	FFPE/FF	MS	44	M	16	PP	1383	6,06	12:00	Pneumonia with aspiration
2	BRS-A2	FFPE/FF	MS	57	M	29	SP	1295	6,5		Euthanasia
3	BRS-C and A	FFPE	MS	61	M	31	SP	1225	6,88	9:15	Euthanasia
4	BRS-F	FFPE/FF	MS	75	M	55	SP	1342	6,53	7:45	Euthanasia
5	BRS-D	FFPE	MS	55	M	32	SP	1125	6,7	6:20	Respiratory insufficiency by pneumonia and urospesis
6	BRS-F	FFPE	MS	56	M	21	SP	1390	6,65	8:00	Pneumonia
7	BRS-C1	FFPE/FF	MS	50	M	24	PP	1292	6,2		Unknown
8	BRS-B	FFPE/FF	MS	47	M	7	SP	1228	6,2	7:15	Urosepsis with organ failure
9	BRS-B links	FFPE	MS	77	F	29	PP	1060	6,5	10:00	Euthanasia
10	MOB	FFPE	NNC	61	F			1452	6,5	6:50	Euthanasia
11	MOB	FFPE	NNC	62	M			1545	6,36	7:20	Unknown
12	MOB	FF	NNC	32	M			1175	NA		E.C.I.
13	lc/pons	FFPE/FF	AD	57	F			1013	6,49	4:05	dehydration
14	lc/pons	FFPE/FF	FTD	63	F			890	5,98	4:00	Gastrointestinal bleeding
15	col-inf/ pons	FFPE/FF	AD	54	F			1027	6,37	6:35	Exhaustion and cachexia
16	col-inf	FFPE/FF	FTD	61	F			1148	6,18	6:15	Lung disorder with fever
17	col-inf	FFPE	AD	60	F			899	6,25	5:10	Cachexia
18	lc/pons	FFPE	ALS	64	F			1076	6,23	9:05	Cardiopulmonal decompensation

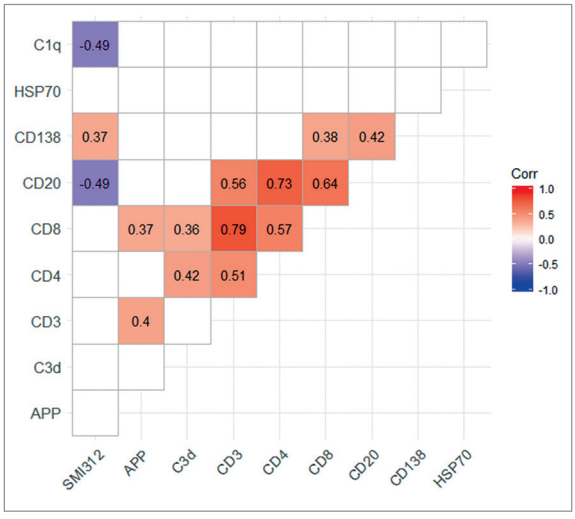
ALS, Amyotrofic Lateral Sclerosis; FTD, Fronto-temporal dementia;AD, Alzheimers disease;
NNC, non-neurological control; F, female; M, male; PMD, post-mortem delay; PP, primary progressive;
SP, secondary progressive.



Supplementary figure 1. Immunoglobulin staining in multiple sclerosis brainstem.
Sections from the multiple sclerosis donor stained for immunoglobulin M (IgM), showing deposition on the perikarya of neurons near a mixed active/inactive lesions. Hematoxylin stains nuclei blue. Scale bars in the low-magnification images, 100µm. Scale bars in the high magnification, 50µm.



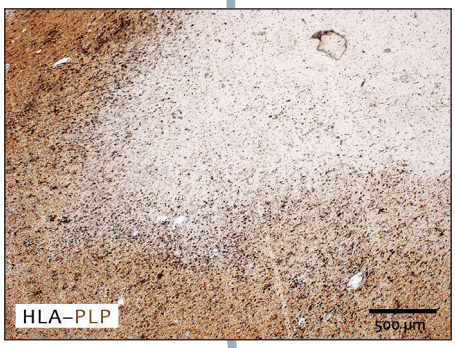
Supplementary figure 2. CD8/CD4 T cell ratio in MS lesions.



Supplementary figure 3. Spearman correlation coefficients for the significant correlations after FDR multiple testing correction.

CHAPTER 3

Progressive multiple sclerosis patients show substantial lesion activity that correlates with clinical disease severity and sex: a retrospective autopsy cohort analysis



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ABSTRACT

Multiple sclerosis (MS) is a highly heterogeneous disease with large inter-individual differences in disease course. MS lesion pathology shows considerable heterogeneity in localization, cellular content and degree of demyelination between patients. In this study, we investigated pathological correlates of disease course in MS using the autopsy cohort of the Netherlands Brain Bank (NBB), containing 182 MS brain donors. Using a standardized autopsy procedure including systematic dissection from standard locations, 3188 tissue blocks containing 7562 MS lesions were dissected. Unbiased measurements of lesion load were made using the tissue from standard locations. Lesion demyelinating and innate inflammatory activity were visualized by immunohistochemistry for proteolipid protein and human leukocyte antigen. Lesions were classified into active, mixed active/inactive (also known as chronic active), inactive or remyelinated, while microglia/macrophage morphology was classified as ramified, amoeboid or foamy. The severity score was calculated from the time from first symptoms to EDSS-6. Lesion type prevalence and microglia/macrophage morphology were analyzed in relation to clinical course, disease severity, lesion load and sex, and in relation to each other. This analysis shows for the first time that (1) in progressive MS, with a mean disease duration of 28.6 ± 13.3 years (mean ± SD), there is substantial inflammatory lesion activity at time to death. 57% of all lesions were either active or mixed active/inactive and 78% of all patients had a mixed active/inactive lesion present; (2) patients that had a more severe disease course show a higher proportion of mixed active/inactive lesions (p=6e-06) and a higher lesion load (p=2e-04) at the time of death, (3) patients with a progressive disease course show a higher lesion load (p=0.001), and a lower proportion of remyelinated lesions (p=0.03) compared to patients with a relapsing disease course, (4) males have a higher incidence of cortical grey matter lesions (p=0.027) and a higher proportion of mixed active/inactive lesions compared to females across the whole cohort (p=0.007). We confirm that there is a higher proportion of mixed active/inactive lesions (p=0.006) in progressive MS compared to relapsing disease. Identification of mixed active/inactive lesions on MRI is necessary to determine whether they can be used as a prognostic tool in living MS patients.

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system, which is characterized by demyelination, inflammation and neuroaxonal damage.[27,39] It is a heterogeneous disease with large inter-individual differences in clinical presentation,[32] radiological appearance of the lesions[17] and response to immunomodulatory therapy[18] as well as sex differences.[23]

In line with the broad clinical spectrum of MS, the pathological manifestation of the disease is also highly heterogeneous between patients and has been at the center of investigation for decades.[12,13,33,36] In biopsy tissue from patients with MS, four immunopathological patterns of MS lesions have been identified based on the presence of immunoglobulin deposits, active complement or evidence of primary oligodendrocyte pathology.[33,36] However, in a post-mortem cohort of chronic MS patients, white matter lesions showed a homogenous pattern of antibody and complement mediated demyelination.[6] Although lesion activity in the chronic late phase of MS does not show the four different patterns, large inter-individual differences in inflammatory and demyelinating activity, meningeal inflammation, degree of axonal loss, and remyelination are still observed in post-mortem MS cohort studies.[9,13,35,38] The pathological and clinical heterogeneity of MS suggest that different pathogenic mechanisms may be at work, with the significant implication that MS patients may require individualized therapeutic approaches. However, pathological correlates for clinical disease course, disease severity and sex-differences in MS are currently not well explored.[13] This is probably due to the fact that world-wide, the availability of clinically and pathologically well characterized MS brain tissue collections of sufficient power are limited.

In this study, we analyzed the heterogeneity in MS lesion activity in relation to clinical disease course, disease severity and sex in the MS autopsy cohort of the Netherlands Brain Bank (NBB), consisting of 182 clinically well-documented MS brain donors. In total, 3188 tissue blocks were analyzed containing 7562 lesions. Based on previously proposed criteria, MS lesions in white and cortical grey matter were categorized according to location, degree of demyelination, and innate inflammatory activity as determined by microglia/macrophage presence and morphology.[25,29,44] Our data show for the first time that demyelinating and innate inflammatory lesion activity is substantial at time of death in progressive MS patients with long-standing disease and that clinical disease course, disease severity and sex strongly correlate with MS lesion characteristics in autopsy tissue.

MATERIAL AND METHODS

Subjects

The cohort comprises 182 MS donors that came to autopsy between 1990 and 2015 in the framework of the NBB. Informed consent was given by the donors for brain autopsy and for the use of material and clinical data for research purposes, in compliance with national ethical guidelines.

MS pathology was confirmed by a certified neuropathologist (Prof. J.M. Rozemuller or Prof. P. van der Valk, VUmc, Amsterdam, The Netherlands). The clinical diagnosis of MS was confirmed for all patients, and the clinical course was defined as relapsing (8 relapsing remitting and 6 progressive relapsing patients), secondary progressive (SP) or primary progressive (PP) by a neurologist (Prof C.H. Polman, VU Medical Center, Amsterdam or Dr. S. Luchetti, Netherlands Institute for Neuroscience, Amsterdam) according to McDonald or Poser criteria. Clinical course was not determined in 12 patients. Patients with clinical or pathological features of acute disseminated encephalomyelitis or neuromyelitis optica or confounding CNS pathologies such as large ischemic, hemorrhagic lesions or cerebral metastasis were excluded. Disability status was determined by retrospective chart analysis using Kurtzke's Expanded Disability Status Scale (EDSS) and the time from first symptoms to EDSS-6 and EDSS-8 was determined. In 14 patients information about the time to EDSS-6 was unavailable. The clinical characteristics of the MS cohort of the NBB are summarized in **Table 1**. No significant differences were found in age at death, post-mortem delay, post-mortem pH and year of autopsy between sexes or disease course types.

Tissue dissection

The NBB autopsy procedures were approved by the Ethical Committee of the VU University Medical Center in Amsterdam, The Netherlands. Blocks were dissected at seven standardized locations from the brainstem (BRS) and eight standardized locations from the spinal cord (SPC), two cervical, two thoracic, two lumbar, two sacral, from 161 and 120 patients, respectively. Visible MS plaques (PLA) were dissected during autopsy from 150 patients. In addition, since 2001 (116 patients), MS lesions were also dissected on post-mortem MRI guidance (MRI) on 1 cm thick coronal brain slices cut throughout the brain.[15] Tissue dissection characteristics are shown in **Suppl. Table 1**. Similar numbers of tissue blocks (18 ± 11 per patient, from which 12 ± 6.9 were from supratentorial locations) were characterized in all clinical patient groups.

Characterization of MS lesions

For each patient, all available archived material was analyzed. Double immunostaining was performed on sections from all dissected tissue blocks to visualize proteolipid protein (PLP) (MCA839G, AbD Serotec, Oxford, UK, with DAB) and human leukocyte antigen (HLA-DR-DQ, referred to as HLA) (M0775, CR3/43, DAKO, Denmark, with DAB-nickel), as previously described.[14,19,20,34] White matter, cortical grey matter and spinal cord MS lesions were characterized according to the system of Van der Valk et al. and Kuhlmann et al.[25,44] Innate inflammatory lesion activity was determined as presence of HLA^+ microglia/macrophages. We used microglia/macrophage morphology to quantify phagocytosing activity since amoeboid and foamy microglia/macrophages are thought to be actively phagocytosing myelin.[5,29]

Reactive sites and four types of white matter lesions were discriminated based on demyelination and presence of HLA^+ microglia/macrophages, while cortical grey matter lesions, identified as demyelinated areas, were classified by location.[4] The criteria for both are given in **Table 2** and

Table 1. Clinical characteristics of multiple sclerosis patients.

	Total	Female	Male	PP	SP	Relapsing
Number patients [a]	182	113	69	56 (F33/M23)	100 (F61/M39)	14 (F10/M4)
Duration of disease, years (mean ± SD)	28.6 ± 13.3	29.2 ± 13.5	27.5 ± 12.8	27.6 ± 11.7	29.9 ± 14.2	24.2 ± 11.6
Time to EDSS-6, years (mean ± SD)	16.3 ± 11.7	16.9 ± 11.8	15.2 ± 11.7	14.0 ± 10.2	17.9 ± 12.7	11.8 ± 4.9
Age at death, years (mean ± SD)	62.0 ± 15.4	63.6 ± 15.6	59.5 ± 14.9	65.3 ± 13.2	59.8 ± 16.5	60.8 ± 15.2
Post-mortem delay, hours (mean ± SD)	9.3 ± 6.9	9.1 ± 6.8	9.7 ± 7.1	8.7 ± 4.4	8.9 ± 5.4	11.6 ± 13.7
Year of autopsy (mean ± range)	2005 (1990-2015)	2004 (1990-2015)	2005 (1991-2015)	2005 (1993-2015)	2005 (1990-2015)	2007 (1997-2012)
Number of tissue blocks dissected per donor (mean ± SD)	18 ± 11	17 ± 11	19 ± 10	18 ± 9.9	18 ± 11	19 ± 9.6
Number of supra-tentorial tissue blocks dissected per donor (mean ± SD)	12 ± 6.9	11 ± 7.4	12 ± 6.2	11 ± 6.6	12 ± 7.2	11 ± 6.4
Cause of death (n)						
Euthanasia	39					
Respiratory failure/ pneumonia	58					
Cardiovascular failure	16					
Sepsis	13					
Cachexia	9					
Suicide	2					
Other [b]	20					
Not reported	25					

PP, primary progressive; SP, secondary progressive; Relapsing; F, female; M, male.
[a] Clinical course was not available for 12 patients; [b] i.e. dehydration, ileus, gastrointestinal bleeding, liver-insufficiency, multi-organ failure, surgery complication, lung embolism.

Figure 9. For active and mixed active/inactive lesions, microglia/macrophage morphology was scored: 0 = thin and ramified; 0.5 = amoeboid with few ramification; 1 = foamy. The microglial/ macrophage activation score (MMAS) was calculated as the average of these scores for each patient.

In previous studies[13] active lesions were further divided into early and late active lesions using the presence of minor myelin proteins within microglia/macrophages. To provide more insight in

Table 2. Definition of lesion scoring system and overall occurrence.

Lesion type	Definition	Score	No. lesions	% Of total lesions	% Patients with lesion type present
Reactive site	No demyelination, aggregates of HLA$^+$ microglia/macrophages	1	770		64,8 (118/182)
White matter					
Active	Demyelination, HLA$^+$ microglia/macrophages throughout the lesion	2	1357	23,8	70,9 (129/182)
Mixed active/inactive (same as chronic active in Kuhlmann, Lassmann, & Brück 2008,[24] includes smoldering in Frischer et.al. 2015[13])	Demyelination, hypocellular and gliotic centre, accumulation of HLA$^+$ microglia/macrophages at the lesion border	3	1873	32,8	77,5 (141/182)
Inactive	Demyelination, hypocellular and gliotic throughout the lesion	4	1561	27,3	86,8 (158/182)
Remyelinated	Partial myelination, sparse HLA$^+$ microglia/ macrophages	6	919	16,1	67,6 (123/182)
Total		2/3/4/6	5710	100	96,7 (176/182)
Cortical grey matter		5			
Leukocortical (mixed grey-white matter)		I	570	31,3	65,0 (104/160)
Intracortical		II	782	42,2	68,1 (109/160)
Subpial		III	457	24,7	43,1 (69/160)
Subpial extending up to the white matter border		IV	43	2,3	10,6 (17/160)
Total		I/II/III/IV	1852	100	79,4 (127/160)

the presence of myelin oligodendrocyte glycoprotein (MOG) in microglia/macrophages in active lesions, co-localization of IBA-1 and MOG is measured in a subset of active (n=5) lesions dissected at a standard location at the level of the Medulla Oblongata. Double sequential immunostaining of MOG (1:200 Abcam, EP4281) with AEC (red), followed by antigen retrieval and then IBA-1 (1:3000 WAKO cat.no. 019-19741) with NBT-BCIP (blue) was performed including controls showing no MOG staining after antigen retrieval. Co-localization was measured using spectral microscopy,[30] shown in **Suppl. Figure 2**. Examples of this classification system and lesion scoring are shown in **Figure 1**, **Figure 9** and **Suppl. Figure 1**.

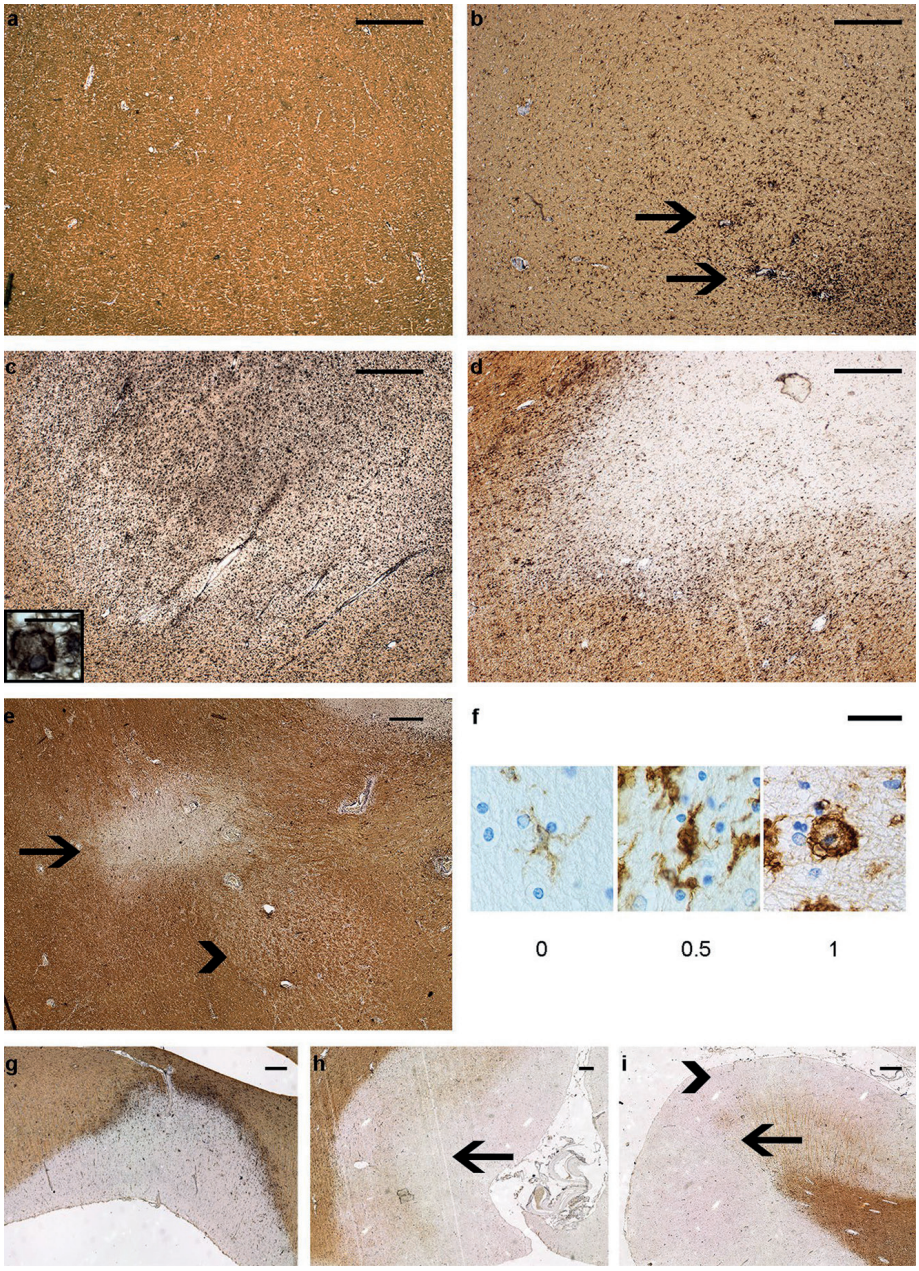

Figure 1. Scoring MS lesion subtypes.
Double immunostaining for human leukocyte antigen (HLA, in black), detecting microglia/macrophages, and proteolipid protein (PLP, in brown), detecting myelin on tissue samples from MS patients. a-e: white matter lesions. a: Normal-appearing white matter (NAWM). b: Reactive site. c: Active lesion, with foamy microglia/macrophages. d: Mixed active/inactive (chronic active) lesion with rounded microglia/macrophages. e: Inactive lesion (arrow) and inactive remyelinated lesion (arrowhead). f: Microglia/macrophage morphology score used for all active (2) and mixed active/inactive (3) lesions. 0: Ramified 0.5: Amoeboid 1: Foamy. g-i: cortical grey matter lesions g: leukocortical lesion (I) h: intracortical lesion (II, arrow). i: subpial lesions (III, arrowhead and IV arrow). Scale bar a-e and g-i: 0.5mm. Scale bar f: 0.025 mm. Scale bar inset in c: 0.020 mm.

Lesion load and proportions of lesion subtype calculations

For each tissue block dissected, all visibly distinct lesions were classified separately as shown in **Suppl. Figure 1**. To avoid sampling bias that may arise when identifying lesions by MRI or macroscopically, lesion load was determined by counting MS lesions in brainstem tissue blocks, as these were dissected at standard locations. One section per block was used and all lesions in the section that were visibly distinct were counted separately. Reactive sites were excluded since it remains unclear whether these represent the initial stage of an MS lesion.[22] Reactive site load was calculated separately as counts of reactive sites present in the brainstem blocks. Where lesions appeared to contain mixed features (e.g. mixed microglial/macrophage morphology) the most preponderant type was recorded. Cortical grey matter lesions were scored as either present or absent, cortical tissue blocks were available for 160 donors. The relative proportions of lesion subtypes were calculated from all lesions scored in both standard locations (BRS and SPC) and PLA and MRI tissue blocks. The number of patients with specific block types available are shown in **Suppl. Table 1**. The calculation of the proportions of lesion subtypes is defined in **Table 3**.

Table 3. Definition of pathological parameters used for analysis.

	Definition	Tissue blocks used for calculations
Lesion load	Counts of all active, mixed active/inactive, inactive and remyelinated lesions	Standardly dissected brainstem tissue blocks (BRS)
Reactive load	Counts of all reactive sites	Standardly dissected brainstem tissue blocks (BRS)
Cortical grey matter lesion presence	Yes/No present	In all supratentorial blocks (PLA+MRI) with cortex
Proportion of active lesions	Active/(active + mixed active/inactive + inactive + remyelinated)	From all tissue blocks dissected
Proportion of mixed active/inactive (chronic active) lesions	Chronic active/(active + mixed active/inactive + inactive + remyelinated)	From all tissue blocks dissected from a donor
Proportion remyelinated lesions	Remyelinated/(inactive + remyelinated)	From all tissue blocks dissected from a donor
Microglia/macrophage activity score (for active and mixed active/inactive lesions)	Average for each patient Ramified = 0 Amoeboid = 0.5 Foamy = 1	From all tissue blocks dissected from a donor

Statistical analysis

Disease severity score was calculated as 5- log (years to EDSS-6 +1), giving a score between 1 (least severe) and 5 (most severe). Lesion load and reactive site load were transformed as log(x+1). Correlations between severity score, lesion load, and proportions of lesion subtypes were tested using Pearson's product moment correlation coefficient. Differences in severity scores, lesion load, reactive site load and lesion subtype proportions were tested against sex, presence of mixed

active/inactive lesions and presence of cortical grey matter lesions using t-test's with unequal variance (n of each group was >30 in these tests). Differences in severity score, lesion load and reactive site load were tested against MS types using Kruskal-Wallis and Wilcoxon tests (non-parametric tests were used here because the relapsing group contained < 25 patients). Proportions of lesion subtypes and the MMAS were tested against MS clinical types and against location using quasibinomial generalized linear models (GLM) and Tukey post-hoc tests. Associations among sex, MS subtypes and presence/absence of cortical grey matter lesions were tested using Fisher's exact test. Correlations with severity score, lesion load and differences between MS subtypes were also tested with linear models including sex as a factor, which showed that sex does not influence these outcomes. All analyses were carried out in the statistical computing environment R.

RESULTS

The MS cohort of the Netherlands Brain Bank consists mainly of patients with (primary or secondary) progressive disease

In 182 MS patients that came to autopsy at the NBB 3188 tissue blocks are dissected and characterized containing 7562 lesions. The clinical disease course of this cohort consists of relapsing (8%), PP (31%) and SP (55%) patients with mean disease duration of 24.2 ± 11.6 years, 27.6 ± 11.7 years and 29.9 ± 14.2 years, respectively and the overall mean disease duration of 28.6 ± 13.3 years (range 2-64 years), indicating that this cohort consists mainly of chronic progressive MS patients (**Table 1**).

MS lesion activity is substantial at time of death in long-term progressive disease

Notably, in the whole NBB cohort, fifty seven percent (57%) of demyelinated white matter and mixed grey-white matter lesions were either active or mixed active/inactive (chronic active), showing that there is considerable inflammatory lesion activity even in autopsy patients with long-term progressive disease (**Table 2**). Furthermore, even in patients with the longest disease duration (42-64yrs), active or mixed active/inactive lesions account for 34% of all lesions, further supporting the notion that lesion activity is substantial in the progressive late phase of the disease. Proportions of lesion subtypes versus years to EDSS-6, years to EDSS-8 and disease duration are shown in **Suppl. Figure 3**.

Lesion load and proportion of mixed active/inactive lesions positively correlates with disease severity

Patients with more severe disease (shorter time to EDSS-6) had a higher lesion load (p=2e-04, R=0.31), and a higher proportion of mixed active/inactive lesions (p=6e-06, R=0.35) (**Figure 2**). No significant correlation was found between the disease severity score and the proportions of active lesions (p=0.17, R=0.11) or remyelinated lesions (p=0.28, R=–0.087, note a single outlier causes a non-significant increase in remyelinated lesions in the most severe patients), or with reactive site load (p=0.10, R=0.14), or microglia/macrophage activity score (p=0.10, R=0.14) (**Figure 2**).

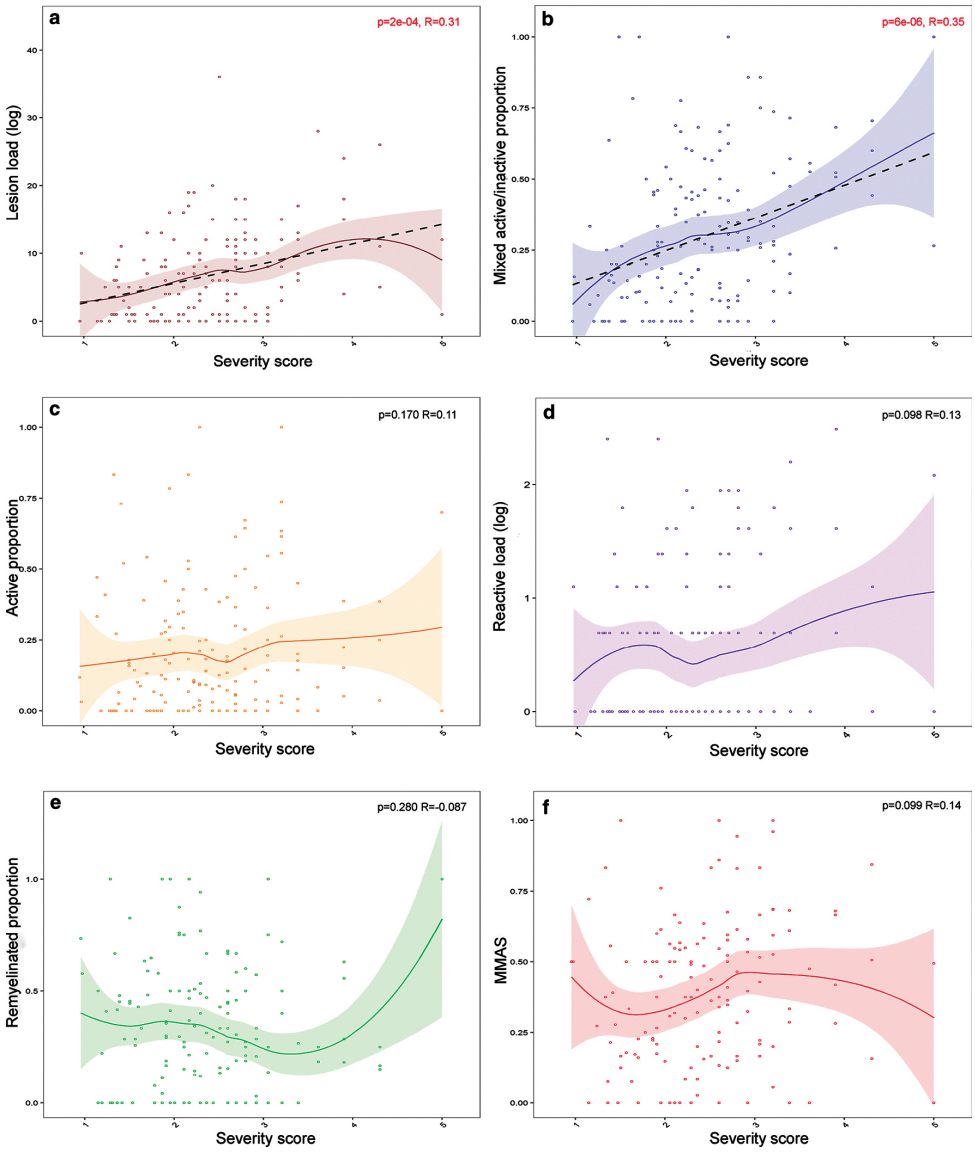

Figure 2. Relationship between lesion activity and clinical disease severity.
Patients with a more severe disease course have a higher lesion load (a) and a higher proportion of mixed active/inactive (chronic active) lesions (b) than patients with a less severe disease course. The disease severity did not correlate with the proportion of active lesions (c), the reactive load (d), the proportion of remyelinated lesions (e) or the MMAS (f). Severity score is calculated as 5 – log (years to EDSS-6 +1), with shorter time to EDSS-6 the patient has a higher severity score. The relationship between each parameter and severity score is shown as a loess-smoothed fit with 95% confidence intervals. Where a significant correlation was found, the straight-line fit is shown (black dotted line). Pearson's product moment correlation coefficient was used.

In 22.5% of MS patients no mixed active/inactive lesions were observed, and these patients had a less severe disease course (p=0.001) and lower lesion load (p= 1.5E-13) compared to patients where mixed active/inactive lesions were present. (**Table 2** and **Figure 3**).

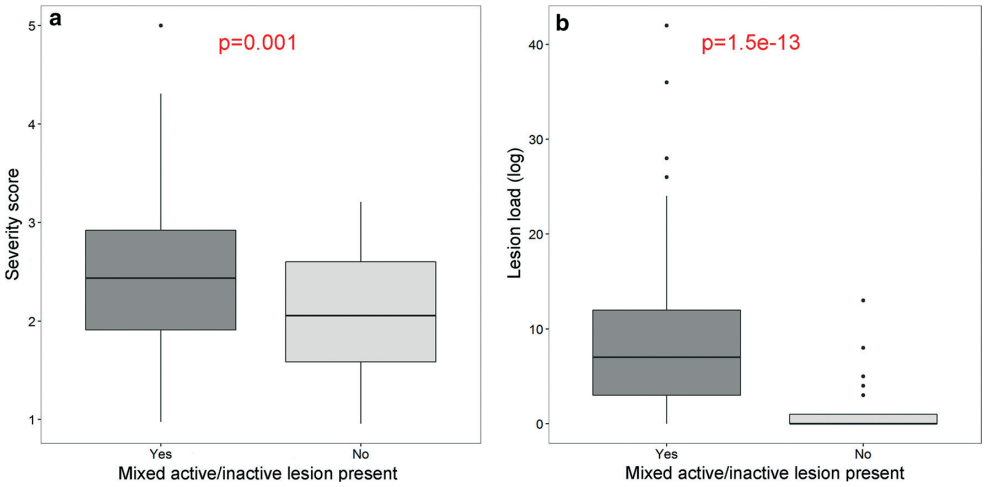

Figure 3. Mixed active/inactive lesion presence relates to severity and lesion load.
Patients with a mixed active/inactive (chronic active) lesion present (n=141) have a more severe disease course (a) and a higher lesion load (b) compared to patients without a mixed active/inactive lesion present (n=41). t-test with unequal variances was used.

In **Suppl. Figure 3** we show all lesion type proportions versus years to EDSS-6, years to EDSS-8 and disease duration. Regardless of the measure of disease severity, patients with more severe disease (shorter time to EDSS-6 or EDSS-8 or shorter disease duration) had relatively more mixed active/inactive lesions, a higher proportion of lesions with foamy microglia/macrophages, and relatively fewer remyelinated lesions. The proportion of active lesions is a constant low percentage (range 16-27 %) in all disease severity and disease duration groups.

Cortical grey matter lesions were observed in 79.4% of patients (**Table 2**) and disease severity did not differ significantly between patients with or without cortical grey matter lesions (p=0.53).

SP and PP patients have a higher lesion load with a higher proportion of mixed active/inactive and lower proportion of remyelinated lesions compared to relapsing patients

SP (n=93) and PP (n=52) patients did not differ significantly in lesion load, reactive site load or in proportions of lesion subtypes (**Figure 4**). However, compared to relapsing (n=14) patients, both SP and PP patients showed a higher lesion load (Kruskal-Wallis; p=0.011, Wilcoxon: SP vs PP p=0.29 SP vs relapsing p=0.004, PP vs relapsing p=0.023), a higher proportion of mixed active/inactive lesions (GLM p=0.006, Tukey post hoc tests SP vs PP p=0.98, SP vs relapsing p=0.01, PP vs relapsing

p=0.01) and PP patients had a lower proportion of remyelinated lesions (GLM *p*=0.03, Tukey post hoc tests SP vs PP *p*=0.28, SP vs relapsing *p*=0.16, PP vs relapsing *p*=0.02). No significant differences were found in reactive site load (Kruskal-Wallis, *p*=0.18), proportion of active lesions (GLM, *p*=0.34) or microglia/macrophage activity score (GLM, *p*=0.79). No difference in presence of cortical grey matter lesions was found between relapsing, PP or SP MS (*p*=0.29).

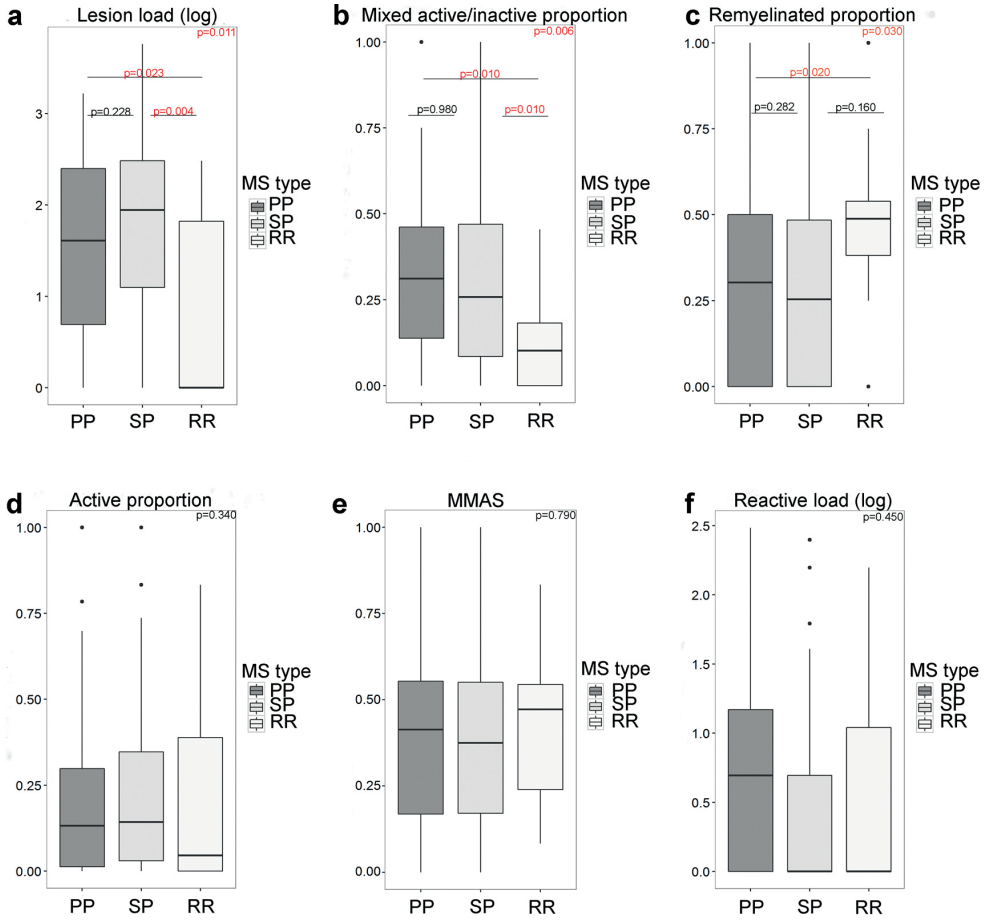

Figure 4. Lesion activity in clinical MS types.
Relapsing patients have a significantly lower lesion load (a), a lower proportion of mixed active/inactive (chronic active) lesions (b) and a higher proportion of remyelinated lesions (c) as compared to PP and SP MS. The proportion of active lesions (d) MMAS (e) and reactive site load (f) were not significantly different between the clinical MS types. Lesion load and reactive load were transformed as log(x+1). The Kruskal-Wallis and Wilcoxon tests of pairs were used for lesion load and reactive load. For lesion subtype proportions and MMAS, a quasibinomial generalized linear model was used with Tukey post-hoc tests.

Males have a higher proportion of mixed active/inactive lesions and a higher incidence of cortical grey matter lesions compared to females

Males had a higher proportion of mixed active/inactive lesions than females (p=0.007). Males had a higher incidence of cortical grey matter lesions (p=0.027) (**Figure 5**). An inverse relationship between cortical grey matter lesion presence and severity score was found in females but not in males (**Suppl. Figure 4**) (GLM, females p=0.004, males p=0.56). Consistent with this, females with cortical grey matter lesions present have a higher disease severity score than females without (p=0.004), while in males there is no difference (p=0.73) (**Suppl. Figure 4**).

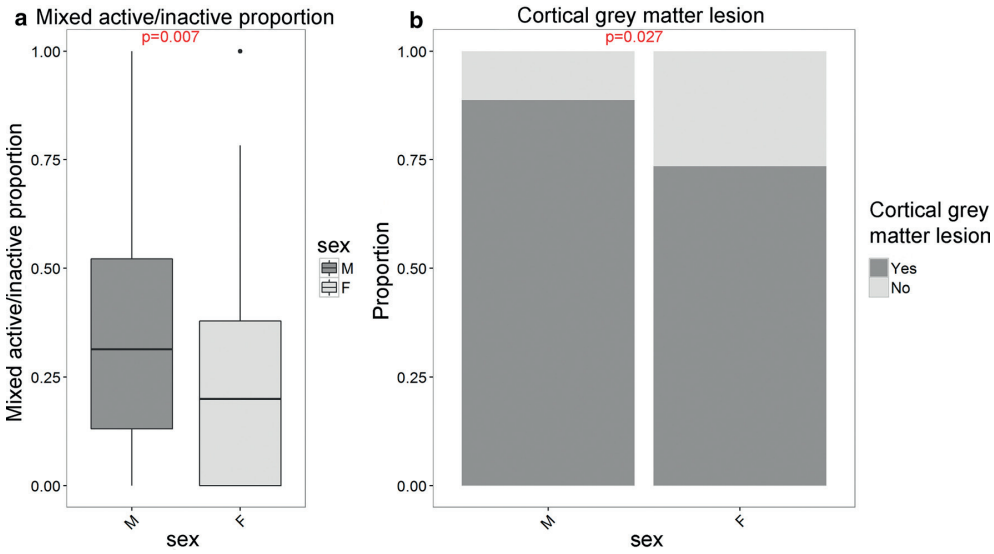

Figure 5. Sex differences in MS lesion activity and cortical grey matter lesions.
Males have a higher proportion of mixed active/inactive (chronic active) lesions (a, t-test with unequal variances was used) and a higher incidence of cortical grey matter lesions (b, Fisher's exact test) as compared to females.

Lesion load correlates with the proportions of lesion subtypes

Lesion load correlated positively with proportion of mixed active/inactive lesions (p=4e-06, R=0.36), microglia/macrophage activity score (p=0.006 R=0.23) and reactive site load (p=5e-06, R=0.35) whereas it correlated negatively with proportion of remyelinated lesions (p=0.002, R=–0.25). No significant correlation was found with proportion of active lesions (p=0.057, R=0.15) (**Figure 6**).

The presence of cortical grey matter lesions relates to lesion load and to the proportions of lesion subtypes in white matter

Patients with cortical grey matter lesions had a higher lesion load (p=4e-06), a higher proportion of mixed active/inactive lesions (p=0.002) and a higher reactive site load (p=2e-04) but a lower

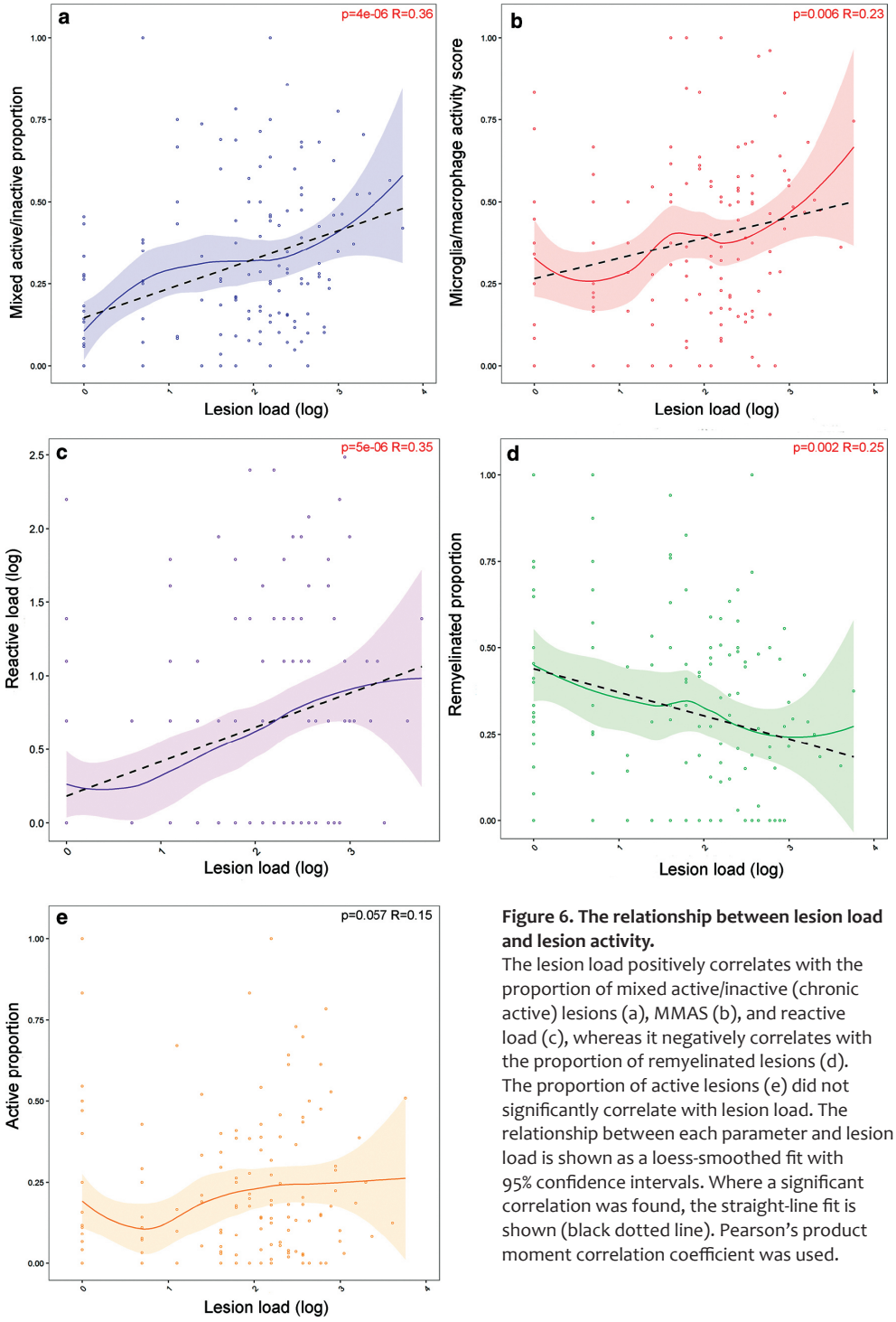

Figure 6. The relationship between lesion load and lesion activity.
The lesion load positively correlates with the proportion of mixed active/inactive (chronic active) lesions (a), MMAS (b), and reactive load (c), whereas it negatively correlates with the proportion of remyelinated lesions (d). The proportion of active lesions (e) did not significantly correlate with lesion load. The relationship between each parameter and lesion load is shown as a loess-smoothed fit with 95% confidence intervals. Where a significant correlation was found, the straight-line fit is shown (black dotted line). Pearson's product moment correlation coefficient was used.

proportion of remyelinated lesions (p=0.032). No relation was found between the presence of cortical grey matter lesions and the proportion of active lesions (p=0.28) or the microglia/macrophage activity score (p=0.13) (**Figure 7**).

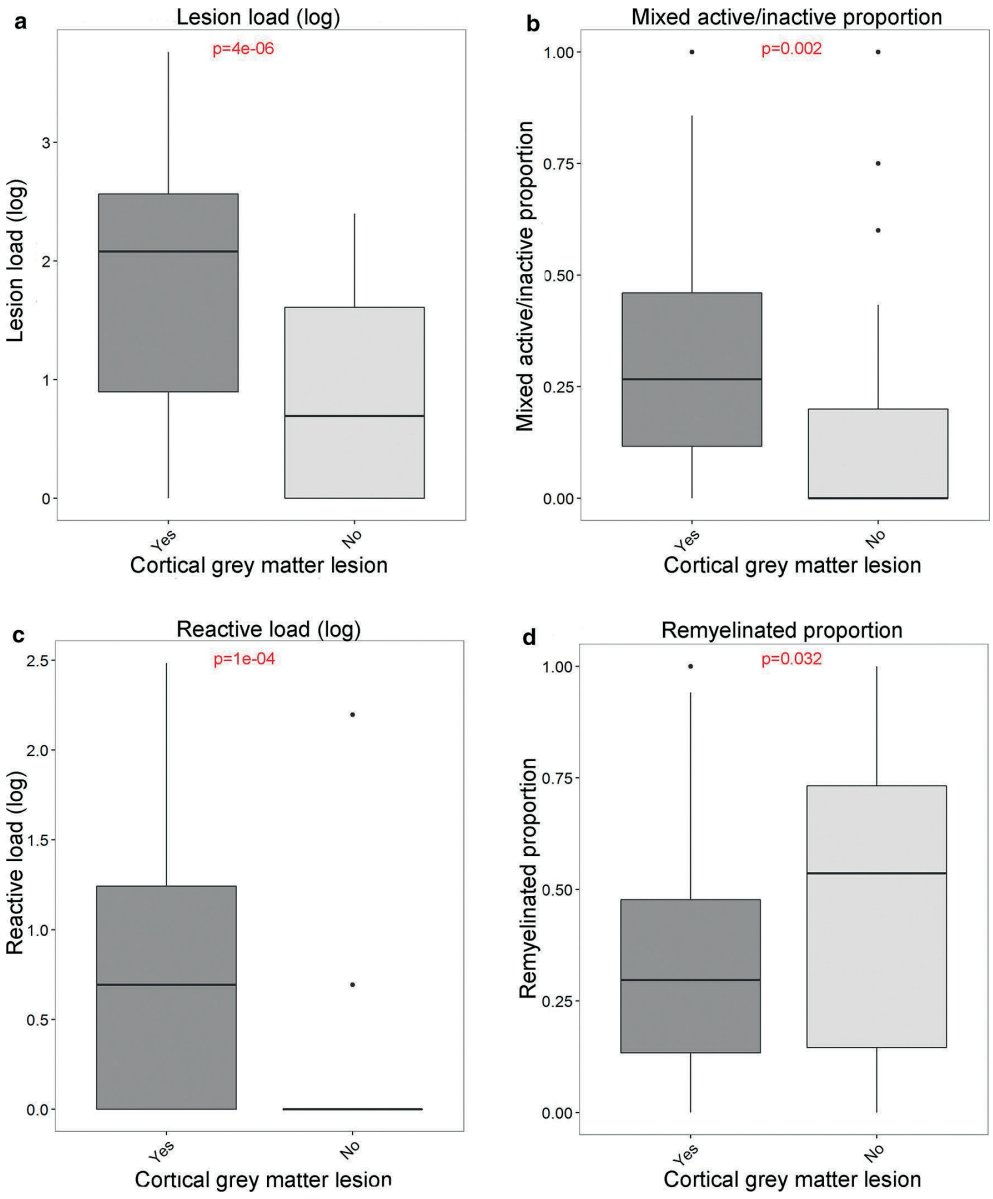

Figure 7. The relationship between the presence of cortical grey matter lesions and white matter lesion activity.

Patients with cortical grey matter lesions have a significantly higher lesion load (a), higher reactive load (c), higher proportion of mixed active/inactive (chronic active) lesions (b) and a lower proportion of remyelinated lesions (d). Lesion load and reactive load were determined in standardly dissected brainstem tissue blocks and proportions of chronic active and remyelinated lesions were determined in white matter. t-test with unequal variance was used.

The correlation of cortical grey matter lesions with lesion load, proportion of mixed active/inactive lesions, reactive site load and proportion of remyelinated lesions, was strongest in type I (leukocortical) (p=3e-08, *p*=0.013, p=9e-05, *p*=0.002 respectively) and II (intracortical) (p=5e-05, *p*=0.003, *p*=0.023, *p*=0.025 respectively) lesions. Subpial III and IV lesions showed a weaker relation with lesion load (*p*=0.003) and no relation with mixed active/inactive (chronic active) lesions (*p*=0.075), reactive sites (*p*=0.16) and remyelinated lesions (*p*=0.17) (**Suppl. Figure 5**).

Relationships between lesion subtypes

The proportion of active lesions is not significantly correlated with the proportion of mixed active/inactive lesions (*p*=0.11, R=−0.12) (**Figure 8**). The proportion of active lesions positively

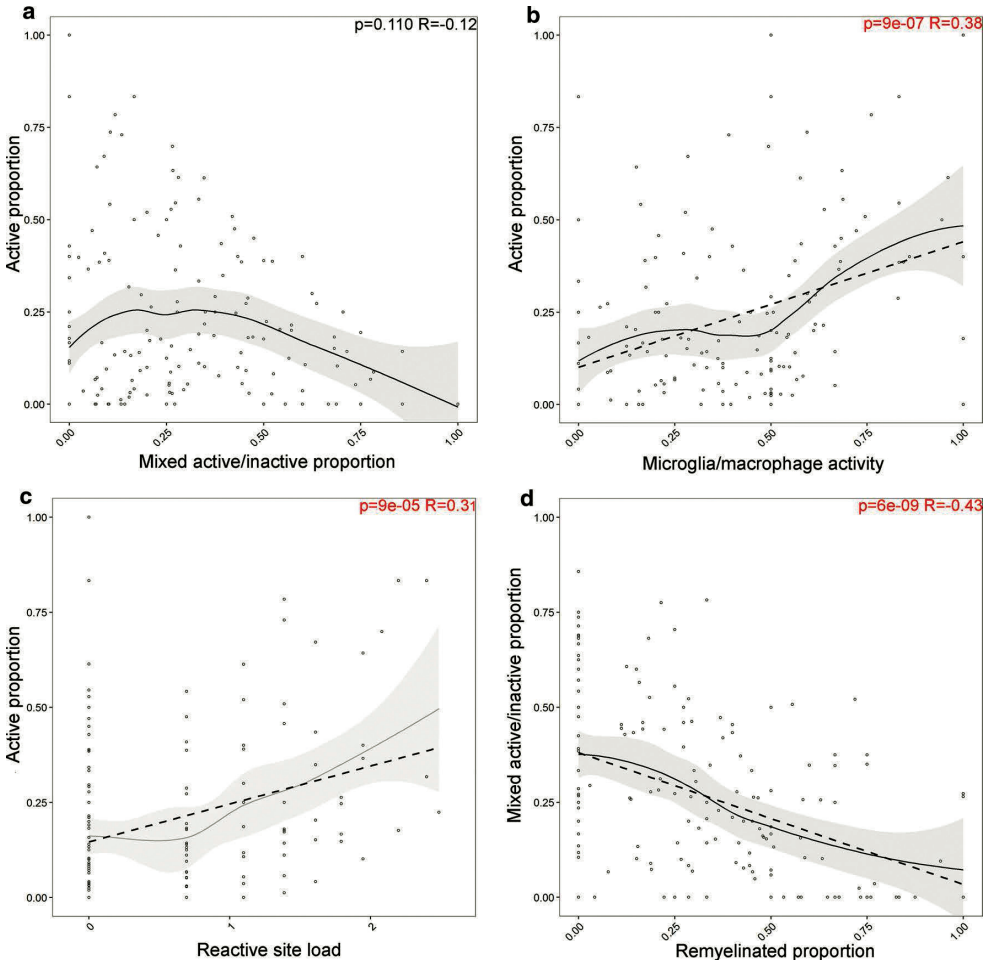

Figure 8. The relationship between active lesion proportion and mixed active/inactive lesion proportion with lesion activity.
The proportion of active lesions significantly correlated with the MMAS (a) and the reactive site load (log transformed) (b). There is no relationship between the proportion of active lesions and mixed active/inactive (chronic active) lesions (c), whereas the proportion of mixed active/inactive lesions inversely correlated with the proportion of remyelinated lesions (d). Pearson's product moment correlation coefficient was used.

correlated with the microglia/macrophage activity score (p=9e-07, R=0.38) and reactive site load (p=9e-05, R=0.31), while the proportion of mixed active/inactive lesions does not show these correlations. However, the mixed active/inactive proportion inversely correlated with proportion of remyelinated lesions (p=6e-09, R=–0.43).

Lesion activity across central nervous system locations

The proportion of mixed active/inactive lesions was significantly lower in spinal cord than in brainstem (p=1e-8). The proportion of remyelinated lesions (22% vs 4%, p=5e-05) was significantly higher in SPC as compared to the BRS. The distribution of lesion subtypes in different tissue samples (SPC, BRS and PLA/MRI samples) is shown in **Suppl. Figure 6**.

Minor myelin protein in microglia/macrophages in active lesions

In a subset of active and mixed active/inactive lesions dissected at a standard location at the level of the Medulla Oblongata (active n=5 and mixed active/inactive n=12) microglia/macrophages showed in variable amount co-localization with MOG. For active lesions mean percentage of IBA-1 signal that colocalized with MOG signal was 13.6 % (range 1,4%-39,2%) whereas for the mixed active/inactive lesions this was 8.8 % (range 0.02-46.3). (**Suppl. Figure 2**).

DISCUSSION

This study demonstrates that the lesion characteristics in post-mortem MS autopsy tissue correlate with clinical disease severity and sex in an extensive collection of MS autopsy patients and highly powered analysis. Lesion inflammatory and demyelinating activity is widely present even in the late stage of disease (mean disease duration of 29 years), with 57% of the 7562 lesions examined being either active or mixed active/inactive (chronic active) at the time of death. This contrasts with previous observations that inflammatory disease processes decline in long-term disease.[13]

This study also shows for the first time that: (1) MS brain donors with shorter time to EDSS-6 have a higher lesion load and a higher proportion of mixed active/inactive lesions at time of death. (2) MS patients with a progressive disease course have a higher lesion load and a lower proportion of remyelinated lesions compared to relapsing MS patients. We confirm that the proportion of mixed active/inactive lesions are increased in progressive compared to relapsing patients.[13] PP and SP patients show comparable lesion activity. (3) Males have a higher incidence of cortical grey matter lesions compared to females. We show for the first time that males have a higher proportion of mixed active/inactive lesions for the whole cohort, where this was previously shown only for one age group.[13] (4) Lesion load is positively correlated with proportion of mixed active/inactive lesions, reactive sites, microglia/macrophages activity and inversely with remyelinated lesions, using a systematic unbiased histological quantification of lesion load by analyzing standardly dissected tissue blocks from the brainstem. (5) The presence of cortical grey matter lesions is related to lesion load, mixed/active inactive lesions, reactive sites and remyelinated lesions by correlating histologically determined presence of cortical grey matter lesions to parameters of lesion activity.

The NBB MS autopsy cohort differs somewhat from the general MS patient population, since it contains a higher proportion of PP patients, being 25% compared to 10% in the general population.[31] The relapsing patients in this cohort consist of a combination of 8 relapsing remitting (RR) and 6 progressive relapsing (PR) patients. Compared to the recently described autopsy MS cohort by Frischer et al. (2015),[13] the NBB cohort contains MS patients with a longer mean disease duration (29 vs 12 years) and higher proportion of PP and SP patients.

In the cohort of Frischer et al. (2015)[13] active lesion, with microglia/macrophages throughout the lesion, were subdivided in early and late active based on the presence of minor myelin proteins within microglia/macrophages. It is suggested that this inclusion of minor myelin proteins reflects recent myelin phagocytosis.[25] Quantification of the inclusion of MOG (a minor myelin protein) within IBA-1 positive cells suggest that 40% of the active lesions show >20% IBA-1 signal co-localized with MOG signal, therefore these can be considered early active. This is a comparable incidence with the data of Frischer et al. 2015[13] showing in the 10-30 years disease duration groups that 25-50% of active lesions can be defined as early active.

This analysis shows for the first time that lesion load correlated strongly with disease severity. This is in line with a number of prospective MRI studies.[7,11,42] We also show for the first time that relapsing patients have a lower lesion load, and a higher proportion of remyelinated lesions and confirm that they have a lower proportion of mixed active/inactive lesions. This is in line with MRI findings showing lower lesion load in RR compared to SP MS.[11,42] Our data show no differences in lesion pathology between PP and SP MS patients, in line with the finding that SP and PP patients do not differ in aspects of clinical disease course once they enter the progressive phase of the disease.[28]

The proportion of mixed active/inactive lesions (chronic active lesions) is a strong predictor of both disease severity and lesion load. These lesions represent ongoing innate demyelinating and inflammatory activity, and include the lesions described as 'smouldering' by Frischer et al.[13] In that study, 'smouldering' lesions were not predominant in very severe patients (disease duration < 1 year), and instead peaked at medium disease duration (around 18 years) before declining. In contrast, we found that the proportion of mixed active/inactive lesions increases monotonically with severity and these lesions are present in all patients with a time to EDSS 6 < 4 yrs. Similar results are shown with time to EDSS-8 and disease duration. These findings suggest that mixed active/inactive lesions are involved in disease progression, and it is plausible that this is due to the accumulation of this lesion type over time. Indeed, active demyelination in mixed active/inactive lesions has been correlated with axonal injury in these lesions[12,13] which could explain the progressive MS clinical course. Patients without a mixed active/inactive lesion present had a less severe disease course and lower lesion load. The development of (PET-)MRI techniques (e.g.[8,10,21]) that allow the specific identification of these mixed active/inactive lesions in living MS patients is needed to determine the evolution of these lesions over time and if these can be used a prognostic tool.

The proportion of remyelinated lesions was higher in relapsing patients compared to PP and SP patients. In addition, the proportion of remyelinated lesions inversely correlated with lesion load, with the proportion of mixed active/inactive lesions and with the presence of cortical grey matter lesions. We hypothesize that the increased chronic lesion activity leads to greater axonal loss, resulting in fewer intact but demyelinated axons available for remyelination when lesions subsequently become inactive. The correlation of lesion activity with axonal injury and loss is currently being investigated in the NBB autopsy cohort.

Interestingly the proportion of active lesions shows no correlation with disease severity and lesion load. Frischer et al.[13] found that the proportion of active lesions was the highest in acute MS patients with very short duration of disease (<1 year) and declined rapidly with disease duration. In that study, MS patients with a disease duration >5 years show a constant low percentage of active lesions in all disease duration groups (on average 25%), which is in line with our data. Because there is no correlation between the proportion of active lesions with disease severity, we hypothesize that activated microglia/macrophages present throughout a (partially) demyelinated lesion, can be both involved in demyelination and/or remyelination. In line with this hypothesis it is very recently shown on PET-MRI that active lesions with microglia/macrophages throughout the lesion are either shrinking or expanding after 1 year follow-up.[3]

The active lesion proportion, MMAS and reactive site load all correlate with each other, suggesting that high microglia/macrophage activity is linked to reactive site and active lesion formation. Mixed active/inactive lesions are thought to derive from active lesions, but in our data there is no correlation between mixed active/inactive lesion proportion and either active proportion or reactive site load, leaving open the possibility that mixed active/inactive lesions and active lesions may form independently.

The reactive site load showed a positive correlation with the lesion load, with the proportion of active lesions and with the presence of cortical grey matter lesions, all suggesting that reactive sites are linked to lesion formation. Interestingly, the reactive site load did not correlate with mixed active/inactive lesion proportion. Indeed reactive sites, which comprise clusters of activated microglia/macrophages, have been previously described as 'pre-active' lesions and suggested to represent the earliest stage of lesion development.[43] They have been found prevalently around active lesions [22] and they have been associated with axons undergoing Wallerian degeneration.[2,22,41] Although these data support the hypothesis that reactive sites may be involved in lesion formation, a recent study showed that microglial clusters are a common hallmark of neuropathology, being present not only in the MS brain but also in non-demyelinating diseases such as stroke.[37] Therefore, it remains to be determined if and how microglial cells arranged in clusters around (partially) myelinated axons in MS develop into a phagocytic state, thereby forming an active demyelinating lesion.

MS patients with presence of an intra-cortical and leuko-cortical grey matter lesion have a higher lesion load, proportion of mixed active/inactive lesions and reactive load and lower proportion

of remyelinated lesions. Apparently, demyelinating and inflammatory lesion activity in the white matter correlates with cortical grey matter pathology. Indeed, a recent study[26] showed the importance of $CCR2^+$ monocytes that are present in mixed/active inactive MS lesions,[40] in the development of cortical demyelination and disease severity in non-human primates with EAE. Interestingly, the presence of subpial MS lesions does not correlate with activity of white matter MS lesions and therefore different pathologic mechanisms may play a role in formation of subpial cortical lesions such as local effects of meningeal inflammation.[35] We show a lower number of subpial lesions than expected based on previous publications,[16] however this is likely because cortex was not sampled from standard locations and therefore we do not draw conclusions from the absolute numbers and did not use the cortical lesion load for analysis.

The spinal cord contains a lower proportion of mixed active/inactive lesions compared to the brain stem, which is in line with observations from Frischer et al. 2015.[13] In the NBB cohort the proportion of remyelinated lesions is also significantly higher in spinal cord than in brain stem. These findings indicate that disease processes are less aggressive in spinal cord than in the brainstem.

Male MS patients show a higher proportion of mixed active/inactive lesions, in line with their more severe clinical disease course.[23] These mixed active/inactive lesions are similar to the "smouldering" lesions previously reported to be increased in male MS patients, but only in the 45-55 age group.[13] Males also had a higher incidence of cortical grey matter lesions than females, suggesting females are more resistant to cortical lesion formation. Indeed, females with cortical grey matter MS lesions have a shorter time to EDSS-6, thus have a more aggressive disease. Males were previously found to have more intra-cortical lesions by MRI.[1] Recently, we found sex differences in progesterone signaling, where progesterone receptor and its synthetic enzyme are increased in lesions and peri-lesional normal appearing white matter (NAWM) in females compared to males, suggesting an important role for progesterone in the sex differences in MS pathology.[34]

CONCLUSION

There is considerable heterogeneity in MS lesion pathology between patients, and lesion characteristics show strong correlations with clinical course, severity and sex. This study shows for the first time that inflammatory lesion activity is substantial at the time of death in progressive MS patients with long-standing disease. In addition, patients that have had a shorter time to EDSS-6 have a higher lesion load and proportion of mixed active/inactive (chronic active) lesions. Males have a higher cortical grey matter lesion incidence and proportion of mixed active/inactive lesions across the whole cohort. Patients with a progressive disease course have a higher lesion load and less remyelinated lesions compared to relapsing patients. Moreover, we confirm that mixed active-inactive lesions are increased in progressive MS patients. Identification of mixed active/inactive lesions on MRI is necessary to determine whether they can be used as a prognostic tool in living MS patients.

Figure 9. Overview of lesion activity scoring and correlates shown in analysis.
Examples are shown of each subtype of white and grey matter lesions, and of microglial/macrophage morphology scoring. Alongside are shown their definitions and significant correlations.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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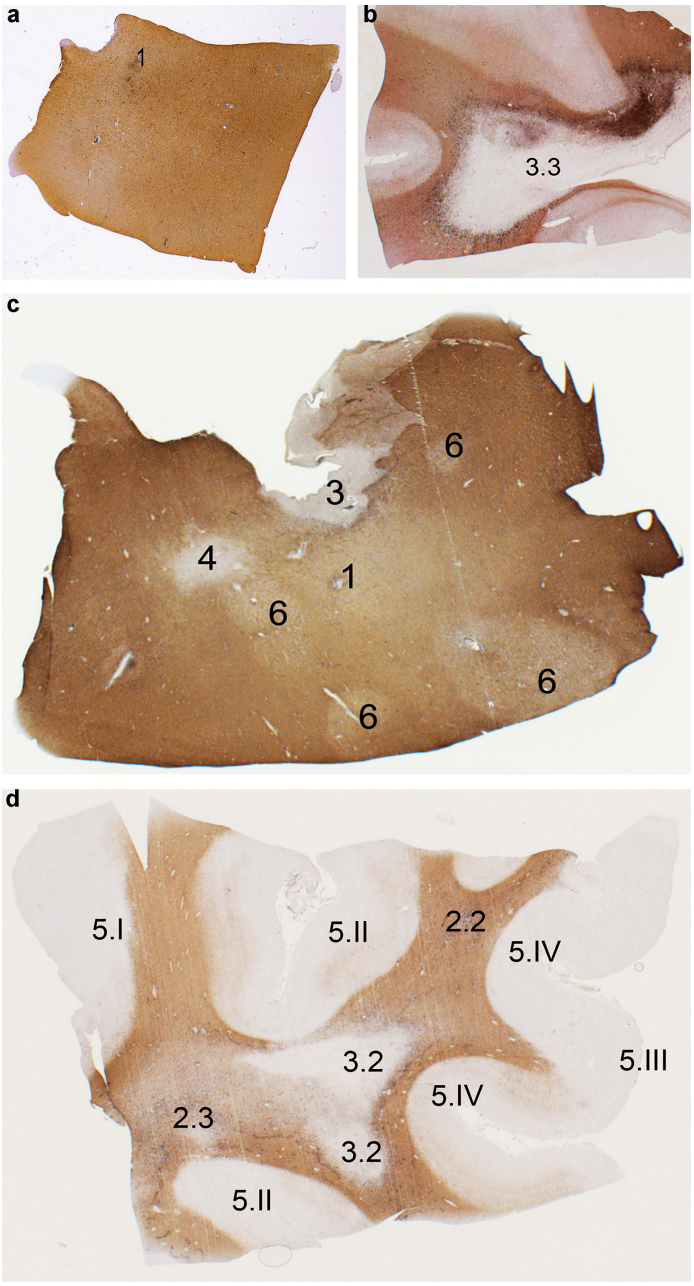
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Characteristics of tissue blocks examined.

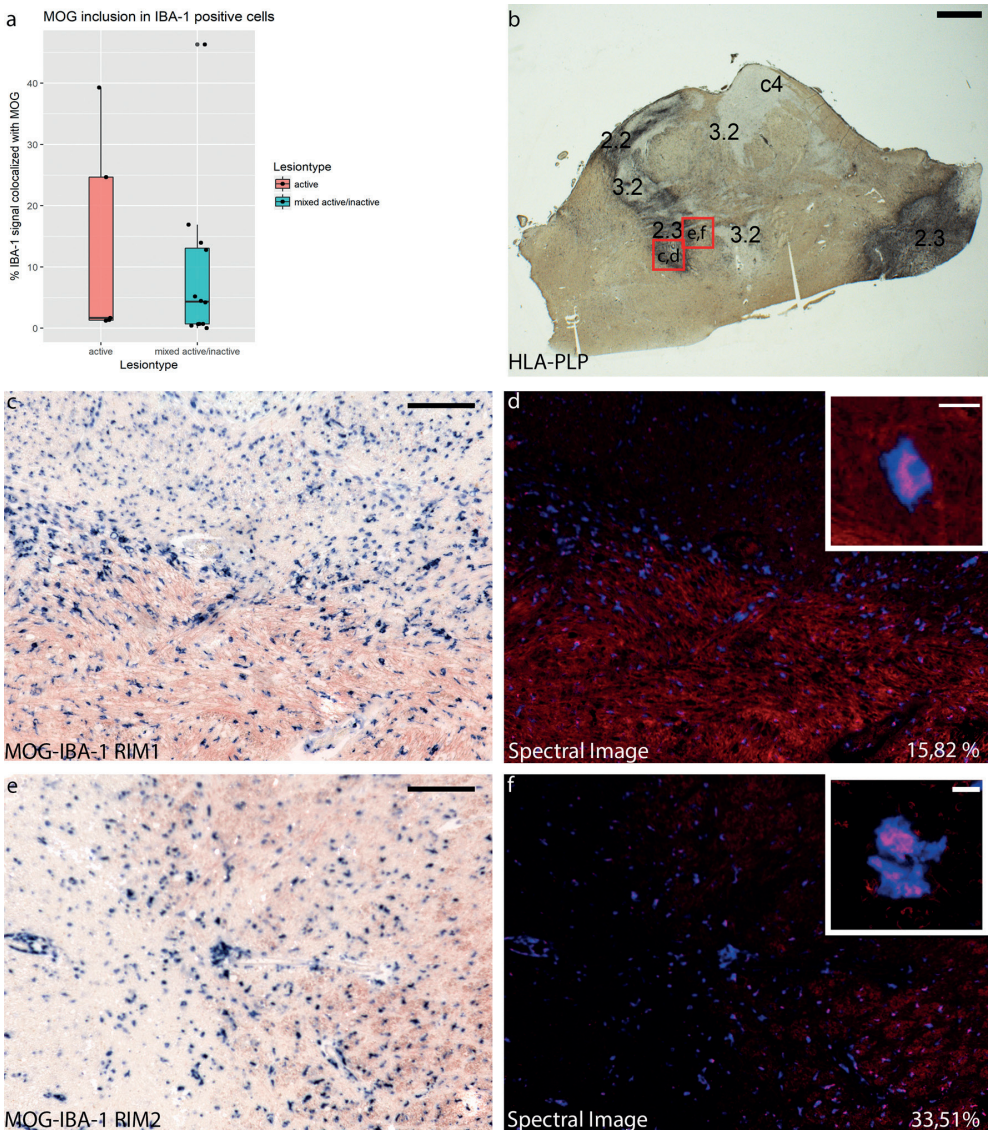
MS type/sex	**No. blocks per case** (mean ± SD)	**Total no. blocks**
all	18 ± 0.8	3188
PP	18 ± 1.3	
SP	18 ± 1.1	
Relapsing	19 ± 2.6	
Females	17 ± 1.0	
Males	19 ± 1.3	
Block type	**No. cases per block type**	**% Cases per block type**
BRS	161	88,5
SPC	120	65.9
PLA/MRI	162	89,0
PLA	150	82,4
MRI	116	63,7
PLA/MRI with cortex	160	88,9

Mean number of blocks per case for clinical subtypes and sex and the number of cases that have specific block types available. BRS: diagnostic blocks dissected at 7 standardized locations from the brainstem. SPC: diagnostic blocks dissected at 6 standardized locations from the spinal cord. PLA: macroscopically visible MS lesions dissected. MRI: MS lesions dissected on post-mortem MRI guidance. PLA/MRI with cortex: tissue blocks with cortex present.



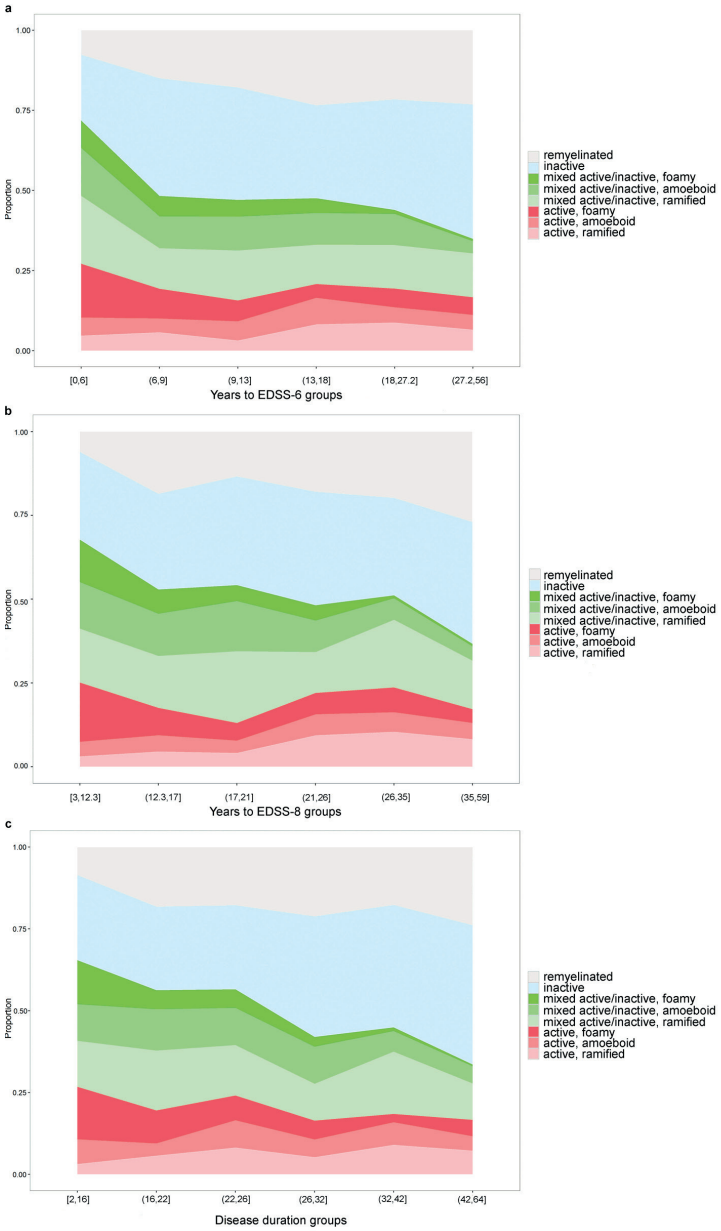
Supplementary Figure 1. Double immunostaining for human leukocyte antigen (HLA, in black) and proteolipid protein (PLP, in brown) on MS brain sections from tissue blocks dissected by the Netherlands Brainbank.

The examples show the annotation of the scoring system, that identifies in a,one reactive site (1); in b, one mixed active/inactive lesion with foamy microglia/macrophages (3.3); in c, one reactive site (1), one mixed active/inactive lesion (3), one inactive lesion (4) and four shadow plaques (6); in d, one active lesion with amoeboid microglia/macrophages (2.2), one active lesion with foamy microglia/macrophages (2.3), two mixed active/inactive lesions with amoeboid microglia/macrophages (3.2), one leukocortical grey matter lesion (5.I), two intracortical grey matter lesions (5.II), three subpial lesions of which one is more superficial (5.III) and two affect multiple layers of the cortex, reaching the border with the white matter (5.IV).

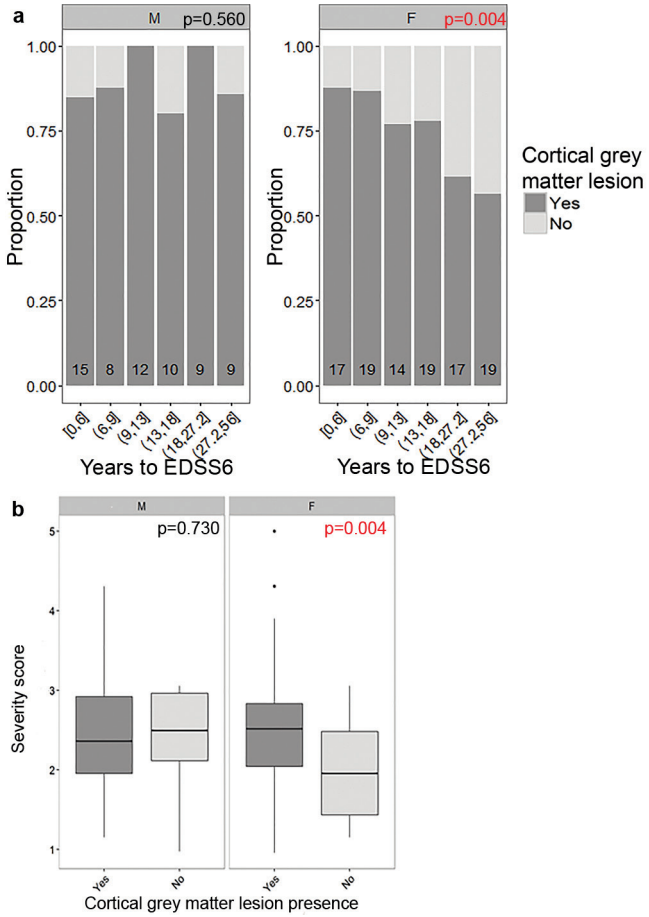


Supplementary Figure 2. Quantification of IBA-1 and MOG co-localization in active and mixed active/inactive lesions.

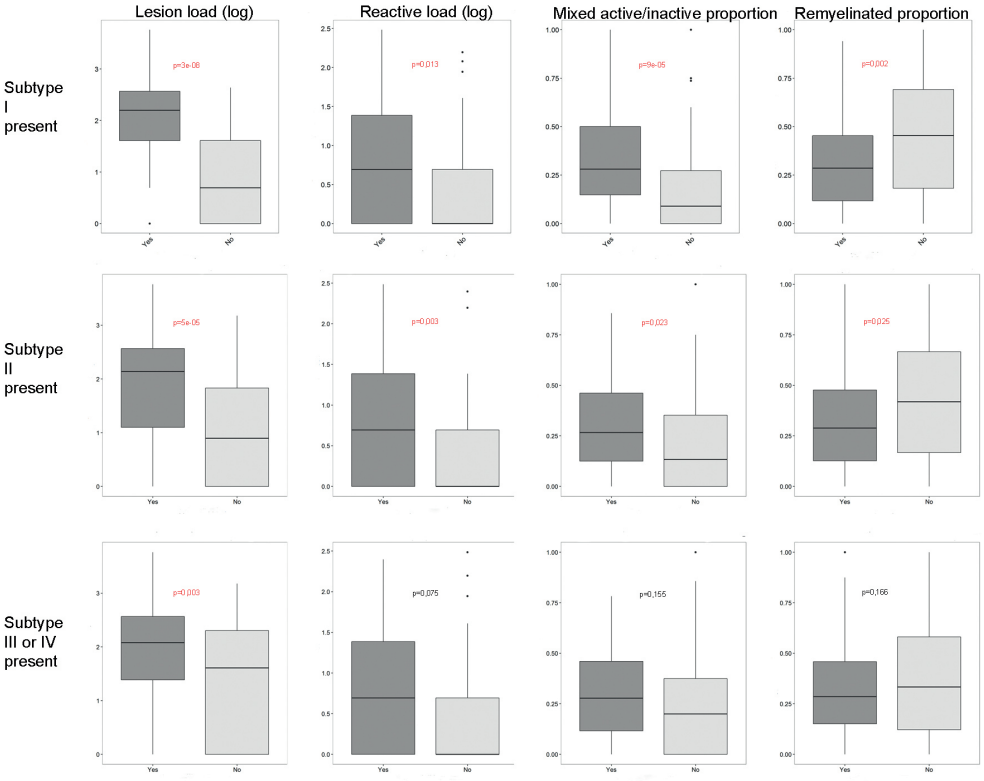
In 5 active and 12 mixed active/inactive lesions dissected from 13 cases at a standard location of the Medulla Oblongata (b) IBA-1 and MOG double immunohistochemistry was performed using NBT/BCIP (blue) and AEC (red) as chromogens, respectively. After the NBT/BCIP reaction, the sections were boiled for 10 min in microwave 700 watt before incubation with second primary antibody. Control sections showed no MOG signal after boiling the sections (not shown). a. percentages of IBA-1 signal co-localized with the MOG signal in active and mixed active/inactive lesions. b. example of HLA-PLP expression from an active lesion in which 2 regions were analyzed. c. images of a double staining of an active lesion are shown in c and e and composites of spectral images with IBA-1 signal in blue and MOG signal in red are shown in d and f. For each lesion or lesion rim, 2 or 3 pictures were captured and co-localization was measured using the set spectra, the mean % of IBA-1 positive signal co-localized with MOG signal was calculated for each lesion. For the active lesion shown here this is (15.82+33.51)/2=24.67%. In 2/5 (40%) of the active lesions, MOG co-localization is >25%. Scale bars represent in b 2.5mm, in c and e 100µm and in d and f 10µm.



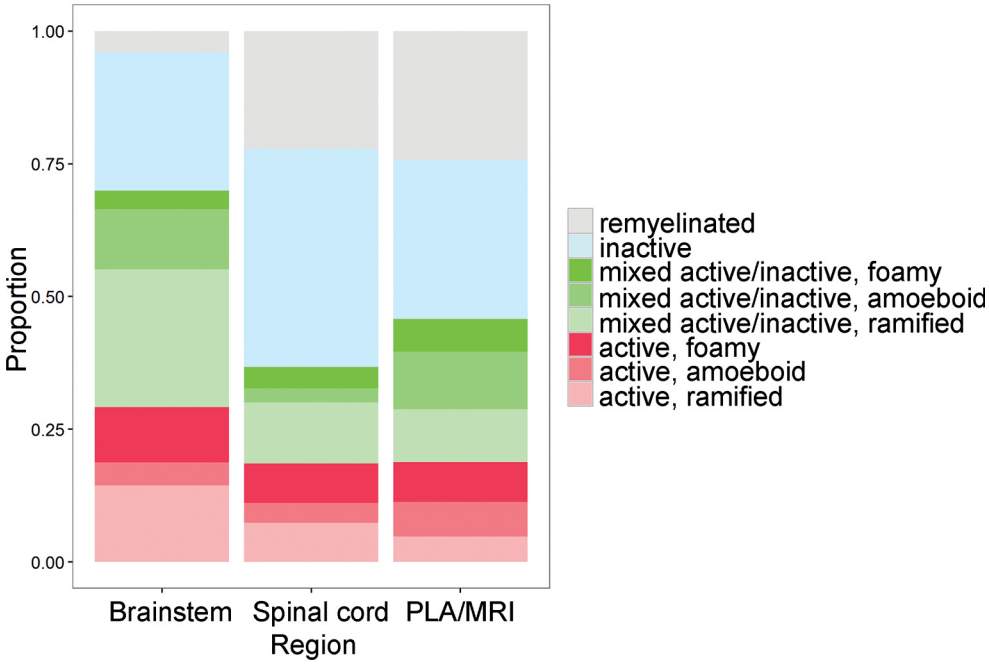
Supplementary Figure 3. Relationship between the proportion of lesion type and disease severity.
a. Lesion type proportions versus years to EDSS-6. Cases have been grouped by years to EDSS-6 as follows: [0,6] n=32; (6,9] n=27; (9,13] n=26; (13,18] n=29; (18, 27.2] n=26; (27.2,56] n=28. b. Lesion type proportions versus years to EDSS-8. Cases have been grouped by years to EDSS-8 as follows: [3,12.3] n=23; (12.3,17] n=25; (17,21] n=21; (21,26] n=25; (26,35] n=19; (35,59] n=22. c. Lesion type proportions versus duration of disease. Cases have been grouped by duration of disease as follows: [2,16] n=32; (16,22] n=32; (22,26] n=24; (26,32] n=30; (32,42] n=27; (42,64] n=27. Mixed active/inactive lesions and lesions with foamy microglia/macrophages are overrepresented in patients with shorter time to EDSS-6 and shorter disease duration, while proportions of inactive and remyelinated lesions are overrepresented in patients with longer time to EDSS-6 and longer disease duration.



Supplementary Figure 4. Relationship between cortical grey matter lesions, sex and time to EDSS-6 in years. a. An inverse relationship was found between grey matter lesion presence and severity in females (GLM, *p*=0.004) but not in males (*p*=0.56). Patients are grouped based on years to EDSS-6 and number of patients per group is shown in the bar. b. Females with cortical grey matter lesions have higher disease severity (*p*=0.004, students t-test), whereas in males no significant difference was found (*p*=0.730, students t-test).



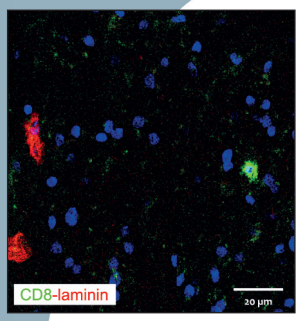
Supplementary Figure 5. Relationship between cortical grey matter lesion subtypes and lesion activity. The presence of leukocortical (type I) and intracortical (type II) lesions is strongly related to lesion load, reactive load, proportions of mixed active/inactive lesions and remyelinated lesions, while subpial III and IV lesions show only a relation with lesion load. Student t-test with unequal variances is used.

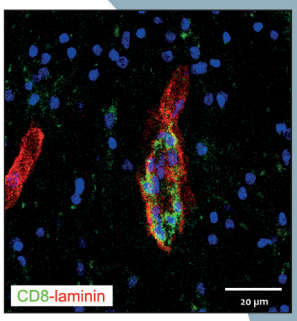


Supplementary Figure 6. Lesion type proportion by sampled location.
Spinal cord and brainstem are both standardly sampled, showing spinal cord has a higher percentage of inactive and remyelinated lesions compared to brainstem. PLA/MRI are not standardly sampled and have a significantly lower proportion of active and mixed active/inactive lesions and a higher proportion of remyelinated lesions, compared to brainstem (quasibinomial generalized linear model).

CHAPTER 4

Tissue resident memory T cells populate the human brain





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# Equal contribution

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ABSTRACT

Most tissues are populated by tissue-resident memory T cells (T_{RM} cells), which are adapted to their niche and appear to be indispensable for local protection against pathogens. Here we show that human white matter-derived brain $CD8^+$ T cells can be subsetted into $CD103^-CD69^+$ and $CD103^+CD69^+$ T cells, both with a phenotypic and transcription factor profile consistent with T_{RM} cells. Specifically, CD103 expression in brain $CD8^+$ T cells correlated with reduced expression of differentiation markers, increased expression of tissue-homing chemokine receptors, intermediate and low expression of the transcription factors T-bet and eomes, increased expression of PD-1 and CTLA-4, and low expression of cytolytic enzymes with preserved polyfunctionality upon activation. Brain $CD4^+$ T cells also display T_{RM} cell-associated markers, but have low CD103 expression. We conclude that the human brain is surveilled by T_{RM} cells, providing protection against neurotropic virus reactivation, whilst being under tight control of key immune checkpoint molecules.

INTRODUCTION

$CD8^+$ T cells have a critical role in immune protection against invading pathogens, in particular viruses. Upon infection, naive T lymphocytes are activated in secondary lymphoid organs and expand to large numbers. After clearance of the infection, some of these activated T cells differentiate into so-called memory T cells. Central memory T cells (T_{CM} cells) circulate through the blood and the secondary lymphoid organs, which collect lymph fluid from the body's peripheral sites. Effector memory T cells (T_{EM} cells) move between the blood and the spleen, and bear the ability to enter non-lymphoid tissues in case of an (re)infectious challenge. More recently, it became clear that tissues, which are common portals of reinfection, are populated by distinct lineages of tissue-resident memory T cells (T_{RM} cells).[1–4] T_{RM} cells orchestrate the response to pathogens (re) encountered at these locations. Using the canonical markers CD69 and CD103, T_{RM} cells have been identified in most murine and human tissues.[5,6]

The central nervous system (CNS) is structurally and functionally unique, but, in common with other tissues, requires efficient immune protection against infections.[7] This is illustrated by the ability of neuropathic viruses to enter the CNS and cause live-threatening infections.[8] The CNS is floating in cerebrospinal fluid (CSF), a functional equivalent of the lymph that is generated in the choroid plexus from arterial blood and reabsorbed into the venous blood at the arachnoid villi. The CSF contains $CD4^+$ and, to a lesser extent, $CD8^+$ T cells, which patrol the boarders of the CNS and provide protection.[9] These cells express CCR7, L-selectin, and CD27, indicating a T_{CM}-cell phenotype.[10] The parenchyma of the CNS was long believed to be an immune-privileged site, separated by tight cellular barriers from the blood and the CSF stream and, thus, being inaccessible for T cells. More lately, $CD8^+$ T_{RM} cells have been identified in the parenchyma of the mouse CNS, where they provide local cytotoxic defense against viral infections.[11–13]

We recently phenotyped human T cells acutely isolated from the post-mortem human brain14. T cells in the corpus callosum had a $CD8^+$ predominance and were mostly located around blood vessels, presumably in the perivascular Virchow-Robin space. Their chemokine receptor profile lacked the lymph node-homing receptor CCR7, but included the tissue homing receptors CX_3CR1 and CXCR3. The absence of the costimulatory molecules CD27 and CD28 suggested a differentiated phenotype,[15,16] yet no perforin and little granzyme B were produced14. These cytotoxic effector molecules are characteristic for circulating effector-type $CD8^+$ T cells, but lack in certain human T_{RM}-cell populations.[17]

We here tested the hypothesis that the $CD8^+$ T-cell compartment in the human brain harbors populations with T_{RM}-cell features and demonstrate the existence of two $CD69^+$ subsets, distinguished by the surface presence of CD103. We provide expression profiles of molecules associated with cellular differentiation, migration, effector functions, and transcriptional control in these cells, as well as cytokine profiles after stimulation. We propose that CD103 expression

reflects antigen- and/or tissue compartment-specific features of these cells. Furthermore, we explored characteristics of the lesser abundant brain $CD4^+$ T-cell fraction and show that they are also enriched for T_{RM} cell-associated surface markers, except for a notably low expression of CD103.

METHODS

Brain donors

Brain tissue samples were obtained from the Netherlands Brain Bank (NBB; www.brainbank.nl). The NBB obtained permission from the donors for brain autopsy and the use of tissue, blood, and clinical information for research purposes (ethical statement available at www.brainbank.nl/media/uploads/file/Ethical-declaration.pdf),[18,19] and all procedures of the NBB have been approved by the Ethics Committee of VU University Medical Center (Amsterdam, The Netherlands). For the present study, eligibility criteria were: acquisition of both a PB sample and a subcortical normal-appearing white matter sample and time between death and end-of-autopsy <12 hours. Donor characteristics are provided in **Table 1**.

Cell isolation

Subcortical white matter samples in a range of 5–10 g/donor were stored at 4°C in Hibernate A medium (Brain Bits LLC, Springfield, IL, USA) until workup. Before workup, a small tissue fragment was snap-frozen in liquid nitrogen and stored at -80°C for immunohistochemistry. The remaining tissue was mechanically dissociated, followed by enzymatic dissociation, as we described previously.[14] After lysis of erythrocytes, mononuclear cells were separated from the suspension by Percoll gradient centrifugation. PBMCs from deceased brain donors, acquired by puncture of either the heart or the iliac artery for collection of 5 ml blood, and from anonymous blood donors (buffy coats; Sanquin Blood Supply Foundation, Amsterdam, The Netherlands) were isolated using standard density gradient centrifugation.

Cell stimulation

To assess intracellular cytokine production, cells were stimulated for 4 hours with phorbol 12-mystrate 13-acetate (PMA)/ionomycin in the presence of brefeldin A, monensin, anti-CD28 (15E8, 2 µg/ml), and anti-CD29 (TS2/16, 1 µg/ml) and compared with a control sample, following earlier published procedures.[20]

Flow cytometry

Cells were stained with antibodies for surface markers and with LIVE/DEAD^{TM} Fixable Red Stain or Fixable Viability Dye eFluor 780 (both Life Technologies, Bleiswijk, The Netherlands) for 30 minutes at 4°C. Subsequently, cells were washed, fixed, and permeabilized, using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Vienna, Austria) or BD Cytofix/Cytoperm^{TM}kit (BD Biosciences, Breda, The Netherlands), followed by intracellular staining with antibodies. Washed

Table 1. Brain donor characteristics.

NBB	Disease	Sex	Age	Cause of Death	pH CSF	PMD
94-325	NO	F	51	Pneumonia	7.05	07:40
95-106	NO	M	74	Myocardial infarction	6.75	08:00
99-051	MS	F	45	Legal euthanasia	6.62	10:55
10-103	NO	F	79	Cardiac insufficiency	6.30	10:30
11-044	NO	M	51	Suicide	7.05	07:45
11-072	NO	F	76	Hepatic failure	6.87	07:15
12-059	NO	F	78	Pneumonia	6.41	04:35
12-104	NO	M	79	Legal euthanasia	6.71	06:30
14-025	FTD	M	65	End stage FTD-ALS	6.34	05:45
14-026	AD	F	80	Dehydration/respiratory tract infection	6.36	04:50
14-030	PD	M	56	Legal euthanasia	ND	07:25
14-031	AD	F	90	Cachexia/dehydration	6.29	06:30
14-032	PD	M	83	Cachexia	6.38	05:10
14-035	PD	M	77	Legal euthanasia	6.69	04:48
14-038	MS	F	35	Legal euthanasia	6.37	10:20
14-039	MS	F	75	Unknown	ND	09:45
14-041	BD	F	79	Renal insufficiency	6.31	08:00
14-043	NO	F	60	Metastasized mammary carcinoma	6.58	08:10
14-045	FTD	F	68	Unknown	6.68	06:05
14-046	AD	F	85	Cachexia/dehydration	6.28	08:00
14-047	AD	M	68	Unknown	6.64	06:10
14-049	AD	F	78	Cachexia/dehydration	6.21	04:45
15-011	MS	F	57	Legal euthanasia	6.40	07:30
15-047	MS	F	50	Legal euthanasia	6.62	09:05
15-064	MS	M	50	Legal euthanasia	6.55	10:50
15-082	MS	F	47	Pneumonia	5.78	08:35

AD, Alzheimer's disease; Age, age at death in years; BD, bipolar disorder; CSF, cerebrospinal fluid; F, female; FTD, frontotemporal dementia; M, male; MS, multiple sclerosis; NBB, Netherlands Brain Bank registration number; ND, not determined; NO, no brain disease; PD, Parkinson's disease; PMD, post-mortem delay = time between death and the end of the autopsy in hours. Legal euthanasia = euthanasia or physician-assisted suicide under the Termination of Life on Request and Assisted Suicide Act of 2002 in The Netherlands.

cells were analyzed at a LSRFortessa^{TM} cell analyzer (BD Biosciences, San Jose, CA, USA). FlowJo software (Tree Star, Ashland, OR, USA) was used for subsequent data analysis. Hierarchical stochastic neighbor embedding (HSNE) analysis was performed with Cytosplore^{+HSNE} software.[21] Specifications of the used antibodies are provided in **Suppl. Table 1**.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded (FFPE) and cryopreserved white matter tissue were used for immunohistochemistry. FFPE sections were deparaffinized, and antigen retrieval was performed in a microwave at 800 W for 10 min in citrate buffer (10 mM citric acid, pH 6). Cryosections

were fixated in 4% paraformaldehyde buffer for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2, 0.2% Triton-X in Tris-buffered saline. The sections were incubated overnight at 4°C with primary antibodies directed against CD3 (ab669, 1:50; Abcam, Cambridge, UK), CD4 (ab133616, 1:200; Abcam), CD8 (ab4055, 1:500; Abcam), or laminin (ab80580, 1:100; Abcam). Alternatively, sections were incubated for 1 h at room temperature with primary antibodies directed against PD-L1 (clone 27A2, 1:100; Origene, Herford, Germany), CD86 (clone BU63 1:00; NSJ Bioreagents, San Diego, CA, USA), or CD103 (ab129202, 1:1,000; Abcam). For immunofluorescence, sections were incubated with biotinylated-anti-mouse (1:400), donkey-anti-rabbit Cy3 (1:400), or donkey-anti-rat-Cy5 (1:400) antibodies, sections were then incubated for 45 minutes with Streptavidin-Alexa 488. For the laminin and CD3 stainings, sections were subsequently incubated with rat-absorbed-biotinylated-anti-mouse antibody (Vector Laboratories). For all sections, a final incubation with Hoechst 1:1,000 for 10 minutes was performed. Negative controls with discard of primary antibody were included. Pictures were taken using Leica TCS SP8 confocal microscope and Leica Application Suite X (Wetzlar, Germany) at 20x and 63x magnification. For immunohistochemistry, binding of biotinylated secondary antibody was visualized with avidin-biotin horseradish peroxidase complex (Vector Elite ABC kit; Vector Laboratories) and 3,3'-diaminobenzidine (EnVision; DAKO) as chromogen. Nuclei were counterstained using hematoxylin. Pictures were taken with a AxioScope microscope (Zeiss, Oberkochen, Germany) at 20x or 40x magnification using a MicroPublisher 5.0 camera (QImaging, Surrey, BC, Canada) and ImagePro Plus 6.3 software (Media-Cybernetics, Rockville, MD, USA).

For immunohistochemical quantification of $CD4^+$ and $CD8^+$ cells, 10x10 tiled bright-field pictures of FFPE sections were taken at 10x magnification. Images covered a median of 42 (IQR 39–48) mm2 overlapping normal-appearing white matter. Images of complete cryostat sections stained for laminin and CD8 were made for proportional quantification of perivascular and parenchymal $CD8^+$ T cells. Cell counts were obtained using image J software.[22,23]

Statistics

All results were analyzed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Distribution of data in dot-plots is provided, as well as bars to indicate median values in the figures. Non-parametric statistical tests were employed. Unpaired samples were analyzed with the Mann-Whitney U test, the paired samples of 2 strata were analyzed with the Wilcoxon Signed Ranks test, or the paired samples of 3 strata were assessed with the Friedman test, utilizing the Wilcoxon signed ranks as post-hoc test. A p-value <0.05 was considered significant.

Data availability

The data that support the findings of this study, as well as specific flow cytometry panels, are available from the corresponding author upon reasonable request.

Flow cytometry analysis of human brain T cells

We designed multicolor flow cytometry panels to simultaneously assess T-cell phenotype, differentiation, activation, exhaustion, senescence, transcriptional regulation, homing characteristics, cytotoxic capacity, and cytokine production in brain isolates. Freshly isolated T cells of subcortical white matter and paired peripheral blood of deceased human brain donors were analyzed using these panels (**Suppl. Figure 1**). For comparison, we analyzed peripheral blood mononuclear cells (PBMCs) of healthy individuals. Blood from deceased donors showed a $CD8^+$ T-cell phenotype congruent with a more terminally differentiated stage, with a distribution profile of differentiation markers similar to living donors (**Suppl. Figure 2**). Despite the variable background of the brain donors, consisting of patients with Alzheimer's disease, Parkinson's disease, dementia, depression, multiple sclerosis, as well as controls with no known neurological disorders (**Table 1**), brain T cells displayed a remarkably consistent phenotype that differed significantly from circulating T cells.

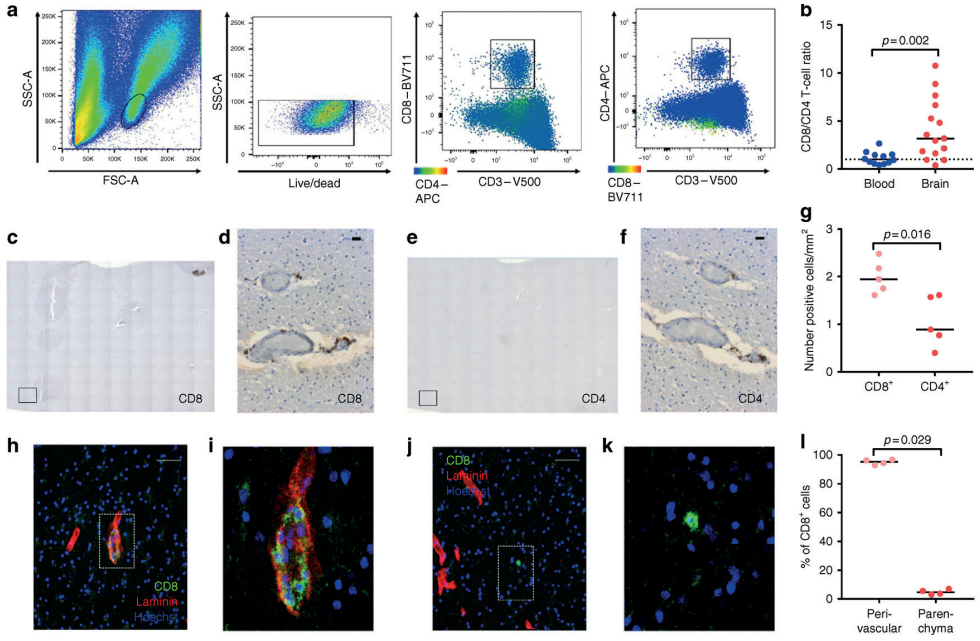

Figure 1. $CD8^+$ and $CD4^+$ T cells populate the human brain.
a. Gating procedure applied to analyze brain $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, eluted from normal-appearing white matter. b. Quantification of the $CD8^+/CD4^+$ T-cell ratio. Immunohistochemical staining of $CD8^-$ (c,d) and $CD4^-$ (e,f) immunoreactive cells in normal subcortical white matter of a donor without brain disease. c,e. Overview of 10x10 tiled images at 10x magnification; the marked square indicates a bright field. d,f. 20x magnification (scale bar = 20 µm). g. Quantification of CD8-immunoreactive and CD4-immunoreactive cells (number/mm_2). h–k. Immunofluorescent staining of CD8 (green), laminin (red), and Hoechst (blue) at 10x magnification (scale bar = 50 µm) (h,j) and a zoom-in (i,k). l. Quantification of CD8-immunoreactive cells co-localizing with laminin (perivascular space) or not co-localizing with laminin (parenchyma). Bars show median values. *p*-values show Mann-Whitney U test.

Human brain white matter contains $CD4^+$ and $CD8^+$ T cells

We analyzed viable T lymphocytes from subcortical white matter and observed both a $CD8^+$ and $CD4^+$ fraction (**Figure 1a**). Approximately three times less $CD4^+$ T cells were retrieved from the tissue when compared to $CD8^+$ T cells (**Figure 1b**). Since isolation procedures may create bias in T-cell proportions,[24] numbers of $CD4^+$ and $CD8^+$ T cells in normal-appearing sub-cortical white matter sections were also quantified with immunohistochemistry (**Figure 1c–f**). We observed a median of 0.89 (interquartile range 0.77–1.57) $CD4^+$ compared to 1.94 (1.75–2.17) $CD8^+$ T cells/mm^2 white matter (**Figure 1g**). Both $CD4^+$ and $CD8^+$ T cells were mostly found in close relationship with blood vessels, and immunofluorescence staining with laminin[25] revealed the majority to reside in the perivascular space (**Figure 1h–l**).

Brain $CD8^+$ T cells bear T_{RM}-cell associated surface markers

Paired blood and brain-derived $CD8^+$ T cells were analyzed for distribution of differentiation markers by HSNE,[21] revealing a segregated clustering of blood-derived and brain-derived $CD8^+$ T cells in the plot (**Figure 2a–d**). As we reported previously,[14] human brain $CD8^+$ T cells are differentiated $CD45RA^-CD45R0^+$ cells that barely express the co-stimulatory molecules CD27 and CD28, weakly bear the IL-7 receptor α-chain (IL-7Rα, CD127), and lack the lymph node-homing receptor CCR7 (**Figure 2e–j**). Staining for the T_{RM}-cell markers CD69 and CD103 (αE integrin) revealed that these cells highly express CD69 (**Figure 2k–m**). While CD69 expression can also indicate cellular activation, brain $CD8^+$ T cells do not express other markers commonly associated with T-cell activation, such as Ki-67 and HLA-DR/CD38 (**Suppl. Figure 3**). This suggests CD69 to reflect a T_{RM}-cell phenotype. About 40% of the $CD8^+$ T cells expressed CD103 (**Figure 2l,m**), and co-expression analysis showed that $CD69^+CD103^-$ and $CD69^+CD103^+$ cells are the dominant $CD8^+$ T-cell populations in the white matter (**Figure 2n**).

CD103 positivity in brain $CD8^+$ T cells correlated with a higher expression of the differentiation markers CD27, CCR7, and IL-7Rα, but a lower expression of CD28 and CD45RA (**Figure 3a–f**). In human skin, $CD8^+$ T_{RM} cells are defined by expression of CD49a ($\alpha_1\beta_1$ integrin).[26,27] Brain $CD8^+$ T cells also expressed CD49a, with high levels on $CD69^+CD103^-$ and even more $CD69^+CD103^+$ cells (**Figure 3g,h**). The expression of the integrin CD49d ($\alpha_4\beta_1$ integrin), which circulating $CD8^+$ T cells employ to cross the blood brain barrier and enter the perivascular space,[28] was slightly lower by brain when compared to blood $CD8^+$ T cells, and equal on brain $CD8^+$ T cells irrespective of the expression of CD69 and CD103 (**Figure 3i,j**). Brain $CD8^+$ T cells did not express the adhesion G protein-coupled receptor GPR56, a surrogate marker for cytotoxic lymphocytes[29] (**Figure 3k,l**). Expression of KLRG1 (killer cell lectin-like receptor subfamily G member 1) and CD57, two molecules found on terminally differentiated/senescent cells with low expression on $CD8^+$ T_{RM} cells,[27,30] was low in brain- compared to blood-derived $CD8^+$ T cells (**Figure 3m–p**). Expression of GPR56 and CD57 was similarly low on all cells (**Figure 3l,p**), while expression of KLRG1 was lower on $CD69^+CD103^+$ when compared to $CD69^+CD103^-$ and $CD69^-CD103^-$ cells (**Figure 3n**).

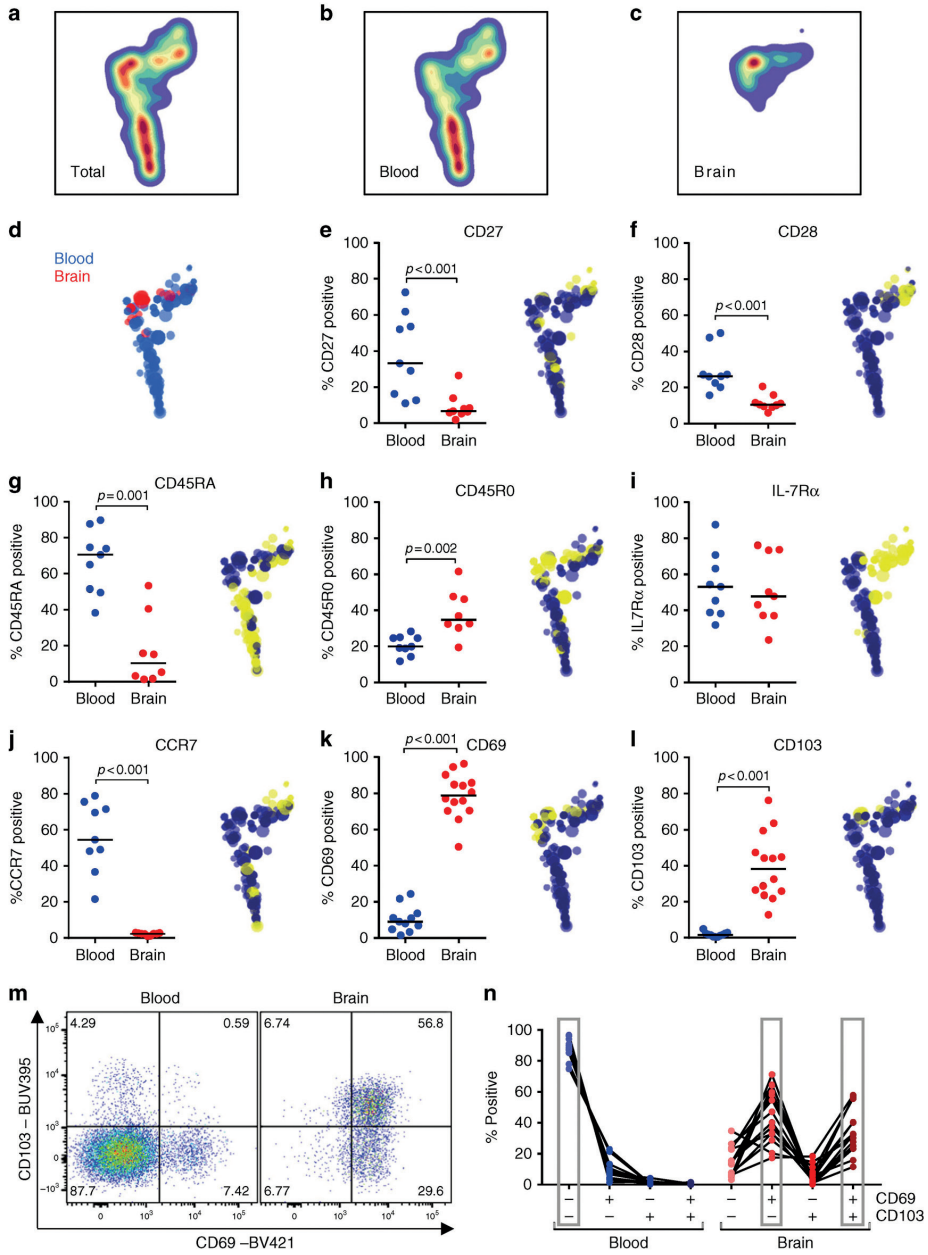

Figure 2. Human brain CD8⁺ T cells express the tissue residence markers CD69 and CD103. a–c. HSNE plot of paired brain-derived and blood-derived CD8⁺ T cells of n=5 donors, based on expression of markers shown in this figure, as well as KLRG1 and GPR56, shows segregated clustering of blood-derived and brain-derived CD8⁺ T cells. d. Distribution of hierarchical clusters in the HSNE plot with the size of the dots indicating hierarchical cluster size. e–l. Quantification of CD8⁺ T cells expressing CD27, CD28, CD45RA, CD45R0, IL-7Rα, CCR7, CD69, and CD103, respectively. In the HSNE plots, yellow dots indicate positive and blue dots negative hierarchical clusters. Clustering of brain CD8⁺ T cells is most prominently characterized by high expression of CD69 and CD103. Bars show median values. *p*-values show Mann-Whitney U test; no brackets indicate no significant difference. m. Dot plot of CD69 and CD103 co-expression in $CD3^+CD8^+$ T cells eluted from blood and brain of a brain donor with Parkinson's disease. n. Co-expression of CD69 and CD103. The dominant phenotype was $CD69^-CD103^-$ in blood and $CD69^+CD103^-$ and $CD69^+CD103^+$ in brain.

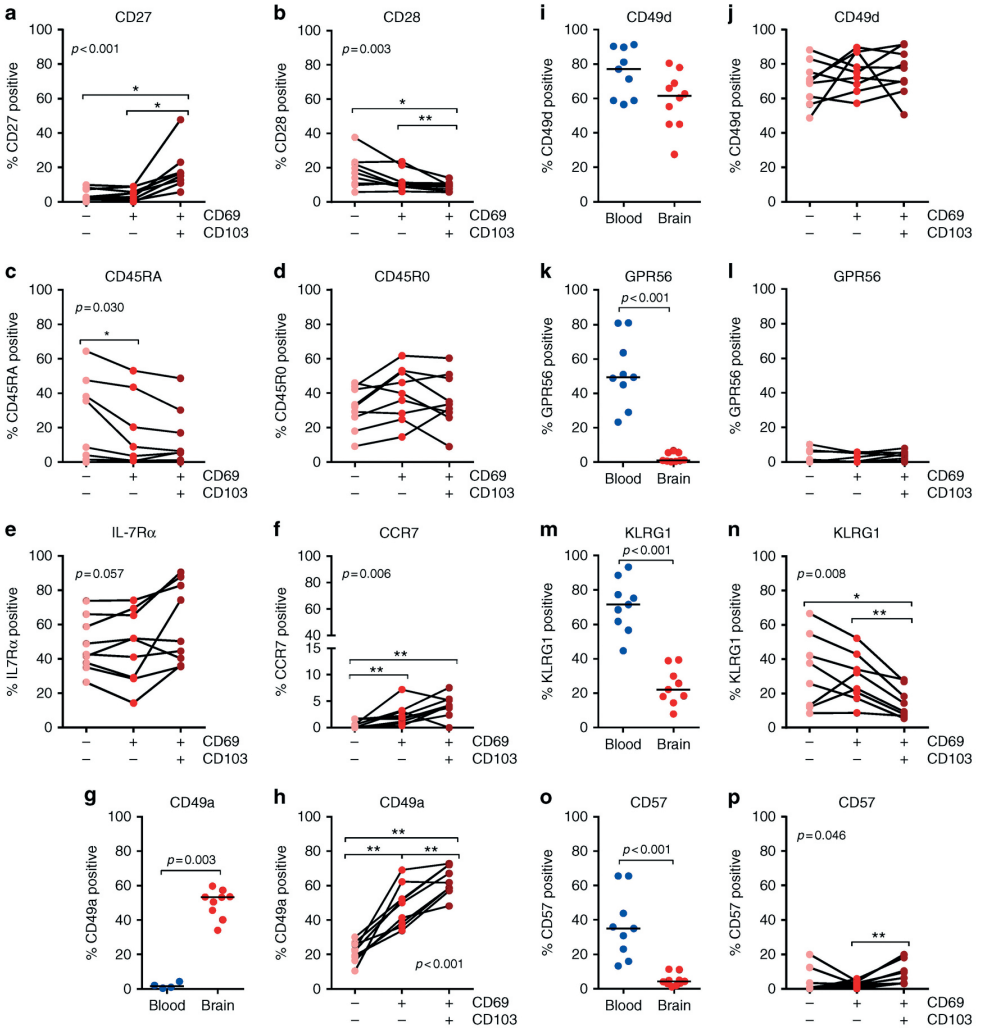

Figure 3. Human brain CD8⁺ T cells distinctly express surface markers, based on CD69 and CD103 co-expression.

a–p. Quantification of CD8⁺ T cells expressing CD27, CD28, CD45RA, CD45R0, IL-7Rα, CCR7, CD49a, CD49d, GPR56, KLRG1, and CD57, respectively, in n=9 donors. Bars show median values. *p*-values show Mann-Whitney U test for unpaired data (g,i,k,m,o) and Friedman test for paired data with Wilcoxon signed ranks as post-hoc test (a–f,h,j,l,n,p) (**p* <0.05, ***p* <0.01); no brackets indicate no significant difference.

Since the anatomical localization of CD8⁺ T cells in the human brain parenchyma requires specific migratory properties, we measured the expression of chemokine receptors that have been implicated in tissue homing. CD69⁺CD103⁺ CD8⁺ T cells were enriched for expression of CCR5, CXCR3, CXCR6, and CX_3CR1 (**Figure 4a–h**). Expression of CXCR3 is critical for the establishment of CD103⁺ T_{RM} cells in the murine skin,[30] and CXCR6 has been identified as part of the core phenotypic profile of T_{RM} cells.[27] Overall, expression of CX_3CR1 and CCR5 were higher in brain CD8⁺ T cells compared to blood CD8⁺ T cells. To further test the assumption of a parenchymal localization of

$CD69^+CD103^+$ $CD8^+$ T cells, we immunohistochemically stained white matter of two brain donors for CD103. As expected, $CD103^+$ T cells localized in the brain parenchyma, but also, among $CD103^-$ T cells, in the perivascular space (**Figure 4i,j; Suppl. Figure 4**). Therefore, CD103 positivity is no exclusive marker for localization in the brain parenchyma, but may reflect a brain $CD8^+$ T-cell subset with a greater propensity to migrate to (inflamed) parenchyma.

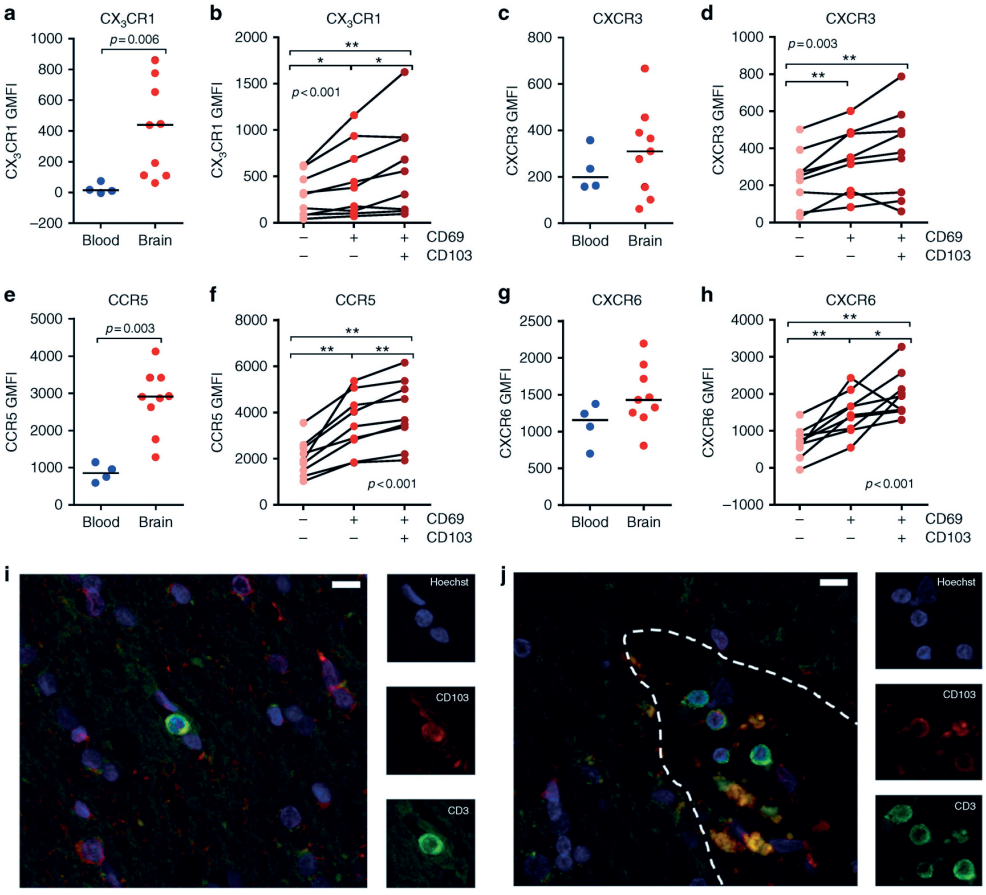

Figure 4. Human brain $CD69^+CD103^+CD8^+$ T cells are enriched for tissue-homing chemokine receptors. a–h. Quantification of $CD8^+$ T-cell expression levels of CX_3CR1, CXCR3, CCR5, and CXCR6 (GMFI, geometric mean fluorescence intensity). Bars show median values. *p*-values show Mann-Whitney U test for unpaired data (a,c,e,g) and Friedman test for paired data with Wilcoxon signed ranks as post-hoc test (b,d,f,h) (*p <0.05, **p <0.01); no brackets indicate no significant difference. i,j. Immunofluorescent staining for CD3 and CD103 of paraffin tissue shows a parenchymal (i) and perivascular (j) localization of CD3 (green) and CD103 (red) immunoreactive cells. Borders of the perivascular space were designated based on histological hallmarks (i.e. lymphocytes in close relationship with the extraluminal side of a blood vessel) and are marked with a dotted white line (scale bar = 10 µm).

Brain CD8$^+$ T cells express T_{RM} cell-associated transcription factors

Control of effector and memory CD8$^+$ T-cell differentiation critically depends on the balance between the T-box transcription factors T-bet and eomes (eomesodermin).[31] We found that most CD8$^+$ T cells eluted from the brain expressed intermediate amounts of T-bet and low amounts of eomes (**Figure 5a,b**).[15] A downregulated expression of eomes has been previously described as a characteristic feature of CD103$^+$ T_{RM} cells,[30,32] with a downregulation of T-bet expression being mandatory for the development of herpes simplex virus-specific CD103$^+$ T_{RM} cells in the murine skin.[32] Likewise, CD103 expression correlated with a low expression of eomes in brain CD8$^+$ T cells (**Figure 5c**), and the proportion of T-bet-intermediate/eomes-low cells was highest in the CD69$^+$CD103$^+$ cell fraction (**Figure 5d**). Hobit (homolog of Blimp-1 in T cells), which is a critical component of murine T_{RM}-cell transcriptional program,[33] yet absent in human lung T_{RM} cells,[34] is not expressed by brain CD8$^+$ T cells (**Figure 5e,f**).

Distinct effector molecules of CD103$^+$ brain CD8$^+$ T cells

We next analyzed the production of cytolytic effector molecules by brain CD8$^+$ T cells directly *ex situ*. In accordance with our previous study,[14] brain CD8$^+$ T cells showed a low expression of granzyme B compared to peripheral blood and virtually no expression of the lytic mediator perforin (**Figure 6a–d**). In contrast, granzyme K is expressed by comparable proportions of CD8$^+$ T cells from blood and brain (**Figure 6e–g**). Both granzyme B and K were predominantly produced by CD69$^-$CD103$^-$ and CD69$^+$CD103$^-$ T cells, with CD69$^+$CD103$^+$ cells showing a clearly smaller positive population for both enzymes (**Figure 6b,f**).

Next, we analyzed cytokines expressed by brain CD8$^+$ T cells upon activation. Almost all brain CD8$^+$ T cells produced IFN-γ (interferon gamma) and TNF-α (tumor necrosis factor alpha) upon stimulation with PMA/ionomycin (**Figure 6h–l**), with the majority being positive for both (**Figure 6r**). CD8$^+$ T cells positive for GM-CSF (granulocyte-macrophage colony-stimulating factor) and IL-17A (interleukin-17A) have been associated with neuroinflammation of multiple sclerosis.[35] A subset of brain CD8$^+$ T cells produced GM-CSF (**Figure 6m–o**), almost exclusively in addition to IFN-γ and TNF-α (**Figure 6R**). We could detect only low proportions IL-17A$^+$ brain CD8$^+$ T cells (**Figure 6p–q**). Expression of CD103 is not affected by stimulation with PMA/ionomycin (**Figure 6s**). Despite a similar production of IFN-γ and TNF-α, slightly more CD103$^+$ CD8$^+$ brain T cells produced GM-CSF when compared to CD103$^-$ cells (**Figure 6o**). Although being attributed pleiotropic functions, GM-CSF is predominantly known as an important activation signal for monocytes/macrophages.[36,37] Therefore, despite producing less cytolytic enzymes directly *ex situ*, CD103$^+$ brain CD8$^+$ T cells displayed after stimulation a polyfunctional cytokine profile (**Figure 6t**) capable to activate myeloid cells.

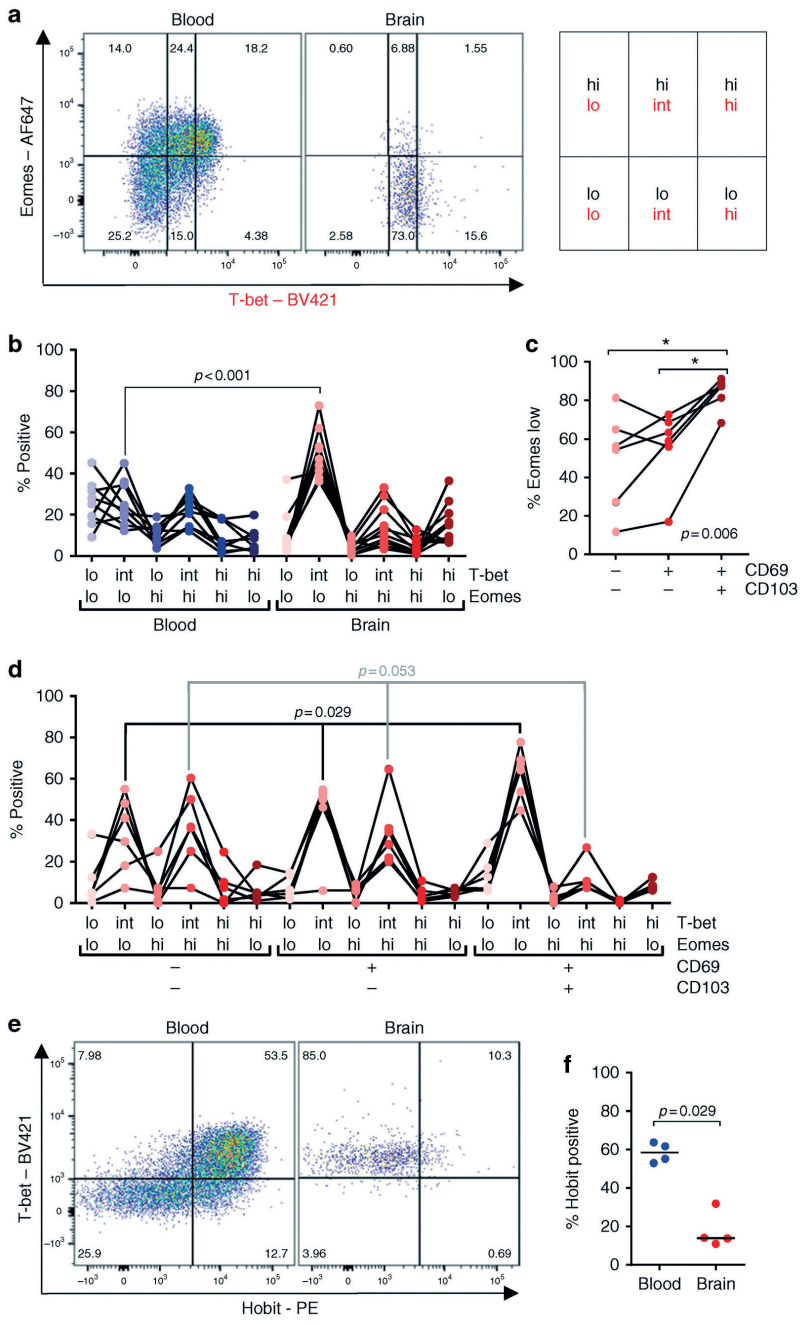

Figure 5. Human brain CD8⁺ T cells show a predominant T-bet-intermediate/eomes-low phenotype.
a. Dot plot showing the gating strategy T-bet and eomes in a paired blood and brain sample. T-bet and eomes co-expression (lo/lo > int/lo > lo/hi > int/hi > hi/hi > hi/lo) correlates in virus-specific CD8⁺ T-cell with differentiation from central memory to terminally differentiated effector cells, respectively.[15] b. Comparison between blood and brain CD8⁺ T_{RM} cells, and stratification based on CD69 and CD103 co-expression of brain eomes low (c) and all T-bet/eomes subsets (d). e. Dot plot of T-bet and Hobit co-expression by CD8⁺ T cells from brain and blood of a donor. f. Quantification of Hobit-positive cells is shown. Bars show median values. *p*-values show Mann-Whitney U test (b,f) and Friedman test with Wilcoxon signed ranks as post-hoc test (c,d) (**p* <0.05, ***p* <0.01); no brackets indicate no significant difference.

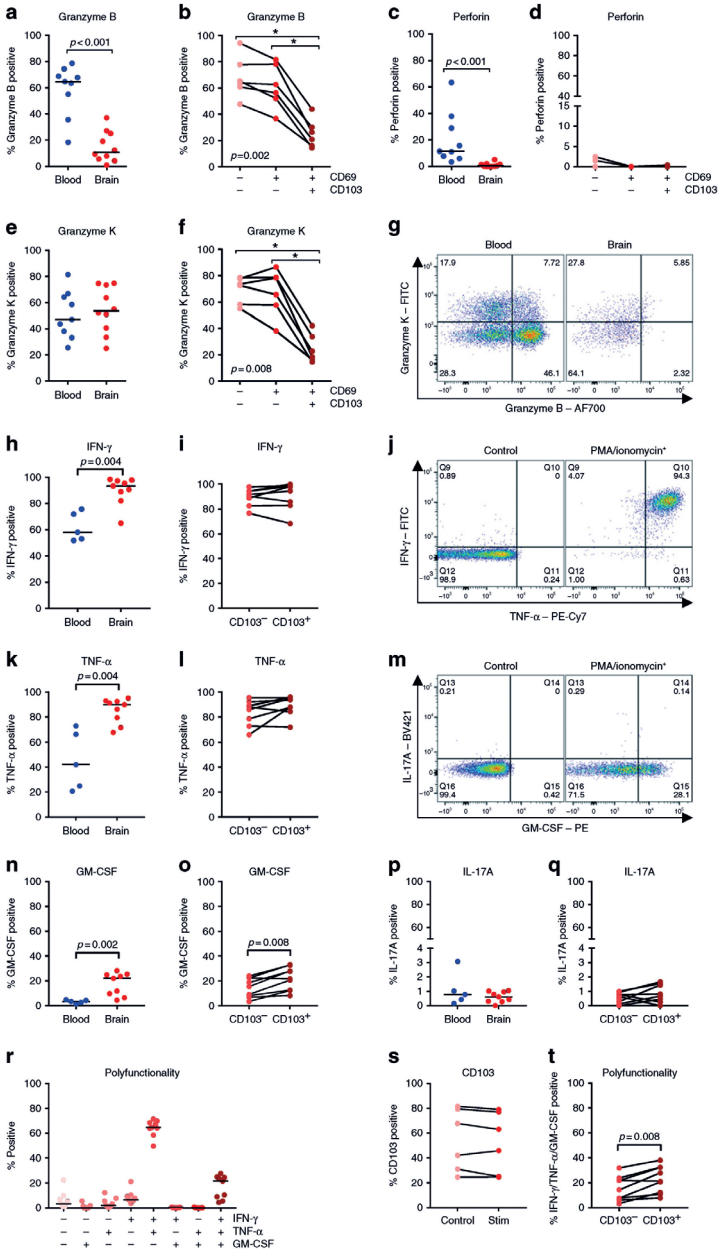

Figure 6. CD103⁺ brain CD8⁺ T cells express few cytolytic enzymes, but show a polyfunctional inflammatory cytokine profile.

a–f. Quantification of the percentage of CD8⁺ T cells directly *ex situ* expressing granzyme B, perforin, and granzyme K, respectively. g. Representative dot plots of $CD3^+CD8^+$ T cells stained for granzyme B and granzyme K. h–i, k–l, n–q. Quantification of the percentage of brain CD8⁺ T cells positive for IFN-γ, TNF-α, GM-CSF, and IL-17A after stimulation with PMA/ionomycin in vitro. j,m. Dot plot of PMA/ionomycin-stimulated $CD3^+CD8^+$ T cells stained for the respective cytokines. r. Quantification of IFN-γ, TNF-α, and GM-CSF co-expression, t stratified for expression CD103. s. CD103 expression in brain CD8⁺ T_{RM} control and PMA/ionomycin-stimulated cells. *p*-values show Mann-Whitney U test (a,c,e,h,k,n,p), Friedman test for paired data with Wilcoxon signed ranks as post-hoc test (b,d,f), or Wilcoxon signed ranks test (i,l,o,q,s,t) (*p <0.05, **p <0.01); no brackets indicate no significant difference.

Brain CD8$^+$ T cells express PD-1 and CTLA-4

Since granzyme B is highly neurotoxic,[38] we hypothesized the levels to be suppressed by signals from the brain microenvironment and analyzed the expression of inhibitory molecules. PD-1 (programmed death-1), a central regulator that preserves CD8$^+$ T cells from overstimulation, excessive proliferation, and terminal differentiation,[39] is highly expressed in brain CD8$^+$ T cells, most prominently in the CD69$^+$CD103$^+$ subset (**Figure 7a–c**). Expression of CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), a homolog of CD28, which blocks T-cell activation,[40] is even more highly expressed in brain CD8$^+$ T cells, again most prominently in the CD69$^+$CD103$^+$ subset (**Figure 7d–f**). This is in line with the high CTLA-4 and PD-1 expression found in the core phenotypic signature of CD103$^+$ T_{RM} cells.[27,30] Systemic treatment with soluble CTLA-4 has been associated with a suppression of CD8$^+$ T-cell granzyme B production in mice infected with lymphocytic choriomeningitis virus.[41] Conversely, in splenocytes of acute Friend retrovirus-infected mice, virus-specific PD-1-positive CD8$^+$ T cells produce the largest amounts of granzyme B.[42] The availability of the ligands for PD-1 or CTLA-4 may determine their effect on granzyme expression. We performed immunohistochemical stainings of normal white matter of two brain donors for CD86 (ligand of CTLA-4) and PD-L1 (ligand of PD-1) and found no immunostaining for either one (**Figure 7g,h**). To assess the effect of inflammation on CD86 and PD-L1 expression, we stained an HLA-DR-positive active demyelinating MS lesion and found microglia-like cells to stain positive for CD86 (**Figure 7i**) and reactive astrocyte-like cells to stain positive for PD-L1 (**Figure 7j**). These findings suggest that CTLA-4 and PD-1 may provide important inhibitory signals to brain CD8$^+$ T cells in inflammatory conditions.

Brain CD4$^+$ T_{RM} cells express low levels of CD103

In accordance with their earlier described T_{EM}-like phenotype,[14] brain CD4$^+$ T cells were high in expression of CD45R0 and showed a low expression of CD27, CD45RA, CCR7, and CD28 (**Suppl. Figure 5a–f**). In peripheral blood, CD4$^+$ T cells with low CD27, CD28, and CCR7 expression associate with viral infections and display cytotoxic functions.[43,44] However, production of lytic mediators granzyme B and perforin by brain CD4$^+$ T cells is low, while granzyme K is expressed at higher levels (**Suppl. Figure 5g–i**). Likewise their CD8$^+$ counterparts, brain-derived CD4$^+$ T cells show a high CD69 expression (**Figure 8a**). The CD69$^+$ CD4$^+$ T-cell fraction is not enriched for activation markers Ki-67, IL-2 receptor alpha-chain (IL-2Rα, CD25), or HLA-DR/CD38 co-expression (**Suppl. Figure 6**). In contrast to brain CD8$^+$ T cells, CD103 was sparsely expressed on brain derived CD4$^+$ T cells (**Figure 8b–d**). A restricted expression of CD103 on CD4$^+$ T_{RM} cells has previously been described in several tissues.[27,45,46]

We analyzed the expression of several other T_{RM}-cell phenotypic markers on CD69$^-$ and CD69$^+$ brain CD4$^+$ T cells (**Figure 8e–l**). CD69$^+$ CD4$^+$ cells are enriched for expression of CD49a, PD-1, and CXCR6, which have been identified as the core-signature of CD4$^+$ T_{RM} cells in multiple tissues.[27] Additionally, a higher expression of CTLA-4, CCR5, and CXCR3 is observed, which was earlier reported in human lung CD69$^+$CD103$^{+/-}$ CD4$^+$ T_{RM} cells.[34] A low expression of CX_3CR1 has been associated with a CD4$^+$

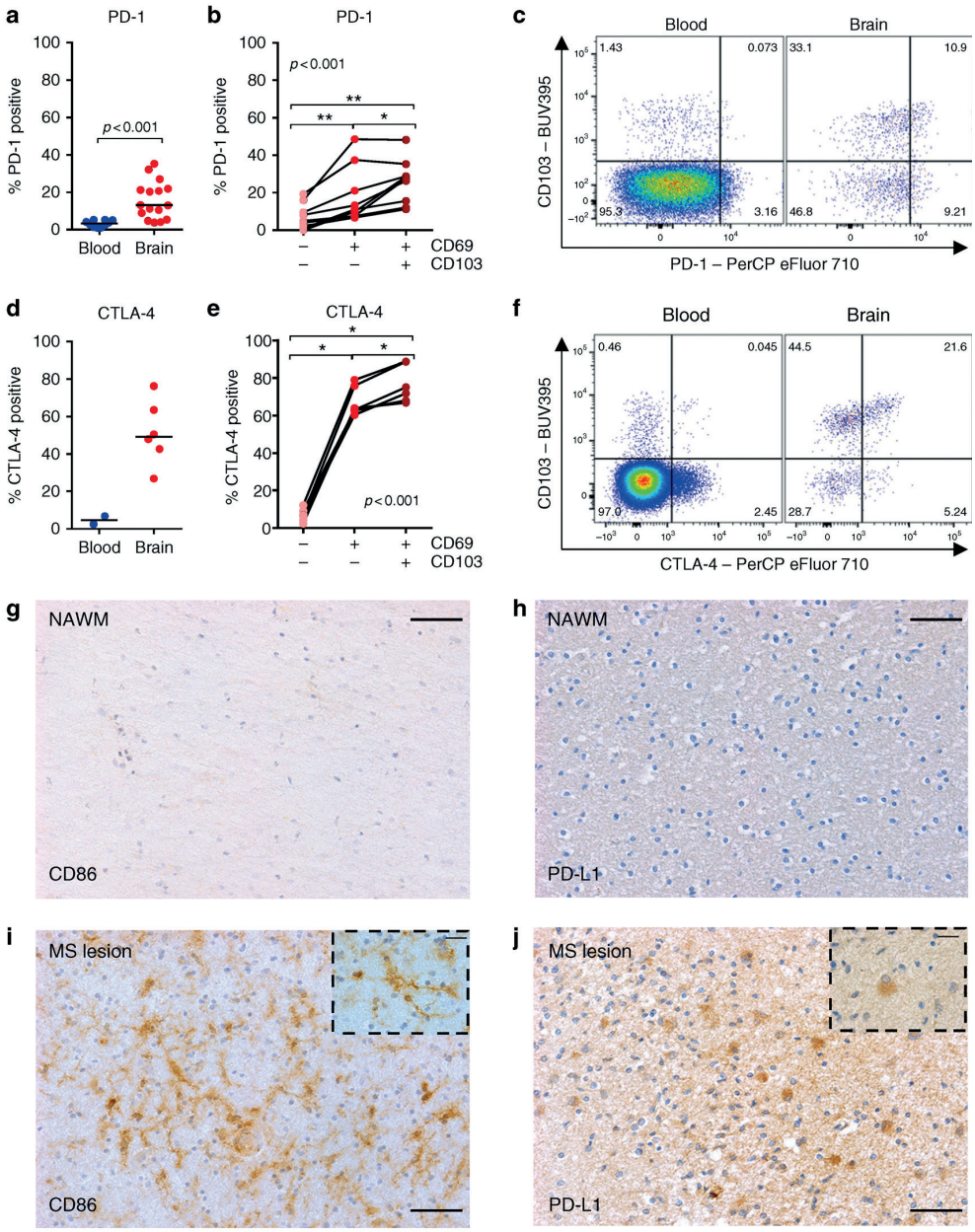

Figure 7. Enrichment CTLA-4 and PD-1 expression on brain CD8⁺ T cells.
a,b,d,e. Quantification of the percentage of $CD8^+$ T cells expressing PD-1 and CTLA-4, respectively. Bars show median values. *p*-values show Mann-Whitney U test for unpaired data (a,d) and Friedman test for paired data with Wilcoxon signed ranks as post-hoc test (b,e) (*p <0.05, **p <0.01); no brackets indicate no significant difference. Representative dot plots of $CD3^+CD8^+$ lymphocytes stained for c PD-1 and CD103 and f CTLA-4 and CD103. Immunohistochemical staining for g CD86 (ligand of CTLA-4, brown) and h PD-L1 (ligand of PD-1, brown) in a donor with Alzheimer's disease and a donor without brain disease showed no staining (scale bar = 50 µm). However, in an HLA-DR-positive active, demyelinating MS lesion of a donor with MS, specific staining for i CD86 and j PD-L1 was found in microglia and astrocyte-like cells, respectively (scale bar = 50 µm; scale bar insert = 20 µm).

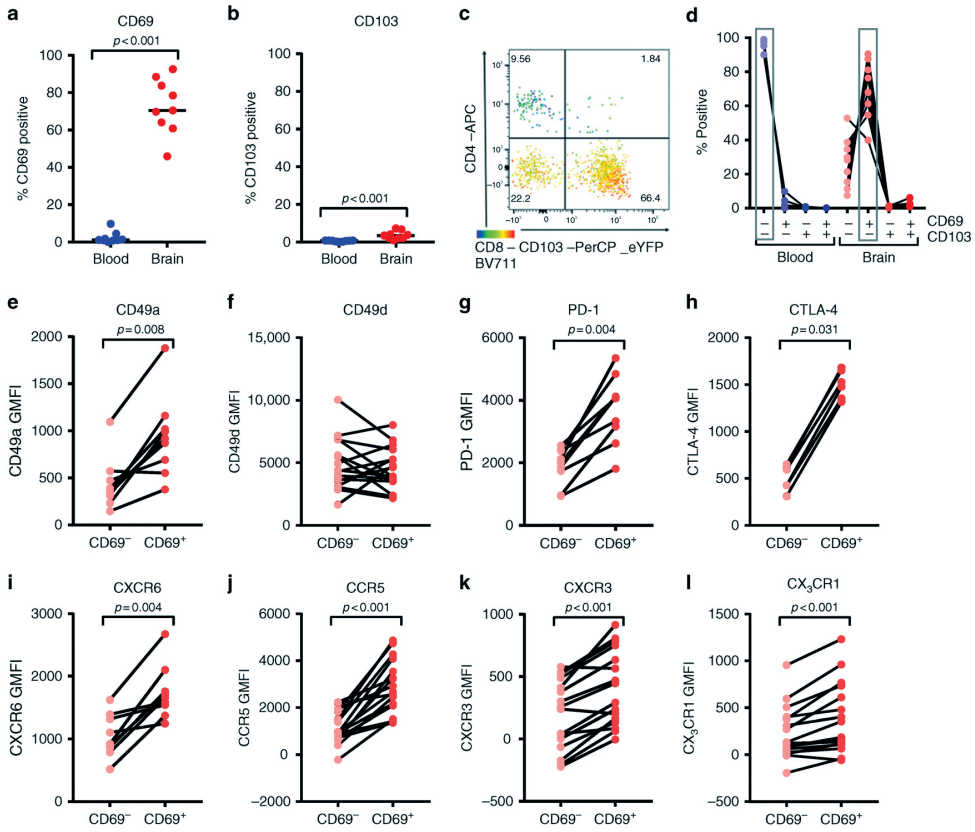

Figure 8. Human brain $CD4^+$ $CD69^+$ T cells are enriched for core phenotypic T_{RM}-cell markers.
a–b,d. Quantification of CD69 and CD103 (co-)expression. *p*-values show Mann-Whitney U test. c. Representative dot plot of CD4, CD8, and CD103 staining of $CD3^+$ $CD69^+$ T cells. e–l. Quantification of $CD4^+$ T-cell expression levels of CD49a, CD49d, PD-1, CTLA-4, CXCR6, CCR5, CXCR3, and CX_3CR1 (GMFI, geometric mean fluorescence intensity). *p*-values show the Wilcoxon signed ranks test.

T_{RM}-cell phenotype.[27] However, the $CD4^+$ T cells we isolated rather displayed an enrichment for CX_3CR1, as we observed for brain $CD8^+$ T cells. $CD4^+$ $CD69^+$ T cells were not enriched for CD49d, which activated $CD4^+$ T cells use to migrate to an inflamed blood brain barrier.[28]

DISCUSSION

Presented here is a phenotypic and functional profile of T cells in the human brain. Among $CD8^+$ cells, expression of differentiation markers, integrins, chemokine receptors, transcription factors, granzymes, cytokines, and immune checkpoint molecules revealed a profile that matches the core phenotypic and transcriptional signature of T_{RM} cells, including the presence of a $CD103^+CD69^+$ and $CD103^-CD69^+$ sub-population. Therefore, we further consider these cells as brain $CD8^+$ T_{RM} cells. About half of all brain $CD8^+$ $CD69^+$ T_{RM} cells express CD103. $CD8^+$ $CD69^+$ T_{RM} cells that express CD103 showed increased expression of chemokine receptors (CX_3CR1, CXCR3, CCR5, CXCR6),

reduced production of cytolytic enzymes (granzyme B and K), and increased presence of inhibitory receptors (CTLA-4 and PD-1), compared to cells that lack CD103. This profile suggests a greater propensity of migration from the perivascular space into the brain parenchyma. $CD4^+$ $CD69^+$ T cells were also enriched for T_{RM} cell-associated integrins, chemokine receptors, and inhibitory molecules, except for CD103. While the finding of phenotypically distinct $CD69^+$ T_{RM}-cell subsets provides a new perspective to the immune surveillance of the human CNS, we do at present only poorly understand the drivers of these diverse phenotypes. Unravelling their kinetics and regulation may provide tools to modulate brain T-cell behavior for the benefit of patients suffering from infectious, inflammatory, or neoplastic conditions in the CNS.

CD69-expressing $CD8^+$ T_{RM}-cell subsets differ across human tissues; in the colon and the lung, most of these cells co-express CD103.[5] The equal distribution between $CD103^-$ and $CD103^+$ $CD8^+CD69^+$ T_{RM} cells that we found in the human brain is also seen in the mouse.[13] We speculate that anatomic compartmentalization may be a contributing factor. The periventricular space is an important immuno-active compartment between the blood–brain barrier and the brain parenchyma, were activated T cells and antigen-presenting cells reside and interact.[7] This is a different, immunologically vibrant microenvironment, compared to the brain parenchyma, where extensive immune activation can be harmful for neurons with a limited regenerative capacity. Mediators of granule exocytosis-mediated cytotoxicity, such as perforin, granzyme A, and granzyme B, are highly neurotoxic.[38,47,48] Release of these lytic enzymes should be under tight control, whilst maintaining the capability to elicit a fast inflammatory response when a neurotropic virus threatens the CNS. This profound inflammatory potential of brain $CD8^+$ T cells is highlighted by the substantial production of IFN-γ, TNF-α, and even GM-CSF upon activation. The polyfunctional inflammatory cytokine profile of human T_{RM} cells was previously also observed for lung $CD103^+$ and $CD103^-$ T_{RM} cells,[49] and mouse cytomegalovirus- and *Toxoplasma gondii*-specific $CD8^+$ brain T_{RM} cells also produced IFN-γ, and TNF-α.[50,51] GM-CSF may be of particular relevance to T_{RM} cells residing in the white matter, since microglia express the GM-CSF-receptor complex and are readily activated by GM-CSF.[52] GM-CSF-immunoreactive $CD8^+$ T cells have also been observed in the context of MS white matter lesions.[35]

Besides anatomic localization, functional differences implicate that antigen exposure may also underlie the distinct features of $CD103^-$ and $CD103^+$ $CD8^+$ T_{RM} cells. We report a consistent profile of surface markers and transcription factors, which points to an earlier phase of differentiation in $CD103^+$ compared to $CD103^-CD8^+$ T_{RM} cells. The exact antigens against which these cells are directed are at present poorly defined, but do most likely comprise neurotropic viruses.[9] Notably, resident $CD8^+$ T cells appear after herpes simplex virus infection in sensory ganglia and trigeminal ganglia, preventing reactivation in latent infection.[53,54] In mice, $CD103^+$ $CD8^+$ T_{RM} cells appeared in the CNS after a vesicular stomatitis virus encephalitis[12] and chronic *Toxoplasma gondii* infection,[51] and congenital infection with mouse cytomegalovirus induced a $CD103^+$ and $CD103^-$ $CD8^+$ T_{RM}-cell population in the brain parenchyma.[50] Contrastingly, autologous Epstein-Barr virus-infected B cell-reactive $CD8^+$ T cells isolated from white matter lesions of MS patients did not show

immunostaining for CD103 *in situ*.[55] Therefore, history of primary infection may also shape the composition of the brain $CD8^+$ T_{RM}-cell pool. The reactivity of these cells to antigens of these and other viruses remains to be determined. In addition to specificity, also the strength of antigenic stimulation during recruitment of $CD8^+$ T cells may influence the composition of brain T_{RM} cells.[56]

Of note, PD-1, an immunological checkpoint for T-cell differentiation, is prominently expressed, with highest levels on $CD103^+$ cells. Presence of PD-1 on the majority of $CD8^+$ T_{RM} cells has been described for other tissues, including eye, skin, lung, liver, and kidney,[5,17,27,57,58] and may be a general characteristic of the transcriptional program of T_{RM} cells. Whether PD-1 has a critical role in restricting the cytotoxic capacity and cytokine production of brain $CD8^+$ T_{RM} cells, thereby controlling excessive immunopathology, remains to be shown. A case of white matter T-cell infiltration with demyelination and macrophage activation in a patient after four courses of the PD-1 immune checkpoint inhibitor nivolumab has been reported.[59] Cases of bilateral, internuclear ophthalmoplegia and cerebellar ataxia have also been reported in PD-1 inhibitor-treated patients, without the neuroradiological substrate being specified60. Of note, human CNS cells express low levels of PD-L1 and PD-L2, the ligands of PD-1, under basal conditions, but upregulate PD-L1 under inflammatory conditions.[61] Likewise, we found no immunostaining for PD-L1 in non-inflamed white matter, with an induction associated with inflammation. PD-1 on $CD8^+$ T_{RM} cells may support CNS homeostasis by preventing uncontrolled T-cell reactivity, which is a risk factor in (auto) inflammatory conditions, including multiple sclerosis.[62]

CTLA-4 is expressed by both $CD4^+$ and $CD8^+$ T cells following activation and has as main function the regulation of CD28 signaling.[40] In murine brain-derived $CD103^+$ $CD8^+$ T_{RM} cells, an upregulation of CTLA-4 has been reported earlier.[11] Downregulation of CD28 with high expression of CTLA-4 reflects a profound inhibition of this co-stimulatory pathway, which may be instrumental in the tight control of T cell-mediated inflammation in the brain parenchyma. Interestingly, treatment of patients with the CTLA-4 immune checkpoint inhibitor ipilimumab has been associated with the occurrence of inflammatory demyelinating white matter lesions with T-cell infiltrates.[63–65] Immunostaining for CD86 was also induced in glia in an inflammatory active MS lesion and absent in non-inflamed tissue. Likewise, mouse microglia did not express CD86 when analyzed directly *ex vivo*, however this was spontaneously upregulated after culture.[66] This suggests a microenvironment *in situ* in which expression of CTLA-4's main ligand is also tightly regulated.

Brain $CD4^+$ T cells also displayed T_{RM}-cell surface markers previously identified on $CD4^+$ T_{RM} cells in other tissues.[27,34] Historically, $CD8^+$ T cells were regarded as the key-players in antiviral immunity, with $CD4^+$ T cells being mediators of the adaptive immune response against extracellular pathogens. In recent years, this dogma has been challenged by several studies: $CD4^+$ T cells facilitate recruitment of other lymphocytes into lymph nodes or sites of infection, provide help to $CD8^+$ T cells and antibody-producing B cells, and offer direct effector function through production of cytokines or lytic enzymes.[67] Although surface markers on brain $CD4^+$ T cells suggest a cytotoxic

phenotype, cytotoxic capacity is restricted. Granzyme B and perforin are highly neurotoxic, while granzyme K by itself mediates limited cytotoxicity and rather acts as proinflammatory signal.[68] The functional role of $CD4^+$ T_{RM} cells in the CNS requires further study.

The major novelty and strength of this study is the analysis of post-mortem brain-derived T cells with flow cytometry directly *ex vivo*. For primary human microglia isolated with a similar procedure, culture *in vitro* modified their phenotype.[69] Comparable effects are unlikely to bias our results. However, previous work in mice showed isolation of T cells from tissue for flow cytometry to affect quantity and phenotypic characteristics when compared to imaging *in situ*.[24] Furthermore, our study was not designed, and thereby neither powered nor matched, to explore differences in T-cell phenotypic profiles between individual brain diseases.

Together, we for the first time demonstrate human brain-derived T cells to harbor T_{RM}-cell features. Gained insights may help understanding how these cells mediate local protection and may be used to study how brain T_{RM} cells contribute to neuroinflammatory and neurodegenerative diseases.

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Conflict of interest

The authors declare no conflict of interest.

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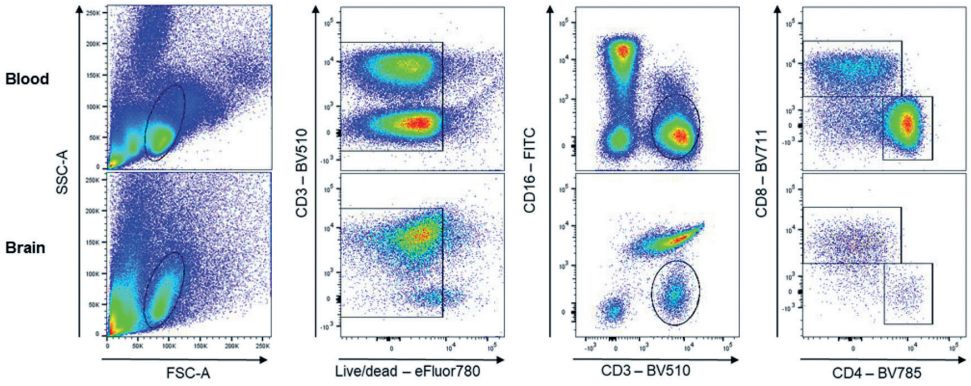
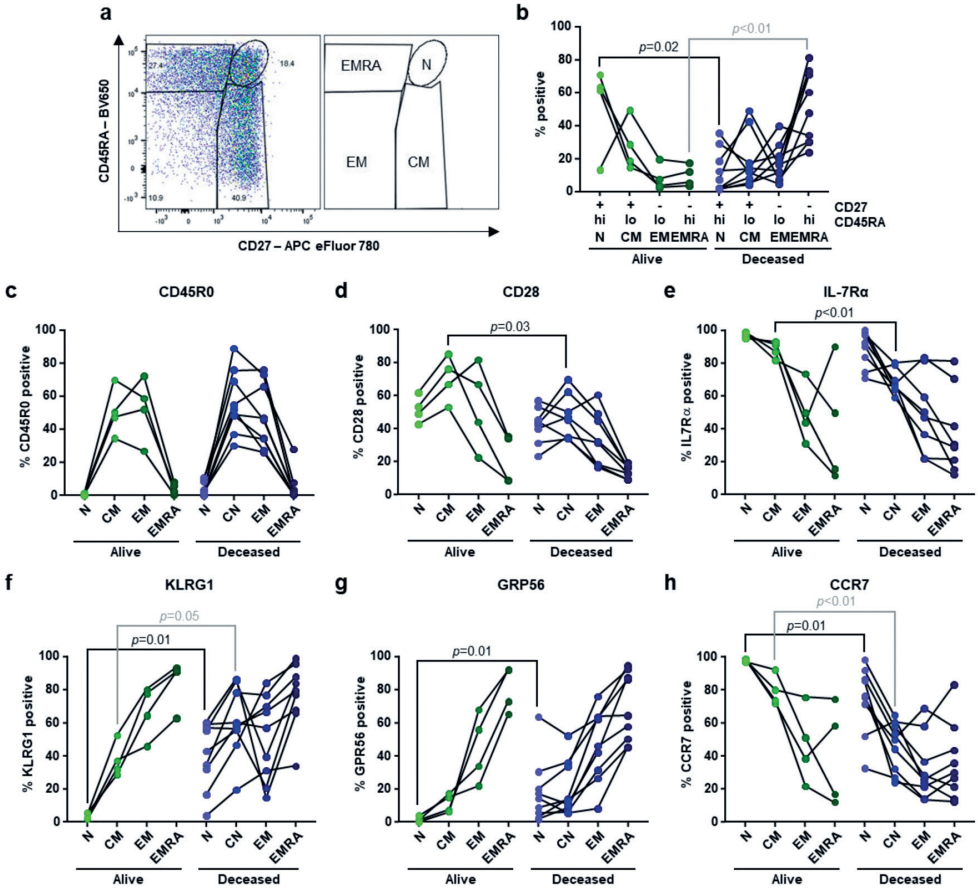
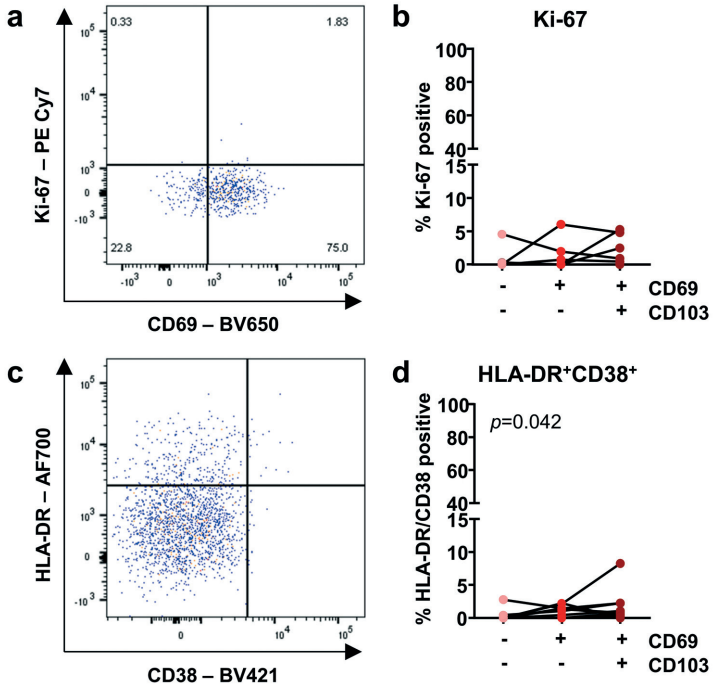


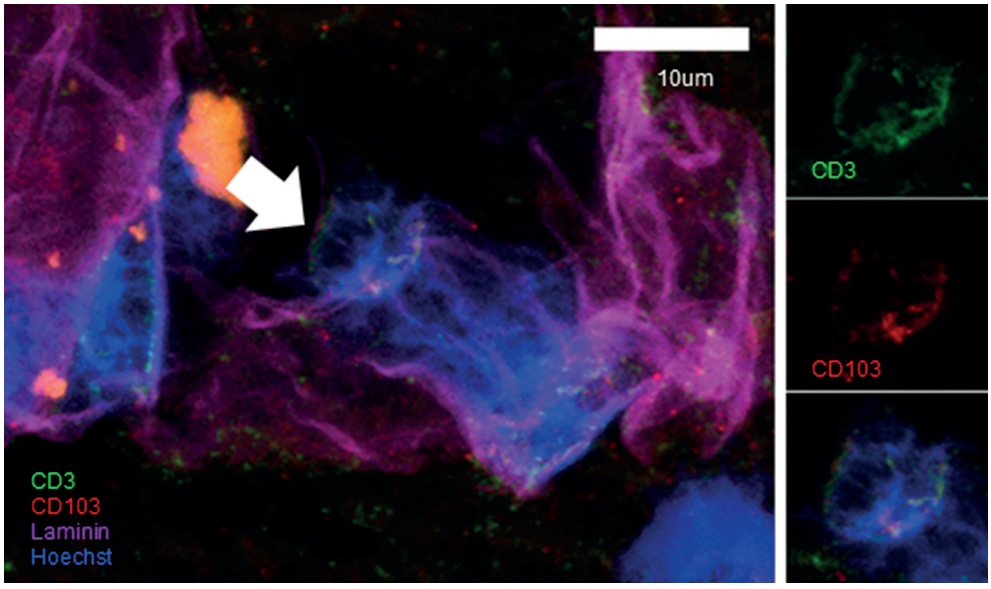
Supplementary Figure 1. Example of $CD8^+$ and $CD4^+$ T-lymphocyte gating strategy.
Paired blood- and brain-derived cells of a donor with Parkinson's disease. Panels from left to right show subsequent within-gate events. Exclusion of duplets (SSC-H*SSC-W and FSC-H*FSC-W) was performed but is not shown. Note the abundant viable brain cell population in the $CD3^+$ fluorescence range, which were not T cells and were gated from the T-cell population by either including an empty channel or by including non-T-cell markers or supra-physiological expression of T-cell markers in the gating procedure (in this example, CD16). Cells and markers were back-gated to ensure no relevant cell populations were lost by this strategy.



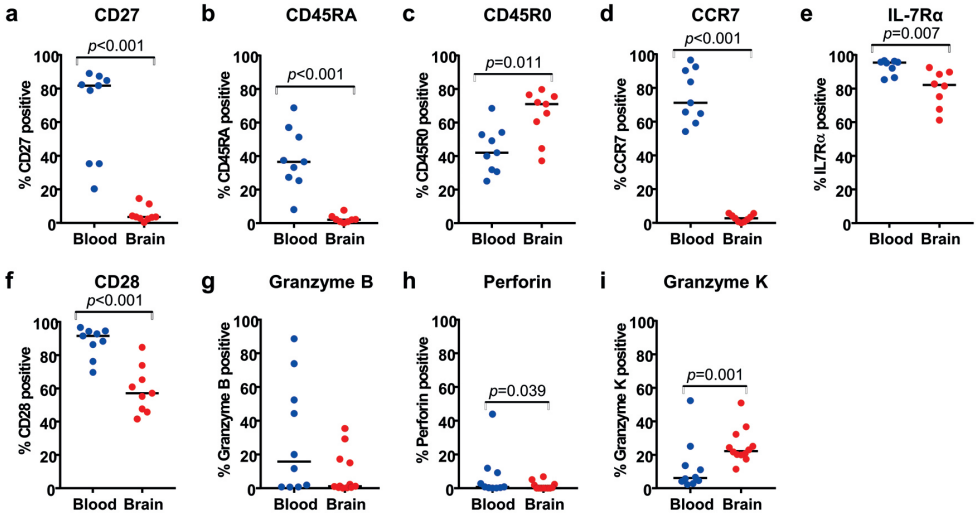
Supplementary Figure 2. T cells from deceased donors shows a further differentiated phenotype compared to healthy controls, with a similar profile of differentiation markers.
Comparison of PBMC from n=4 healthy controls with post-mortem blood of n=8–9 brain donors. (a) Gating strategy. (b) Quantification of $CD8^+$ T cells with a $CD27^+CD45RA^+$ (N, naïve), $CD27^+CD45RA^-$ (CM, central memory), $CD27^-CD45RA^-$ (EM, effector memory), and $CD27^-CD45RA^+$ (EMRA, $CD45RA^+$ effector memory) phenotype.[1] (c–h) Expression of CD45Ro, CD28, IL-7Rα, KLRG1, GPR56, and CCR7 stratified for CD27 and CD45RA co-expression, respectively. *p*-values show Mann-Whitney U test; the absence of brackets indicates the absence of a significant difference. Note the general similar profiles, although some differences exist. It is not clear whether these are due to post-mortem effects, age, (terminal) disease state, or the general higher level of antigen experience in brain donors.



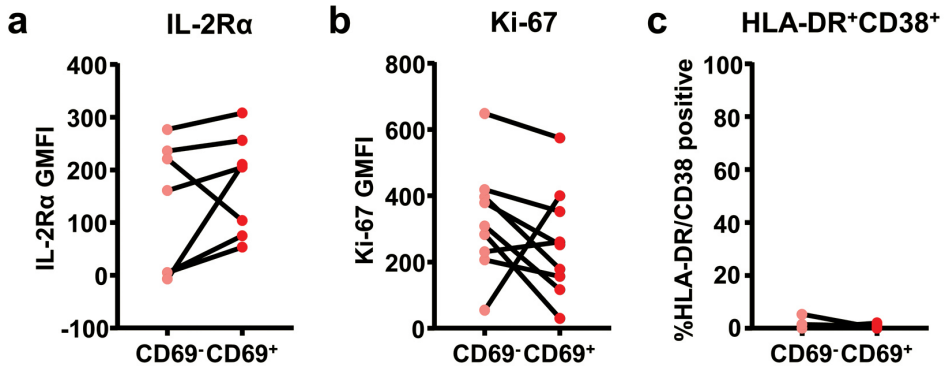
Supplementary Figure 3. Brain CD8$^+$ T cells contain very low proportions of Ki-67$^+$ and HLA-DR$^+$CD38$^+$ cells. Staining of brain CD8$^+$ T cells for Ki-67 (a) and HLA-DR/CD38 co-expression (c) in a donor, with quantification ((b) and (d), respectively). *p*-values show Friedman test (post-hoc Wilcoxon signed ranks showed no significant differences); no brackets indicate no significant difference. Note the substantial CD69 expression, while few cells are either Ki-67$^+$ or HLA-DR$^+$CD38$^+$.



Supplementary Figure 4. CD3 CD103 double-positive T cells reside in the perivascular space. Triple immunofluorescent labeling for CD3 (green), CD103 (red), and laminin (magenta) with Hoechst. The arrow marks a CD3 and CD103-positive cell in close association with the perivascular space (scale bar = 10 µm).



Supplementary Figure 5. Differentiated CD4$^+$ T cells populate the human brain.
Quantification of CD27, CD45RA, CD45Ro, CCR7, IL-7Rα, CD28, granzyme B, perforin, and granzyme K expression by brain-derived CD4$^+$ T cells and peripheral blood-derived CD4$^+$ T cells. *p*-values show Mann-Whitney U test, the absence of brackets indicates no significant difference.



Supplementary Figure 6. Human brain CD4$^+$ T-cell expression of activation markers IL-2Rα, Ki-67, and HLA-DR/CD38, stratified for CD69 expression.
The absence of brackets indicates the absence of a significant difference tested with the Wilcoxon signed ranks test.

Supplementary Table 1. Specifications of antibodies used for flow cytometry.

Specificity	Clone	Fluorochrome	Manufacturer
CD3	UCHT1	V500	BD
CD3	7D6	PE Cy5.5	eBioscience
CD4	SK3	BV421	BD
CD4	OKT4	BV510	Biolegend
CD4	SK3	APC	BD
CD8	RPA-T8	BV711	Biolegend
CD8	RPA-T8	BV785	Biolegend
0316	CLB-Fc-gran/1, 5D2	FITC	Sanquin
0325	BC96	APC-eFluor780	eBioscience
OD27	0323	APC-eFluor780	eBioscience
CD38	HIT2	BV421	Biolegend
CD45RA	HI100	BV650	BD
CD45R0	UCHL1	BV785	Biolegend
CD49a/ITGA1	TS2/7	APC-Vio770	Miltenyi Biotec
CD49D/VLA4	9F10	PE-Cy7	Biolegend
OD57	HCD57	PE	Biolegend
0369	FN50	BV650	Biolegend
CD69	FN50	BV421	Biolegend
CD69	FN50	BV786	BD
CD103	Ber-Act8	BUV395	BD
CD103	Ber-Act8	PerCP-eFluor710	eBioscience
IL-7Rα (CD127)	eBioRDR5	PE-Cy7	eBioscience
CCR5 (CD195)	Hek/1/85a	AF700	Biolegend
CCR7 (CD197)	150503	BUV395	BD
CXCR3 (CD183)	G025H7	PerCP-Cy5.5	Biolegend
CXCR6 (CD186)	K041E5	PE-Cy7	Biolegend
CX_3CR1	2A9-1	PE	eBioscience
Eomes	WD1928	AF647	eBioscience
GM-CSF	BVD2-21C11	PE	BD
GPR56	CG4	PE	H.H. Lin, Chang Gung University, Tao-Yuan, Taiwan[2]
Granzyme B	GB11	AF700	BD
HLA-DR	LN3	AF700	eBioscience
Hobit[A]	Hobit/1	PE	K.P.J.M. van Gisbergen, Sanquin Research, Amsterdam, The Netherlands[3]
IFN-γ	25723.11	FITC	BD
IL-17A	BL-168	BV421	Biolegend
Ki-67	B56	PE-Cy7	BD
KLRG1	13A2	AF488	H. Pircher, University of Freiburg, Germany[4]
Perforin	DG9	PerCP-eFluor710	eBioscience
T-bet	4B10	BV421	Biolegend
TNF-α	MabII	PE-cy7	eBioscience

[A] Mouse IgM antibody detected with mouse-anti-mouse IgM-PE.

SUPPLEMENTARY REFERENCES

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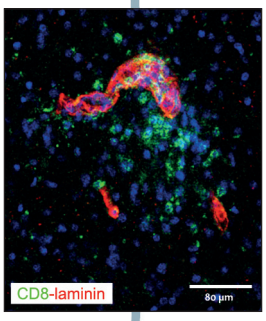
Addendum: letters to the editor of *Brain*

Tissue-resident CD8$^+$ memory T cells in multiple sclerosis
Brain. 2021 Jan; 144(1): e7
Hohlfeld, R, Beltran E, Gerdes LA, Dornmair K

Reply: Tissue- resident CD8$^+$ memory T cells in multiple sclerosis
Brain. 2021 Jan; 144(1): e8
Smolders J, **Fransen NL**, Huitinga I, Hamann J

CHAPTER 5

Tissue resident memory T cells invade the brain parenchyma in multiple sclerosis white matter lesions



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ABSTRACT

Background | Multiple sclerosis is a chronic inflammatory, demyelinating disease, although it has been suggested that in the progressive late phase, inflammatory lesion activity declines. We recently showed in the Netherlands Brain Bank multiple sclerosis-autopsy cohort considerable ongoing inflammatory lesion activity also at the end stage of the disease, based on microglia/macrophage activity. We have now studied the role of T-cells in this ongoing inflammatory lesion activity in chronic multiple sclerosis autopsy cases.

Methods | We quantified T-cells and perivascular T-cell cuffing at a standardized location in the medulla oblongata in 146 multiple sclerosis, 20 neurodegenerative control and 20 non-neurological control brain donors. In addition, we quantified $CD3^+$, $CD4^+$, and $CD8^+$ T-cells in 140 subcortical white matter lesions. The location of $CD8^+$ T cells in either the perivascular space or the brain parenchyma was determined using CD8/laminin staining and confocal imaging. Finally, we analyzed $CD8^+$ T-cells, isolated from fresh autopsy tissues from subcortical multiple sclerosis white matter lesions (n=8), multiple sclerosis normal-appearing white matter (n=7), and control white matter (n=10), by flow cytometry.

Results | In normal-appearing white matter, the number of T-cells was increased compared to control white matter. In active and mixed active/inactive lesions, the number of T-cells was further augmented compared to normal-appearing white matter. Active and mixed active/inactive lesions were enriched for both $CD4^+$ and $CD8^+$ T-cells, the latter being more abundant in all lesion types. Perivascular clustering of T-cells in the medulla oblongata was only found in cases with a progressive disease course and correlated with a higher percentage of mixed active/inactive lesions and a higher lesion load compared to cases without perivascular clusters in the medulla oblongata. In all white matter samples, $CD8^+$ T-cells were located mostly in the perivascular space, whereas in mixed active/inactive lesions, 16.3% of the $CD8^+$ T-cells were encountered in the brain parenchyma. $CD8^+$ T-cells from mixed active/inactive lesions showed a tissue-resident memory phenotype with expression of CD69, CD103, CD44, CD49a, and PD-1 and absence of S1P1. They upregulated markers for homing (CXCR6), re-activation (Ki-67), and cytotoxicity (GPR56), yet lacked the cytolytic enzyme granzyme B.

Conclusions | These data show that in chronic progressive multiple sclerosis cases, inflammatory lesion activity and demyelinated lesion load is associated with an increased number of T-cells clustering in the perivascular space. Inflammatory active multiple sclerosis lesions are populated by $CD8^+$ tissue-resident memory T-cells, which show signs of re-activation and infiltration of the brain parenchyma.

INTRODUCTION

Relapsing–remitting multiple sclerosis is at its onset characterized by a relatively high frequency of clinical exacerbations and gadolinium-enhancing lesions on brain MRI.[31,56,53] These phenomena are believed to reflect waves of inflammatory cells trafficking from the circulation through the blood brain barrier into the CNS causing focal demyelinating lesions. Invading T cells have been proposed to play a central role in the early disease process of MS.[11] At later stages, both relapse rate and lesion enhancement often decline to near zero.[31,57,54] Patients with advanced multiple sclerosis often experience a continuous deterioration of neurological functions referred to as progressive multiple sclerosis.[53] Clinical trials of drugs targeting circulating leukocytes are mostly negative on their primary endpoints in progressive multiple sclerosis. Advanced multiple sclerosis is therefore sometimes referred to as a disease driven by neurodegenerative mechanisms, rather than inflammatory mechanisms.[29]

When performing an immunohistochemical analysis of a large collection of post-mortem multiple sclerosis-brains, we encountered mixed active/inactive (previously referred to as chronic active) lesions containing HLA^+ macrophages in 78% of brain donors with advanced MS.[27] In this autopsy cohort, the percentage of mA/I lesions correlated with a faster accrual of multiple sclerosis-related disability.[27] Earlier studies suggest a role of T cells in this ongoing inflammatory response in chronic multiple sclerosis patients, as perivascular inflammatory infiltrates are found in association with mixed active/inactive lesions, specifically in secondary progressive multiple sclerosis cases.[39] These T cells mostly express CD8[5,19,16,35,28] and display signs of clonal expansion.[4] Whether T cells also critically contribute to the ongoing inflammation in advanced multiple sclerosis, where signs of trafficking immune cells through the blood-brain barrier are generally lacking, is uncertain.

Recently, we characterized T cells in normal human brain white matter by flow-cytometric analysis of rapid post-mortem autopsy tissue.[44,45] We observed low numbers of predominantly $CD8^+$ T cells, almost exclusively residing in the perivascular space (PVS), with a phenotype resembling the core profile of tissue-resident memory T (T_{RM}) cells.[44] In several tissues, viral antigen-specific $CD8^+$ T_{RM} cell populations that arise after a first viral infection are characterized by a set of surface markers including the C-type lectin CD69 and the alpha E integrin CD103. These cells do not recirculate but are retained locally to become reactivated in the case of renewed viral exposure.[51] CNS T_{RM}-cell populations also develop after experimental neurotropic virus infection.[1,41,43] Evidence suggests that MS-associated T-cell populations express markers associated with T_{RM} cells. Sørensen et al. found both CXC chemokine receptor (CXCR)3 and C-C chemokine receptor (CCR)5 expression by perivascular cuff and lesional T cells.[47] Machado-Santos et al. described a loss of the recirculation markers sphingosine-1-phosphate receptor 1 (S1P1) and CCR7, and an upregulation of CD103 and CCR5, but not CD69, by lesional $CD8^+$ T cells.[28] Contrastingly, Van Nierop et al. found no expression of CD103 but rather expression of CD69 by $CD8^+$ T cells.[35] Whether white matter lesion-associated T cells are bona fide T_{RM} cells and contribute as such to chronic multiple sclerosis lesion activity remained to be consolidated.

We here used a combination of immunohistochemistry and flow cytometry to study the localization, quantity, and phenotype of $CD8^+$ T cells in association with multiple sclerosis normal-appearing white matter and white matter lesions.

MATERIAL AND METHODS

Donors and tissue characteristics

One hundred and forty six multiple sclerosis brain donors from the Netherlands Brain Bank were included in the analysis of T cells in multiple sclerosis lesions. Informed consent was given by the donors for brain autopsy and for the use of material and clinical data for research purposes, in compliance with national ethical guidelines. The Netherlands Brain Bank autopsy procedures were approved by the Medical Ethics Committee of the VU Medical Center, Amsterdam, The Netherlands. The donors came to autopsy between 1991 and 2015. The clinical diagnosis of MS was confirmed for all patients, and the clinical course was defined as relapsing (for both relapsing–remitting and progressive relapsing cases), secondary progressive, or primary progressive by a certified neurologist according to McDonald or Poser criteria. The diagnosis MS was confirmed by a certified neuropathologist.

T cells and perivascular cuffing were examined in the standardly dissected brainstem at the level of the medulla oblongata. The brainstem allowed a standardized comparison between multiple sclerosis autopsy cases in a functional important white matter tract. Brainstem tissue blocks were obtained from 146 MS cases, 20 Alzheimer's disease cases (Braak score >5), and 20 non-neurological controls (**Table 1**). To correlate T-cell numbers with stages of multiple sclerosis lesion pathology, subcortical white matter lesions were studied. Subcortical white matter tissue blocks from 57 MS donors containing 140 multiple sclerosis white matter lesions were obtained together with subcortical white matter from 20 Alzheimer's disease cases (Braak score >5) and

Table 1. Donor and sample information for immunohistochemistry.

Diagnosis	**Cases** (n)	**Age** (years)	**Sex** (F/M)	**PMD** (h:min)	**pH value**	**Brain weight** (g)	**Disease duration** (years)
Multiple sclerosis	146	64.6 ± 13.1	94/52	8:58 ± 6:12	6.5 ± 0.3	1202 ± 146	30.0 ± 13.4
Relapsing-remitting	15	64.2 ± 16.3	10/5	11:12 ± 13:18	6.5 ± 0.4	1214 ± 100	24.9 ± 11.8
Primary progressive	49	67.9 ± 13.0	31/18	8:06 ± 2:27	6.5 ± 0.3	1194 ± 132	28.6 ± 12.3
Secondary progressive	82	62.8 ± 12.2	53/29	9:04 ± 5:45	6.5 ± 0.3	1186 ± 141	31.8 ± 14.2
Neurodegenerative controls (Alzheimer's disease)	20	65,2 ± 6,35	11/9	5:37 ± 1:41	6.5 ± 0.2	1125 ± 183	-
Non-neurological controls	20	63.6 ± 11.0	14/6	9:08 ± 4:19	6.5 ± 0.4	1270 ± 175	-

Values are provided as mean ± SD (standard deviation). PMD = post-mortem delay.

18 non-neurological controls. Treatment status for immunomodulatory therapies (fingolimod, natalizumab, or interferon beta) in the year before death was obtained from the clinical files. From the in total 146 multiple sclerosis cases, one had received fingolimod.

Biopsy tissue sections containing inflammatory active multiple sclerosis lesions from six donors were made available by the Institute for Neuropathology, University Hospital Münster, Germany) upon approval by the local Medical Ethics Committee. For donor information, see in **Suppl. Table 1**.

Characterization of multiple sclerosis lesion activity and perivascular T cell cuffing

All brainstem and subcortical white matter tissue sections were immunostained for proteolipid protein (PLP) and human leucocyte antigen (HLA-DR/DQ, referred to as HLA) as previously described.[27,15] Reactive, active, mixed active/inactive, inactive, and inactive remyelinated lesions were distinguished. Lesions were annotated, and adjacent sections were stained for CD3 and counter-stained with cresyl violet. All tissue sections were systematically examined with the microscope at x20 magnification, while the observer was blind for the clinical disease course. Perivascular T cell cuffing was considered present when more than one ring of $CD3^+$ T cells was present in the PVS.[39]

Immunohistochemistry and quantification of T-cell numbers

Adjacent 8-µm formalin-fixed, paraffin-embedded sections were immunostained for CD3, CD4, and CD8 without nuclear counterstaining. Antigen retrieval was accomplished with microwave treatment at 700 W. Endogenous peroxidase activity and non-specific binding were blocked as described previously.[15] Sections were incubated with a primary antibody overnight in blocking buffer at 4˚C (details on primary antibodies and concentrations are provided in **Suppl. Table 2**). The appropriate biotinylated, secondary antibody was applied, followed by conjugation with avidin-biotin horseradish peroxidase (HRP) complex (Vector Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Alternatively, an anti-rabbit secondary antibody directly conjugated with HRP (REAL™ EnVision™ Detection System; DAKO, Santa Clara, CA, USA) was applied to the CD8-stained sections. Visualization was established with 3,3-diaminobenzidine chromogen.

Brightfield tiled images of tissue sections were taken at an Axioscope microscope (Zeiss, Oberkochen, Germany) while using a green filter to obtain monochromatic light with a x10 Zeiss Plan-Neofluar objective and a black and white camera (XC-77; Sony, Towada, Japan). To outline the lesions and the normal-appearing white matter, an overlay was made in Adobe Photoshop CC (version 19.1.2; Adobe Systems, San Jose, CA, USA) of the CD3-, CD4-, and CD8-stained sections with the corresponding images of the HLA-PLP-stained section. Areas of interest were manually outlined with Image-Pro Plus 6.3 software (Media Cybernetics, Rockville, MD, USA). For each staining, the background optical density (OD) was measured for all sections. Subsequently, the OD threshold for a positive cell signal was set at four times the average background. OD particles

with size between 10 and 100 µm2,[13] exceeding the OD threshold, were counted as $CD3^+$, $CD4^+$, or $CD8^+$-positive T cells using Image Pro-Plus.[61] An example of the mask, created for the particle analysis of CD3 and CD4 immunostainings, is shown in **Suppl. Figure 1**. All masks were visually inspected for disturbances.

Axonal quantification by Bielschowsky silver staining

Axonal density in pyramidal tract normal-appearing white matter was examined in 76 MS and 13 control cases. Bielschowsky silver staining was performed by placing 8-µm thick paraffin-embedded, formalin-fixed tissue sections in Milli-Q water, followed by preheated 20% silver nitrate solution, in a dark stove at 40°C. Then, 32% ammonium hydroxide was added until the solution turned colorless, and sections were incubated at 40°C. Next, the sections were placed in 1% ammonium hydroxide solution, and developer solution was added to this solution. The sections were placed in the developer–silver nitrate–ammonium hydroxide solution, followed by fixation in 5% sodium thiosulfate. A grid count was performed at x40 magnification to quantify axonal density using the Axioscope microscope with a micropublisher 5.0 RTV digital CCD camera (Qimaging, Surrey, BC, Canada) and Image-Pro Plus 6.3 software.

S1P1 immunohistochemical quantification

Biopsy and autopsy WM sections were immunostained for S1P1 as described above and in **Suppl. Table 2**. Tiled images were taken. Using Fiji just Image J,[40] the white matter tissue surface area was calculated and the number of positive cells was manually counted.

Immunofluorescence and confocal imaging

Immunofluorescent double-labeling was performed to analyze multiple sclerosis lesions and perivascular T-cell clusters. Antigen retrieval and incubation with primary antibodies was performed as described above. Sections were then incubated with secondary antibodies directly labeled with Alexa fluorophore Cy3 or Cy5. Alternatively, sections are incubated with biotinylated, secondary antibodies, followed by incubation with streptavidin labeled with Cy3 or Cy5. Anti-CD44 rabbit polyclonal antibody staining was enhanced by Tyramide Signal Amplification (PerkinElmer, Waltham, MA, USA). Finally, all sections were incubated with Hoechst (33342; Thermo Fisher Scientific, Waltham, MA, USA). Confocal imaging was performed using the Leica microscope TSA SP8 X at x20, x40, x63 magnification (Leica Microsystems, Wetzlar, Germany) using Leica Applications Suite X software.[15] For $CD8^-$ and laminin-stained sections, tiled confocal images of tissue sections were made at x20 magnification (an example shown in **Suppl. Figure 2**).

Assessment of T-cell location

Localization of T cells was assessed in normal-appearing white matter, active, mixed active/inactive, and inactive lesions as described above. Using Image-J, tissue sections were systematically examined, and the number of parenchymal and perivascular T cells was counted as described in.[44] For donor and tissue information, see **Suppl. Table 3**.

Isolation of T cells from fresh autopsy material

White matter from multiple sclerosis and control cases and macroscopically visible multiple sclerosis lesions were dissected at autopsy and stored at 4°C in Hibernate A medium (Invitrogen, Carlsbad, CA, USA). A small tissue sample was snap-frozen in liquid nitrogen and stored at -80°C for immunohistochemistry. The remaining tissue was mechanically dissociated as previously described.[44,45] Mononuclear cells were separated from the suspension by Percoll (GE Healthcare, Little Chalfont, UK) gradient centrifugation, followed by CD11b magnetic activated cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany), as described previously.[44,45,33,36] After CD11b cell sorting, the flow-through containing T cells was cryopreserved. The frozen tissue sample was sectioned at 20 µm, immunostained for HLA and PLP, and lesions were characterized as described above and in **Suppl. Table 2**. Donor and sample characteristics are described in **Suppl. Table 4**. None of the multiple sclersosis cases received immunomodulatory therapies in the year before autopsy.

Flow-cytometric analysis

Cells were stained with antibodies for surface markers and LIVE/DEAD fixable red (Life Technologies, Bleiswijk, The Netherlands) for 30 min at 4°C. Subsequently, cells were washed, fixated, and permeabilized, followed by intracellular staining (Foxp3/Transcription Factor Staining Buffer Set, Thermo Fisher Scientific). Washed cells were analyzed at an LSRFortessa cell analyzer (BD Biosciences, San Jose, CA, USA). FlowJo software (version 10; Tree Star, Ashland, OR, USA) was used for subsequent data analysis. The guidelines for the use of flow cytometry and cell sorting in immunological studies were followed.[9] Specifications of the used antibodies are provided in **Suppl. Table 5**. Gating strategies are shown in **Suppl. Figure 3A**. T-distributed stochastic neighbor embedding (t-SNE) analysis was performed with Cytosplore^{+HSNE} software.[55]

Cell sorting and quantitative RT-PCR

Cryopreserved samples were thawed, washed, blocked with 10% normal horse serum, and stained directly with cocktails of fluorescently-conjugated antibodies described in **Suppl. Table 5**. CD20$^+$ B cells, naïve, memory, effector CD8$^+$ T and NK cells from blood and CD103$^-$ and CD103$^+$CD69$^+$CD8$^+$ T_{RM} cells from brain were sorted on a BD FACS Aria II cell sorter (BD Biosciences, Franklin Lakes, NJ). Gating strategies are shown in **Suppl. Figure 3B**. Total RNA was isolated with the RNeasy® mini kit (QIAGEN, Hilden, Germany), and cDNA was synthesized using the RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Relative gene expression levels were measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR) using Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus^{TM} system (Applied Biosystems) with the cycle threshold method. Donor and sample characteristics are provided in **Suppl. Table 6**, and primers are described in **Suppl. Table 7**.

Microarray data analysis

RNA expression levels for CXCR6 and CXCL16 from laser-dissected tissue from mixed active/inactive and inactive demyelinated lesions were obtained from a microarray dataset.[21] Adjusted *p*-values are shown in **Figure 5**.

Statistical analysis

All analysis is performed in GraphPad Prism 6 or 7 (GraphPad Software, San Diego, CA, USA). When data were not normally distributed non-parametric tests, either Kruskal-Wallis or Mann-Whitney U test were performed. Dunn's test was used for multiple comparison. When data were normally distributed one-way ANOVA test was used together with Tukey post-hoc test.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

RESULTS

Multiple sclerosis normal-appearing white matter is enriched for T cells

We first quantified the presence of $CD3^+$ T cells in normal-appearing white matter of the pyramidal tract at the level of the medulla oblongata of multiple sclerosis, Alzheimer's disease, and non-neurological control brains (**Figure 1A**). Donor and sample characteristics were comparable for all three groups (**Table 1** and **Suppl. Table 1**). However, brain weight was significantly lower in both, the multiple sclerosis and the Alzheimer's disease cases compared to the non-neurological controls, indicating brain tissue loss in the diseased brains (**Suppl. Figure 4**). In multiple sclerosis brains, more T cells were encountered compared to Alzheimer's disease and control brains (**Figure 1B**). We then assessed the relation between the number of T cells and axonal loss in pyramidal tract normal-appearing white matter. Axon density was significantly reduced in the multiple sclerosis cases compared to the controls, indicating axonal loss in multiple sclerosis normal-appearing white matter (**Figure 1C**). Axon density did not correlate with the number of T cells in multiple sclerosis normal-appearing white matter (**Figure 1D**).

Inflammatory active white matter lesions are enriched for $CD4^+$ and $CD8^+$ T cells

In sections of subcortical white matter of controls, Alzheimer's disease cases, and multiple sclerosis cases containing different lesion types, both lesional and perilesional white matter were delineated, and $CD3^+$ T cells were quantified (**Suppl. Figure 1**). T-cell counts in pyramidal tract and subcortical white matter were comparable for non-neurological controls, however T-cell counts were higher in (perilesional) subcortical white matter compared to the pyramidal white matter tract in both, the MS and AD cases (cf. **Figure 1B and E**).

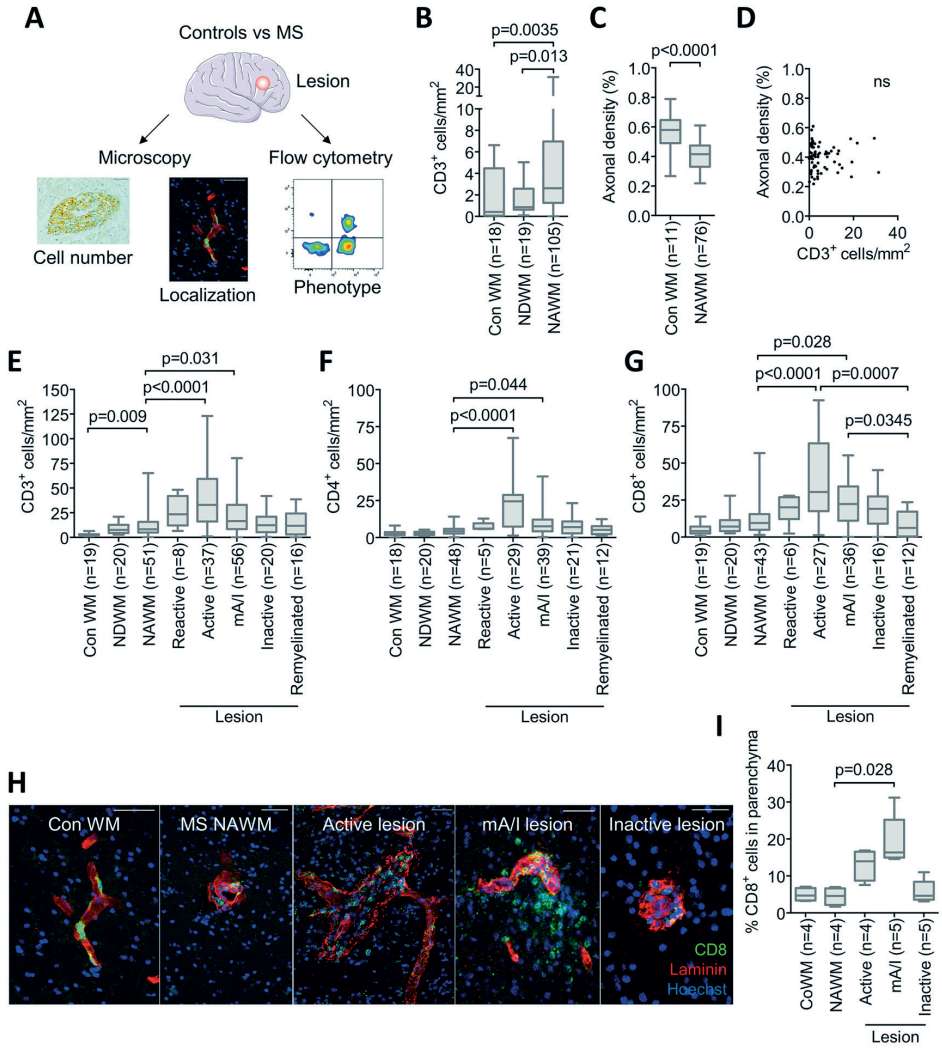


Figure 1. T-cell numbers are increased in multiple sclerosis normal-appearing white matter and further enhanced in inflammatory active and mixed active/inactive lesions.

(A) Illustration of experimental set-up. (B) Number of $CD3^+$ cells in the pyramid tract of non-neurological control (Con) white matter, Alzheimer's disease neurodegenerative white matter, and multiple sclerosis normal-appearing white matter at a standardly dissected location at the level of the medulla oblongata (Kruskal-Wallis test p=0.0002). (C) Axonal density in control white matter and multiple sclerosis normal-appearing white matter in the pyramid tract. (D) Correlation of axonal density with the number of T cells in multiple sclerosis normal-appearing white matter in the pyramid tract. (E) Number of $CD3^+$ cells in subcortical white matter lesions (Kruskal-Wallis test p<0.0001). (F) Number of $CD4^+$ cells in subcortical white matter lesions in active and mixed active/inactive lesions compared to normal-appearing white matter (Kruskal-Wallis test p<0.0001). (G) Number of $CD8^+$ cells in subcortical white matter in active and mixed active/inactive lesions compared to normal-appearing white matter (Kruskal-Wallis test p<0.0001). (H) Confocal images of CD8 and laminin immunofluorescent staining from control white matter, normal-appearing white matter, active lesion, mixed active/inactive lesion, and inactive lesion. Scale bars = 50 µm. (I) Percentage of $CD8^+$ cells located in brain parenchyma (Kruskal-Wallis test p=0.0042). Note that the quantification of control white matter $CD8^+$ cells in brain parenchyma has been published previously (Smolders et al., 2018) and is shown here for comparison. Kruskal-Wallis and Dunn's post-hoc test were used and p-values are shown in the plots. ConWM = control white matter; mA/I = mixed active/inactive; MS = multiple sclerosis; NAWM= normal-appearing white matter; NDWM = neurodegenerative white matter.

Perilesional white matter and all multiple sclerosis lesion types contained more T cells compared to control white matter, but comparable to neurodegenerative control white matter. When compared to multiple sclerosis perilesional white matter, both active and mixed active/inactive lesions were enriched for $CD3^+$ T cells (**Figure 1E**). This enrichment was observed both for $CD4^+$ and $CD8^+$ T cells (**Figure 1F and G**), without a skewing of the CD8/CD4 ratio between lesion types (**Suppl. Figure 5A**). Interestingly, the number of $CD8^+$ T cells was reduced in remyelinated areas (shadow plaques) compared to inflammatory active and mixed active/inactive lesions (**Figure 1G**). Furthermore, the CD8/CD4 ratio was variable between donors, but consistent between the different regions of individual donors (**Suppl. Figure 5A and B**). Interestingly, multiple sclerosis donors with a low CD8/CD4 ratio showed a higher percentage of inactive remyelinated areas in all dissected tissue blocks compared to donors with a high CD8/CD4 ratio (**Suppl. Figure 5C**). Because $CD8^+$ T cells were, next to active lesions, also more closely associated with mixed active/inactive lesions, our further analysis focused on $CD8^+$ T cells.

$CD8^+$ T cells are restricted to the PVS, except in inflammatory active and mixed active/inactive lesions

We next assessed the localization of $CD8^+$ T cells in multiple sclerosis normal-appearing white matter, mixed active/inactive lesions, and inactive lesions, by staining the laminin-gamma subunit in basement membranes. The PVS, the Virchow–Robin space, is the only compartment in the human body delineated by two basement membranes, covered on the luminal side by specialized endothelium and on the parenchymal side by the glia limitans.[48] These basement membranes contain various matrix proteins, including different laminin chains and collagen type IV.[22] In multiple sclerosis normal-appearing white matter, $CD8^+$ T cells were found almost exclusively in the PVS, comparable to control white matter (**Figure 1H**). In active and mixed active/inactive lesions, an increased proportion of $CD8^+$ T cells infiltrated the brain parenchyma (median 13.9% and 16.5%, respectively). In inactive lesions, the percentage of parenchymal $CD8^+$ T cells was comparable to the MS NAWM (**Figure 1I**).

Progressive multiple sclerosis donors show perivascular cuffing of T cells

In the multiple sclerosis normal-appearing white matter, we occasionally observed large clusters of T cells restricted to the PVS, which were previously characterized as perivascular cuffs.[39] We systematically scored all medulla oblongata sections for the presence of perivascular T-cell cuffing (**Figure 2A**). T-cell cuffs were only encountered in the progressive MS donors (**Figure 2B**). Interestingly, donors with perivascular cuffs showed a higher number of demyelinated lesions in the brainstem and a higher percentage of mixed active/inactive lesions (**Figure 2C and D**). There were no significant differences in the percentage of remyelinated or active lesions or the number of reactive sites in the brainstem and the total disease duration or time from first symptoms until the patient needed a walking aid (data not shown).

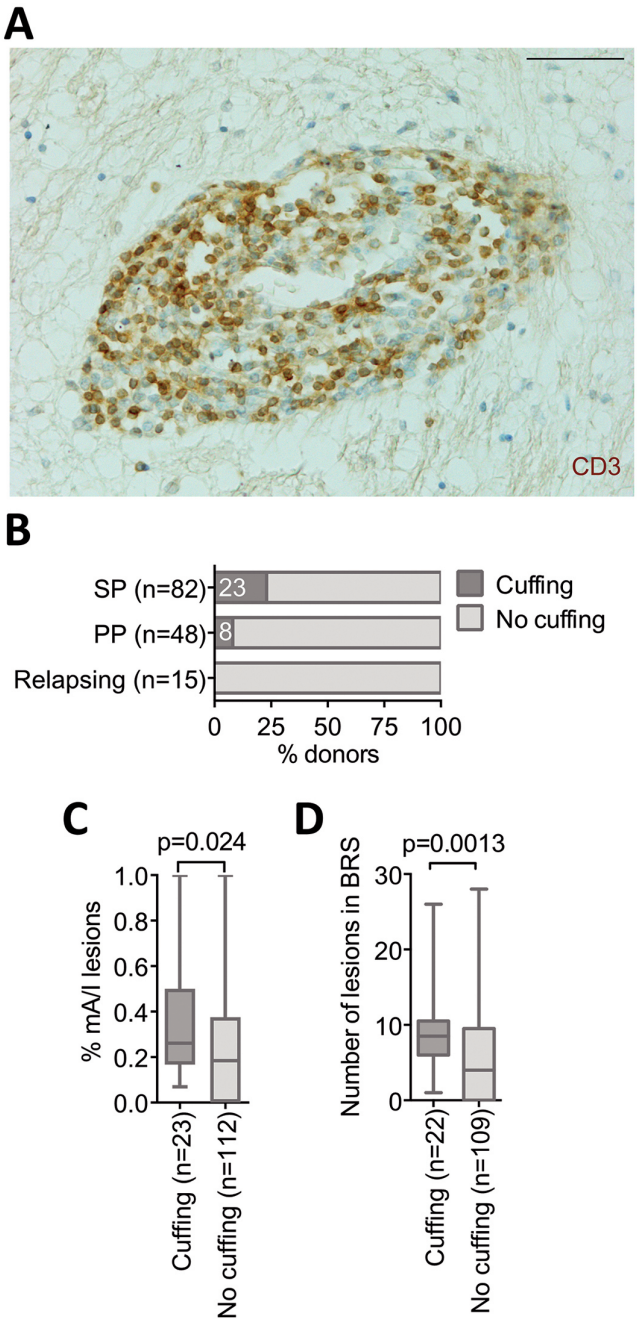


Figure 2. Clinical and pathological correlates of perivascular cuffing of $CD3^+$ cells at a standardly dissected location at the level of the medulla oblongata.

(A) $CD3^+$ perivascular cuff showing more than one ring of $CD3^+$ cells around a blood vessel at the level of the medulla oblongata. Scale bar = 50 µm. (B) Multiple sclerosis cases with perivascular cuffing more often show a progressive multiple sclerosis disease course (chi-square test p=0.017). (C, D) Multiple sclerosis cases with perivascular cuffing show a higher brainstem demyelinated lesion load (C) and a higher percentage of mixed active/inactive lesions (D). Mann-Whitney-U test was used, and p-values are shown in the plots. BRS = brainstem; mA/I = mixed active/inactive; PP = primary progressive; SP = secondary progressive.

Multiple sclerosis lesion T cells show a CD8$^+$ T_{RM}-cell surface marker profile

We next analyzed rapidly-isolated post-mortem mononuclear cells for the expression of phenotypic markers that we recently identified on human brain T_{RM} cells by flow cytometry.[44] These included the canonical markers CD69 and CD103,[51] but also programmed death receptor 1 (PD-1), very late antigen-1 (VLA-1, CD49a), and the general memory marker CD44.[24] Cells were acquired from fresh multiple sclerosis tissue blocks containing either mixed active/inactive (n=3) or active (n=5) lesions, multiple sclerosis normal appearing white matter (n=7), and control white matter tissue (n=10) (**Figure 3A**, donor and sample information in **Suppl. Table 4**).

Virtually all T cells isolated from multiple sclerosis lesions were CD69$^+$, with both a CD103$^+$ and a CD103$^-$ fraction present (**Figure 3B**). There was a lower fraction of CD69$^-$CD103$^-$ CD8$^+$ T cells in multiple sclerosis lesions compared to control white matter (**Figure 3C**).

Because the presence of CD103 impacts on the phenotype of brain T_{RM} cells,[59,44] we stratified further analyses for expression of CD103. Expression of the T_{RM} markers PD-1, CD49a, and CD44 was equally high on CD8$^+$ T cells from all three categories of tissue subtypes sampled (**Figures 3B, D, E**). Interestingly, CD49a is a receptor for collagen type IV and CD44 can act as a receptor for laminin, among others, and both molecules may hereby modulate homing of brain T_{RM} cells in the PVS.[54] CD44$^+$ lymphocytes could also be observed in close contact with laminin in the PVS with immunohistochemistry in both normal-appearing white matter and inactive centers of multiple sclerosis lesions (**Figure 3F-G**). Perivascular T-cell clusters showed high expression of CD44 (**Figure 3H**).

Circulating T cells are almost absent in chronic multiple sclerosis autopsy lesions

Next we compared the presence of S1P1$^+$ circulating T cells and the CD103$^+$ T_{RM}-cell subset in both inflammatory active lesions from early multiple sclerosis biopsy cases and chronic progressive multiple sclerosis autopsy cases. In both lesion stages, we encountered CD103$^+$ T_{RM} cells in the brain parenchyma (**Figure 4A**). In the early multiple sclerosis lesion stages, a relatively smaller proportion of CD3$^+$ T cells was CD103$^+$ compared to the chronic progressive multiple sclerosis autopsy cases (**Figure 4B**), which suggests a smaller pool of CD103$^+$ T_{RM} cells in early multiple sclerosis lesions. We stained for the recirculation marker S1P1 and found, in accordance with earlier studies,[12,6,28] no staining of S1P1$^+$ T cells in the parenchyma or PVS of biopsy or autopsy material. Almost all S1P1$^+$ cells in biopsy samples were located directly adjacent to the luminal side of the endothelium (**Figure 4C**). However, where few intravascular S1P1$^+$ T cells were found in inflammatory active lesions from chronic progressive multiple sclerosis autopsy, they were present at high number in lesions from early multiple sclerosis biopsies (**Figure 4C and D**). Although a post-mortem effect cannot be excluded, desensitization of S1P1 is an important mechanism for effector T cells to migrate from the circulation into tissues.[2] Close contact with the endothelium suggests that T cells in early multiple sclerosis biopsies may reflect an expanded local pool of intravascular effector-type T cells, which may lose S1P1 upon infiltration of the PVS. This interpretation would

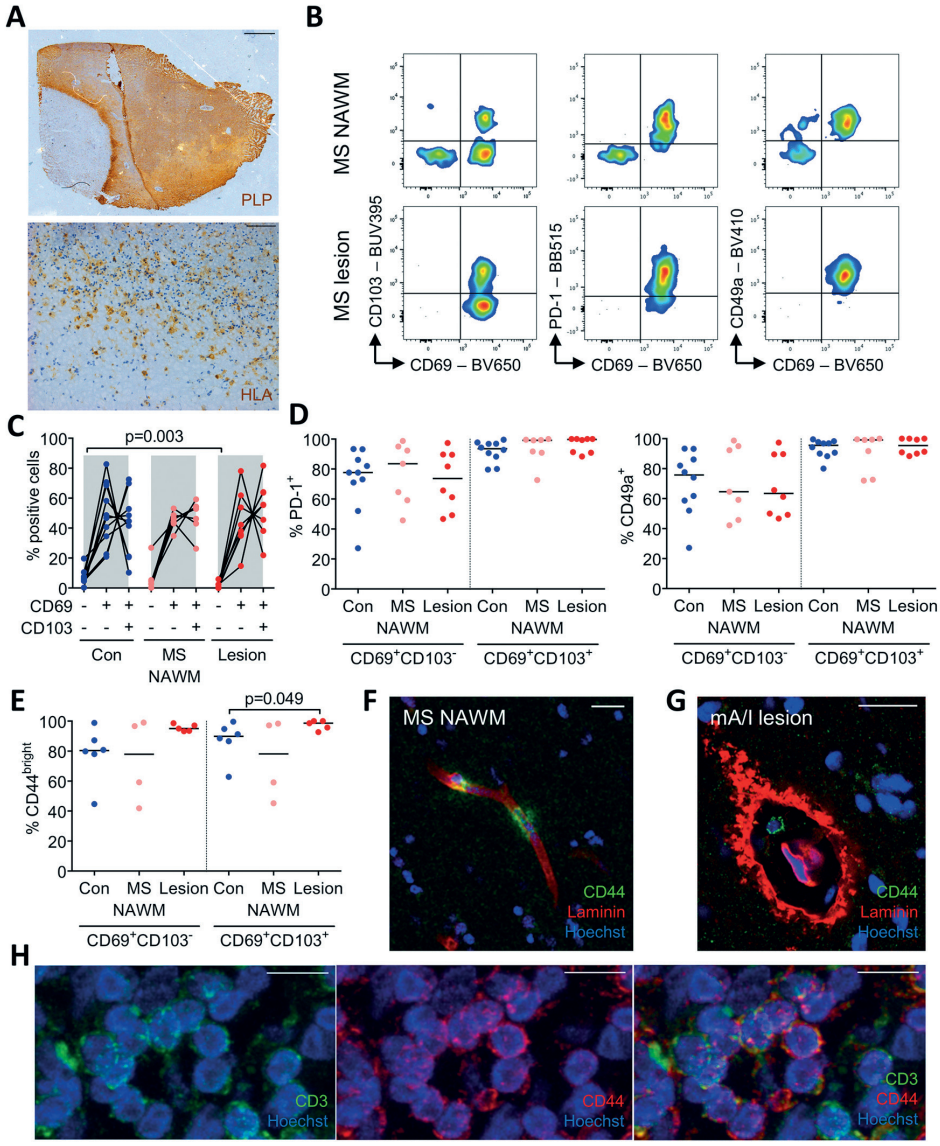


Figure 3. CD8$^+$ T cells in multiple sclerosis lesions show a T_{RM}-cell surface marker profile.
(A) An example of PLP and HLA staining of an multiple sclerosis mixed active/inactive lesion dissected for cell isolation. Scale bar = 1 mm (PLP) and 100 µm (HLA). (B) Flow-cytometric plots for CD103, CD69, PD-1, and CD49a expression on CD8$^+$ T cells from multiple sclerosis normal appearing white matter and lesions. (C) Percentage of single- and double-positive cells for CD69 and CD103 in control white matter, multiple sclerosis normal-appearing white matter, and multiple sclerosis lesions. (D) Percentage of PD-1$^+$ and CD49a$^+$ CD8$^+$ T cells is comparable in control white matter, multiple sclerosis normal-appearing white matter, and multiple sclerosis lesions. PD-1 and CD49a expression is increased in CD103$^+$ cells. (E) Multiple sclerosis normal-appearing white matter and multiple sclerosis lesion CD8$^+$ T_{RM} cells are CD44bright. (F) In the PVS of multiple sclerosis normal-appearing white matter, CD44$^+$ T cells are located in close contact to the CD44 ligand, laminin. Scale bare = 25 µm. (G) In the inactive center of mixed active/inactive lesions, CD44$^+$ T cells are present in the PVS. The basal lamina in inactive centers appears fibrotic. Scale bar = 25 µm. (H) T-cell clusters in multiple sclerosis perivascular cuffs show high CD44 expression. Scale bar = 10 µm. Con = control; mA/I = mixed active/inactive; MS = multiple sclerosis; NAWM = normal appearing white matter; NDWM = neurodegenerative white matter.

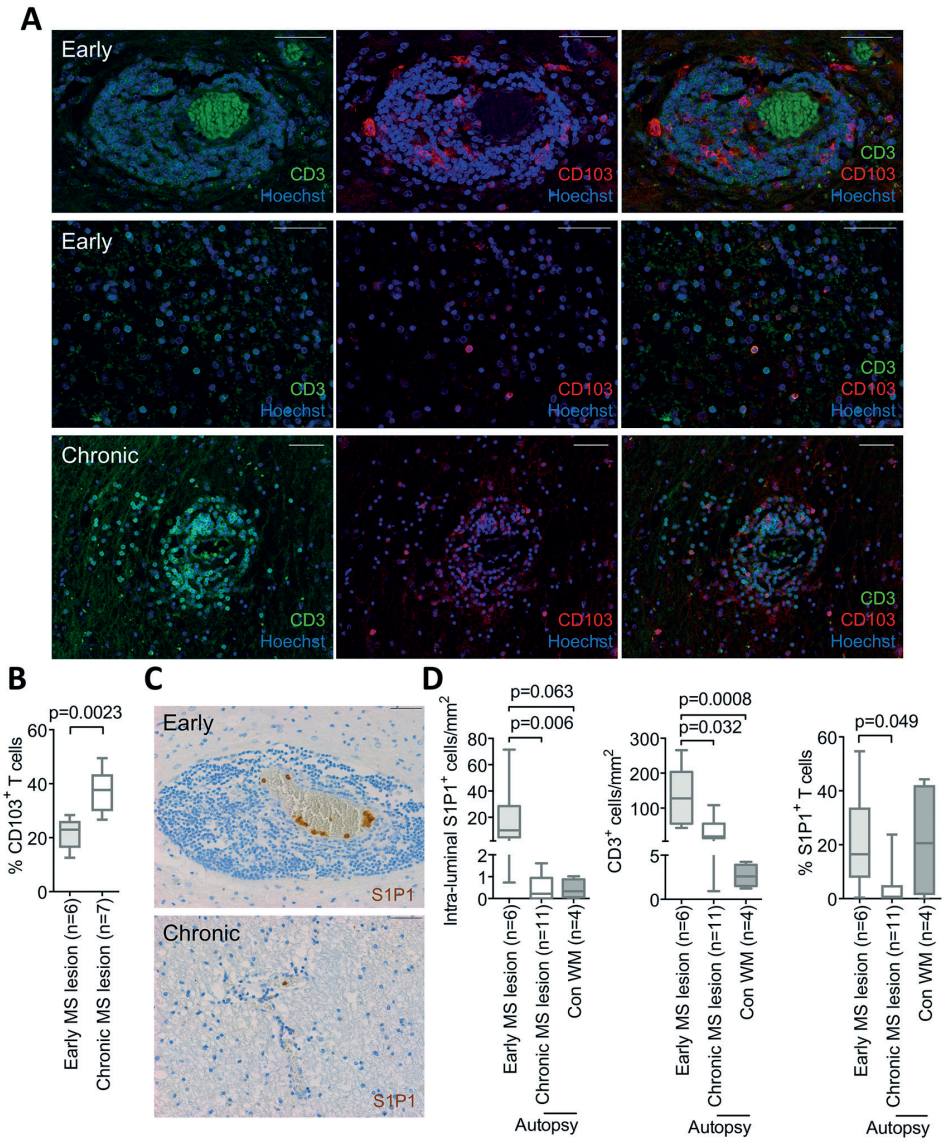


Figure 4. CD103$^+$ T cells infiltrate the brain parenchyma in early biopsy and chronic autopsy multiple sclerosis lesions, while circulating S1P1$^+$ T cells are almost absent in chronic autopsy lesions.
(A) Confocal images of CD3 and CD103 immunofluorescent staining showing CD103$^+$ T cells in both, the PVS and the brain parenchyma in both early and chronic multiple sclerosis lesions. The bright green signal inside the blood vessel lumen is autofluorescence from erythrocytes. Scale bars = 50 µm. (B) The percentage of CD103$^+$ T cells was increased in chronic multiple sclerosis lesions (Mann-Whitney U test p=0.0023) (C) S1P1$^+$ are present mostly in early MS lesions and located within the blood vessel lumen, confirming these are circulating T cells. Scale bars = 50 µm. (D) The absolute number of S1P1$^+$ cells in blood vessels is decreased in chronic multiple sclerosis autopsy lesions compared to early multiple sclerosis biopsy lesions that are both inflammatory active (Kruskal-Wallis test p=0.0017). The total number of T cells was decreased in chronic multiple sclerosis lesions compared to early multiple sclerosis lesions (Kruskal-Wallis test p=0.0001). The relative number of S1P1$^+$ T cells was lower in chronic multiple sclerosis lesions compared to early multiple sclerosis lesions (Kruskal-Wallis test p=0.049). Dunn's post-hoc test was used and p-values are shown in the plots. Con WM = control white matter; MS = multiple sclerosis.

support a role for non-T_{RM} cells in early multiple sclerosis, which was not observed in end-stage multiple sclerosis.

t-SNE analysis identifies exclusively $CD8^+$ T_{RM}-cell clusters in multiple sclerosis cases

To evaluate the presence of small T-cell clusters lacking a T_{RM} cell phenotype in lesions, which may get lost in conventional gating strategies, we generated t-SNE plots of the total $CD3^+CD8^+$ T-cell fraction. Only in the control donors, unique clusters of cells displaying a non-T_{RM} cell phenotype were encountered, characterized by a lack of CD69 expression (**Figure 5A, cluster 1 and 2**). These cells showed low expression of T_{RM} cell surface markers and, in cluster 2, high expression of molecules related to cytotoxicity (granzyme B and GPR56). In multiple sclerosis normal-appearing white matter and lesions, dominant T-cell clusters showed a T_{RM} cell phenotypic profile with clusters 5 and 9 being characterized by high expression of CD103.

$CD8^+$ T_{RM} cells in multiple sclerosis lesions upregulate the tissue homing receptor CXCR6

CXCR6 is a core T_{RM} cell marker and a chemokine receptor mediating tissue infiltration of $CD8^+$ T cells.[24] The percentage of $CXCR6^+$ $CD8^+$ T cells was increased in multiple sclerosis lesions compared to control white matter in both the $CD103^+$ and $CD103^-$ $CD8^+$ T-cell population (**Figure 5B and C**). In addition, whole tissue gene expression microarray analysis of laser-dissected control white matter as well as mixed active/inactive and inactive multiple sclerosis lesions[21] revealed increased expression of CXCR6 in the rim of mixed active/inactive lesions (**Figure 5D**). The ligand of CXCR6, CXCL16, is also upregulated in the rim of mixed active/inactive lesions, indicated by quantitative RT-PCR[20] and by microarray analysis (**Figure 5D**). CXCR6–CXCL16 interaction may mediate homing of $CD8^+$ T cells in the inflamed parenchyma, because these are found in the inflammatory active rim of mixed active/inactive lesions in close contact to $CXCL16^+$ cells [median (interquartile range, IQR) 42.8% (34.9–43.9%) of parenchymal T cells; n=3] (**Figure 5E and F**).

Lesional $CD8^+$ T_{RM} cells show signs of re-activation without notable cytotoxicity

To assess the activation status of brain T_{RM} cells, we stained for Ki-67. Ki-67 is a marker for T cells undergoing antigen-specific proliferation *in vitro*.[46] In cells isolated from lesions, higher expression of Ki-67, as measured by the geometric mean fluorescence intensity (GMFI), was observed when compared to control white matter with flow-cytometry (**Figure 6A and B**), which can indicate an increased rate of recent reactivation. The T-cell clusters in the PVS contained $CD3^+$ T cells positive for Ki-67 with immunohistochemistry (**Figure 6C and D**).

Next, we analyzed expression of the cytotoxic enzyme granzyme B, which was earlier observed with immunohistochemistry in $CD8^+$ T cells in active lesions.[35,28] Using immunohistochemical staining, we encountered low numbers of granzyme B^+ cells in inflammatory active multiple sclerosis lesions [median (IQR) = 0.017 (0.012–0.026) per mm^2 in 12 tissue sections containing

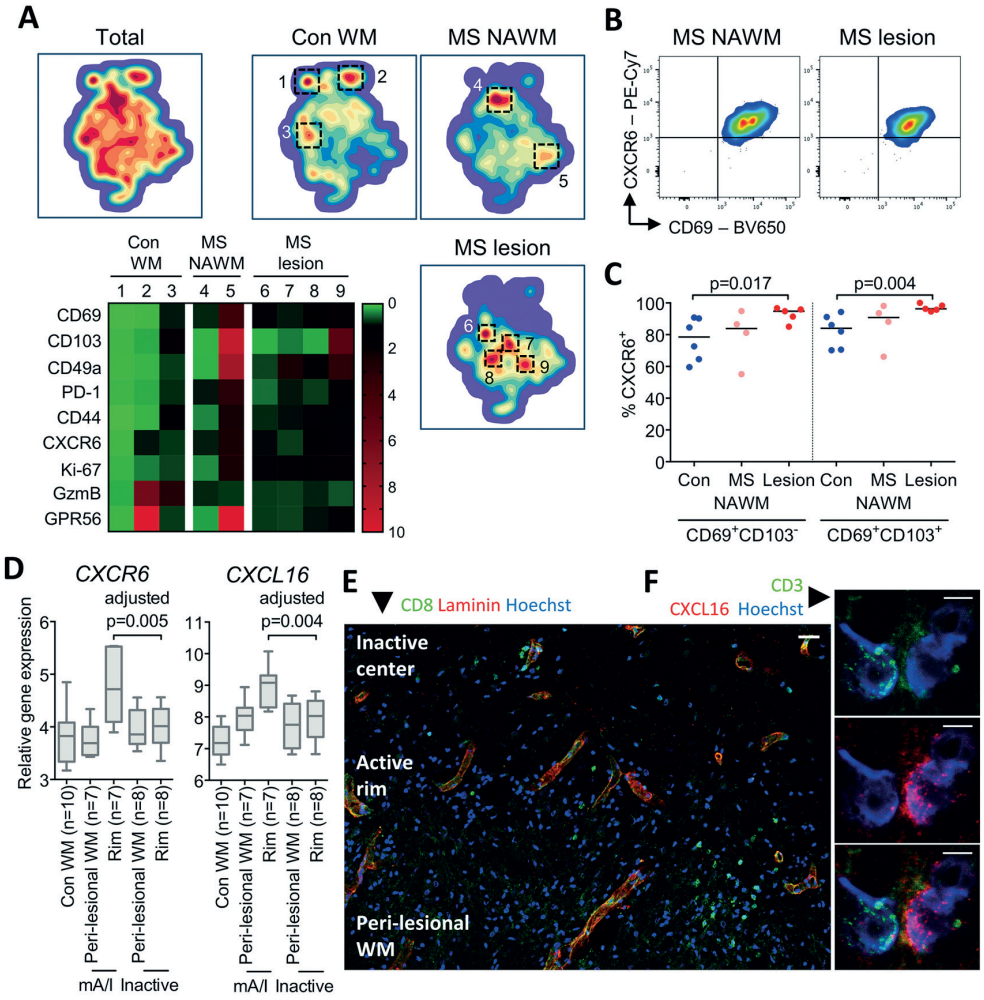


Figure 5. Multiple sclerosis normal-appearing white matter and multiple sclerosis lesions show a distinct T_{RM} cell surface marker profile with a higher percentage of CXCR6$^+$ CD8$^+$ T cells in multiple sclerosis lesions. (A) t-SNE analysis of all CD3$^+$CD8$^+$ T cells reveals a distinct cell clusters in control white matter, multiple sclerosis normal-appearing white matter, and multiple sclerosis lesions. The heatmap shows the expression pattern in the indicated T cell clusters. (B) Flow-cytometric plots for CXCR6 and CD69 expression on CD8$^+$ T_{RM} cells in control white matter, normal appearing white matter, and multiple sclerosis lesion samples. (C) CXCR6 is upregulated in multiple sclerosis lesions in both CD103$^-$ and CD103$^+$ cells (Kruskal-Wallis test p=0.0357). (D) Tissue mRNA gene expression levels from mixed active/inactive lesions shows upregulation of CXCR6 and its ligand, CXCL16. (E) CD8$^+$ T cells are found in brain parenchyma in the mixed active/inactive lesion rim. Scale bar = 50 µm. (F) CD3$^+$ T cell in close contact to a CXCL16$^+$ cell in an inflammatory active multiple sclerosis lesions. Scale bar = 5 µm. Mann-Whitney U and Kruskal-Wallis test were used, and (adjusted) p-values are shown in the plots. Con WM = control white matter; mA/I = mixed active/inactive; MS= multiple sclerosis; NAWM = normal-appearing white matter.

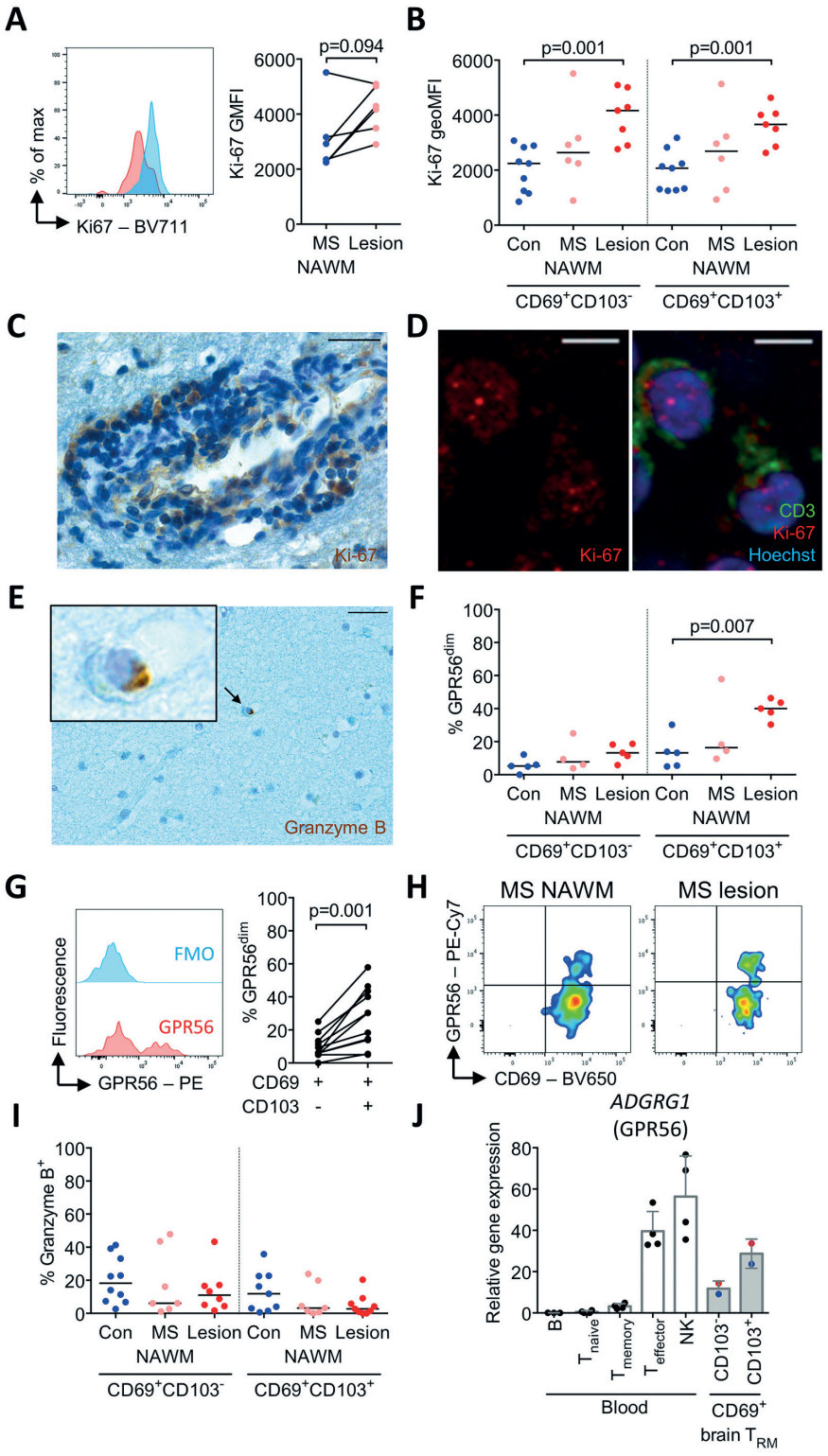
inflammatory active MS lesions; **Figure 6E**], while these tissue sections contained high number of $CD3^+$ cells [median (IQR) = 36.9 (11.1–39.6) per mm^2]. We observed with flow cytometry equally low numbers of granzyme B^+ $CD8^+$ T cells in fractions from control white matter and multiple sclerosis normal-appearing white matter and lesions (**Figure 6F**). In accordance with earlier data, lowest granzyme B expression was observed in the $CD103^+$ T_{RM} cell subset.[44] Therefore, our data do not support an upregulation of granzyme B as cytotoxic effector molecule by lesional T_{RM} cells.

The adhesion G protein-coupled receptor GPR56 is expressed at high levels on circulating cytotoxic lymphocytes, where it inhibits immediate effector functions.[7] Previously, we found almost no $GPR56^{high}$ expression on $CD8^+$ brain T_{RM} cells.[44] In multiple sclerosis lesions, we saw an expansion of a $GPR56^{dim}$ subset in the $CD8^+CD69^+CD103^+$ T_{RM}-cell fraction (**Figure 6G–I**). This $CD103^+$ cell-restricted expansion was confirmed by quantitative RT-PCR (**Figure 6J**). Expression of GPR56 may reflect cytotoxic capacity of multiple sclerosis lesion brain T_{RM} cells but may also restrict granzyme B expression.

Perivascular cuffs show signs of T_{RM} cell reactivation

In search for the site of T_{RM} cell reactivation, we stained the perivascular cuffs for antigen presentation and reactivation markers. As shown above, $Ki\text{-}67^+$ $CD3^+$ T cells were observed in these clusters (**Figure 6C and D**), suggesting that reactivation could occur in these perivascular cuffs. Within the perivascular T cell cuffs, large populations of $CD3^+CD103^+$ T_{RM} cells were observed (**Figure 7A**). A median proportion of 73.1% (IQR 64.7-84.6%) of these T_{RM} cells showed expression of E-cadherin, the ligand for CD103, which may be involved in clustering of these cells[54] (**Figure 7B**; n=3). In the perivascular cuffs, we found $CD103^+$ T_{RM} cells in close proximity to HLA^+ (**Figure 7C and D**) and $CD163^+$ (**Figure 7E**) perivascular macrophages and $CD20^+$ B cells (**Figure 7F**), which could both be involved in antigen presentation and reactivation of T_{RM} cells within the PVS in chronic multiple sclerosis cases and accumulating in mixed active/inactive lesions.

Figure 6. $CD8^+$ T_{RM} cells in multiple sclerosis lesions are reactivated and show increased expression of GPR56 without upregulation of granzyme B. **(→ p. 142).**
(A) Ki-67 signal in $CD8^+$ T_{RM} cells from multiple sclerosis normal-appearing white matter and multiple sclerosis lesions. (B) In multiple sclerosis lesion $CD8^+$ T_{RM} cells upregulate Ki-67 expression compared to controls, both in $CD103^+$ and $CD103^-$ T_{RM} cells (Kruskal-Wallis test p=0.0081). (C) T cells within perivascular cuffs are $Ki\text{-}67^+$. Scale bar = 25 µm. (D) Expression of nuclear Ki-67 in $CD3^+$ T cells. Scale bar = 5 µm. (E) Immunohistochemistry of a granzyme B^+ cell in a subcortical white matter multiple sclerosis lesion. Scale bar = 25 µm. (F) There is no difference in the percentage of granzyme B^+ cells derived from multiple sclerosis lesions compared to control white matter. (G) Brain T_{RM} cells show $GPR56^{dim}$ expression, with a higher percentage of positive cells in $CD103^+$ compared to $CD103^-$ T_{RM} populations. (H) Some multiple sclerosis normal-appearing white matter and multiple sclerosis lesion T_{RM} cells show $GPR56^{dim}$ expression (I) The percentage of $GPR56^{dim}$ cells is increased in $CD69^+CD103^+$ $CD8^+$ T_{RM} cells derived from multiple sclerosis lesions compared to control white matter. (J) Relative gene expression for *ADGRG1* (GPR56) in FACS-sorted $CD20^+$ B cells, naïve, memory, effector $CD8^+$ T cells, and NK cells from peripheral blood and $CD103^-$ and $CD103^+$ $CD69^+CD8^+$ T_{RM} cells from brain samples showing increased *ADGRG1* expression in $CD103^+$ cells. Sample 1 (●) and sample 2 (●) were pooled cells from derived from five and three donors, respectively. Mann-Whitney U test was used, and p-values are shown in the plots. Con = control;GMFI = geometric mean fluorescence intensity; MS = multiple sclerosis; NAWM = normal-appearing white matter.


A
% of max
Ki67 – BV711
Ki-67 GMFI
p=0.094
MS Lesion
NAWM
B
Ki-67 geoMFI
p=0.001
p=0.001
Con MS Lesion Con MS Lesion
NAWM NAWM
CD69+CD103-
CD69+CD103+
C
Ki-67
D
Ki-67
CD3
Ki-67
Hoechst
E
Granzyme B
F
% GPR56dim
p=0.007
Con MS Lesion Con MS Lesion
NAWM NAWM
CD69+CD103-
CD69+CD103+
G
Fluorescence
FMO
GPR56
GPR56 – PE
% GPR56dim
p=0.001
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CD103 - +
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GPR56 – PE-Cy7
CD69 – BV650
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CD69+CD103+
J
ADGRG1
(GPR56)
Relative gene expression
B
Tnaive
Tmemory
Teffector
NK
CD103-
CD103+
Blood
CD69+
brain TRM

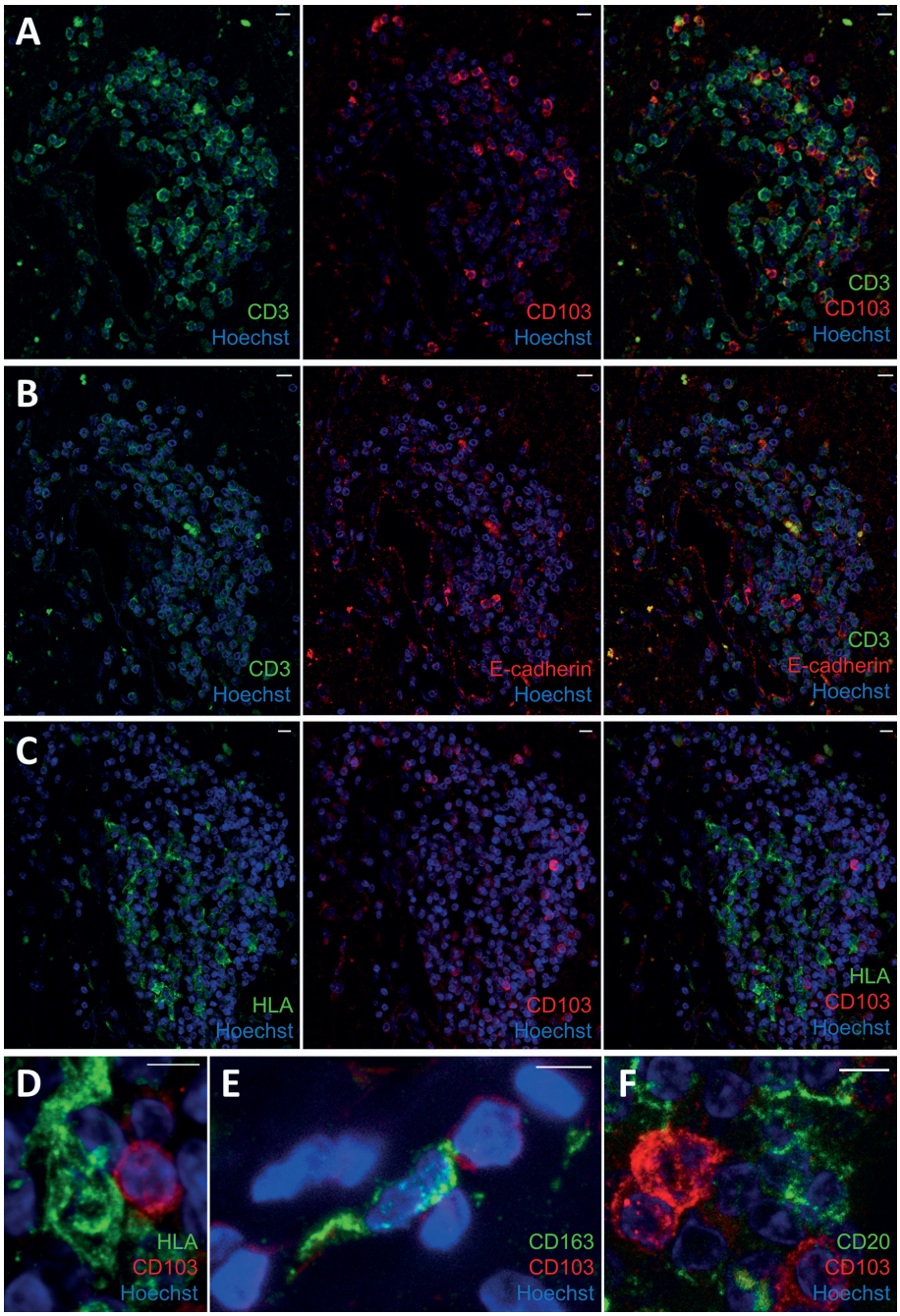

Figure 7. Perivascular cuffs consist of CD103$^+$ T_{RM} cells in close contact with perivascular macrophages and B cells suggesting antigen presentation and reactivation of T_{RM} cells in the perivascular space.
(A) CD3$^+$ T cells in perivascular clusters express CD103. (B) CD3$^+$ T cells in perivascular clusters express the ligand for CD103, E-cadherin. (C) HLA$^+$ perivascular macrophages are present in perivascular cuffs. (D-F) In perivascular cuffs, CD103$^+$ T_{RM} cells are found in close proximity to HLA$^+$ and CD163$^+$ perivascular macrophages (D and E) and to CD20$^+$ B cells (F). Scale bars = 10 μm in A-C and 5 μm in D-F.

DISCUSSION

Here we provide an extensive analysis of the phenotypic profile and spatial localization of human brain T cells in relation to the post-mortem pathology of advanced multiple sclerosis. We show that the number of T cells is increased in multiple sclerosis normal-appearing white matter and further enhanced in inflammatory active multiple sclerosis white matter lesions. In line with previous reports,[44,45,35,28] we show these are mostly $CD8^+$ T_{RM} cells, which lack characteristics of circulating lymphocytes. We expand previous work by elaborating on the phenotypic and functional profile of these cells and associating their characteristics with the localization in the PVS. In multiple sclerosis normal-appearing white matter, $CD8^+$ T_{RM} cells were retained in the PVS, likely mediated by high expression of CD44 and CD49a. We found that clustering of $CD8^+$ T_{RM} cells in perivascular cuffs is only observed in donors with progressive multiple sclerosis. In mixed active/inactive lesions, $CD8^+$ T_{RM} cells also localized in the brain parenchyma. This is possibly mediated by high expression of the tissue homing receptor CXCR6 by lesional $CD8^+$ T_{RM} cells, since its ligand CXCL16 also shows increased expression in the mixed active/inactive lesion rim. $CD8^+$ T_{RM} cells in multiple sclerosis lesions showed a higher expression of Ki-67, presumably reflecting recent re-activation. We observed antigen-presenting cells in conjunction with Ki-67^+ T_{RM} cells in the perivascular cuffs, suggesting these are potential hotspots of this re-activation. We further observed a low expression of granzyme B, which could be due to the high expression of inhibitory GPR56. The quantities, localization, and activation state of these cells, together with the association of perivascular T-cell clusters with lesion load, mixed active/inactive lesions, and progressive disease, suggest that re-activation of $CD8^+$ T_{RM} cells in the PVS is a key mechanism in the maintenance of white matter lesion activity in advanced progressive multiple sclerosis.

Only in demyelinated white matter lesions did we observe substantial numbers of $CD8^+$ T_{RM} cells outside the PVS. Generating cytotoxic mediators, such as granzyme B, is an effector mechanism of reactivated T_{RM} cells.[34] Surprisingly, we did not find an enrichment of granzyme B^+ cells in the cell fraction isolated from lesions. This finding contrasts with an enrichment for granzyme B^+ T cells in multiple sclerosis lesions reported by Van Nierop et al.[35] A sampling error is unlikely, since only few granzyme B^+ cells were stained with immunohistochemistry in lesions as well. This does not suggest an upregulation of the granzyme B–perforin axis in chronic multiple sclerosis lesions as was observed in early multiple sclerosis diagnostic biopsy samples.[23] In general, brain $CD103^+$ T_{RM} cells show low expression of granzyme B and are almost devoid of perforin.[44,45] One of the reasons for this lack of cytotoxicity could be the expression of intermediate levels of GPR56 by multiple sclerosis lesional $CD103^+$ T_{RM} cells. GPR56 is expressed at high levels on circulating lymphocytes with cytotoxic capacities, where it inhibits effector functions.[7] In normal white matter, human post-mortem $CD8^+$ T_{RM} cells produce more granzyme K than granzyme B,[44,45] which could also serve as an effector molecule in multiple sclerosis lesions. Furthermore, $CD8^+$ T_{RM} cells express high levels of the inhibitory receptor PD-1. Previously, we observed expression of its ligand, programmed death-ligand 1 (PD-L1), in multiple sclerosis lesions but not in control white matter.[44]

In polyomavirus-infected mice, expression of PD-L1 was observed on myeloid cells, microglia, and astrocytes.[43] Conversely, blockade of the PD-1 system in treatment of human melanoma patients with immune checkpoint inhibitors has been associated with the occurrence of multiple sclerosis-like demyelinating lesions.[30,14] Cytokine production by activated $CD8^+$ T_{RM} cells could contribute to the maintenance of mixed active/inactive lesions. Human white matter $CD8^+$ T_{RM} cells are potent producers of IFNγ, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor upon activation.[44] Also, Fas ligand-mediated killing of Fas-expressing oligodendrocytes around multiple sclerosis lesions could be a potential mechanism by which $CD8^+$ T cells contribute to demyelination in chronic multiple sclerosis lesions.[10,35,15,57]

In multiple sclerosis normal-appearing white matter, $CD8^+$ T_{RM} cells were observed in their normal compartment, the PVS. Within this compartment, accumulation of inflammatory cells in perivascular cuffs has since long been recognized as an important pathological feature of multiple sclerosis.[38] In our dataset, perivascular cuffing at the medulla oblongata was only observed in progressive multiple sclerosis. Earlier studies found perivascular cuffing to be more prevalent in brain donors with secondary progressive MS compared to primary progressive MS.[39] We now show these cuffs to contain T cells expressing the T_{RM}-cell marker CD103. Gray et al. earlier argued that T_{RM}-cell clustering with antigen-presenting cells, $CD163^+$ perivascular macrophages and B cells, could be the first step of ectopic lymphoid structure formation found in several inflammatory diseases.[17] In post-mortem multiple sclerosis studies, ectopic lymphoid structures are prevalent in the meninges of progressive donors[42,18,28] and may reflect mechanisms similarly to the role of perivascular cuffing. This idea is further supported by studies that show that the PVS and the meningeal compartment are connected.[11,25,26]

There are some limitations to our work. First, cells were isolated from tissue blocks containing both multiple sclerosis normal-appearing white matter and multiple sclerosis lesions, which may dilute differences in T-cell phenotypes between both locations. Since we did not use enzymes during the isolation procedure, we likely isolated only a proportion of cells from the brain tissue but on the other hand did not skew phenotypes by enzymatic digestion. Earlier studies showed that isolation procedures target subsets of tissue-resident cells and create skewing in phenotypes found.[50] These aspects are covered by the combination with immunohistochemistry to validate our findings. Last, the overall numbers of cells sampled from tissue blocks are quite low, when compared to flow cytometric studies on peripheral blood mononuclear cells. This did not allow sequencing of the brain T_{RM} cell transcriptome. In particular, the number of $CD4^+$ T cells we isolated for flow cytometry studies was relatively low for reliable assessment. Strengths of our work are the well-characterized donor cohort and lesions, the combination of flow cytometry and immunohistochemistry, and the sample size.

Interestingly, in subcortical white matter from neurodegenerative controls we observed a comparable number of T cells compared to multiple sclerosis cases. This finding may relate to the

lower weight of the Alzheimer's disease brains and/or the neurodegenerative process and is in line with two earlier reports on T cell numbers in relation with Alzheimer's disease pathology.[62,32] It would be interesting to study differences in effector functions in Alzheimer's disease-associated T_{RM} cells potentially involved in a neuroinflammatory response in neurodegenerative diseases. However, our data suggest that in advanced multiple sclerosis cases, the T_{RM} cell response in normal-appearing white matter is not solely part of a neuroinflammatory response to the axonal damage, since we did not find a correlation with axonal density.

Furthermore, genetic risk factors for multiple sclerosis in immune-related genes,[3,11] effectiveness of therapies directed at circulating lymphocytes in the earlier stage of the disease,[60] and pathological features of chronic multiple sclerosis, like the lymphocytic infiltrates found in both PVS and meninges that mimic other immune-mediated diseases,[8] all lead us to think of multiple sclerosis as a primary immune-mediated disease. Therefore, a main open question is which antigen is presented to the $CD8^+$ T_{RM} cells in the PVS of these chronic progressive multiple sclerosis brain donors. In other human and animal tissues investigated, T_{RM}-cell populations appear to control local viral infections.[51] In models of CNS neurotropic virus infection, local brain $CD8^+$ T_{RM}-cell populations are generated against vesicular stomatitis virus,[58] mouse polyomavirus,[43] West Nile virus,[1] and non-replicating adenoviruses.[41] This supports an anti-viral response as a potential driver of $CD8^+$ T_{RM} cell recruitment in the CNS, which may also be applicable to multiple sclerosis lesions. In a mouse model for multiple sclerosis, it was recently shown that viral infections during early life precipitate brain auto-immune disease by an increased recruitment of T_{RM} cells into the brain.[49] In accordance, electron microscopy studies showed paramyxovirus-like fuzzy filament in nuclei of lymphocytes and macrophages in multiple sclerosis perivascular cuffs,[52] which could reflect the initiation of an antiviral T-cell response.[37] Identification of the exact cells expressing viral antigens and provoking subsequent $CD8^+$ T_{RM} cell expansion could provide a critical avenue towards understanding the cause of multiple sclerosis. However, as low numbers of perivascular $CD8^+$ T_{RM} cells are also observed in donors without multiple sclerosis,[44] the antigen presented to T_{RM} cells in multiple sclerosis could be present both in donors with and without multiple sclerosis. Differences in genetic and environmental background could in this scenario accumulate in the destructive immune response seen in multiple sclerosis.

Altogether, our findings point to the PVS as new frontier for progressive multiple sclerosis treatment development. Understanding the mechanisms which are involved in $CD8^+$ T_{RM} cells expanding in the PVS, entering mixed active/inactive lesions, and drive ongoing inflammatory lesion activity could provide targets for new disease-modifying therapies with efficacy in progressive multiple sclerosis.

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Competing interests

The authors report no competing interests. IH received lecture and/or consultancy fee from Biogen and Novartis. JS received lecture and/or consultancy fee from Biogen, Merck, Novartis, and Sanofi-Genzyme.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Donor and sample information for subcortical WM lesions and biopsies.

Diagnosis	Cases (n)	Age (years)	Sex (F/M)	PMD (h:min)	pH value	Brain weight (g)	Disease duration (years)
MS autopsies	57	62.3 ± 12.3	36/21	9:45 ± 7:04	6.5 ± 0.2	1178 ± 135	28.6 ± 13.4
RR	2	65.0 ± 12.7	1/1	8:35 ± 2:14	6.7± 0.6	1193 ± 37	16.0 ± 0.0
PP	19	66.9 ± 11.1	10/9	9:29 ± 6:25	6.5 ± 0.2	1193 ± 144	26.3 ± 9.7
SP	36	62.5 ± 13.2	25/11	9:58 ± 7:39	6.5 ± 0.3	1170 ± 135	30.5 ± 14.9
MS biopsies	6	47.5 ± 15.2	3/3	–	–	–	–
Neurodegenerative controls (Braak 5)	20	73.1 ± 11.28	10/10	5:22 ± 1:20	6.44 ± 0.28	1150 ± 145	–
Non-neurological controls	18	67.8 ± 11.5	9/9	8:06 ± 3:15	6.6 ± 0.3	1297 ± 159	–

Provided is the mean ± SD (standard deviation). F, female; M, male; PMD, post-mortem delay; PP, primary progressive; RR, relapsing–remitting; SP, secondary progressive.

Supplementary Table 2. Specification of antibodies and procedures for immunohistochemistry.

Antigen	Product code, company	Host	Clonality	Clone	Conc.	Tissue fixation	Antigen retrieval method
CD3	Ab699, Abcam	Mouse	Mono	PS1	1:50	FFPE and FF (10 min acetone)	Citrate pH 6.0
CD3	A0452, DAKO	Rabbit	Poly		1:100	FFPE	Citrate pH 6.0
CD4	Ab13361, Abcam	Rabbit	Mono	EPR6855	1:500	FFPE	Citrate pH 6.0
CD8	Ab4055, Abcam	Rabbit	Poly		1:200 – 1:500	FFPE and FF (10 min acetone)	Citrate pH 6.0
CD20	M0755, DAKO	Mouse	Mono	L26	1:100	FFPE	Citrate pH 6.0
CD44	Ab157107, Abcam	Rabbit	Poly		1:2000	FFPE and FF (10 min acetone)	Citrate pH 6.0
CD103	Ab12920, Abcam	Rabbit	Mono	EPR4166 (2)	1:200	FFPE and FF (10 min acetone)	Citrate pH 6.0
CD163	NB110-40686, Novus Biologicals	Mouse	Mono	EDHu-1	1:200	FFPE	Citrate pH 6.0
CXCL16	500-P200, PeproTech	Rabbit	Poly		1:100	FF (10 min acetone)	NA
E cadherin	Ab40772, Abcam	Rabbit	Mono	EP700Y	1:100	FFPE	Citrate pH 6.0
Granzyme B	MA5-11587, Invitrogen	Mouse	Mono	GZB01	1:100	FFPE	Tris-NaCl (10 mM) + EDTA (1 mM) ph 9.0
HLA	M0755, DAKO	Mouse	Mono	CR3/43	1:1000	FF (30 min 4% PFA/0.9% NaCl)	NA
Ki-67	Novacastra	Rabbit	Poly		1:200	FFPE	Citrate pH 6.0
Laminin gamma-1	Ab8058, Abcam	Rat	Mono	A5	1:100	FF (10 min acetone)	NA
PLP	MCA839G, Biorad	Mouse	Mono	Plpc1	1:3000	FF (30 min 4% PFA/0.9% NaCl)	NA
S1P1	PK-AB718-4809, Promokine	Rabbit	Poly		1:1000	FFPE	Citrate pH 6.0

FF, fresh-frozen; FFPE, formalin-fixed, paraffin-embedded; mono, monoclonal; PFA, paraformaldehyde; poly, polyclonal.

Supplementary Table 3. Donor and sample information for the assessment of T-cell location.

Tissue	Cases (n)	Age (years)	Sex (F/M)	PMD (h:min)	pH value	Brain weight (kg)	MS subtype	Disease duration (years)
Control WM	4	78.0 ± 1.4	3/1	7:12 ± 2:27	6.6 ± 0.3	1,129 ± 44	–	–
NAWM	4	73.5 ± 13.3	4F	7:56 ± 1:46	6.6 ± 0.5	1,164 ± 91	SP: 2 PP: 2	31.5 ± 18.4
MS lesion (active)	4	68.25 ± 13.6	2/2	10:06 ± 0:26	6.4 ± 0.08	1,239 ± 179	SP: 3 PP: 1	40.0 ± 15.6
MS lesion (mA/I)	5	60.6 ± 9.7	3/2	9:31 ± 1:18	6.4 ± 0.2	1,099 ± 54	SP: 4 RR: 1	26.8 ± 12.5
MS lesions (inactive)	5	66.8 ± 16.8	3/2	9:34 ± 1:41	6.6 ± 0.6	1,191 ± 118	PP: 2 SP: 2 RR: 1	41.2 ± 23.1

Provided is the mean ± SD (standard deviation). F, female; M, male; PMD, post-mortem delay; PP, primary progressive; RR, relapsing–remitting SP, secondary progressive.

Supplementary Table 4. Donor and sample information for flow-cytometric analysis.

Donor	Samples	Diagnosis	Age (years)	Sex	PMD (h)	pH value	Disease duration (years)	MS type	Cause of death
1	Control WM	FTD-Picks disease	81	F	4:10	6.3			General deterioration, cachexia
2	Control WM	Depression	61	F	4:45	6.8			Euthanasia
3	Control WM	NDC	96	F	8:30	6.7			Stopped drinking and food intake
4	Control WM	NDC	72	M	6:55	6.8			Secondary renal insufficiency and pleural effusion
5	Control WM	Metabolic disorder	87	M	6:20	6.5			Hemodynamic instability with lactate acidosis
6	Control WM	NDC	97	M	5:10	6.7			Dehydration, kidney failure
7	Control WM	NDC	86	M	6:45	6.4			Endovascular aneurysm repair with iatrogenic infection in abdomen
8	Control WM	NDC	95	F	4:20	6.6			Ileus caused by tumor
9	Control WM	AD	73	M	7:20	6.1			Cachexia
10	Control WM	NDC	101	F	6:00	6.7			Heart failure
11	MS NAWM MS lesion (A, Fo)	MS	67	F	5:45	6.6	6	PP	Euthanasia
12	MS NAWM MS lesion (A, Fo) MS lesion (mA/I, Ro)	MS	50	F	10:15	6.4	13	SP	Euthanasia
13	MS NAWM MS lesion (A, Fo)	MS	53	F	5:50	6.8	16	PP	Euthanasia
14	MS NAWM MS lesion (A, Fo)	MS	67	M	7:55	6.4	11	PP	Euthanasia
15	MS NAWM MS lesion (mA/I, Fo)	MS	56	M	6:15	6.2	19	SP	Respiratory insufficiency due to MS, assumed pneumonia
16	MS NAWM MS lesion (mA/I, Fo)	MS	63	M	10:00	6.5	30	na	Aspiration pneumonia and sepsis
17	MS NAWM	MS	67	F	6:40	6.6	39	na	Sepsis from infected bile ducts
18	MS lesion (A, Fo)	MS	70	M	5:10	6.8	40	SP	Dehydration, decompensation, palliative sedation

A, active; F, female; Fo, foamy; FTD, frontotemporal dementia; M, male; na, clinical disease course not available; NDC, non-demented control; PMD, post-mortem delay; Ro, rounded.

Supplementary Table 5. Specification of antibodies used for flow cytometry and cell sorting.

Specificity	Clone	Fluorochrome	Manufacturer
CD3	SK7	PerCP-Cy5.5	BD Bioscience
CD3	UCHT1	APC	eBioscience
CD3	SK7	PE-Cy5.5	eBioscience
CD4	RPA-T4	FITC	eBioscience
CD4	OKT4	BV510	Biolegend
CD4	RPA-T4	AF700	BD Bioscience
CD8	RPA-T8	BV785	BD Bioscience
CD8	RPA-T8	PE-Cy7	eBioscience
CD8	SK1	FITC	BD Bioscience
CD20	L27	APC	BD Bioscience
CD27	O323	APC-Fire750	Biolegend
CD44	IM7	PE-Dazzle594	Biolegend
CD45RA	L48	PE-Cy7	BD Bioscience
CD49a (VLA-1)	SR84	BV421	BD Bioscience
CD56	MY31	PE	BD Bioscience
CD69	FN50	BV650	Biolegend
CD69	FN50	APC-Cy7	BD Bioscience
CD103	Ber-ACT8	BUV395	BD Bioscience
CD103	B-Ly7	PE	BD Bioscience
CXCR6 (CD186)	K041E5	PE-Cy7	Biolegend
GPR56	CG4	PE	Biolegend
Granzyme B	GB11	AF700	BD Bioscience
Ki-67	Ki-67	BV711	Biolegend
PD-1 (CD279)	EH12.1	BB515	BD Bioscience

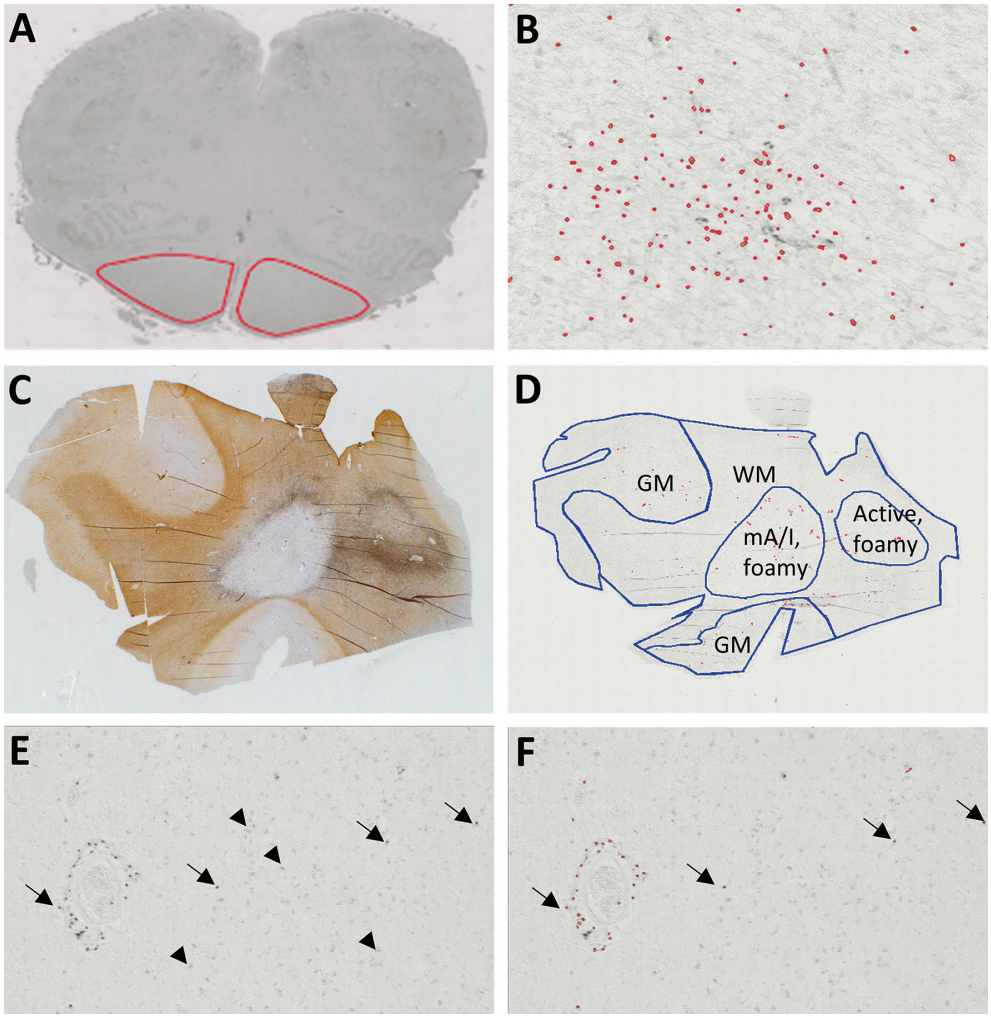
Supplementary Table 6. Donor and sample information for quantitative RT-PCR.

Donor	Sample	$CD69^+CD103^-$ cells	$CD69^+CD103^+$ cells	Diagnosis	Age (years)	Sex	PMD (h)	pH value	Cause of death
1	1	1,828	804	NDC	95	F	4:20	6.6	Ileus caused by tumor
2	1	4,071	1,964	NDC	72	M	6:55	6.8	Secondary renal insufficiency and pleural effusion
3	1	649	594	NDC	82	F	6:20	na	Physical deterioration, cachexia, dehydration
4	1	109	105	NDC	94	M	5:30	6.6	Metastatic lung cancer
5	1	599	31	NDC	98	F	7:20	6.2	Old age
6	2	2861	1901	DEM	85	M	5:10	6.24	Pulmonary carcinoma
7	2	4336	3563	DEM	68	F	6:05	6.68	Epileptic seizure
8	2	1170	1086	NDC	91	F	9:30	6.36	Old age

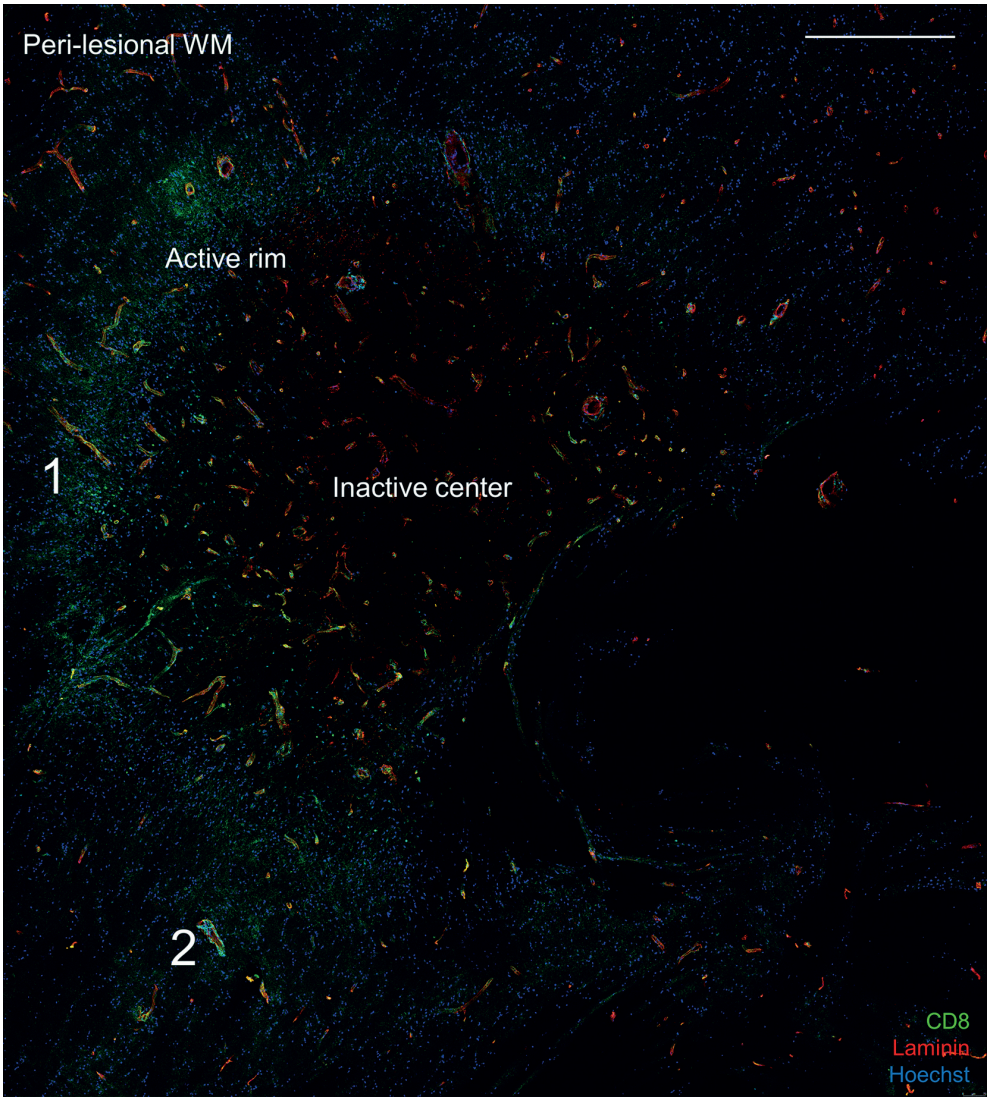
$CD69^+CD103^-$ cells (total sample 1 7,256, sample 2 8,367) and $CD69^+CD103^+$ cells (total sample 1 3,498, sample 2 6,550) were pooled for analysis. F, female; M, male; na, not available; NDC, non-demented control; DEM, dementia; PMD, post-mortem delay.

Supplementary Table 7. Primer pairs used for quantitative RT-PCR.

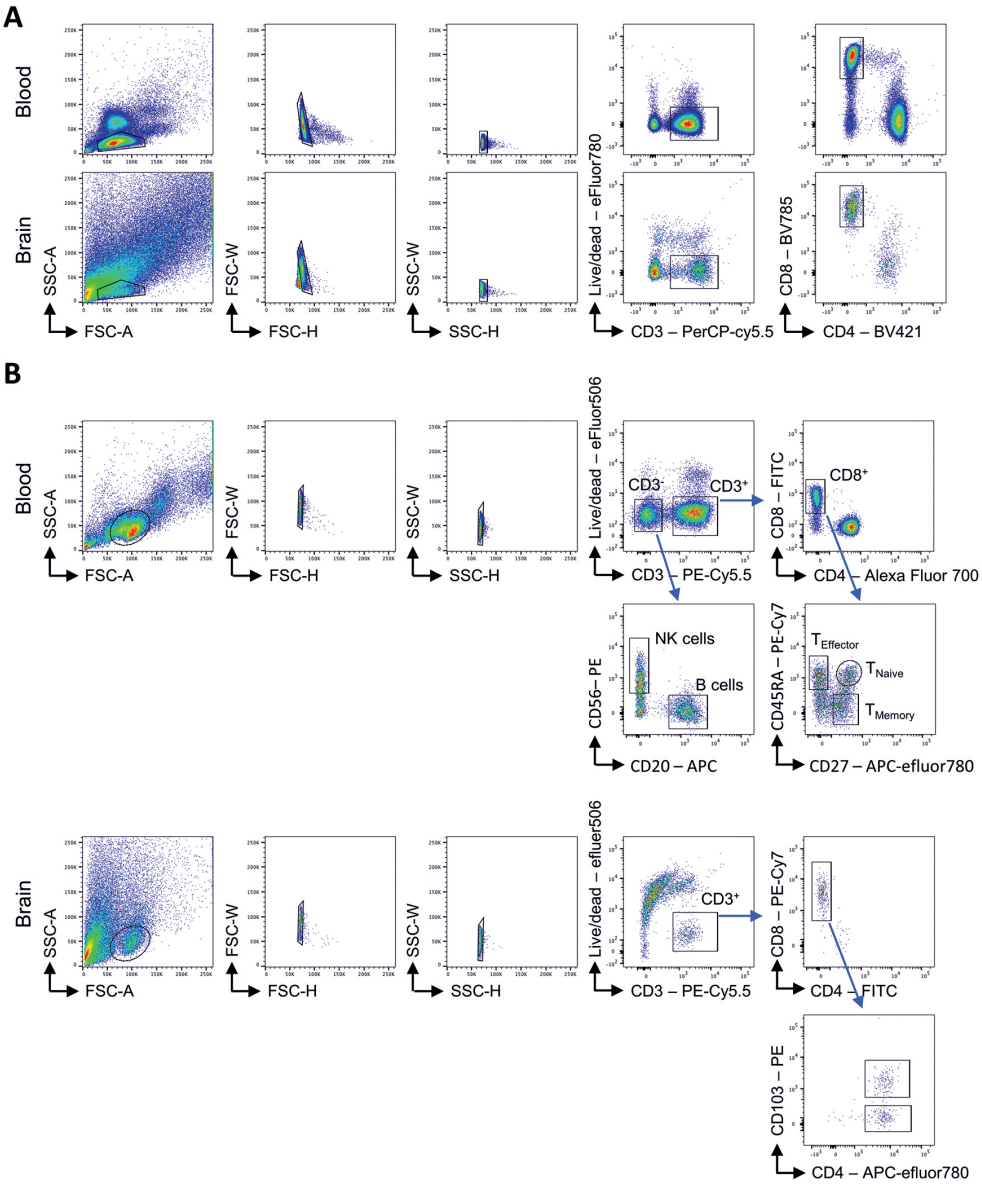
Gene (protein)	Primer pairs
ADGRG1 (GPR56)	Forward 5'-GATTGCTGGCCTGTTGTAG-3' Reverse 5'-GAATGATGGCTCCCTGTCC-3'
GAPDH	Forward 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse 5'-GAAGATGGT GATGGGATTTC-3'



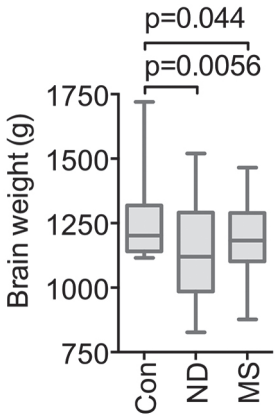
Supplementary Figure 1. Examples of mask calculation.
(A) Example of a black and white image of a medulla oblongata section stained for CD3, where WM pyramid tract is outlined. (B) Example of the mask setting for $CD3^+$ particles showing sensitivity and specificity of the mask. (C) Example of a macroscopic image of an HLA-PLP-stained section. (D) Example of the outline in a black and white image of a CD3-stained section using overlay with the HLA-PLP macroscopic picture. (E) Example of a black and white image of the CD4 immunohistochemistry showing perivascular $CD4^+$ lymphocytes (arrow) and $CD4^+$ ramified cells (arrow head) in the brain parenchyma. The latter show a less intense $CD4^+$ staining and likely are microglia/macrophages. (F) Example of the mask for the CD4 immunohistochemistry showing the specificity for the detection of $CD4^+$ lymphocytes.



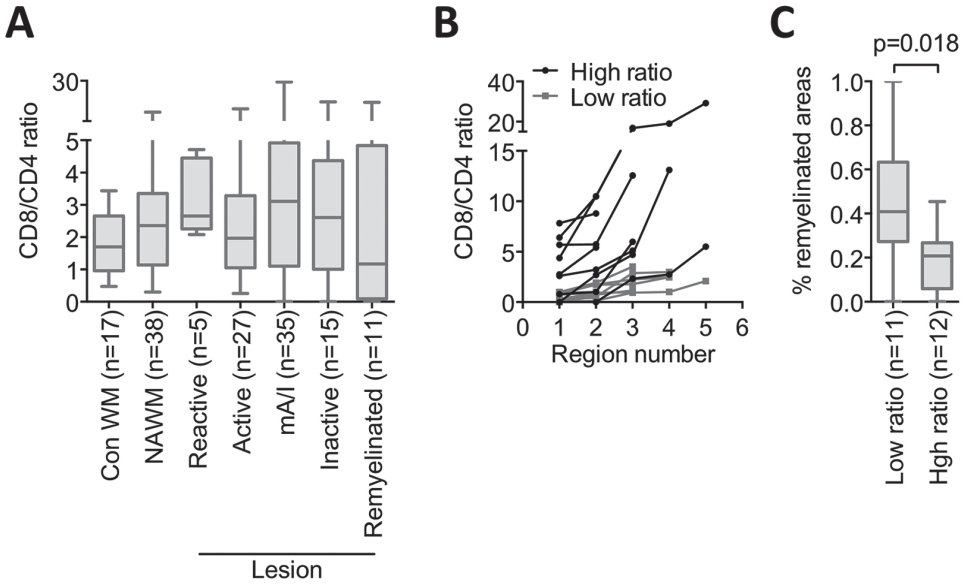
Supplementary Figure 2. Tiled confocal images from mA/I MS lesion with laminin, CD8, and Hoechst staining.
Region 1 shows that CD8 T cells enter brain parenchyma in chronic active lesion rim, while region 2 shows a small perivascular cuff located in NAWM outside active lesion rim. Scale bar is 0.5 mm.



Supplementary Figure 3. Gating strategy used for flow-cytometry and cell sorting analysis.
Representative dot plots showing the gating strategy of flow cytometry from left to right: cell gating by forward scatter (FCS) and sideward scatter (SSC), FCS-width/height duplet exclusion, SSC-width/height duplet exclusion, gating of viable $CD3^+$ cells, gating of $CD8^+CD4^-$ events. Blood cells were used to locate lymphocytes. Cells and markers were back-gated to ensure no relevant cell populations were lost by this strategy. (B) Representative dot plots showing gating strategy of cell sorting analysis: $CD3^-CD56^-CD20^+$ B cells, $CD27^+CD45RA^+$ naïve, $CD27^+CD45RA^-$ memory, $CD27^-CD45RA^+$ effector $CD3^+CD8^+$ T cells, and $CD20^-CD3^-CD56^+$ NK cells were sorted from human blood as well as $CD103^-$ and $CD103^+CD69^+CD8^+$ T_{RM} cells were sorted from human brain.



Supplementary Figure 4. Brain weight in MS cases, neurodegenerative controls, and non-neurological controls. One-way ANOVA test *p*=0.0084 with Tukey post-hoc test MS versus non-neurological controls *p*=0.044 and neurodegenerative controls versus non-neurological controls *p*=0.0056.



Supplementary Figure 5. Brain CD8/CD4 ratio is associated with the percentage of remyelinated regions in MS-autopsy cases.
(A) CD8/CD4 ratio did not alter between lesion types. (B) Donors with a high CD8/CD4 ratio and a low CD8/CD4 ratio show a consistent ratio in all regions, including lesions and perilesional WM that were measured. (C) Overall percentage of inactive shadow plaques in all dissected tissue blocks from these donors (Luchetti et al. 2018) was increased in donors that showed a low CD8/CD4 ratio. Mann–Whitney test was used and *p*-value is shown in plot.

Addendum: letters to the editor of *Brain*

Tissue-resident CD8$^+$ memory T cells in multiple sclerosis

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Sir,

We read with interest the article by Fransen and colleagues in Brain.[1] The authors studied the role of $CD8^+$ T cells in a large cohort of chronic multiple sclerosis autopsy cases from the Netherlands Brain Bank. In all white matter samples, $CD8^+$ T cells were conspicuous in the perivascular space (PVS; Virchow-Robin space). A large proportion of the $CD8^+$ cells displayed the phenotype of tissue-resident memory cells (T_{RM}) ($CD69^+$ $CD103^{+/-}$ $S1P1^-$ $CCR7^-$ $CXCR6^+$), similar to observations previously reported by another group of investigators.[2]

T_{RM} cells have emerged as an important subset of memory T cells. Unlike central memory and effector memory T cells, T_{RM} do not recirculate but are sessile residents in various tissues, including the brain, where they provide a first line of protection, especially against local viral spread.[3,4]

Fransen et al. suggest that (re-)activation of $CD8^+T_{RM}$ cells in the PVS "is a key mechanism in the maintenance of white matter lesion activity in advanced progressive multiple sclerosis".[1] Recruitment of $CD8^+$ T_{RM} might reflect an antiviral response that drives or facilitates the autoimmune process in a similar way as recently described in an animal model of multiple sclerosis.[5]

It is interesting to compare the findings by Fransen et al. in late chronic multiple sclerosis with our observations in subjects with early prodromal (subclinical) multiple sclerosis.[6] In our cohort of monozygotic twins who are clinically discordant for multiple sclerosis, we identified a subgroup of clinically healthy co-twins who show evidence of "subclinical neuroinflammation" on MRI or CSF analysis. Using single-cell transcriptomics (RNA-seq) we found that activated, clonally expanded $CD8^+T_{RM}$ cells are conspicuous components of the CSF from subjects with subclinical neuroinflammation (prodromal multiple sclerosis).[6]

The anatomy of the PVS and especially its connection with the CSF are complicated and controversial.[7] Nevertheless, there are obvious links and parallels between the $CD8^+$ T_{RM} observed in the PVS of autopsy cases with late-stage chronic multiple sclerosis,[1] and the $CD8^+$ T_{RM} detected in the CSF of subjects with early prodromal multiple sclerosis.[6] We conclude that $CD8^+$ T_{RM} are not just involved in the chronic late phase of multiple sclerosis, but are key players even in the earliest detectable (prodromal) stage of the disease process.

Funding

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Competing interests

The authors report no competing interests.

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Reply:

Tissue-resident CD8$^+$ memory T cells in multiple sclerosis

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We thank Hohlfeld and colleagues (2020) for their appreciation and discussion of our work. Our data provide an extension of the data earlier reported by Machado-Santos et al. (2018).[1] We showed that white matter perivascular $CD8^+$ T cells of multiple sclerosis donors co-expressed CD69 with variable levels of CD103 and CD49a, PD-1, and CXCR6,[2] characterizing them as tissue-resident memory T (T_{RM}) cells.[3]

Studying multiple sclerosis lesional T cells in brain autopsy tissue, we focused our work on the latest stages of multiple sclerosis. However, we fully agree that brain T_{RM} cells may also be relevant in the first stages of multiple sclerosis.[4] Since all patterns of white matter lesions in early multiple sclerosis are associated with profound infiltration of T cells,[5] an important question is how perivascular T_{RM} cells relate numerically to T cells infiltrating from the circulation through inflamed post-venular endothelium. Of note, Machado-Santos et al. and we observed in early multiple sclerosis lesions derived from diagnostic biopsies $CD103^-$ positive lymphocytes in both perivascular cuffs and brain parenchyma.[1,2] The sequence of events in which these resident and infiltrating populations are recruited to the perivascular space and multiple sclerosis lesions, and how their origins and clonality relate to each other is not understood.

Within and beyond this context, the observation of Beltrán et al. (2019)[6] concerning $CD8^+$ T_{RM} cells in CSF of twins discordant for multiple sclerosis is intriguing. It suggests that specific multiple sclerosis-related $CD8^+$ T_{RM}-like cells are recruited into the CSF as one of the earliest events in the disease. Potentially, viral infections account for the establishment of multiple sclerosis-associated brain $CD8^+$ T_{RM} populations as suggested by Steinbach et al. (2019).[7] Of note, where normal-appearing white matter and multiple sclerosis white matter lesions are dominated by $CD8^+$ T_{RM} cells, $CD4^+$ central memory T (T_{CM})-like cells dominate the CSF.[8] Although less prevalent numerically, $CD4^+$ T cells also accumulate in association with active and mixed active/inactive white matter le- sions.[2] Additionally to white matter and CSF T-cell pools, meningeal infiltrates containing T cells characterize the disease process of multiple sclerosis and associate with cortical demyelination already prevalent early in disease.[9] A detailed phenotypic profiling of these different T-cell fractions in the context of multiple sclerosis pathology is still is to be performed.

We fully agree with Hohlfeld and colleagues in their interpretation that further studies on the interplay between different CNS resident and infiltrating T cell subsets, and their temporal evolution during the onset and course of multiple sclerosis, may provide valuable insights to the nature and pathophysiology of multiple sclerosis. That is, both in early and advanced multiple sclerosis.

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Competing interests

The authors report no competing interests. IH received lecture and/or consultancy fee from Biogen and Novartis. JS received lecture and/or consultancy fee from Biogen, Merck, Novartis, and Sanofi-Genzyme.

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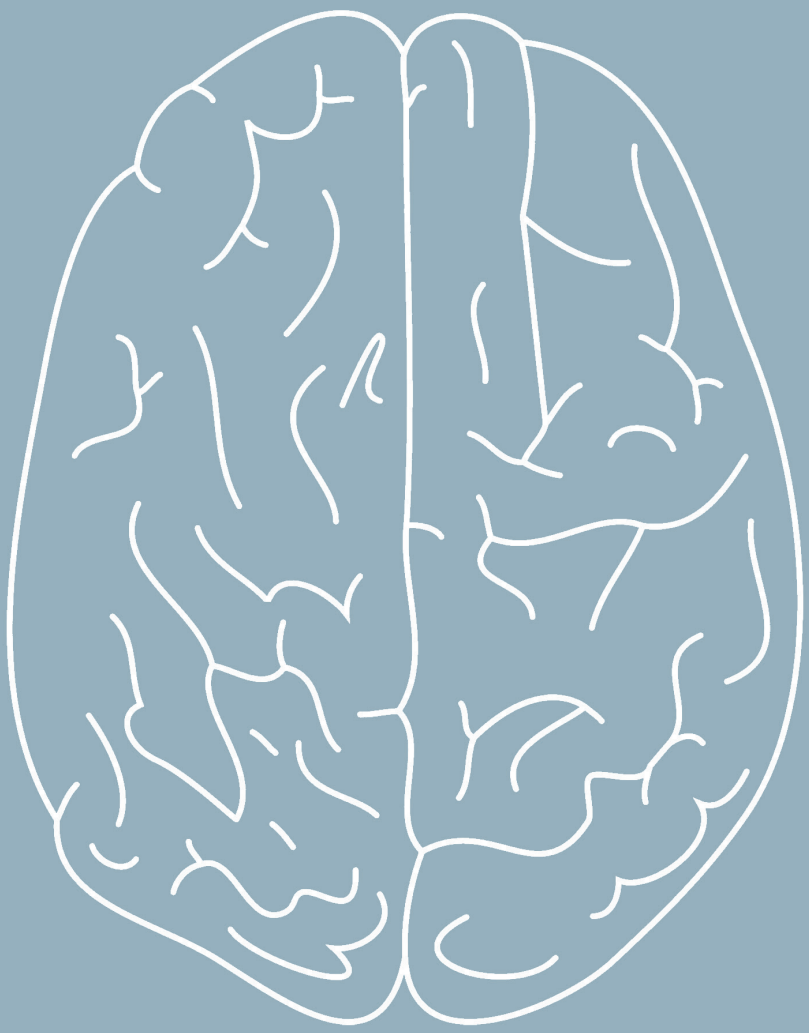
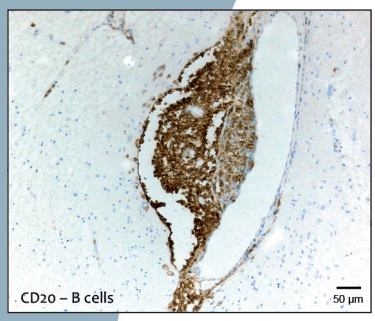
PART 2

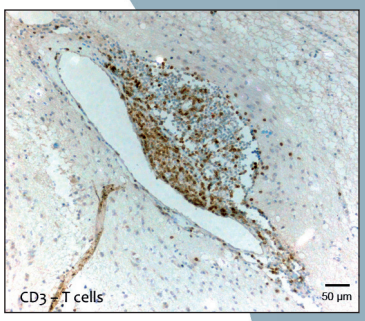
HETEROGENEITY OF THE IMMUNOPATHOLOGY IN ADVANCED MULTIPLE SCLEROSIS



CHAPTER 6

Absence of B cells in brainstem and white matter lesions associates with a less severe disease and absence of oligoclonal bands in multiple sclerosis





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ABSTRACT

Objective | To determine whether B-cell presence in brainstem and white matter (WM) lesions is associated with poorer pathological and clinical characteristics in advanced multiple sclerosis (MS) autopsy cases.

Methods | Autopsy tissue of 140 MS and 24 control cases and biopsy tissue of 24 MS patients was examined for $CD20^+$ B cells and $CD138^+$ plasma cells. Presence of these cells was compared to pathological and clinical characteristics. In corresponding cerebrospinal fluid (CSF) and plasma, immunoglobulin (Ig)G ratio and oligoclonal band (OCB) patterns were determined. In a clinical cohort of 73 patients, the presence of OCBs was determined at diagnosis and during follow-up.

Results | In 34% of active and 71% of mixed active/inactive lesions, B cells were absent, which correlated with less pronounced meningeal B-cell infiltration (p<0,0001). Absence of B cells and plasma cells in brainstem and WM lesions was associated with a longer disease duration (p=0,001), less frequent secondary progressive MS compared to relapsing and primary progressive MS (p<0,0001 and p=0,046 respectively), a lower proportion of mixed active/inactive lesions (p=0,01) and a trend for a lower number of T cells in MS WM lesions. Moreover, a lower CSF IgG ratio (p=0,006) and more frequent absence of OCBs (p<0,0001) were noted. In a clinical cohort, numbers of patients without OCBs in CSF were increased at follow-up (27,4%).

Conclusions | Absence of B cells is associated with a favorable clinical and pathological profile. This finding may reflect extremes of a continuum of genetic or environmental constitution, but also a regression of WM humoral immunopathology in the natural course of advanced MS.

INTRODUCTION

Multiple sclerosis (MS) is a heterogeneous disease differing in clinical disease course,[1] radiological appearance of the lesions,[2] and response to immunomodulatory therapies.[3] Interestingly, variability between MS patients is observed in the involvement of humoral immunity in the disease. At time of diagnosis, 10% of MS patients show absence of oligoclonal bands (OCBs) consisting of intrathecally produced immunoglobulin (Ig)Gs.[4] Absence of OCBs is associated with a decreased number of lesions on magnetic resonance imaging (MRI) and a more benign disease course.[4,5] Furthermore, the presence of OCBs in patients with clinically isolated syndrome is associated with an increased risk for clinically definite MS and with a increased risk of disability progression.[6,7]

In contrast to its limited presence in early MS,[8] advanced progressive MS is characterized by extensive cortical demyelination.[9] Active cortical demyelination is observed in conjunction with the presence of meningeal follicle-like inflammatory structures.[10–12] The distinct zones of B cells, plasma cells, and T cells resemble tertiary lymphoid structures.[12,13] The presence of these follicle-like structures associate with more severe disease, reflected by an earlier disease onset and faster accumulation of disability and earlier death.[11,12] Recently, Reali et al. reported that the density of meningeal B cells correlates with extensive axonal loss and white matter (WM) lesion area, but also with density of B cells in WM perivascular space.[14]

Besides cortical demyelination, demyelinating WM lesions also add up to disease severity in donors with advanced MS. In MS-autopsy cases, the presence of active and mixed active/inactive lesions has been reported to be substantial and correlate with a short time to reaching EDSS-endpoints, a shorter time to death, an unfavorable profile of risk factors for adverse outcomes, and an unfavorable profile of genetic risk factors for adverse outcomes.[15,16] Furthermore, we showed these active lesions to be populated by infiltrating T cells with a dominant tissue-resident memory T cell fraction showing signs of recent re-activation.[17,18] Frischer et al. quantified the presence of B cells in MS WM lesion and found these to be predominantly present in perivascular cuffs and meninges, and less frequently in the parenchyma.[19] Presence of B cells was found most frequent in acute lesions in relapsing–remitting donors and less frequently in progressive patients.[19] IgG-producing cells and IgG deposits are regularly found in MS WM lesions.[20,21] Furthermore, the number of B cells reported in late MS-autopsy lesions is highly variable between cases.[19,22]

The correlation of B-cell presence in WM lesions with clinical endpoints and risk-factors as well as with meningeal B cell-infiltration has been limitedly explored. Here we investigated the clinical and pathological characteristics of Netherlands Brain Bank (NBB) MS-autopsy cases in association with B-cell infiltration of brainstem and subcortical WM lesions.

MATERIAL AND METHODS

Donor and sample characteristics

141 MS-brain donors and 24 non-neurological controls from the NBB-autopsy cohort (Amsterdam, The Netherlands) were included for the analysis of B cells and plasma cells. Donors came to autopsy between 1991 and 2015, and were diagnosed with MS according to the then diagnostic criteria by their treating physicians. Clinical files were collected post-mortem by the NBB. By retrospective chart analysis, the clinical diagnosis of MS was confirmed for all patients, and the clinical course was defined as either relapsing–remitting (RR), secondary progressive (SP), or primary progressive (PP) by a neurologist.[23] No MRI data were available. None of the donors received MS disease-modifying therapies in the year before autopsy, except for one (B cell-positive) donor on fingolimod. Detailed donor and tissue characteristics are described in **Suppl.Table 1**, and treatment status is provided in **Suppl. Table 2**. The pathological diagnosis of MS was confirmed for all cases by a certified neuropathologist.[15] All donors were analyzed for anti-myelin oligodendrocyte glycoprotein (MOG) and anti-aquaporin (AQP)4 antibodies using cell-based assays (**Suppl. Figure 1**).[24–26]

For the immunohistochemical part of this study, three types of tissues were analyzed for the presence of B cells and plasma cells: (1) standardly dissected tissue blocks at the level of the medulla oblongata (MO) from 140 MS autopsy cases, (2) subcortical WM lesions from 73/140 MS-autopsy cases (158 WM lesions with a median two lesions per donor for both the donors with and without B cells) and 24 non-neurological control donors, and (3) early MS-biopsy WM lesions (N=28) from 24 MS patients, to explore how findings in post-mortem autopsy samples of donors with advanced MS correlate with findings at the earliest stages of MS. These sections were made available by the Institute for Neuropathology, University Hospital Münster (Münster, Germany). Additional information on the analysis and selection of the different tissue samples is described in the Supplementary Methods.

A CSF sample was acquired with a lumbar puncture from 73 MS patients with average disease duration of 11.7 ± 8.5 (mean ± SD) years. These patients visited the MS Center Amsterdam (Amsterdam, The Netherlands) for analysis of cognitive complaints, which is a common symptom in MS.[27] Information on OCB pattern at time of diagnosis was collected by a retrospective chart analysis. Patients characteristics are provided in **Table 1**.

Standard protocol approvals, registrations, and patient consents

Informed consent was given by the donors of the Netherlands Brain Bank for brain autopsy and for the use of material and clinical data for research purposes. NBB autopsy procedures have been approved by the medical ethics committee of Amsterdam UMC, location VUmc, Amsterdam, The Netherlands. Sampling of biopsies and CSF has been approved by the medical ethics committee of the University Hospital Münster and Amsterdam UMC, location VUmc, respectively.

Immunohistochemistry

Immunohistochemistry of the autopsy tissue samples was performed on 8-µm thick formalin-fixed paraffin-embedded tissue sections. All brainstem and subcortical WM tissue sections were immunostained for myelin (proteolipid protein, PLP) and human leukocyte antigen (HLA-DR/DQ, referred to as HLA) as previously described.[15,16] Lesions were annotated and sections were stained for CD20, CD138,[29] and CD3 as described in the Supplementary Methods. For CD138, an image of the positive control in tonsil is provided in **Suppl. Figure 2**.

OCB and IgG measurement in CSF and plasma

A selection of 16 NBB MS cases without presence of B cells and CD138$^+$ plasma cells in perivascular space and parenchyma of both MO and subcortical active WM lesions and 16 MS cases with B cells and CD138$^+$ plasma cells at these locations was made to analyze post-mortem cerebrospinal fluid (CSF) samples. One case was excluded due to CSF anti-MOG positivity (**Suppl. Figure 1**). Paired plasma samples were available from 20 of these MS autopsy cases (10 with B cells and 10 without B cells). Additionally, paired CSF and serum samples of 73 MS patients were analyzed. In all samples, IgG levels were determined with nephelometry, and the presence of OCBs was analyzed with isoelectric focusing followed by IgG immunoblotting.[30]

Statistical analysis

Statistical analysis were performed in GraphPad Prism 8 (8.1.1, April 2019; GraphPad, San Diego, CA, USA). Proportional differences between two or more strata were tested with the Fisher's exact and Chi-Square test, respectively. Brainstem lesion load and reactive site load were log transformed. Normally distributed data were analyzed using a Students t-test. Non-parametric Mann–Whitney U test was used when data was not normally distributed data. For disease duration and age at death a survival, analysis was performed using the Gehan–Breslow–Wilcoxon test.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

B cells are present in early-MS biopsy lesions and in active and mixed active/inactive MS-autopsy lesions

Of the NBB MS autopsy cohort, we analyzed the material of N=140 donors for the current study. First, we analyzed the presence of B cells and CD138$^+$ plasma cells in the MO, since this is one of the few standardly-dissected regions in the NBB MS-autopsy protocol, which contains both WM and grey matter (GM) brain parenchyma and meninges. B cells were more often present in the perivascular space (p=0.004) and meninges (p<0.0001), compared to the brain parenchyma (17%,

51%, and 4% of cases, respectively **Figure 1A-B**). In the MO collection, 85 sections contained MS lesions, and 53 sections did not contain MS lesions. B cells and CD138$^+$ plasma cells were found more frequently (p=0.028 and p=0.038, respectively) in sections with MS lesions (21% had B cells and 19% had plasma cells), compared to normal-appearing MO tissue sections (9% had B cells and 8% had plasma cells) (**Figure 1C-D**).

To investigate the association with WM lesion characteristics, we scored the presence of B cells and CD138$^+$ plasma cells in subcortical WM from 24 non-neurological controls and 73 MS-autopsy cases, containing 158 MS lesions (10 reactive, 41 active, 66 mixed active/inactive, 25 inactive, and 16 remyelinated). Moreover, we determined the presence of B cells and CD138$^+$ plasma cells in 28 early MS biopsies from WM lesions, which were all active. In the non-neurological controls, we identified B cells (2-5 cells per section) in the meninges in 4% (2 out of 24) of the cases. B cells were more frequently found in early biopsy (93%; p<0.0001) and in active (66%; p<0.0001) and mixed active/inactive (29%; p<0.0001) autopsy MS lesions compared to control WM. Notably, in 34% of the active autopsy lesions, no B cells were identified. In reactive (10%), inactive (8%), and remyelinated lesions (6%), B cells were not significantly enriched, compared to control WM (**Figure 1E**). In control WM and meninges, no CD138$^+$ plasma cells were identified. CD138$^+$ plasma cells were found in early biopsy lesions (56%; p=0.004) and in all autopsy MS-lesion subtypes – reactive (10%; p=0.002), active (22%; p<0.0001), mixed active/inactive (8%; p=0.007), inactive (24%; p<0.0001), remyelinated (19%; p<0.0001), compared to control WM (**Figure 1F-I**).

The presence of B cells and CD138$^+$ plasma cells is a general donor characteristic

We next assessed the presence of B cells and CD138$^+$ plasma cells within multiple locations (parenchyma, perivascular space, and meninges) and tissue blocks (MO and subcortical WM) from the same donors (**Figure 2A-B**). The presence of B cells in the perivascular space was associated with the presence of B cells in the meninges (92% and 43% in donors with and without perivascular B cells, respectively; p<0.0001; **Figure 2D)**. This is in accordance with Reali et al., who also observed a positive correlation between B-cell counts in meninges and perivascular space of MS spinal cords.[14] Furthermore, the presence of B cells in the MO was associated with the presence of B cells in subcortical WM (50% and 27% in donors with and without MO B cells, respectively; p=0.001; **Figure 2E**).

Limited presence of B cells in MS-autopsy cases associates with a favorable clinical and pathological profile

To assess whether the presence of B and CD138$^+$ plasma cells in MO and subcortical WM lesions correlates with more severe MS, likewise earlier reported for meningeal B-cell infiltrates, we compared donors with and without B cells at these locations. B cells were frequently encountered in perivascular clusters with T cells (**Figure 3A**). Cases without B cells at the MO showed less often perivascular cuffing of T cells in the MO (11% and 35%; p<0.0001, **Figure 3B**). Cases without B cells in subcortical WM showed a trend for a lower number of T cells in subcortical MS lesions (median

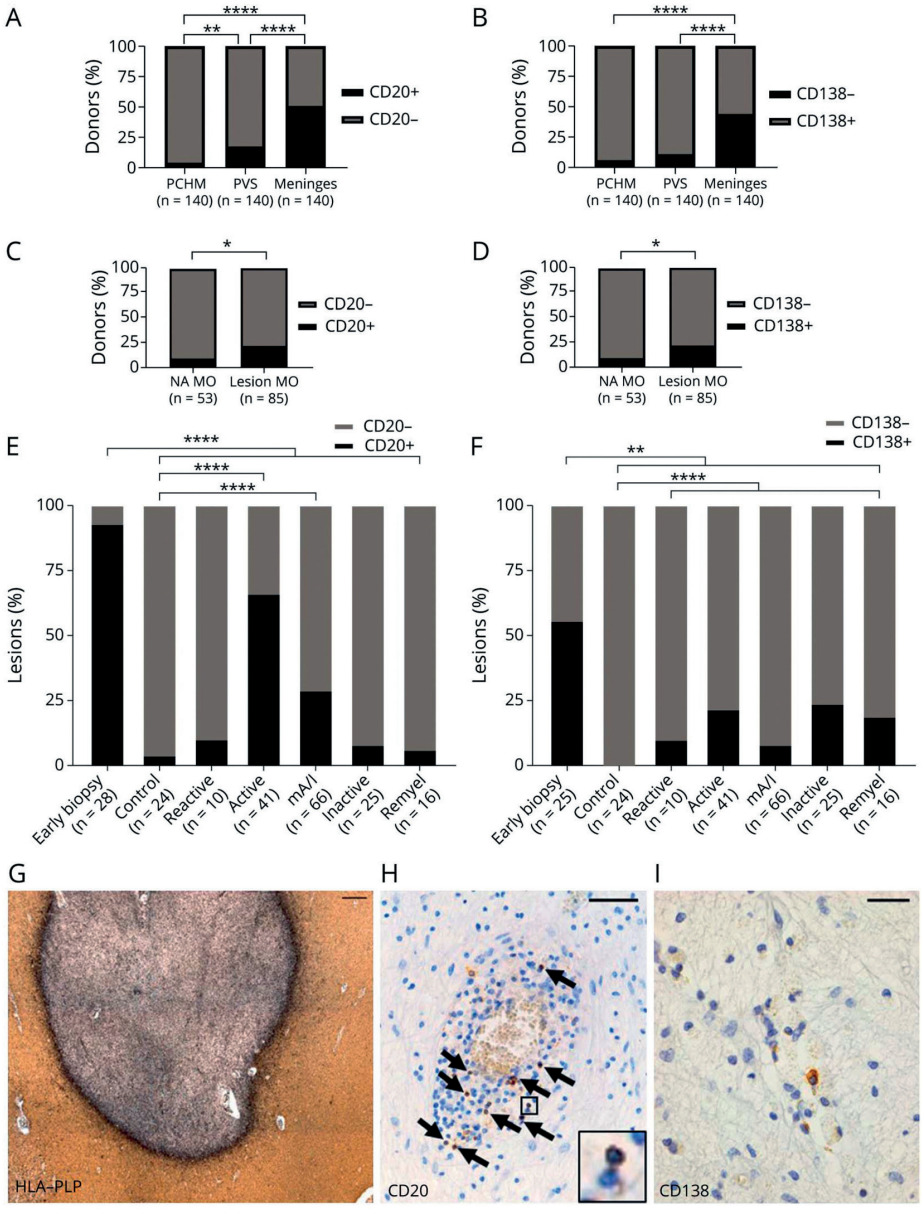

Figure 1. B cells are enriched in biopsy lesions and active and mixed active/inactive lesions at autopsy. (A/B) B cells and plasma cells were enriched in perivascular space and meninges of the MO, compared to MO parenchyma. (C/D) MO lesions contained more frequently B cells and plasma cells, compared to the normal-appearing MO (NA MO). MS lesion subtypes were analyzed in the subcortical WM. (E) Early MS biopsy lesions significantly more often contained B cells, compared to all autopsy lesions. In active and mixed active/inactive autopsy lesions, B cells were significantly more often present, compared to control WM. (F) Early MS-biopsy lesions significantly more often contained plasma cells, compared to autopsy lesions. Plasma cells were significantly more often present in all MS lesion types, compared to control WM. (G) Example of an inflammatory active MS lesions of a secondary progressive MS brain donor with MS for 27 years, stained for HLA (black) and PLP (brown). Scale bar is 500 µm. (H/I) In the perivascular space, B cells ($CD20^+$, panel H, scale bar is 50 µm) and a plasma cell ($CD138^+$, panel I, scale bar is 25 µm) were present (both brown color). * $p<0.05$, ** $p<0.01$, and **** $p<0.0001$.

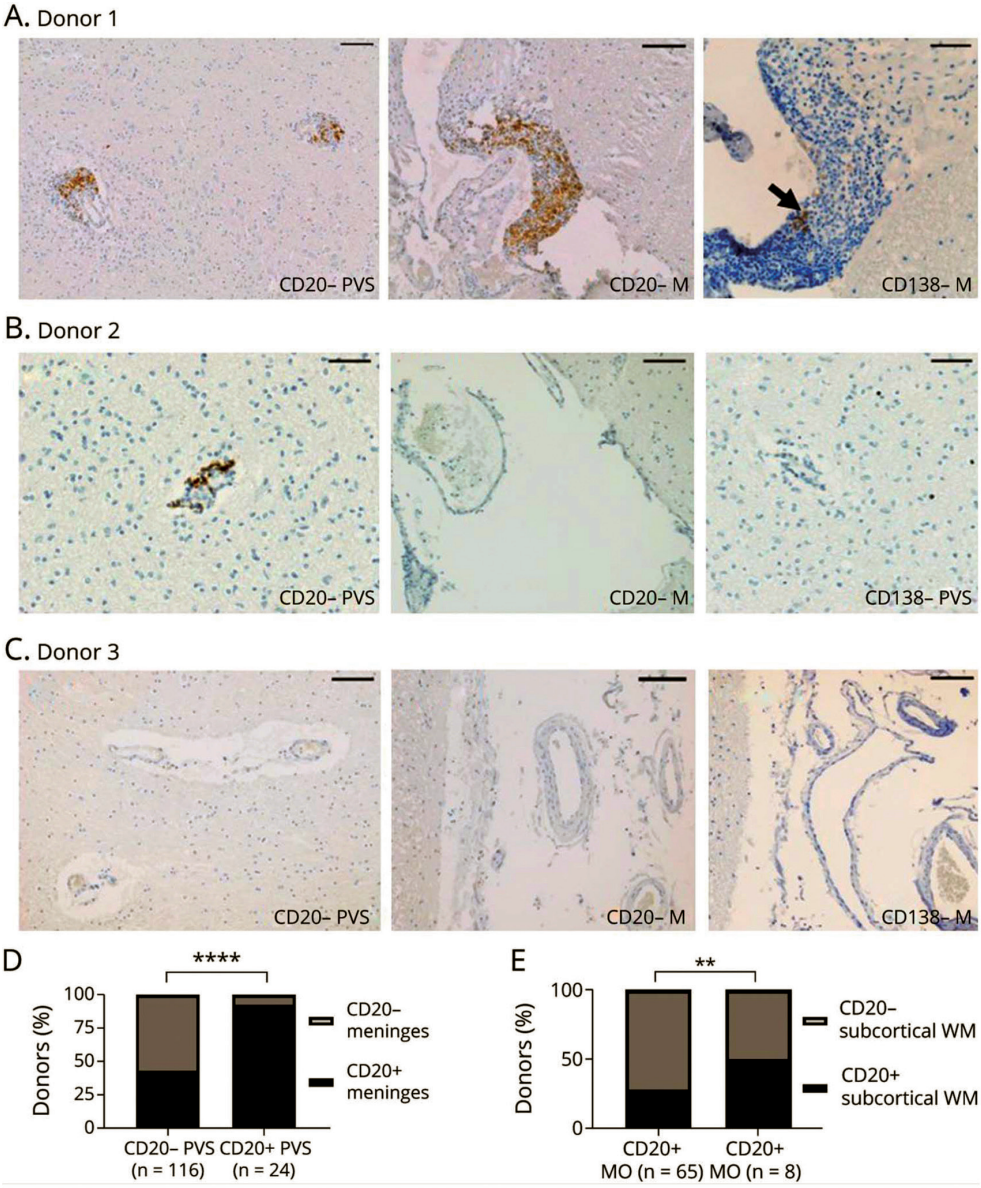

Figure 2. B-cell and plasma cell presence in meninges and perivascular space is consistent within donors. (A) In the MO of donor 1 with 27 years of secondary progressive MS, B-cell and plasma cell infiltrates were identified in both the perivascular space (PVS) and meninges (M). Scale bars are 100 µm for CD20 and 50 µm for CD138. (B) In the MO of donor 2 with a primary progressive disease course and a disease duration of 2 years, B cells were detected in the perivascular space but not in the meninges, and no plasma cells were identified. Scale bars are 50 µm for CD20 and CD138 in PVS and 100 µm for CD20 in meninges. (C) In the MO of donor 3 with a relapsing disease course for 38 years, no B cells or plasma cells were identified in both the meninges and perivascular space. Scale bars are as in A. (D) The absence of B cells in the perivascular space (PVS) is associated with the absence of B cells in the meninges. (E) The absence of B cells in the MO is associated with the absence of B cells in subcortical WM. ** $p<0.01$, **** $p<0.0001$.

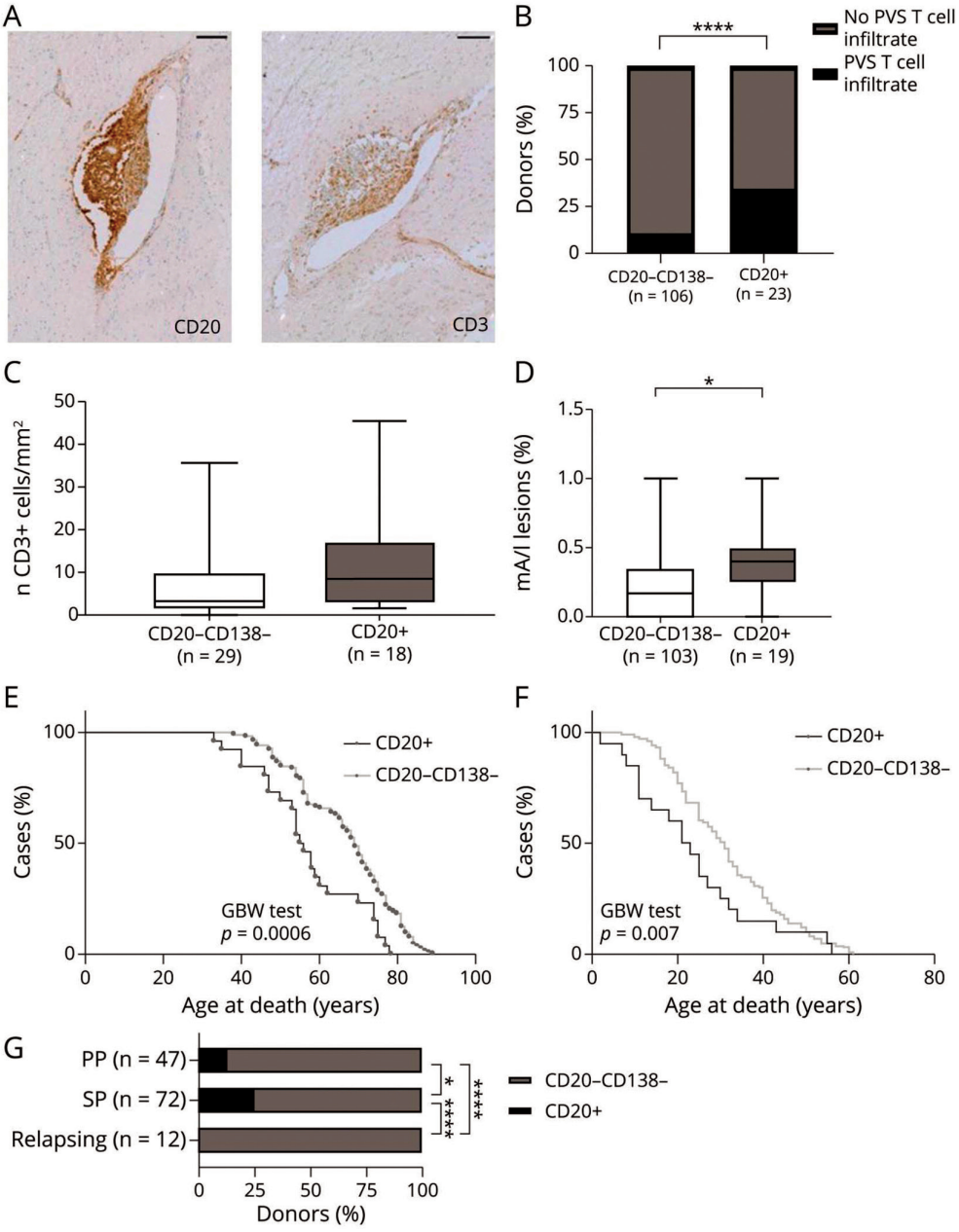

Figure 3. MS cases with limited presence of B cells show a favorable pathological and clinical profile.
(A) B cells were often encountered in perivascular clusters together with T cells. Scale bars are 100 µm. (B) MS cases with limited presence of B cells showed less often perivascular clustering of $CD3^+$ T cells, (C) a trend for a lower number of $CD3^+$ T cells in MS lesions, (D) a lower percentage of mA/I lesions, (E) a higher age at death, (F) a longer disease duration, and (G) more often a secondary progressive disease course. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

3.3 vs 8.3 cells/mm^2; p=0.06; **Figure 3C**) and a lower overall percentage of mixed active/inactive lesions (mean 23.7% vs 39.6%; p=0,01; **Figure 3D**), compared to MS donors with B cells. Clinically, they showed a higher age at death (median 69.0 vs 55.5; p=0,0006; **Figure 3E**) and a less severe clinical disease course, defined as a longer disease duration (median 31.0 vs 22.0; p=0.007; **Figure 3F**), and they more often had a persistent relapsing or primary progressive course, compared to a secondary progressive course (100% and 87% vs 75%; p<0.0001 and p=0.046; **Figure 3G**). There was no difference in brainstem lesion load, reactive site load, percentage of inactive remyelinated areas, and incidence of cortical GM lesions between MS cases with and without B cells at the investigated locations (**Suppl. Figure 3**).

MS autopsy cases with limited presence of B cells show a lower intrathecal IgG production and lack more often OCBs

Since our data suggest an association between the presence of B cells in meninges and MO/subcortical WM, as well as an association with a more severe pathological and clinical profile, we explored its relevance for intrathecal B-cell activation. Since an increased intrathecal IgG production and OCB presence are highly correlating biomarkers of MS,[31] and presence of OCB's is associated with adverse outcomes,[6,32] we explored whether these CSF biomarkers were associated with B-cell and CD138$^+$-plasma cell presence in MO and subcortical WM lesions. We conducted an extreme-of-outcomes-analysis by selecting 16 cases with B cells and 16 cases without B cells and CD138$^+$ plasma cells in both MO and subcortical WM lesions. One MS case with B cells and CD138$^+$ plasma cells was excluded prior to OCB analysis since anti-MOG antibodies were detected in the post-mortem CSF. There was no significant association of IgG index and OCB presence (**Figure 4A**) with post-mortem delay. The pH of post-mortem CSF showed a positive correlation with IgG index (Spearman's correlation R=0.498; p=0.035), but not with OCB presence of presence of B cells (**Suppl. Figure 4**). Selected cases lacking B cells and CD138$^+$ plasma cells in MO and subcortical WM lesions showed a lower CSF IgG level (median 0.04 vs 0.07; p=0.02, **Figure 4B**) and a lower IgG CSF/serum ratio (median 0.003 vs 0.008; p=0.007, **Figure 4C**), indicating a lower intrathecal IgG production compared to MS cases with B cells. CSF OCBs were absent in 37% of cases without B cells and CD138$^+$ plasma cells, while all cases with B cells displayed CSF OCBs (p<0.0001; **Figure 4D**). This observation suggests that these MS cases with limited presence of B cells and CD138$^+$ plasma cells in MO and subcortical WM lesions are characterized by an overall altered CSF IgG clonality and lower IgG production. This observation is again in line with the strong correlation reported by Reali et al. between meningeal and perivascular B-cell presence.[14] Additionally, the IgG index, but not the presence of OCBs, was positively correlated with the number of T cells in subcortical WM (**Suppl. Figure 5A**). Other pathological endpoints did not correlate with IgG production or clonality (**Suppl. Figure 5B-D**).

OCBs can disappear over time in MS patients

In MS autopsy cases selected for absence of B cells and CD138$^+$ plasma cells, the prevalence of OCBs was lower than the expected 90% OCB-positivity of MS patients at diagnosis.[33] This difference

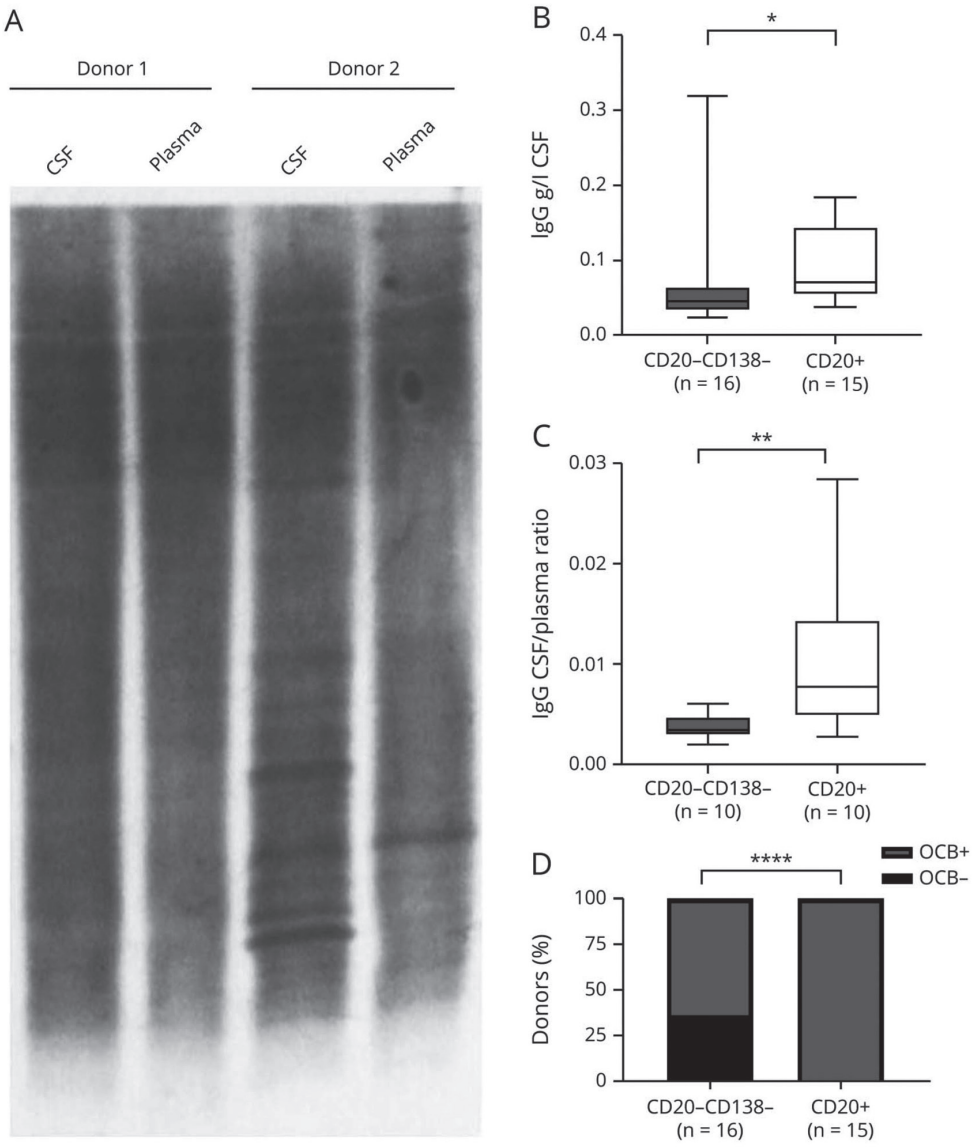

Figure 4. MS cases with limited B cells show lower intrathecal IgG-production and more often lack OCBs. (A) Example of OCB-patterns in paired CSF and plasma for a donor without (donor 1) and a donor with (donor 2) CSF-unique OCBs. MS cases with limited presence of B cells show (B) a lower concentration of IgG in post-mortem CSF and (C) a lower CSF/plasma IgG ratio, suggesting a lower intrathecal IgG production, and (D) more lack CSF OCBs. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

could be explained by selection of MS donors with an extreme profile of genetic or environmental factors,[16] but also by a decline of the intrathecal humoral immune response overtime in chronic MS. In our current study, two of six MS cases without OCBs at autopsy had an elevated IgG ratio at diagnosis without information on OCBs, one of six donors had normal diagnostic CSF examination, and no information was available for the three other donors. To explore whether a dynamic course of OCB pattern throughout the disease course of MS can be a plausible explanation of our findings,

Table 1. Clinical cohort of MS patients with OCB examinations.

Diagnosis	**Cases** (n (%))	**Age at OCB** (years)	**Sex**	**MS type** (%)	**Disease duration at OCB** (years)	**Treatment (%)**
All MS	73	49.2 ± 10.0	46F/27M	RR 68 SP 19 PP 5 various 8	11.7 ± 8.5	49 DMT 51 none
OCB-positive	53 (72.6)	48.3 ± 9.7	32F/21M	RR 75 SP 19 PP 8 various 2	11.4 ± 8.5	53 DMT 47 none
OCB-negative	20 (27.4)	51.5 ± 10.7	14F/6M	RR 55 SP 20 various 25	13.2 ± 8.7	40 DMT 60 none
All OCB-negative						
At diagnosis OCB-positive	6 (30)	49.5 ± 9.7	5F/1M	BD 17 RR 67 SP 17	11.0 ± 7.0	83 DMT 17 none
Previously elevated IgG, OCB unknown	4 (20)	60.0 ± 9.6	2F/2M	CIS 25 RR 50 SP 25	17.3 ± 5.7	25 DMT 75 none
At diagnosis OCB-negative	4 (20)	49.0 ± 10.6	3F/1M	CIS 50 RR 50	6.0 ± 9.4	25 DMT 75 none
Not reported	6 (30)	49.5 ± 12.1	4F/2M	RR 50 SP 33 NR 17	16.0 ± 10.1	17 DMT 83 none
OCB-negative, at diagnosis positive						
Case 1		56	F	RR	20	GLA
Case 2		54	F	RR	8	DMF
Case 3		37	F	RR	13	DMF
Case 4		52	F	BD	4	IFNβ
Case 5		38	F	RR	2	DMF
Case 6		60	M	SP	16	none

Provided is the mean ± SD (standard deviation). BD, Balo's disease; CIS, clinically isolated syndrome; DMF, dimethyl fumarate; F, female; GLA, glatiramer acetate; IFNβ, interferon-β; M, male; NR, not reported; PP, primary progressive; RR, relapsing–remitting; SP, secondary progressive; various, BD, CIS and, NR.

the presence of OCBs was determined in a clinical cohort of 73 MS patients that underwent a lumbar puncture after an average disease duration of 11.7 ± 8.5 (mean ± SD) years. In 27.4% (20 out of 73) of the MS patients, OCBs were absent. In six of the 20 MS patients without OCBs at follow-up, OCBs were present at time of diagnosis (**Table 1**). Although laboratory differences can be confounders, these data support the hypothesis that the contribution of B cells to MS pathology may decline during the course of MS.

DISCUSSION

We here demonstrate absence of B cells and CD138$^+$ plasma cells in 34% of the active WM lesions of advanced MS cases in the NBB autopsy cohort. Cases without B cells at MO or subcortical WM showed a more favorable pathological profile as indicated by a lower number of T cells in MS lesions, a lesser frequency of perivascular cuffing of T cells, and a lower percentage of mixed active/inactive lesions. Clinically, they manifested with a less frequent secondary progressive disease course, a longer disease duration, and a lower percentage of mixed active/inactive lesions, compared to the MS donors with B cells in MO or subcortical WM lesions. Further, a selected subgroup of MS patients without WM B cells and CD138$^+$ plasma cells had a lower intrathecal IgG production and lacked more often unique OCBs in post-mortem CSF. Our findings indicate that, besides an important role of meningeal B cells in cortical pathology of advanced progressive MS, B-cell infiltration in WM is also a detrimental phenomenon at the later stages of MS.

In MS and also other autoimmune diseases, B cells have been described to play an important role in antigen presentation and cytokine production, which induces the activation and proliferation of T cells.[34–37] MS cases with B cells show an increased number of T cells in their MS lesions suggesting increased T-cell activation. We and others previously showed that re-activated tissue resident-memory T cells (T_{RM}) are associated with the ongoing inflammatory lesion activity in WM lesions from advanced MS cases.[19,22,38] In MS lesions, these re-activated T_{RM} cells are often encountered in clusters in the perivascular space together with B cells[17,39] suggesting that antigen presentation and reactivation of T_{RM} cells induced by B cells potentially occurs at this location.[17,18,40] This illustrates that besides IgG production, B cells may have different functional roles in MS WM lesions.

We show that CD138$^+$ plasma cells are present more often in MS lesions compared to control and normal-appearing WM in line with earlier reports, however, only in a low percentage of the MS autopsy cases.[20,41] Interestingly, CD138$^+$ plasma cells were most often present in inactive lesions compared to the other lesion subtypes. Prineas et al.[41] previously showed in a detailed electron microscopy study of the perivascular space in MS tissues that high numbers of plasma cells are present in inactive lesion areas. This suggests that CD138$^+$ plasma cells play a less prominent role compared to B cells in the ongoing microglial activity of MS lesions. Ocrelizumab and rituximab, which show an effect on disease progression in MS, are directed against circulating CD20$^+$ B cells but do not affect CD138$^+$ plasma cells.

A large heterogeneity in the number of B cells and the presence of IgG depositions in MS lesions has been described over the past decades. In both, early MS biopsies as well as late MS autopsies, the presence[42–44] and absence[42,45] of IgG deposits in MS lesions has been described. Also the number of B cells in MS autopsy lesions is highly heterogenous between MS cases. In 34% of the inflammatory active MS lesions in autopsy tissue, we identified no B cells and we showed that presence of B cells correlates between different location (MO and subcortical WM) and compartments (parenchyma, perivascular space, meninges) in an individual donor.

In a selected subgroup of MS cases without WM B cells, a lower intrathecal IgG production and a more frequent absence of OCBs was found. The presence of OCBs in 60% in these MS cases is lower compared to clinical MS cohorts, where 90% showed OCBs at diagnosis4. Possibly, we now selected an extreme subgroup of MS cases with a genetic profile at one side of a continuum that restricts involvement of B cells in MS lesion pathogenesis.[28] Alternatively, since we identified B cells in 92% of the early MS biopsy lesions, and the MS cases with limited B-cell presence in autopsy tissue had a longer disease duration and older age, B-cell involvement in WM lesion activity might be extinguishing over time. Accordingly, Frischer et al. reported higher numbers of perivascular B cells in donors with relapsing and progressive disease, when compared to inactive disease.[19] We provided some support for this hypothesis, by observing in a clinical cohort the absence of OCBs in 27.4% of patients after a disease duration of 11.7 ± 8.5 (mean ± SD) years. In six of these patients without OCBs, their presence at diagnosis could be validated. In four of these patients, the elevated IgG index at diagnosis was validated. These data require careful interpretation, since comparison with historical data on OCB presence may be inaccurate. It is not likely that treatment with disease-modifying therapies confounds these results. In clinical studies, the presence of CSF OCBs was not-affected by highly efficacious therapies as fingolimod,[46] rituximab,[47] and alemtuzumab,[48] while treatment with natalizumab[49,50] and cladribine[51] was associated with reduced OCBs. Treatment with dimethyl-fumarate has not been associated with lower CSF IgG production.[52] Although loss of CSF OCBs has been described in a cohort of interferon-beta and glatiramer acetate-treated MS patients,[53] this has not been observed in controlled studies. Whether the absence of perivascular B cells truly is a biomarker for the regression of WM inflammatory disease activity in advanced MS remains to be determined. Regarding cessation of disease-modifying therapies in advanced MS, this could be a clinically useful hypothesis to pursue.

Our study has some limitations. Due to the structure of the NBB donor program, we could only investigate presence of B cells at selected locations in WM and meninges. Bias in our data by sample and site selection cannot be excluded, which may be partially overcome by selecting a standardly dissected location and comparing multiple locations within the same donor. The extreme outcome analysis, comparing donors with or without B cells at multiple locations in a dichotomous approach, providing a rather crude estimate of biological associations than correlation analyses. However, due to limited availability of material, this was for the current research question the most feasible approach.

In sum, we here demonstrate in an advanced MS-autopsy cohort that absence of B cells at the MO and subcortical WM is associated with a favorable clinical and pathological profile. This finding may reflect extremes of a continuum of genetic or environmental constitution, but also a regression of WM humoral immunopathology in the natural course of advanced MS.

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SUPPLEMENTARY MATERIAL

Supplementary methods

Sample selection

For the immunohistochemical part of this study, three types of tissues were analyzed:

1. Standardly dissected tissue blocks at the level of the medulla oblongata (MO) were systematically examined for B cells and plasma cells. This approach allowed a standardized comparison between MS cases in the MS-autopsy cohort. The MO was selected since (1) it is one of the few regions standardly and unbiasedly dissected in the NBB-autopsy protocol, (2) it contains white matter, grey matter, and meninges and hereby covers relevant tissue compartments for MS, (3) brainstem lesions as presenting clinical symptom or on MRI scans are associated with a poor prognosis in MS patients and therefore are clinically very relevant to study.[1] MO tissue blocks were obtained from 140 MS donors, and 1 MO tissue section was analyzed per case. B-cell and plasma cell presence or absence was scored for three regions within the MO section: the meninges, the perivascular space, and the parenchyma. B cells and plasma cells were considered present when >1 individual cell could be identified in the section. The scoring was performed by an observer that was blinded for the MS-lesion characterization of these sections. MS cases with B cells in the MO (n=24) were compared to the cases without both B cells and plasma cells in the MO (n=107). MO sections that contained MS lesions and sections without any MS lesions were compared. The presence of infiltrates of B cells was scored separately when clusters of B cells were identified in the perivascular space or the meninges.[2–4]
2. In addition to the MO, to assess the consistency of findings when sampling tissue at another location, 158 subcortical white matter (WM) lesions from 73 MS cases and subcortical white matter from 24 non-neurological controls was examined for the presence of B cells and plasma cells. The total WM per section was systematically scored, and in all positive sections, the presence of B cells and plasma cells in WM lesions was scored separately for each lesion.
3. To explore how findings in post-mortem autopsy samples of donors with advanced MS correlate with findings at the earliest stages of MS, 28 biopsies of WM lesions from 24 early MS patients were scored for the presence of B cells and plasma cells.

MS lesion characterization

Reactive, active, mixed active/inactive, inactive, and inactive remyelinated lesions were distinguished using HLA and PLP immunohistochemistry as previously described.[5,6] In short, reactive sites are characterized by normal-appearing myelin with increased number of activated microglia/macrophages, active lesions show partial loss of myelin with microglia/macrophages throughout the lesion area, mixed active/inactive lesions are characterized by a demyelinated inactive center with a rim of activated microglia/macrophages, and inactive and remyelinated lesions are characterized by partial loss of myelin without any microglia/macrophages. The characterization of MS lesion subtypes in the autopsy lesions is comparable to the characterization performed in the early biopsy lesions according to Kuhlmann et al.[7]

Immunohistochemistry

Lesions were annotated, and adjacent sections were stained for CD20 (M0755, concentration 1:100; Agilent, Santa Clara, CA, USA), CD138 (MCA2459, concentration 1:500; Biorad, Hercules, CA, USA), and CD3 (GA503, concentration 1:100; Agilent) using previously described protocols.[6,8] Biopsy lesions were all characterized as active as described in Kuhlmann et al.[7] For the biopsy lesions, 4-µm thick sections were cut, and immunohistochemistry for CD20 (M0755, concentration 1:700) and CD138 (MCA2459, concentration 1:500) was performed. Images of CD20 and CD138 immunostainings were taken using an Axioscope microscope with a micropublisher 5.0 RTV digital CCD camera (Qimaging, Surrey, BC, Canada) and the Image-Pro Plus 6.3 software (Media Cybernetics, Rockville, MD, USA). The number of T cells in MS lesions and subcortical WM were assessed using black and white images from tissue sections and particle analysis as previously described.[8] Perivascular T-cell cuffing was assessed based on CD3 immunohistochemistery, where cuffing was considered when >1 ring of $CD3^+$ cells was present, as previously described in Fransen et al. 2020.[8]

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Supplementary Table 1. Donor and sample information for immunohistochemistry.

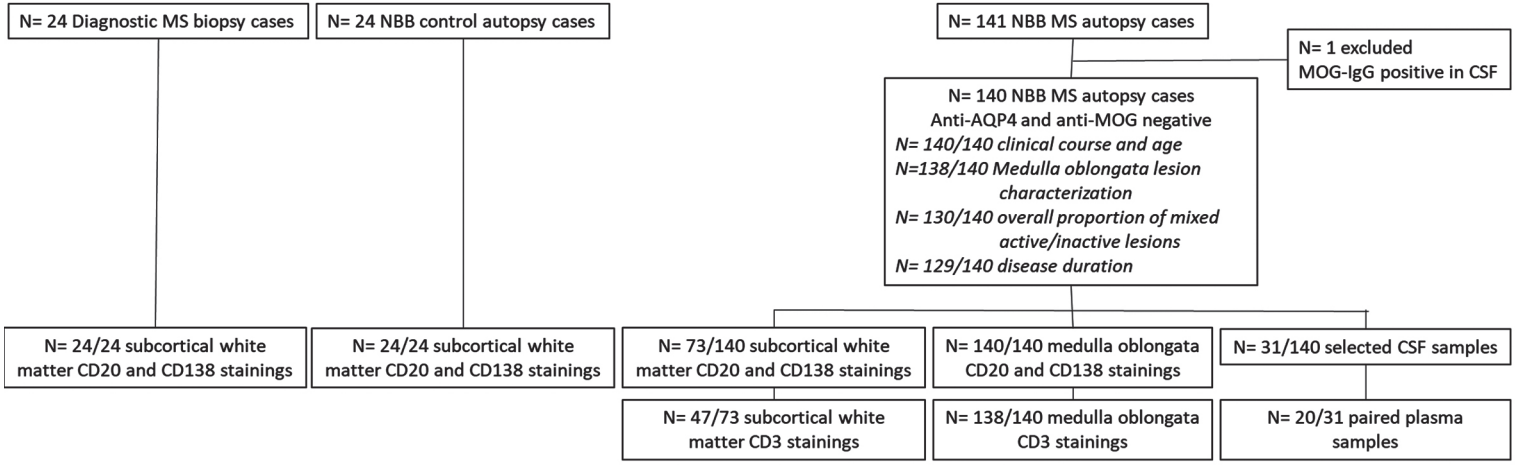
Diagnosis	Cases (n)	Age (years)	Sex (F/M)	PMD (h:min)	pH value	Brain weight (g)	Disease duration (years)
MS	140	64.8 ± 13.0	88/52	9:13 ± 6:40	6.5 ± 0.3	1192.0 ± 135.4	30.1 ± 13.2
RR	13	64.8 ± 16.0	8/5	11:52 ± 14:12	6.5 ± 0.4	1202.6 ± 102.0	25.8 ± 11.5
PP	49	67.9 ± 12.8	30/19	8:39 ± 4:32	6.5 ± 0.3	1193.8 ± 132.3	28.6 ± 12.3
SP	78	62.8 ± 12.3	50/28	9:07 ± 5:53	6.5 ± 0.3	1189.2 ± 143.0	31.8 ± 13.9
Biopsy MS donors	24	45.0 ± 13.9	18/6	–	–	–	–
Non-neurological controls	24	69.0 ± 12.7	13/11	7:53 ± 0:13	6.3 ± 0.3	1264.4 ± 142.6	–

Provided is the mean ± SD (standard deviation). F, female; M, male; PMD, post-mortem delay; PP, primary progressive; RR, relapsing–remitting; SP, secondary progressive.

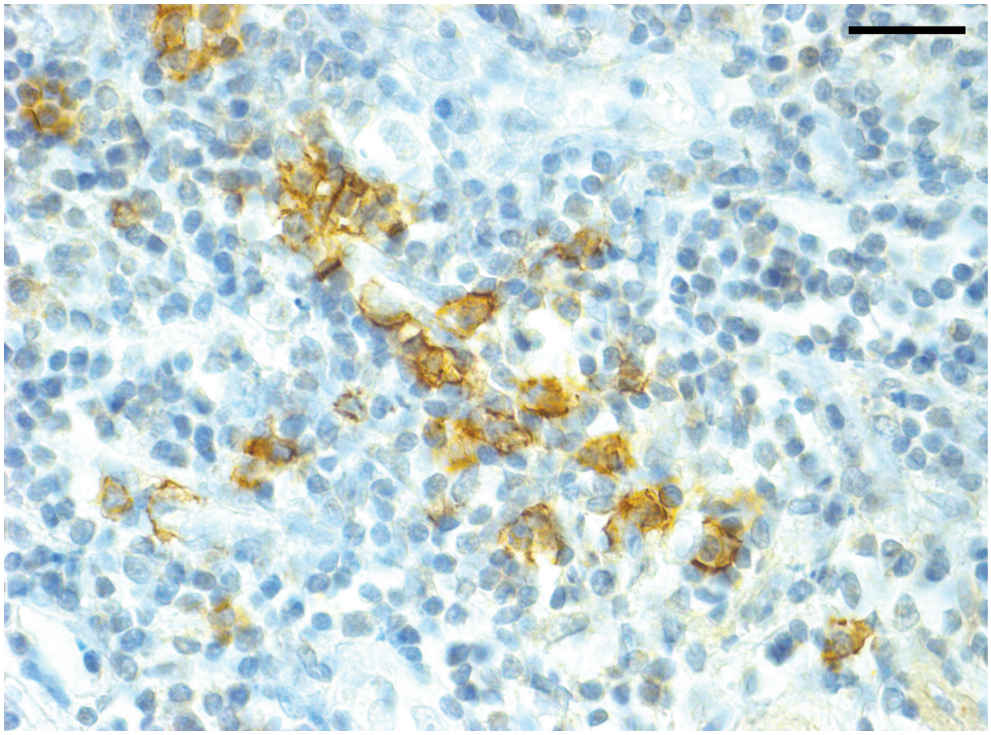
Supplementary Table 2. Disease-modifying therapy status of the MS-autopsy cohort.

Therapy	Total (%)	B cell positive (%)	B/plasma cell negative (%)	OCB positive	OCB negative
Interferon-beta	25/136 (18)	6/21 (29)	17/106 (16)	5/25 (20)	0/6 (0)
Natalizumab	1*/136 (0)	0/21 (0)	1/106 (1)	1/25 (4)	0/6 (0)
Fingolimod	2*/136 (1)	1/21 (5)	1/106 (1)	2/25 (8)	0/6 (0)
Glatiramer acetate	3/136 (2)	0/21 (0)	2/106 (2)	1/25 (4)	0/6 (0)

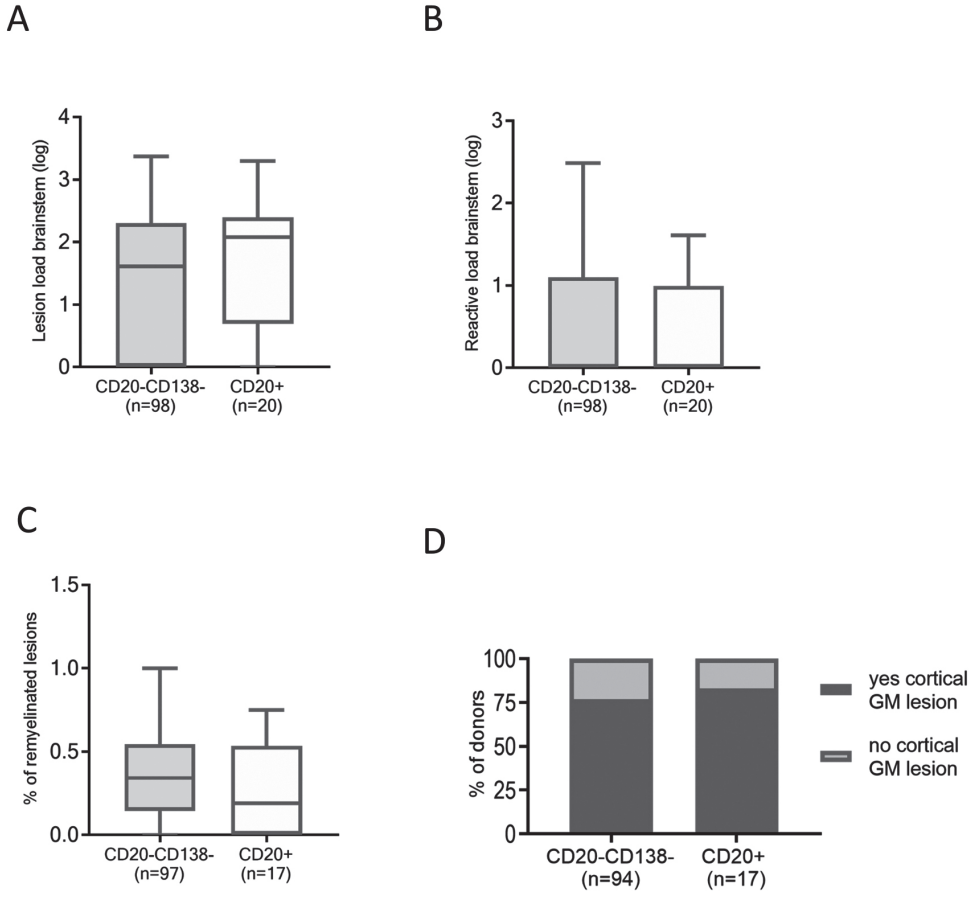
Of 4 MS autopsy cases included in the study, no information on therapy status could be obtained from the clinical files. B-cell and plasma cell status was obtained at the standardized location of the MO.
*One B cell-negative patient was treated with natalizumab 9 years before death and switched to fingolimod 3 years before death, with discontinuation of treatment 1 year prior to death. One B cell-positive patient received DMT (fingolimod) 1 year before death. All other patients did not receive therapy in the year before death.



Supplementary Figure 1. Flowchart of donor and sample inclusion.

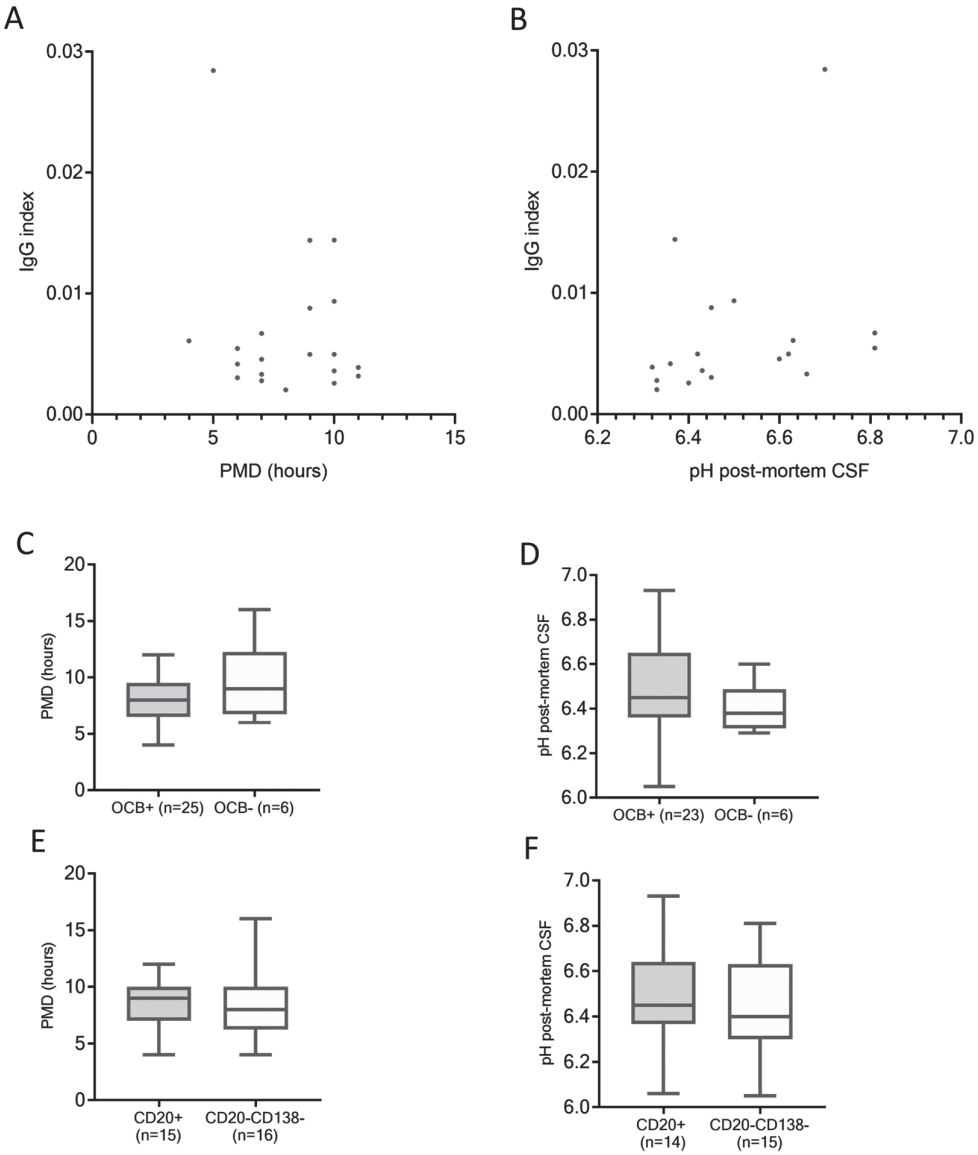


Supplementary Figure 2. Control staining of tonsil tissue for CD138^{+} plasma cells (scale bar is 25 µm).

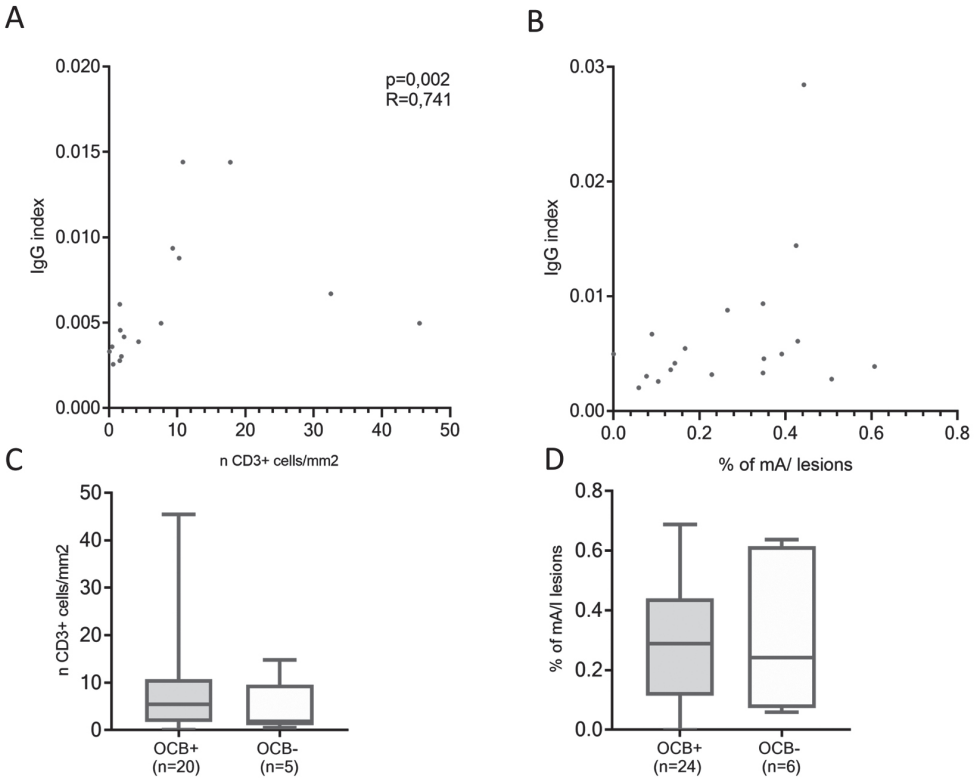


Supplementary Figure 3. Pathological parameters that were not significantly different between MS cases with and without B cells.

(A) Number of lesions in the brainstem, (B) Number of reactive sites in the brainstem, (C) Percentage of remyelinated areas, and (D) Incidence of cortical grey matter (GM) lesions.



Supplementary Figure 4. Correlation of post-mortem delay (PMD, left column) and CSF pH (right column). With (A,B) IgG index, (C,D) presence of OCB in the post-mortem CSF samples, and (E,F) presence of B cells in the MO.



Supplementary Figure 5. Association of (A,B) IgG index and (C,D) OCB presence with (A,C) number of T cells in subcortical white matter and (B,D) the percentage of mixed active/inactive (mA/I) lesions.

CHAPTER 7

Sex differences in neuroinflammation and neuroactive steroids synthesis in the multiple sclerosis cortical grey matter

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Submitted

ABSTRACT

Background | There are sex differences in MS lesion pathology in brain autopsy tissue, where males are more susceptible to develop mixed active/inactive white matter lesions and cortical grey matter lesions compared to females. Previously we showed in white matter lesions that progestogen synthesis and signaling is increased in females compared to males.

Objective | Here we aim to identify sex differences in progestogen and androgen response in the normal appearing cortical grey matter and their potential neuroprotective effects in MS.

Methods | The superior temporal gyrus was obtained from 40 MS cases (20F/20M) and 35 non-neurological controls (20F/15M) from the Netherlands Brain Bank. Cortical grey matter was analyzed for lesion content and activity. Gene-expression of progestogen-, androgens, GABA- and glutamate synthesizing enzymes and reuptake transporters, anti-inflammatory cytokines and T cell response genes was analyzed by q-PCR

Results | Males more often showed leukocortical lesions in the superior temporal gyrus compared to females. The neurosteroidogenic enzymes STS, AKR1C1 and AKR1C2 showed increased RNA expression in female normal appearing cortex compared to female controls. CD8 and IFNG mRNA expression was increased in male normal appearing cortex compared to male controls.

Conclusion | In female MS normal appearing cortical grey matter allopregnanolone and 3aDIOL synthesis is induced, but not in males, while CD8 and interferon gamma expression is increased in males compared to females. This may contribute to their increased susceptibility for the development of leukocortical MS lesions.

INTRODUCTION

MS is a heterogenous disease with well-known sex differences in the clinical disease course. Female MS patients more often show a relapsing-remitting and a more benign disease course compared to males.[1] Males develop MS at an older age and they faster reach a more severe disability.[2] Male MS patients also show a higher incidence of cognitive decline compared to females.[3,4] In MRI studies it has been shown that males show more destructive white matter lesions that evolve into black holes suggestive of more axonal damage,[5] and males also more often develop cortical grey matter lesions compared to females.[6] We and others showed that in advanced MS autopsy cases males have more ongoing inflammatory and demyelinating lesion activity in the white matter[7,8] and a higher incidence of cortical grey matter lesions compared to females.[8]

That sex steroids are contributing to these clinical and pathological differences between males and females is supported by the observation that MS relapses decrease during pregnancy and increase again post-partum, when oestrogen and progesterone levels rapidly decrease.[2] More pregnancies are also associated with a decreased risk of a first demyelinating event.[9] Not only sex steroids produced in peripheral tissues target the CNS in MS, but also steroids produced within the CNS, "neurosteroids", can impact on MS lesion pathology.[10] There is a large body of in-vitro and in-vivo evidence suggesting that neurosteroids, especially progestogens and androgens, inhibit demyelination, promote remyelination and have anti-inflammatory and neuroprotective effects in models for MS.[10–12]

We recently found altered expression of progestogen synthetic enzymes and their receptors in white matter MS lesions, showing in females there is an increased progestogen signaling whereas this was not increased in males.[13] Potentially, a lack of progestogen signaling in males facilitates ongoing inflammatory and demyelinating lesion activity in white matter and may also be responsible for the increased susceptibility for the development of cortical grey matter lesions in males.

Reactivation of $CD8^+$ T_{RM} cells has been suggested to play a pivotal role in this ongoing inflammatory lesion activity in advanced MS[14–16] and ongoing inflammatory lesion activity in white matter and $CD8^+$ T cell activation are suspected to be related to the development of cortical grey matter lesions.[17,18] It has been shown that both progestogen and allopregnanolone have an inhibitory effect on the $CD8^+$ T cell and interferon response.[11,19] Furthermore it has been shown that progesterone and allopregnanolone treatment induces a less pro-inflammatory cytokine production by microglia and macrophages.[11,12] This would inhibit the ongoing inflammatory lesion activity and potentially the development of cortical grey matter lesions in MS.

Also allopregnanolone and the androgen DHEA have shown to increase the expression and release of the neuroprotective protein BDNF in several experimental systems.[20,21] BDNF is a neurotrophin

which is synthesized by neurons and glia cells and supports cell survival and neuronal synaptic plasticity.[22]

Finally, allopregnanolone and 3-alpha-DIOL bind the GABA-A receptor, which is the principal mediator of inhibitory transmission in the CNS and allopregnanolone and 3-alpha-DIOL binding enhances inhibitory transmission. Therefore alterations in brain levels of allopregnanolone may produce changes in neuronal excitability, and potentially protect neuronal networks against excitotoxic damage.[23]

This all suggests that the synthesis of both progestogens and androgens will have several neuroprotective and anti-inflammatory effects. However, if progestogen and androgen synthesis is indeed altered in cortical grey matter from males and females with MS and by which pathways this affects the susceptibility for the development of cortical grey matter lesions in males remains poorly understood. Therefore, we here aim to identify sex differences in progestogen and androgen synthesis in cortical grey matter and the potential neuroprotective and anti-inflammatory effects by studying expression of genes for steroid synthesis, GABA/Glutamate synthesis and re-uptake, the neuroprotective protein BDNF, anti-inflammatory cytokines and the CD8 T cell response.

MATERIALS AND METHODS

Donors and sample information

In total 40 MS brain donors (20M/20F) and 35 non-neurological control donors (15M/20F) were included in this analysis. Informed consent was given by the donors for brain autopsy and for the use of material and clinical data for research purposes, in compliance with national ethical guidelines. The Netherlands Brain Bank autopsy procedures were approved by the Medical Ethics Committee of the VU Medical Center, Amsterdam, The Netherlands. The donors came to autopsy between 1995 and 2013. The clinical diagnosis of MS was confirmed for all patients, and the clinical course was defined as relapsing, secondary progressive (SP), or primary progressive (PP) by a certified neurologist according to McDonald or Poser criteria. The diagnosis MS was confirmed by a certified neuropathologist. Donor and tissue characteristics are described in **Table 1**.

The standardly dissected superior temporal gyrus allowed a standardized comparison between MS and controls and males and females. The location and activity of cortical MS lesions was characterized according to previously published criteria[24,25] and gene-expression levels were analyzed from the tissue blocks.

Cortical MS lesion characterization in the superior temporal gyrus

Frozen tissue from superior temporal gyrus tissue blocks was sectioned. 20um thick sections were cut using a cryostate. Sections were fixed using 4% PFA and 0,9% NaCl for 30 minutes at room temperature. Immunohistochemistry for HLA (DAKO, M0775, 1:1000) and PLP (Serotec,

Table 1. Donor and tissue information.

Diagnosis	MS Males	NNC Males	MS vs Co (*p*-value)	MS Females	NNC Females	MS vs Co (*p*-value)
Cases (n)	20	10		20	15	
Age (years)	62,1 ± 12,0	74,1 ± 12,6	0.02	67,0 ± 1 4,0	73,2 ± 12,7	0.19
PMD (h:min)	8:43 ± 1:33	6:32 ± 1:20		8:24 ± 2:09	8:48 ± 5:19	
pH value	6,43 ± 0,3	6,46 ± 0,2	0.80	6,43 ± 0,2	6,6 ± 0,4	0.09
Brain weight (g)	1222 ± 124,8	1355,9 ± 136,0	0.01	1158,0 ± 120,4	1164,2 ± 85,4	0.87
Disease duration (years)	31,3 ± 14,6	–	–	29,4 ± 12,9	–	–
Time to EDSS-6 (years)	18,1 ± 14,3	–		14,9 ± 11,3	–	–
MS type		–			–	
Relapsing	2	–		4	–	
SP	12	–		8	–	
PP	5	–		7	–	
NA	1			1		
Lesion load in brainstem (n)	8,6 ± 6,4	–		7,3 ± 7,7	–	
Proportion of mA/I lesion (% of lesions in total dissected tissue blocks)	28,6 ± 18,4	–		24,4 ± 24,9	–	
Presence of cortical grey matter lesions (% of donors)	95%	–		74%	–	
Cortical grey matter lesion (% of donors)	45%	–		25%	–	
Leukocortical (% of donors)	25%	–		5%	–	
Intracortical (% of donors)	15%	–		5%	–	
Subpial (% of donors)	10%	–		20%	–	
Microglia activation in cortex (% of donors)	10%	–		5%	–	

Provided is the mean ± SD (standard deviation). M, male; F, female; NNC, non-neurological control; PMD, post-mortem delay; PP, primary progressive; RR, relapsing–remitting; SP, secondary progressive. T-test *p*-values are shown.

MCA839G, 1:500) was performed as previously described.[26] Lesions in the cortical grey matter were characterized as leukocortical, intracortical or subpial as described in Luchetti et al.[25] and Fransen et al.[26] Images of the cortical MS lesions in superior temporal gyrus were taken using the Axioscope microscope with a micropublisher 5.0 RTV digital CCD camera (Qimaging, Surrey, BC, Canada) and the Image-Pro Plus 6.3 software.

RNA isolation and qPCR analysis

From these tissue blocks 10-15 mg of exclusively cortical grey matter tissue, from either NAGM or cortical grey matter lesions, was dissected using a scalpel and stored in ice cold TRIsure. RNA was isolated as previously described[26] RNA samples were dissolved in 20 µl of water and RNA yield was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was performed as previously described,[26] by incubation in a reaction mixture of RNA and gDNAse Wipeout Buffer, followed by incubation in a mixture of Quantiscript Reverse Transcriptase, Quantiscript Buffer and RT-Primer Mix (Qiagen Benelux, Venlo, The Netherlands). Finally, RT transcriptase was inactivated. Primers were designed using the primer designer from Integrated DNA Technologies, Inc. NCBI blast was performed for suitable primer sequences using OligoAnalyzer 3.1 (Integrated DNA Technologies, Illinois, USA). Primer sequences and characteristics are shown in **Suppl. Table 1**. Specificity was tested on cDNA derived from pooled RNA of both brain and spleen samples. Dissociation curves were examined and the PCR product sizes were determined by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Quantitative PCR was performed as previously described using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with 2 ng total RNA per reaction. Analysis was performed according to the manufacturer's protocol at the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Target genes were normalized to the geometric mean of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and elongation factor 1 alpha (EEF1A1) mRNA expression. All primers showed amplification and dissociation curves in the tested samples. SULT2A1 was excluded from further analysis since low relative expression values were detected in only 7 out of 65 samples. Primer characteristics are shown in **Suppl. Table 1**.

Immunohistochemistry for AKR1C2/AKR1C3 in normal appearing MS cortex

Formalin fixed paraffin embedded cortical tissue sections from MS brain donors were obtained from the Netherlands Brain Bank. Immunohistochemistry for AKR1C (rabbit anti rat AKR1C type 9 full protein which corresponds to human AKR1C type 1-3, kindly provided by Prof. T. Penning University of Pennsylvania School of Medicine, USA) was performed according to previously a described protocol.[13,26] Briefly, paraffin sections were deparaffinized and rehydrated in an ethanol series and then rinsed in distilled water, then underwent an antigen retrieval treatment (10 minutes in the microwave at 90°C in 0.01 M Citrate Buffer, pH6). After a blocking step with Tris-buffered saline (TBS) containing 0.5% bovine serum albumin (BSA), 0.1% Triton X-100 and 10% normal horse serum, AKR1C primary antibody (1:1000) was incubated overnight at 4°C. For visualization a DAKO

Real ENVision detection system peroxidase/DAB (cat. K5007, DAKO, Denmark) was used, following the manufacturer's instructions. Images were taken as described previously.[15,26]

Statistical analysis

All statistical analysis were performed in R Studio (Version 1.2.5033). Proportional differences in cortical lesion presence between males and females were tested with Fisher's exact tests. Clinical and pathological characteristics were compared using student t-test. Gene-expression data was log-transformed and compared between groups using One-Way ANOVA test. FDR multiple testing correction was performed for each set of genes (progestogen and androgen synthetic enzymes, GABA-Glutamate synthetic enzymes and re-uptake transporters and the inflammatory response genes). Post-hoc analysis was performed using the glht function using the multcomp package, performing Tukey post-hoc test for the comparison of expression in control cortex, NAGM and cortical lesion between and within males and females. Significance level was 0.05. Correlation analysis for gene-expression data was performed using the psych and ggcorrplot packages and analyzed using Pearson correlation coefficient. FDR multiple testing correction was performed on all correlation tests. For the correlation analysis significance level was 0.01.

RESULTS

Cortical grey matter lesions in female and male superior temporal gyrus

In 40 temporal gyri from 20 females and 20 males cortical demyelination was characterized using HLA and PLP immunohistochemistry as previously described.[8,26] They were characterized as either normal appearing (**Figure 1A**) leukocortical (**Figure 1B**), intracortical (**Figure 1C**) or subpial (**Figure 1D**). Leukocortical lesions were more often identified in males (p=0.03) (**Figure 1 E**). The presence of intra-cortical lesions and subpial lesions was not significantly different between males and females in this selection of MS brain donors (**Figure 1 F-G**). Interestingly brain weight at autopsy was higher in control males compared to control females in line with previous reports.[27] In MS males there was significantly lower brain weight compared to controls while in females this was not the case, suggesting more pronounced brain tissue loss in male MS patients compared to females.

Progestogen and androgen are more induced in female normal appearing cortex in MS, while this is not induced in males

Gene-expression of progestogen synthesizing enzymes and progesterone receptor and androgen synthetic enzymes was analyzed in the superior temporal gyri from 40 MS and 35 control cases. The gene STS showed increased expression in the normal appearing cortical grey matter from females (FDR p=0,006) (**Figure 2A**). The genes AKR1C1 and AKR1C2 show increased expression in normal appearing cortex from MS females (FDR p=0,04 and 0,03 respectively) (**Figure 2B-C**). The AKR1C3 gene did not show significant different expression levels between MS and control normal appearing cortex in both males and females (**Figure 2D**). PGR, which codes for the nuclear progesterone receptor, shows no increase in expression between female normal appearing

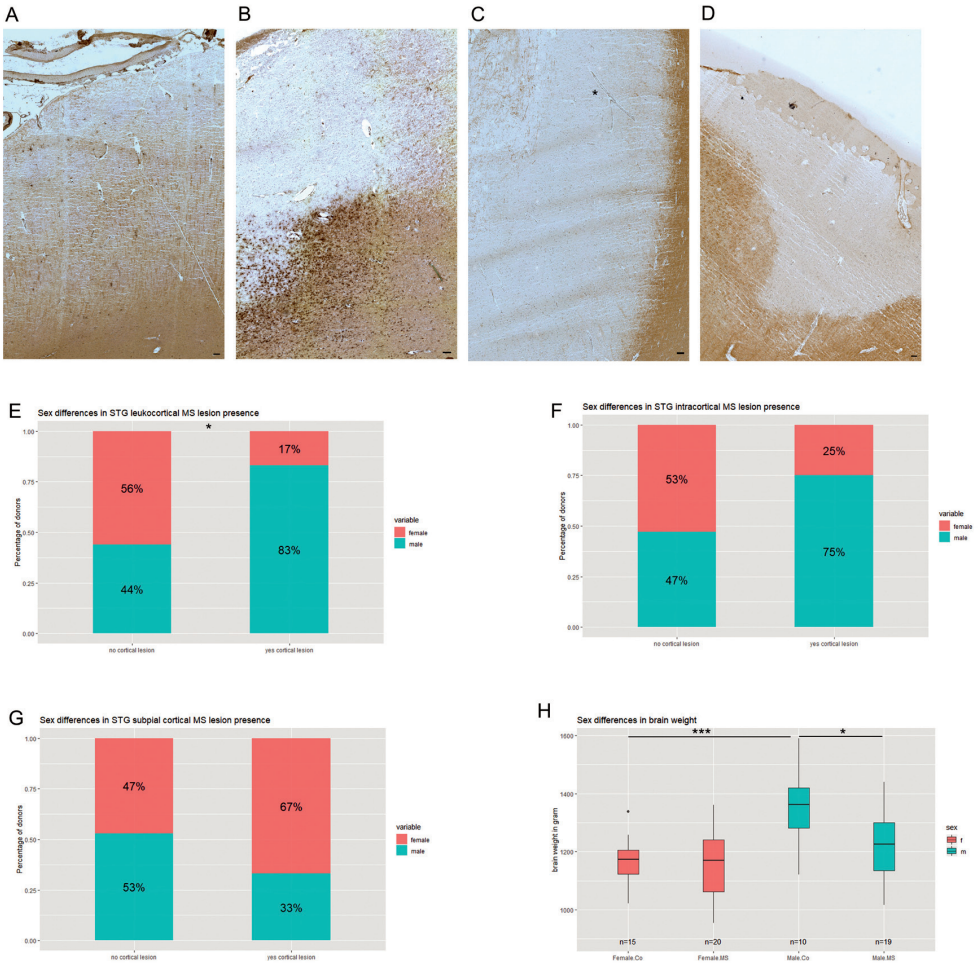

Figure 1. Cortical demyelination in the superior temporal gyrus in MS females and males.
HLA-PLP staining of fresh frozen superior temporal gyrus tissue showing cortical demyelinated lesions, HLA is black and PLP is brown; A. Normal-appearing cortical grey matter B Leukocortical lesion C Intracortical lesion D. Subpial cortical lesion. E. Males more often show the presence of leukocortical lesions in the superior temporal gyrus compared to females. F The presence of intracortical lesions was comparable between males and females. G. The presence of subpial lesions was comparable between males and females. H. non-neurological control males show a higher brain weight compared to non-neurological control females, in MS males there is a significant decrease in brain weight compared to controls while there is no significant difference in females. * $p<0.05$, *** $p<0.001$. Scale bars are 100um.

grey matter compared to controls, however there is a decreased signaling in the cortical lesions compared to the normal appearing grey matter (FDR $p=0,04$) (**Figure 2E**). The increased expression of progestogen synthetic enzymes in the female normal appearing cortical grey matter suggests an increased progesterone signaling in females with MS, while this is not increased in males with MS.

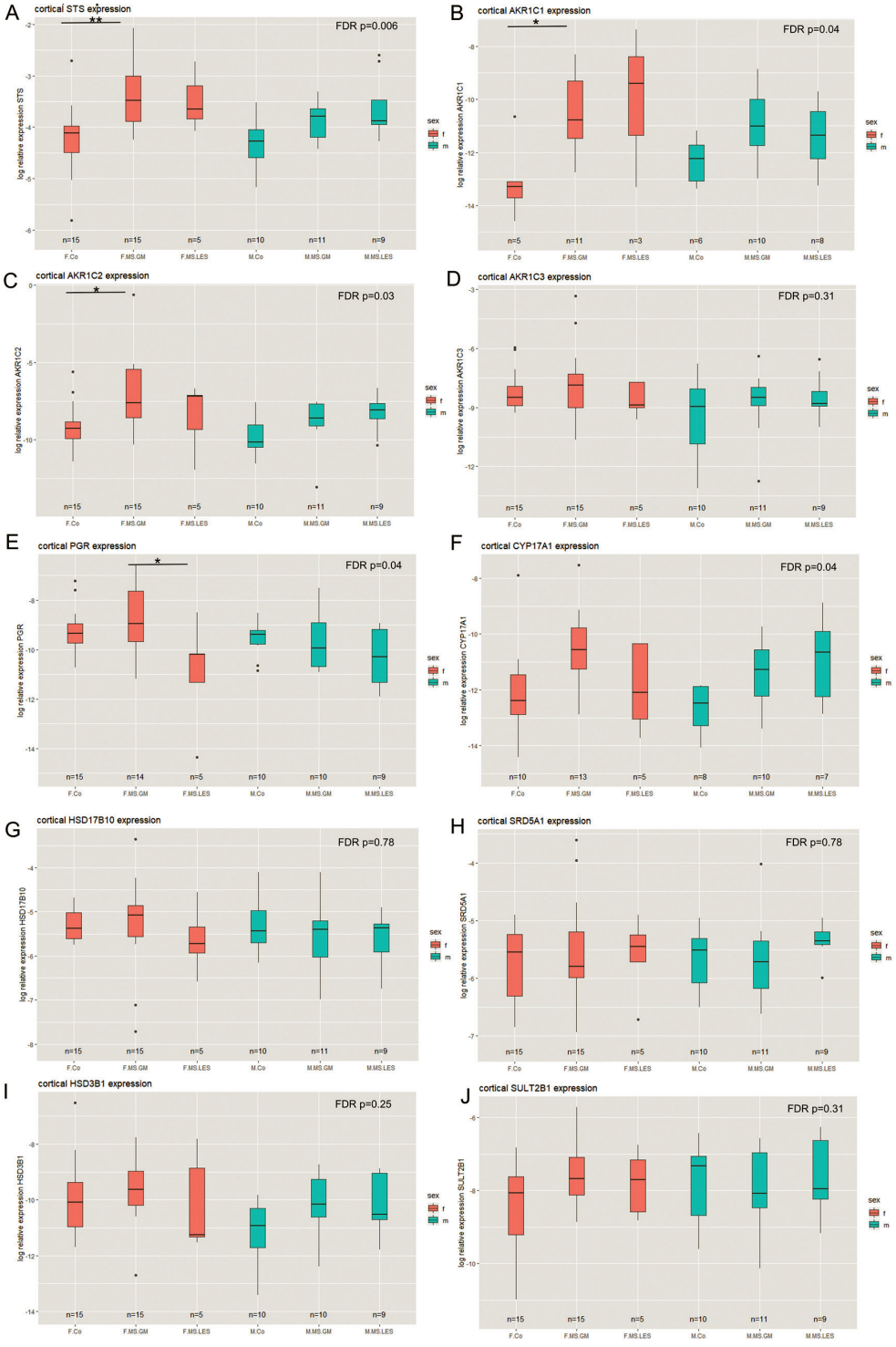
A
cortical STS expression
FDR p=0.006
log relative expression STS
B
cortical AKR1C1 expression
FDR p=0.04
log relative expression AKR1C1
C
cortical AKR1C2 expression
FDR p=0.03
log relative expression AKR1C2
D
cortical AKR1C3 expression
FDR p=0.31
log relative expression AKR1C3
E
cortical PGR expression
FDR p=0.04
log relative expression PGR
F
cortical CYP17A1 expression
FDR p=0.04
log relative expression CYP17A1
G
cortical HSD17B10 expression
FDR p=0.78
log relative expression HSD17B10
H
cortical SRD5A1 expression
FDR p=0.78
log relative expression SRD5A1
I
cortical HSD3B1 expression
FDR p=0.25
log relative expression HSD3B1
J
cortical SULT2B1 expression
FDR p=0.31
log relative expression SULT2B1
sex
f
m
F.Co
F.MS.GM
F.MS.LES
M.Co
M.MS.GM
M.MS.LES

Figure 2. Progesterone receptor and synthetic enzymes and androgen synthetic enzymes show increased expression in normal appearing cortical grey matter from MS females while there is no increase in MS males.
A. STS expression is increased in NAGM from MS females compared to female and male controls. B. AKR1C1 expression is increased in NAGM from MS females compared to control females. C. AKR1C2 expression is increased in NAGM from MS females compared to females and male controls. D. AKR1C3 did not show significant different expression levels. E. PGR expression is decreased in cortical grey matter lesions from MS females compared to NAGM. CYP17A1 (F), HSD17B10 (G), SRD5A1 (H), HSD3B1 (I), SULT2B1 (J) shows no differences in gene expression between males and females with MS. One way ANOVA and FDR multiple testing correction is performed and shown in the graph. Tuckey post-hoc *p*-values are shown in the graph. * $p<0.05$, ** $p<0.001$.

CYP17A1 gene expression was highest in female normal appearing cortical grey matter, however this was not significantly increased compared to female controls or female MS lesions (**Figure 2F**). HSD17B10, SRD5A1, HSD3B1 and SULT2B1 did not show a significant difference in expression levels between MS and controls in both males and females (**Figure 2E, G-J**).

An overview of the neurosteroid synthetic pathway with a summary of the results found in our analysis of sex differences in neurosteroid synthetic enzyme expression in normal appearing cortical grey matter are shown **Figure 3**.

Immunocytochemistry of AKR1C in cortical grey matter

Immunohistochemistry using an antibody which binds both the enzymes AKR1C2 and AKR1C3, shows expression in the human cortex by different cell types, likely representing astrocytes and neurons (**Figure 4A-B**).

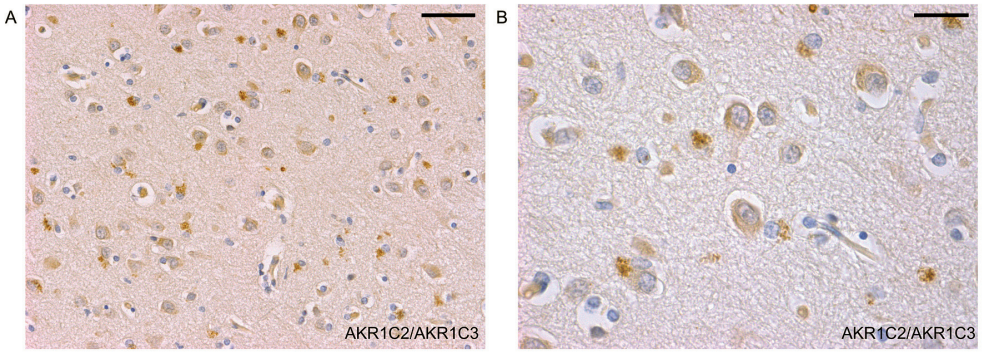

Figure 4. Immunohistochemistry for AKR1C2/AKR1C3 in the normal appearing cortex shows it is expressed by astrocytes.
A. Immunohistochemistry of AKR1C2/AKR1C3 in the normal appearing cortical grey matter from a female with multiple sclerosis. Scale bar is 50um B. AKR1C2/AKR1C3 is expressed by astrocytes in the cortical grey matter, here shown in layer 5. Scale bar is 25 µm.

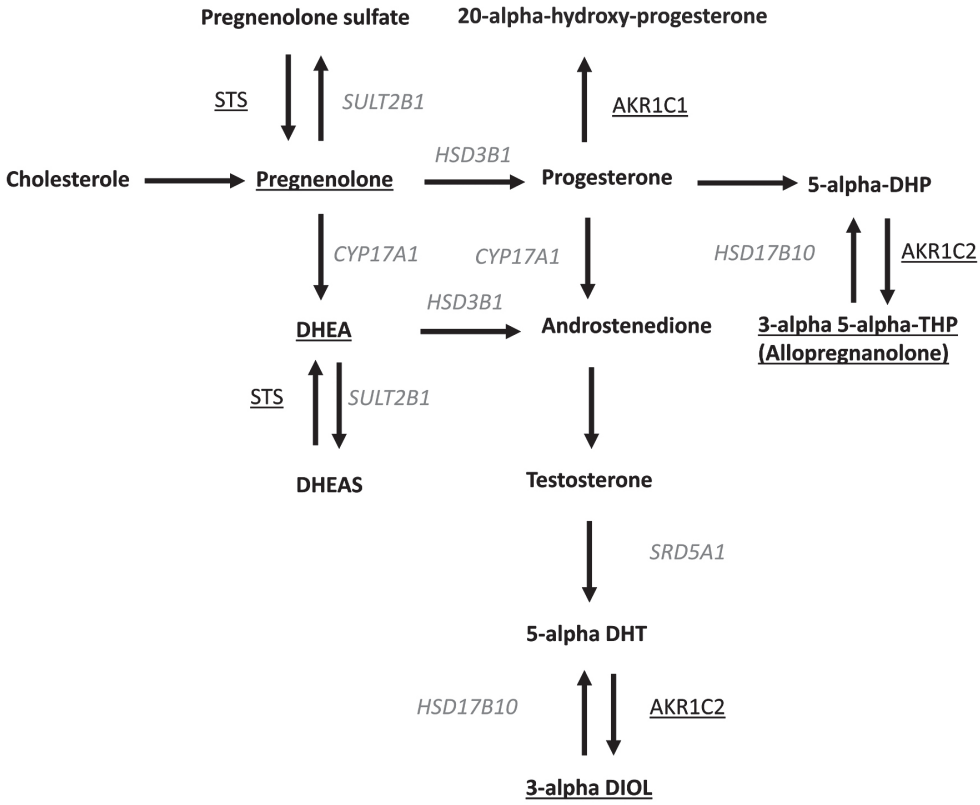

Figure 3. In MS normal appearing cortical grey matter from females there is increased pregnenolone and allopregnanolone synthesis and increased DHEA and 3alpha DIOL synthesis while this is not increased in males.

STS encodes for the enzyme that catalyzes the desulfation of sulfated steroid precursors, such as DHEAS and PREGS to the free steroids. The genes AKR1C1, AKR1C2, AKR1C3 encode for aldo-keto reductases that catalyze NADPH dependent reductions of steroids. AKR1C1 plays an important role in reductive inactivation of progesterone into 20α-DHP. AKR1C2 preferentially reduces 5α-DHP into the potent progesterone metabolite allopregnanolone and it reduced DHT to the metabolite 5α-androstane-3α,17β-diol (3α-diol). The enzyme encoded by the CYP17A1 gene has 17 alpha(α)-hydroxylase activity, and converts pregnenalone to 17-hydroxypregnenolone and progesterone to 17-hydroxyprogesterone. CYP17A1 also has 17,20-lyase activity, which converts 17-hydroxypregnenolone to dehydroepiandrosterone (DHEA).[13] HSD17B10 is involved in the synthesis of HSD10 protein which generates the potent androgen dihydrotestosterone (DHT) from 3α-DIOL and inactivates allopregnanolone. The enzyme SRD5A1 converts testosterone and progesterone to the 5alpha reduced metabolites, the potent 5-α-DHT or 5α-DHP respectively.[13] The enzyme encoded by the HSD3B1 gene catalyzes the conversion of pregnenolone to progesterone and DHEA to androstenedione. The sulfotransferase enzyme encoded by the SULT2B1 gene catalyzes the sulfate conjugation of pregnenolone and DHEA which regulates the circulation, transport and action of these steroids. STS, AKR1C2 and AKR1C1 are increased in female normal appearing cortical grey matter while this was not significantly increased in males.

GABA and glutamate synthesis and glutamate re-uptake

We analyzed if sex differences in excitoxicity in the cortical grey matter can be expected based on alterations in gene expression of GABA and glutamate synthesis and glutamate re-uptake. The RNA expression of GABA synthetic enzymes, GAD1 and GAD2 did not differ between MS and controls in males and females (**Figure 5A and B**). GLUL is involved in glutamate synthesis and gives rise to the glutamate precursor, glutamine.[28] Neurons take up glutamine and convert it to glutamate using GLS. There is no alteration seen in glutamate synthetic enzymes GLUL and GLS between males and females and MS and controls (**Figure 5C and D**). Gene expression of the glutamate transporters, GLAST1, GLT1, EAAT2 and EAAT3 involved in glutamate re-uptake did not show a significant difference between MS and controls in males and females (**Figure 5E-H**). However, GLAST1 showed higher expression in normal appearing cortical grey matter in MS from both males and females compared to controls, however this was not significantly different after multiple testing correction.

CD8 T cell, interferon gamma and cytotoxicity

We hypothesize that cortical demyelination in MS is related to the ongoing inflammatory lesion activity in the white matter.[8] CD8 gene expression is increased in male normal appearing cortex compared to female normal appearing cortex and compared to male control cortex (FDR p=0,03) (**Figure 6A**). Also, interferon-gamma (IFNG), which is exclusively expressed by T cells in the brain, is increased in the male normal appearing cortex compared to the male controls, while this was not increased in females (FDR p=0,03) (**Figure 6B**). The cytolytic enzyme GZMK, which is produced by brain resident $CD8^+$ T cells upon activation,[29] did not show a significant difference in expression in male normal appearing cortex compared to the control cortex (**Figure 6C**).

Anti-inflammatory and neuroprotective cytokines

We in addition analyzed if altered progestogen synthesis in females associates with the expression of the neuroprotective BDNF and with anti-inflammatory cytokine production by microglia. The expression of BDNF was not significantly different between MS and controls in both males and females (**Figure 6D**). Although IL10 shows the highest expression in male normal appearing cortical grey matter, this is not significant different after multiple testing correction (**Figure 6E**) Also, TGFbeta did not show a significant difference between MS and controls in both males and females (**Figure 6F**).

Progestogen and synthetic enzymes positively correlate with increased glutamate re-uptake

To indicate whether the progestogen and androgen synthesis impacts on the GABA and glutamate synthesis and re-uptake and the inflammatory response genes that we analyzed we performed a correlation analysis. Pearson correlation with FDR multiple testing correction was performed and all correlation coefficients of the significant correlations with p<0.01 are shown in **Figure 7**. This shows that there is no correlation between gene expression and age or pH of post-mortem

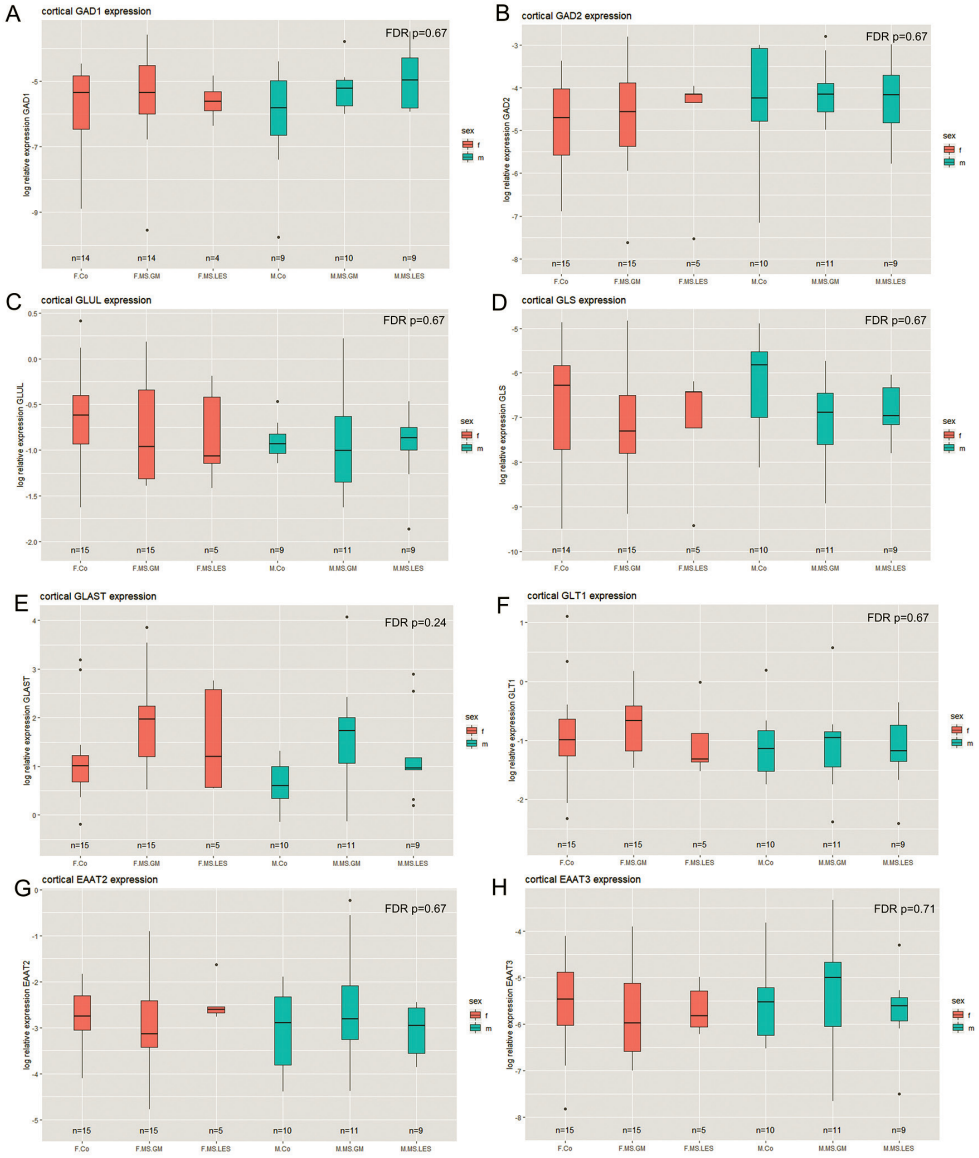

Figure 5. Glutamate and GABA synthetic enzyme and glutamate transporter genes show unaltered expression in cortical grey matter from females and males with multiple sclerosis.
There is no alteration in GABA synthetic enzyme genes GAD1 (A) and GAD2 (B). There is no alteration in glutamate synthetic enzyme genes GLUL (C) and GLS (D). There is no alteration in glutamate transporter genes GLAST (E), GLT1 (F), EAAT2 (G) and EAAT3 (H). One-way ANOVA and FDR multiple testing correction is performed and shown in the graphs.

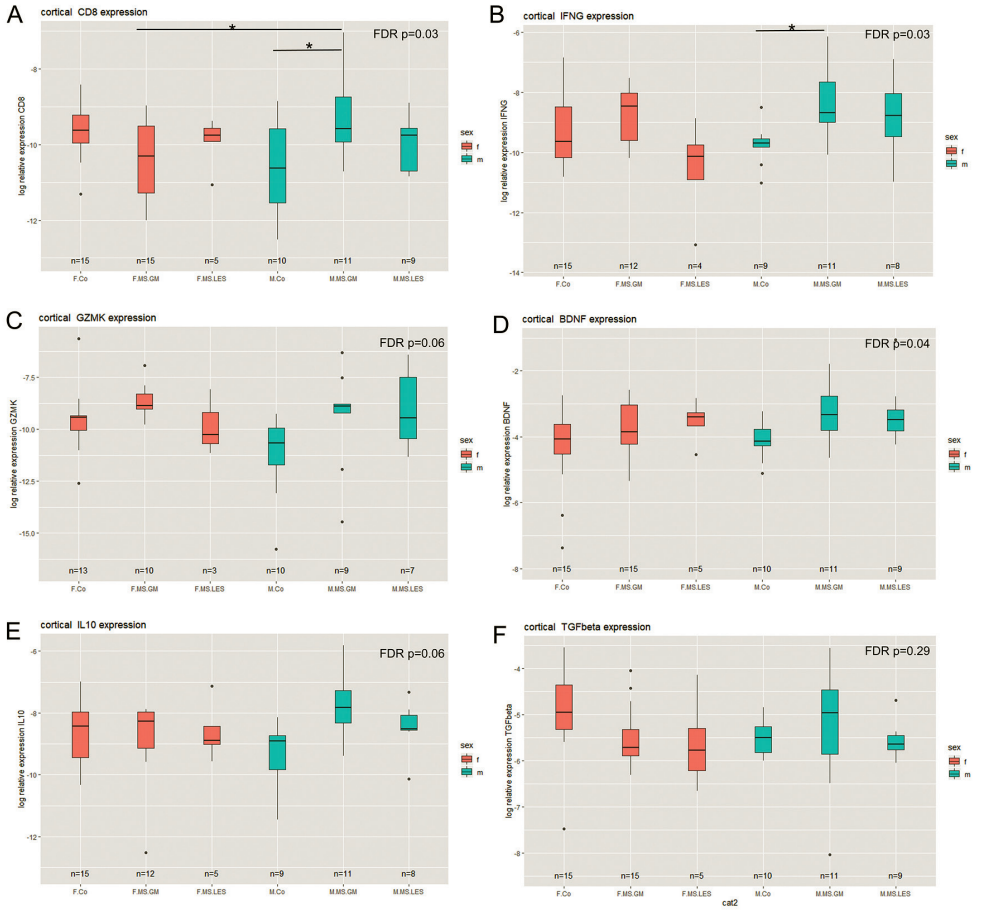

Figure 6. CD8 and IFNG expression is increased in the NAGM from MS males, while there is no increase in females.

A. CD8 expression is increased in NAGM from MS males compared to normal appearing grey matter from MS females and to control males. B. IFNG expression is increased in NAGM from MS males compared to cortex from controls males. C. GZMK gene expression is unaltered in the cortex from MS females and males. D. BDNF is increased in cortex from MS males compared to cortex from control females, however there is no significant increase compared to control males. E. IL10 expression is unaltered in cortex from MS females and males. F. TGFbeta expression is unaltered in cortex from MS females and males. One way ANOVA and FDR multiple testing correction is performed and shown in the graph. Tuckey post-hoc *p*-values are shown in the graph. * $p<0.05$.

CSF. Progestogen and androgen synthetic enzymes positively correlate with each other. AKR1C2 positively correlates with AKR1C3, HSD3B1, CYP17A1 and STS. HSD3B1 positively correlates with CYP17A1 and STS. CYP17A1 positively correlates with STS. This suggests that induction of progestogen and androgen metabolites is occurring parallel.

Progestogen and androgen synthetic enzymes AKR1C2 and HSD3B1 positively correlate with glutamate synthetic enzyme GLUL and the glutamate re-uptake genes GLAST and GLT1 were

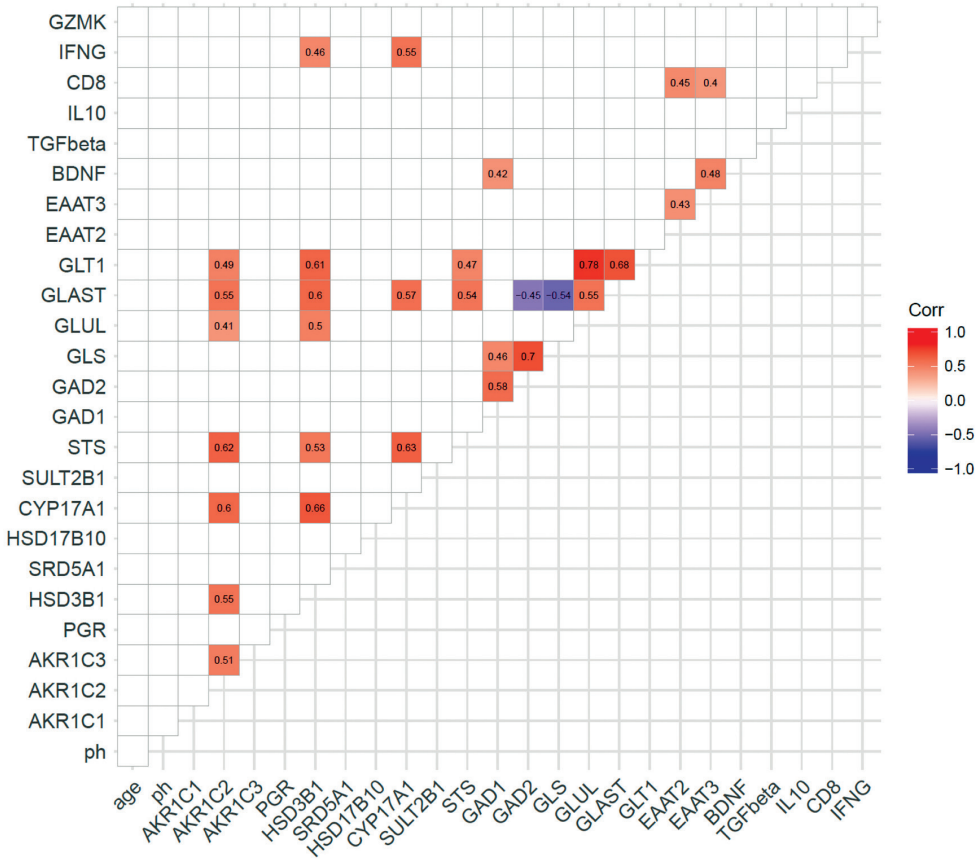

Figure 7. Correlation analysis shows progestogen and androgen synthetic enzyme expression is positively correlated with glutamate re-uptake gene expression in cortical grey matter.
Pearson correlations coefficients are shown for correlations with FDR adjusted *p*-values of $p<0.01$. Progestogen and androgen synthetic enzymes are positively correlated with each other. Progestogen and androgen synthetic enzymes are positively correlated with glutamate re-uptake genes GLAST and GLT1 and glutamate synthesis GLUL. Progestogen and androgen synthetic enzymes are positively correlated with interferon-gamma gene expression.

positively correlated with AKR1C2, HSD3B1, CYP17A1 and STS. The progestogen and androgen synthetic enzymes HSD3B1 and SULT2B1 are positively correlated with IFNG expression. Furthermore, also the GABA and glutamate synthetic enzymes and glutamate re-uptake genes were correlated with each other. GABA synthesis and glutamate reuptake were positively correlated with BDNF expression. Finally, CD8 expression was positively correlated with glutamate re-uptake effectors EAAT2 and EAAT3 (**Figure 7**).

DISCUSSION

Here we demonstrate sex differences in the presence of cortical grey matter lesions in the superior temporal gyrus and in the induction of progestogens and androgens in normal appearing cortical grey matter in MS. Males showed a higher susceptibility for developing leukocortical lesions than females. Females showed a higher expression of the steroid synthetic enzymes STS, AKR1C1 and AKR1C2 in normal appearing cortex compared to control females, suggesting increased production of allopregnanolone and 3-α-DIOL in the female cortex in MS. In MS males there was no alteration in these enzymes compared to male controls. In particular, the induction of AKR1C2, responsible for allopregnanalone and 3α-DIOL synthesis, is of interest since these are both neuroactive steroids that act as GABA receptor agonists. There was no alteration in GABA and glutamate synthesis between males and females and also no alteration in glutamate re-uptake transporters. However, the glutamate re-uptake transporter genes GLT1 and GLAST positively correlated with progesterone, allopregnanolone and androgen synthesis. This suggests that increased allopregnanolone and androgen synthesis are correlated with increased re-uptake of glutamate and potentially could protect against excitotoxic damage. Furthermore, males showed a higher expression of CD8 and interferon gamma in the normal appearing cortical grey matter in MS, while this was not increased in female normal appearing cortex. The reduced neuroactive steroid synthesis and the increased CD8 T cell and interferon levels in normal appearing cortical grey matter in MS males potentially contributes to their increased tendency to develop leukocortical MS lesions.

Cortical grey matter lesions are present from the earliest disease stages.[30,31] We previously showed that the incidence of both leukocortical and intracortical lesions correlates with the percentage of mixed active/inactive lesions in the white matter suggesting that cortical demyelination is related to the inflammatory lesion activity in the white matter.[8,17] This supports the suggestion that neuronal damage may occur as a result of axonal degeneration in the inflammatory active white matter lesions.[31] In line with this, the inflammatory lesion activity in white matter correlated with disease progression in MS since progressive MS cases show a higher percentage of mixed active/inactive or smoldering lesions compared to the acute or relapsing MS cases.[7,8] In these two studies males showed a higher percentage of mixed active/inactive lesions in the white matter. In line with previous pathological and MRI analysis[6,8] we here show that in a standardly dissected cortical region males more often have a leukocortical lesion. The presence of intracortical and subpial lesions did not significantly differ between females and males in this sample of MS brain donors. This all suggests the increased susceptibility for the development of cortical demyelination in males is closely related to the increased ongoing inflammatory lesion activity in the white matter.

We previously showed that in mixed active/inactive white matter lesions from females there is increased progestogen synthesis and signaling, while in the mixed active/inactive lesions from males there is increased oestrogen signaling.[13] Unpublished gas-spectometry data show that in mixed active/inactive lesions from females there are increased levels of allopregnanolone and progesterone while this is not increased in males. We now demonstrate that STS, AKR1C1 and

AKR1C2 show increased expression in the cortex from MS females. The enzyme encoded by STS catalyzes the desulfation of sulfated steroid precursors, both from progestogens, androgens and estrogens to the free steroids, suggesting a general push toward neurosteroid synthesis. AKR1C1 plays an important role in reductive inactivation of progesterone into 20α-DHP while AKR1C2 preferentially reduces 5α-DHP into the potent progesterone metabolite allopregnanolone and it also reduces DHT to the metabolite 3-α-DIOL.[13,33] Therefore the gene expression data for neurosteroid synthetic enzymes in MS normal appearing cortical grey matter suggest there is increased synthesis of the neuroactive allopregnanolone and 3-α-DIOL in the MS female cortex, while this is not increased in males.

We were interested in potential protective effects of increased allopregnanolone and 3-α-DIOL signaling in the normal appearing cortical grey matter in females on the development of cortical MS lesion pathology. It has been suggested that the reactivation of $CD8^+$ T cells in MS white matter lesions is related to the ongoing inflammatory and demyelinating lesion activity in progressive MS.[14,15] Furthermore, it has been shown that increased numbers of cytotoxic T cells are also present in cortical demyelinated lesions, suggesting $CD8^+$ T cell reactivation is involved in cortical demyelination.[17,34] We show males have a higher expression of CD8 and interferon gamma in the normal appearing cortical grey matter compared to controls while in females this was not increased. This suggests that in females the CD8 T cell response may be inhibited compared to males.

Interestingly, allopregnanolone affects CD8 T cell activity by binding to the GABA-A surface receptor.[35] GABA-A agonists have been shown to inhibit antigen specific T cell proliferation.[19] Suggesting that allopregnanolone could inhibit a cytotoxic $CD8^+$ T cell response. It remains to be determined if the difference in $CD8^+$ T cell and IFNG expression in the NAGM between males and females is a consequence of the altered allopregnanolone synthesis. We did not see a significant negative correlation between the allopregnanolone synthetic enzymes and CD8 expression, indicating that also other factors then sex steroids alone (e.g. differences in inflammatory environment) influence the CD8 T cell response in MS.

Microglia and macrophages also express a functional GABA-A receptor and activation of this receptor is known to result in reduced production of inflammatory cytokines. GABA-A agonist treatment altered macrophages and dendritic cell activation which resulted in an altered T cell response following antigen presentation.[12] Here we analyzed the expression of the anti-inflammatory cytokine IL10 and TGFbeta in cortex. In vitro studies have reported both increased and decreased IL10 expression after progesterone treatment.[11] However, we did not see an alteration in anti-inflammatory cytokine genes in MS compared to controls in both males and females.

It has been demonstrated in different experimental systems that allopregnanolone increases mRNA and protein expression of the neurotrophin BNDF which is produced by neurons and glial

cells.[20,21] Although it is likely that allopregnanalone exerts its neuroprotective effects through an increased production of BDNF, we did not see a significant difference in BDNF expression between males and females and we did not encounter a positive correlation between allopregnanalone synthesis and BDNF expression. BDNF expression is increased around MS lesions,[36] possibly in normal appearing cortical grey matter sex differences were therefore not detected. Also the number of cortical MS lesions that we now analyzed is quite low to detect sex differences in BDNF expression.

Since allopregnanolone and 3-α-DIOL are agonists for the GABA-A receptor on neurons,[33,37] the neuroprotective effect of allopregnanolone in different brain diseases is also suspected to be mediated by the ability to attenuate excitotoxicity which is associated with brain injury.[33] It has been shown that MS cortical grey matter has increased expression of glutamate receptors and transporters compared to controls.[38–40] Since oligodendrocytes are most vulnerable to excessive glutamate, increased re-uptake of glutamate is a potential protective mechanism in MS.[41,42] Since allopregnanolone and 3-α-DIOL are expected to alter neurotransmitter expression and release[33] we analyzed gene expression of GABA and glutamate synthesis and glutamate re-uptake. However, we did not see a significant difference between males and females in the GABA and glutamate synthesis and glutamate re-uptake genes. Although not significant after multiple testing correction, glutamate re-uptake gene GLAST seems to be increased in the normal appearing grey matter from both females and males with MS compared to controls. This suggests there is increased glutamate re-uptake in the normal appearing cortex in multiple sclerosis, in line with previous reports.[32] Interestingly we showed that progestogen and androgen synthetic enzymes AKR1C2, HSD3B1, CYP17A1 and STS are positively correlated with glutamate re-uptake genes GLAST and GLT1. Indicating that allopregnanolone and 3-α-DIOL synthesis can potentially impact on glutamate re-uptake, thereby ameliorating the excitotoxicity[43,44] and potentially preventing cortical demyelination in females.

Altogether these data suggest that allopregnanolone and 3-α-DIOL synthesis, in contrast to females, is not induced in the normal appearing cortex in males, potentially resulting in decreased glutamate re-uptake and an increased $CD8^+$ T cell and interferon response, thereby making the normal appearing cortical tissue in males with MS more susceptible for the development of leukocortical demyelinated lesions. These results together with our earlier findings in mixed active/inactive lesions,[45] indicate that supplementation with progesterone and allopregnanolone in MS patients might be effective in preventing ongoing inflammatory lesion activity and cortical demyelination and thereby effecting the progression of the disease. There is one clinical trial reported with progesterone treatment in MS which was targeted to prevent post-partum relapses (POPARTMUS), where 12 weeks treatment with 10 mg nomegestrol acetate was compared with placebo, however it is reported that this showed no reduction of post-partum relapses, although the analysis has never been published.[46,47] Interestingly the Cochrane review on clinical trials for the administration of progesterone in traumatic brain injury concluded progesterone treatment

improved neurological outcome after traumatic brain injury, but larger clinical trials are needed for a definite conclusion.[48] This suggests the investigation of the effect of progesterone and/or allopregnanolone treatment on disease progression in female and male MS patients is still promising and needs to be considered in further studies.

Acknowledgments

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Supplementary Table 1. Primer characteristics for qPCR analysis.

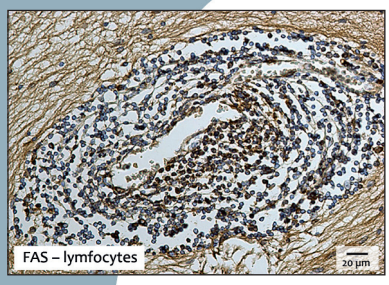
NAME	SEQUENCE (5' > 3')	Transcriptcode
HSD3BF	CGTATAAGCCACTCTACAGC	NM_000862
HSD3BR	CCACAAGGGAACCAACC	NM_000862
AKR1C1F	CACAGCTTGTGTAAGACTG	NM_001353
AKR1C1R	GCTTCAATTGCCAATTTGGT	NM_001353
AKR1C2F	GTTGTTTGAAAGTGTGTAGCA	NM_001354
AKR1C2R	GTCTTCACTTGGCTGGCA	NM_001354
AKR1C3F	GGAGGGCTTTGCCTGATGT	NM_003739
AKR1C3R	GCTAAACAGGACGGATTTAAGT	NM_003739
SRD5A1F	TCCTCGTCCACTACGGGCAT	NM_001047
SRD5A1R	ACGCTTTCCTCCTCGCATC	NM_001047
HSD17B10F	CCAGCGAGTTCTTGATGTG	NM_004493, NM_001037811
HSD17B10R	GCAGTGTTGATGATGACCC	NM_004493, NM_001037811
CYP17A1F	GTCATCTCCTTGATCTGCT	NM_000102
CYP17A1R	CTCAGGTTGTCTATGATGC	NM_000102
SULT2A1F	TGAGGAGCTGAAACAGGA	NM_003167
SULT2A1R	AAGTTCAGTTCTTCGGGTTC	NM_003167
SULT2B1F	CGTTGTGGTCTCCCTCTATCA	NM_004605, NM_177973
SULT2B1R	AACTGCACTTCGCCTTTGAG	NM_004605, NM_177973
STSF	GGGGATGCTGTTGAGGAA	NM_000351
STSR	GTCCGATGTGAAGTAGATGAGG	NM_000351
PGRF	TCAGGCTGTCATTATGGTGTCC	NM_001271162.2
PGRR	GCAGTCATTTCTTCCAGCACAT	NM_001271162.2
GAD1F	CTAAGAACGGTGAGGAGCAAAC	NM_000817.3
GAD1R	TGGTGTGGGTGATGAAAGTCC	NM_000817.3
GAD2F	CCACACAAGATGATGGGAGTC	NM_001134366.2
GAD2R	TGCTCAAGGTAGGAGGC	NM_001134366.2
GLAST-1F	GCTGTGGTGATTGGCATAATCA	NM_001166695.3
GLAST-1R	CAGCTGTCACTCGTACAATTTTGC	NM_001166695.3
GLT1F	ATACCATTGACTCCCAGCATCG	NM_001252652.2
GLT1R	GAGTTGCTTTCCCTGTGGTTCTT	NM_001252652.2
EAAT2F	CTGCCGGATAGTGCTGAAG	NM_004171
EAAT2R	CTCTGAGCCAAGATGACTGTC	NM_004171
EAAT3F	AAGGATGCGAGTGGAAGC	NM_004170
EAAT3R	AGTTGAGAGGTTGCTGTGTTC	NM_004170
GLSF	GCTTTCCATGTTGGTCTTCC	NM_014905.5
GLSR	ACACTGTTGCCCATCTTATCC	NM_014905.5
GLULF	CGACCTTGATATTCCACCCTT	NM_001033044.4
GLULR	TTGAGCCCCTCCTAGTTCTTC	NM_001033044.4
BDNFF	AAAAGGCATTGGAACTCCCAG	NM_001709.5

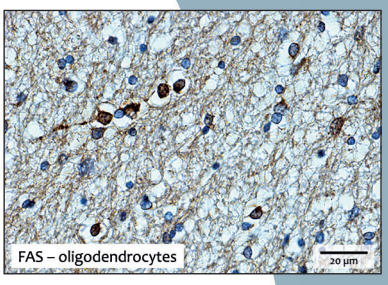
Supplementary Table 1. (continued)

NAME	SEQUENCE (5' > 3')	Transcriptcode
BDNFR	GCCAGCCAATTCTCTTTTTGC	NM_001709.5
TGFbetaF	TGTGACAGCAGGGATAACAC	NM_000660.7
TGFbetaR	ATGAGAAGCAGGAAAGGCC	NM_000660.7
IL10F	GAGAACCAAGACCCAGACATC	NM_000572
IL10R	CCTTGCTCTTGTTTTCACAGG	NM_000572
CD8F	TCACCCTTTACTGCAACCAC	NM_001770
CD8R	TTGCACAGGGTTAGACGTATC	NM_001770
IFNGF	GCAAGATCCCATGGGTTGTGT	NM_000619
IFNGR	CTGGCTCAGATTGCAGGCATA	NM_000619
GZMKF	ACGGCGACCCTTTTATCAC	NM_002104
GZMKR	AGCGTGGAAGACACCTTTAC	NM_002104
EF1αF	AAGCTGGAAGATGGCCCTAAA	NM_001402.6
EF1αR	AAGCGACCCAAAGGTGGAT	NM_001402.6
GAPDH	TGCACCACCAACTGCTTAGC	NM_001357943.2

CHAPTER 8

Post-mortem multiple sclerosis lesion pathology is influenced by single nucleotide polymorphisms





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ABSTRACT

Over the last few decades several common single nucleotide polymorphisms (SNPs) have been identified that correlate with clinical outcome in multiple sclerosis (MS), but the pathogenic mechanisms underlying the clinical effects of these SNPs are unknown. This is in part due to the difficulty in the functional translation of genotype into disease-relevant mechanisms. Building on our recent work showing the association of clinical disease course with post-mortem MS lesion characteristics, we hypothesized that SNPs that correlate with clinical disease course would also correlate with specific MS lesion characteristics in autopsy tissue. To test this hypothesis 179 MS brain donors from the Netherlands Brain Bank MS autopsy cohort were genotyped for 102 SNPs, selected based on their reported associations with clinical outcome or their associations with genes that show differential gene expression in MS lesions. Three SNPs linked to MS clinical severity showed a significant association between the genotype and either the proportion of active lesions (rs2234978/FAS and rs11957313/KCNIP1) or the proportion of mixed active/inactive lesions (rs8056098/CLEC16A). Three SNPs linked to MS pathology-associated genes showed a significant association with either proportion of active lesions (rs3130253/MOG), incidence of cortical grey matter lesions (rs1064395/NCAN) or the proportion of remyelinated lesions (rs5742909/CTLA4). In addition, rs2234978/FAS T-allele carriers showed increased FAS gene expression levels in perivascular T cells and perilesional oligodendrocytes, cell types that have been implicated in MS lesion formation. Thus, by combining pathological characterization of MS brain autopsy tissue with genetics, we now start to translate genotypes linked to clinical outcomes in MS into mechanisms involved in MS lesion pathogenesis.

INTRODUCTION

Multiple sclerosis (MS) is a heterogeneous disease with large inter-individual differences in disease course and response to immunomodulatory therapies.[40,41] The pathogenic mechanisms underlying these differences between MS patients remain largely unknown.[23,41] Over the past decades several common genetic variants have been associated with clinical outcome of MS in candidate gene and genome-wide association studies (GWAS).[4,6,7,57] On their own the identified common genetic variants show a minor effect on the clinical outcome and therefore they have no clinical predictive utility, but nevertheless they possess an important translational potential.[3,26,27,32] The genes and associated biological pathways implicated in clinical outcome by genetic association may represent targets for interventions that are likely to have greater effects than the naturally occurring variant.[28,32]

However, the genes and biological pathways associated with the identified variants remain largely unknown because of the inability to translate genotype into disease-relevant mechanisms.[3,26,28] Here, by quantitative pathological characterization and gene expression studies of autopsy tissue, we aim to translate genotypic information into pathogenic mechanisms.

Recently, we and others showed that MS lesion characteristics in autopsy tissue are associated with the clinical disease course of MS, in which the proportion of mixed active/inactive (chronic active) lesions was significantly associated with disease progression.[23,41] This chronic lesion activity is not identifiable with commonly used MRI techniques, although recent 7T MRI and PET-MRI studies have shown the prognostic potential of these approaches for detection and quantification of these mixed active/inactive lesions in living MS patients.[15,42]

Here, we have genotyped 179 MS brain donors from the Netherlands Brain Bank autopsy cohort for 67 SNPs that were previously associated with clinical or MRI outcomes in GWAS studies[4,6,7,57] and 35 SNPs in genes associated with MS pathology in previous studies, for example genes that were found upregulated in the area around these mixed active/inactive lesions in a microarray analysis.[33] We identified six variants that showed a significant association with either the proportion of active, mixed active/inactive or remyelinated lesions or the incidence of cortical grey matter lesions in autopsy tissue, of which one, shows increased mRNA levels in brain autopsy tissue. With these findings we now begin to translate genotypic effects on clinical outcome in MS into pathological mechanisms. Improving our understanding of the pathological mechanisms that underlie the differences in clinical outcomes will help us to identify biomarkers to improve the prognosis and development of therapies in MS patients.

MATERIALS AND METHODS

Donor and tissue characteristics

In total, 179 MS brain donors from the Netherlands Brain Bank (NBB) were included in this study. Informed consent was given by the donors for brain autopsy and for the use of material and clinical data for research purposes, in compliance with national ethical guidelines. The NBB autopsy procedures were approved by the Medical Ethics Committee of the VU University Medical Center, Amsterdam, The Netherlands. The donors came to autopsy between 1990 and 2015. The diagnosis MS was confirmed by a certified neuropathologist (Prof. J.M. Rozemuller or Prof. P. van der Valk, VU University Medical Center, Amsterdam, The Netherlands). Cases with confounding CNS pathology (bleeding, infarction and metastasis) were excluded (n=6). Tissue was dissected from standard locations in the brainstem and spinal cord. Visible MS plaques were dissected during autopsy and since 2001 MS lesions were also dissected on post-mortem MRI guidance from 1 cm thick coronal brain slices. Donor and tissue characteristics are shown in **Table 1** and described in Luchetti et al. 2018.[41]

Table 1. Donor and tissue characteristics of the post-mortem tissue investigated in this study.

	Cases (n)	**Age** (mean ± SD)	**Disease duration** (mean ± SD)	**Tissue blocks examined per case** (mean ± SD)	**Total number of tissue blocks** (n)	**Total number of lesions** (n)
Total	179	62.5 (13.7)	28.5 (13.3)	17.6 (10.8)	3148	8462
SP	99	60.4 (13.4)	29.8 (14.2)	17.8 (11.2)	1766	4955
PP	54	66.0 (13.2)	27.5 (11.9)	17.7 (10.1)	954	2632
Relapsing	14	61.8 (15.1)	24.2 (11.6)	19.1 (9.55)	268	414
Type not determined	12	65.8 (14.9)	24.5 (14.5)	13.3 (11.9)	160	461
Male	68	60.0 (13.5)	27.5 (12.9)	19.1 (10.5)	1297	3532
Female	111	64.1 (13.7)	29.1 (13.6)	16.7 (10.9)	1851	4930

SP indicates secondary progressive and PP primary progressive MS. MS disease course was not reported in 12 cases.

Pathological characterization of MS lesions

The visualization, definition, and calculation of proportion of lesion subtypes is described in Luchetti et al. 2018.[41] In short, double immunostaining was performed on sections from all formalin fixed and paraffin embedded tissues blocks that were dissected from a donor to visualize proteolipid protein (PLP) (MCA839G, AbD Serotec, Oxford, UK, with DAB) and human leukocyte antigen (HLA-DR-DQ, referred to as HLA) (M0775, CR3/43, DAKO, Denmark, with DAB-nickel), as previously described.[41] On average 19.4 ± 12.4 (mean ± SD) tissue blocks were characterized per case. The different lesion characteristics are presented in **Figure 1**.

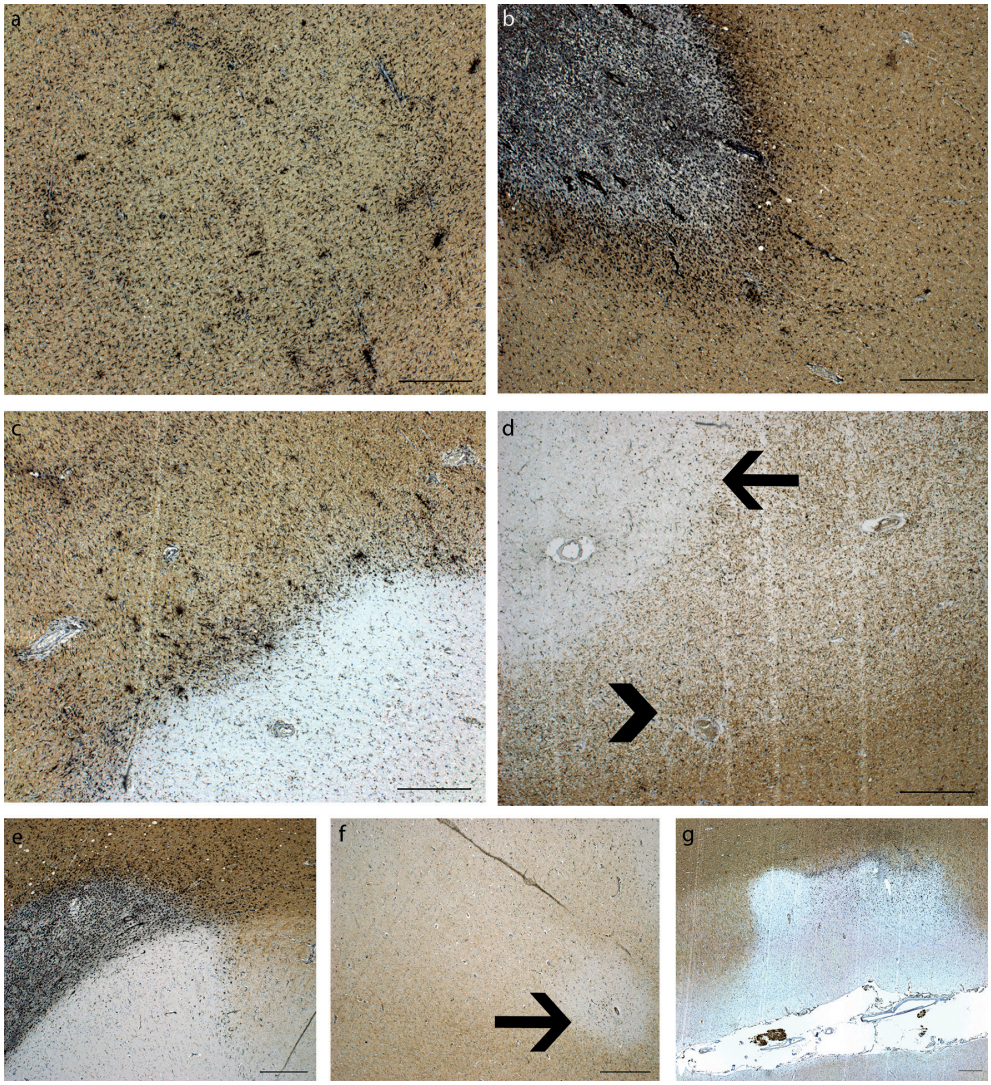

Figure 1. Pathological characterization of MS lesions.
HLA-PLP immunohistochemistry of MS autopsy tissue showing the MS lesion characteristics. A. Reactive site with increased density of HLA^+ cells with microscopically intact myelin. B. Active lesion with partial demyelination and HLA^+ cells throughout the lesion. C. Mixed active/inactive (chronic active) lesion with inactive demyelinated center and a rim of HLA^+ cells. D. Inactive lesion (arrow), demyelinated with sparse HLA^+ cells. Remyelinated lesion (arrow head), partial myelination with sparse HLA^+ cells. E. Leukocortical lesion. F. Intracortical lesion. G. Subpial cortical lesion. Scale bar represents 500 µm.

SNP selection

SNPs were selected that were associated with either multiple sclerosis severity score (MSSS), expanded disability status scale (EDSS) or MRI outcomes in three GWAS[4,6,7] and one candidate gene SNP study.[57] Strong linkage disequilibrium (LD) was defined as r^2>0.8 and strong LD SNPs

were identified for the selected SNPs using the SNPsnap database from Broad Institute.[45] When two selected SNPs were in strong LD with each other, one of the two SNPs was selected for genotyping (the SNP with highest MAF was selected). SNPs that had a minor allele frequency (MAF) of <0.02 in the GO-NL database were excluded. In total 69 SNPs were selected in this way. Second, genes were identified that were associated with the MS lesion characteristics in autopsy tissue in micro-array analyses of laser dissected mixed active/inactive, inactive and remyelinated lesions and normal appearing white matter.[33] Furthermore genes that were previously associated with MS lesion characteristics or related to either microglia/macrophage, T and B cell response or neurodegenerative processes were identified. SNPs in these genes that were either functionally studied or associated with auto-immune, neurological or psychiatric diseases were identified. In total, 36 SNPs were selected in this way. **Suppl. Table 1** shows the 105 SNPs selected for genotyping and their association with MS with literature references. None of the 105 selected SNPs were in strong LD with each other and MAF was >0.02 in the GO-NL database.

Genotyping

DNA was isolated from whole blood or frozen cerebellar tissue. DNA of nine MS cases was isolated from formalin-fixed paraffin cerebellar tissue using DNeasy Blood & Tissue Kit Qiagen.

Genotyping was performed by LGC genomics (Teddington, UK) using PCR-based KASP^{TM} genotyping assays. Fluorescence-based competitive allele-specific assays were used (www.lgcgroup.com/genomics). Fifty-one assays were ready made by LGC and for 54 assays were custom made based on 120 base pair sequences flanking the location of the SNP based on hg19 reference genome with annotation of all SNPs with a MAF of >0.1 to prevent primer design at these sites. All SNPs were in Hardy-Weinberg-equilibrium (HWE) and MAF in the study population is comparable to the GO-NL reference population. MAF and HWE are shown in **Suppl. Table 2**.

Linkage disequilibrium and GTEx single tissue eQTL analysis

SNPs in strong LD (r^2>0.8) with SNPs that showed a significant association with MS lesion characteristics were determined by searching the SNiPA database (http://snipa.helmholtz-muenchen.de/snipa3/index.php?task=proxy_search) using the hg19 reference genome. The settings were genome assembly 'GRCh37', variant set '1000 Genomes, Phase 3 v5', population 'European', genome annotation 'Ensembl 87'. The rs numbers of these strong LD SNPs are shown in **Suppl. Table 3**. Using the GTEx portal (www.gtexportal.org) single tissue *cis* eQTLs were identified for all strong LD SNPs. Single tissue *cis* eQTLs in brain samples and EBV transformed cells (lymphocytes and fibroblasts) were considered relevant to MS pathogenesis.[19,29]

Quantitative PCR

40 MS cases that had frozen standardly dissected temporal gyri available were used for qPCR analysis of gene expression. Donor characteristics are shown in **Suppl. Table 4**. Frozen tissue from standardly dissected superior temporal gyri was sectioned. Sections of 20 µm were made and

10-50 mg of tissue was collected in the cryostat and stored in ice cold TRIsure. After addition of chloroform and centrifugation the aqueous phase was removed and mixed with an equal volume of Isopropanol. Samples were then centrifuged and supernatant was removed from pellet and the pellet was washed twice with 75% Ethanol. RNA samples were dissolved in 20 µl of water and RNA yield was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was performed in a reaction mixture of 10 µl containing 200 ng RNA and gDNAse Wipeout Buffer, incubated for 2 minutes at 42°C and a mixture of Quantiscript Reverse Transcriptase, Quantiscript Buffer and RT-Primer Mix (Qiagen Benelux, Venlo, The Netherlands), incubated for 30 min at 42°C. RT transcriptase was inactivated by incubation for 3 minutes at 95°C. Primers were designed using the primer designer from Integrated DNA Technologies, Inc. NCBI blast was performed for suitable primer sequences using OligoAnalyzer 3.1 (Integrated DNA Technologies, Illinois, USA). Primer sequences and characteristics are shown in **Suppl. Table 5**. Specificity was tested on cDNA derived from pooled RNA of both brain and spleen from MS and control donors. Dissociation curves were examined and the PCR product sizes were determined by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Quantitative PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with samples containing equal cDNA concentrations of 10 ng/µl resulting from 2 ng total RNA per reaction. Analysis was performed according to the manufacturer's protocol at the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Target genes were normalized to the geometric mean of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and elongation factor 1 alpha (EEF1A1) mRNA expression. qPCR experiments are performed in duplicate.

Characterization of tissue used for RNA isolation and relative expression analysis

Frozen sections (20 µm) from the standardly dissected superior temporal gyri used for qPCR analysis, were fixed for 30 min in 4% paraformaldehyde. Sections were incubated with 3% H_2O_2 solution for 20 min to quench endogenous peroxidase activity. Then sections were incubated with 10% normal horse serum for 30 min to block non-specific binding of secondary antibodies. HLA-PLP double immunohistochemistry was performed and the tissue was characterized as described above and in more detail in Luchetti et al. 2018.[41]

FAS expression in proteomics data from peripheral T cells

Peripheral lymphocyte mass spectrometry values for FAS in different subpopulations of peripheral T cells were extracted from www.immprot.org. Data is shown in **Suppl. Table 6**.[52]

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence was performed for FAS (mouse B-10 Santa Cruz or HPA027444), CD4 (ab133616, Abcam) and NOGO-A (11C7, gift from prof. M. Schwab, Zurich[9]) on formaldehyde fixed and paraffin-embedded sections (8 µm) containing mixed active/

inactive and active MS lesions. Sections were deparaffinized with xylene and rehydrated. Antigen retrieval was performed using citrate buffer at pH 6 (microwave, 10 min at 700 W). Endogenous peroxidase activity and aspecific binding of secondary antibodies was blocked as described above. For fluorescent imaging, sections were incubated with primary antibodies overnight at 4°C. CD4, NOGO-A and FAS rabbit antibody were visualized using directly labeled secondary antibodies with Alexa 488 fluorophore or Cy3 fluorophore. FAS antibody (B-10) was visualized by incubation with an appropriate biotinylated secondary antibody followed by incubation with streptavidin-labeled Cy3 antibody for 45 min. All sections were finally incubated with Hoechst for 10 min. Z stack images were taken using a Leica SP8 confocal microscope and Leica Application Suite X (2017) at 63x magnification. Negative controls with the omission of primary antibody were included.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells and subcortical white matter-derived single cell fractions were isolated after rapid post-mortem autopsies of NBB brain donors as described previously.[55,56] Donor and sample characteristics are listed in **Suppl. Table 7**. Cells were stained with fixable viability dye eFluor 780 (Life Technologies) and the following antibodies: CD3 PE-Cy5.5, clone SK7, (Invitrogen), CD20 APC, clone L27, CD25 FITC, clone 2A3, CD69 BV395, clone FN50, CD127 PE, clone HIL-7R-M21, (BD Biosciences), CD4 BV510, clone RPA-T4, CD8a BV785, clone RPA-T8, and FAS PE-Cy7, clone DX2, (Biolegend), and analyzed on a Fortessa LSR™ cell analyzer (BD Biosciences, San Jose, CA, USA). Data were analyzed with FlowJo software 10.5 (Tree Star, Ashland, OR, USA).

Statistical analysis of SNP-pathology relationships

The associations of genotype with MS lesion characteristics were analyzed as follows. For all tests the numbers in each group corresponding to each genotype are given in **Suppl. Table 8**.

Lesion load was log-transformed and analyzed with linear models. Presence of cortical grey matter lesion is a binomial yes/no measure and so was analyzed with binomial generalized linear models (GLM). Lesion classification was treated as a yes/no outcome for each lesion type (Luchetti et al.2018) and so yes/no counts for each patient were used in a quasibinomial GLM (quasibinomial models allow for additional variance due to between-patient variation).

In all models the pathological characteristic was the dependent variable and genotype or genotype and sex were independent categorical variables. Multiple testing correction was performed over all tests of genotype effect on MS lesion characteristics (a total of 612 tests) using the Benjamini-Hochberg method. All analyses were carried out in R.[49] For linear models, significance was calculated using F-tests for linear models and likelihood ratio chi-squared tests for GLMs using R package *car*.[22] Graphs of pathological characteristics versus genotype show means and 95% confidence intervals calculated by the LMs and GLMs using R package *effects*,[21] except for presence of cortical grey lesions where 95% confidence intervals were calculated using R package *binom* using the 'bayes' method.

The correlation of log transformed relative gene-expression levels with genotypes were analyzed using a linear model including the presence of an MS lesion and meninges in the tissue block from which RNA was isolated as factors in the model. From all genotypes with sufficiently large (n> 5) genotyping groups, relative gene-expression levels of the genes they were previously associated to, were compared. rs11957313/C5orf58 was excluded from analysis since the groups were too small.

RESULTS

SNP genotyping of the Netherlands Brain Bank MS cohort

179 multiple sclerosis autopsy cases were included in the analysis (**Table 1**). 102 genotyping assays were successfully performed, giving results that were in Hardy-Weinberg equilibrium. Two pre-designed assay's failed on all samples (rs1557351, rs12202350[4]) and these were excluded from analysis. rs1065761 showed no minor allele carriers and is, therefore excluded, from analysis. Furthermore, the minor allele frequency (MAF) in the NBB cohort is comparable with the Dutch reference population (**Suppl. Table 2**). MAF was above >0.02 for all SNPs.

Six SNPs were associated with MS lesion characteristics

In order to link genotype to pathological parameters we analyzed the correlation of allelic distributions for each SNP with the proportion of lesion subtypes that was scored for each donor. As described in Luchetti et.al. 2018, reactive, active, mixed active/inactive, inactive, remyelinated and cortical demyelination were examined in all autopsy cases as shown in **Figure 1.** On average 17.6 (S.D. 10.8) dissected tissue blocks were examined per case and in total 3148 tissue blocks containing 8462 lesions were examined.[41] Total lesion load, presence of cortical grey matter lesions and the proportions of lesion subtypes, either active, mixed active/inactive (also known as chronic active) or remyelinated and the microglia/macrophage activity score (as calculated in Luchetti et al. 2018) were tested for correlation with genotype for each SNP.

Three SNPs previously associated with the clinical outcomes in MS showed a significant association with post-mortem MS lesion characteristics (**Figure 2A**). Rs2234978/FAS associated with the proportion of active lesions (false discovery rate (FDR) p=0.02). Heterozygotes (T:C) and homozygotes (T:T) for this minor allele had a higher proportion of active lesions compared to major allele homozygotes (C:C). rs11957313/KCNIP1 associated with the proportion of active lesions (FDR p=0.047). Heterozygotes (G:A) and homozygotes (A:A) for this minor allele had a lower proportion of active lesions compared to the major allele homozygotes (G:G). rs8056098/CLEC16A associated with the proportion of mixed active/inactive lesions (FDR p=0.047). Homozygotes for the minor allele (A:A) had a lower proportion of mixed active/inactive lesions compared to homozygotes for the major allele (G:G) and compared to heterozygotes (G:A).

Three SNPs that were located in genes previously associated with MS pathological characteristics showed a significant relation with post-mortem MS lesion characteristics (**Figure 2B**). rs1064395/NCAN associated with the incidence of cortical grey matter lesions (FDR *p*=0.010). Heterozygotes (G:A) had a higher incidence of cortical lesions compared to major allele homozygotes (G:G). rs3130253/MOG associated with the proportion of active lesions (FDR *p*=0.02). Heterozygotes (G:A) for the minor allele had a higher proportion of active lesions compared to major allele homozygotes (G:G). rs5742909/CTLA4 associated with the proportion of remyelinated lesions (FDR *p*=0.047). Heterozygotes (C:T) had a higher proportion of remyelinated lesions compared to major allele homozygotes (T:T). Results for the six SNPs showing a significant association are shown in **Table 2 and Suppl. Table 8**.

Of the SNPs with a significant effect on a pathological outcome, sex was not significantly associated with the exception of rs1064395/NCAN versus the incidence of cortical grey matter lesion presence. There sex was a nominally significant covariate, before multiple testing correction (sex: *p*= 0.02; χ^2=5.4 on 1 d.f.; genotype: *p*= 1.5E-5; χ^2=22.1 on 2 d.f.). However genotype was still equally significant in this model. The effect of genotype on cortical grey matter lesions is plotted separately for the sexes (**Suppl. Figure 1**).

Single tissue *cis* eQTLs for significant SNPs

In order to investigate the functional implication of pathology-associated SNPs, we set out to investigate possible effects of the identified SNPs and SNPs with strong linkage disequilibrium (LD) on transcript abundance, demonstrated by expression quantitative trait loci (eQTL). All SNPs in strong LD (r^2>0.8) with the SNPs found to be significantly associated in the current study were identified and are listed in **Suppl. Table 3**. Using the GTEx database[59] single tissue *cis* eQTLs were identified for these SNPs. Only significant eQTLs in brain and EBV transformed lymphocytes and fibroblasts were considered relevant to MS pathology.[19,29] rs2234978/FAS showed 216 eQTLs, rs3130253/MOG showed 489 eQTLs, rs1064395/NCAN showed 9088 eQTLs, rs11957313/KCNIP1 showed 1 eQTLs, rs8056098/CLEC16A showed 42 eQTLs, and rs5742909/CTLA4 showed no eQTLs. When considering only the protein coding genes in MS relevant tissues significant associations of genotype with increased or decreased RNA expression levels were found as follows. rs1064395/NCAN is associated with expression levels of six genes (CILP2, HAPLN4, ZNF101, LPAR2, TSSK6, TM6SF2), rs2234978/FAS is associated with two genes (FAS and ACTA2), rs3130253/MOG is associated with one gene (HLA-A) and rs11957313/KCNIP1 is associated with one gene (C5orf58). rs5742909/CTLA4 and rs8056098/CLEC16A did not show significant eQTLs in the selected tissue types. The eQTL boxplots for the ten protein coding genes associated with the significant SNPs and their SNPs in strong LD are displayed in **Suppl. Figure 2**.

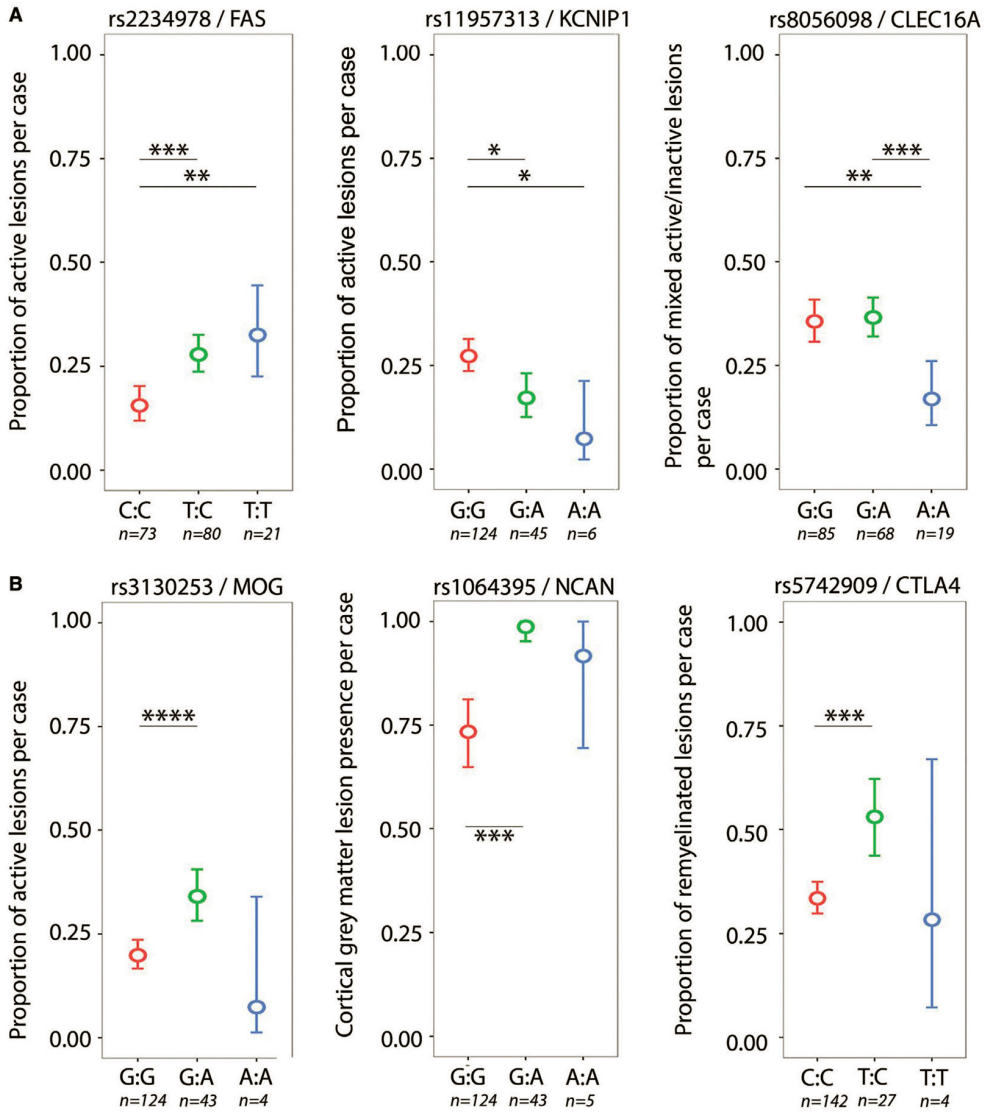

Figure 2. SNPs significantly associated with MS lesion pathology.
A. SNPs previously associated with clinical or MRI measures for disease severity. rs2234978/FAS, heterozygotes (T:C) and minor allele homozygotes (T:T) showed a higher proportion of active lesions compared to major allele homozygotes (C:C). rs11957313/KCNIP1, heterozygotes (G:A) and minor allele homozygotes (A:A) showed a lower proportion of active lesions compared to major allele homozygotes (G:G). rs8056098, minor allele homozygotes (A:A) showed a lower proportion of mixed active/inactive lesions compared to heterozygotes (G:A) and major allele homozygotes (G:G). b: SNPs located in genes previously associated with MS pathological characteristics. rs3130253/MOG, heterozygotes (G:A) showed a higher proportion of active lesions compared to major allele homozygotes (G:G). rs1064395/NCAN, heterozygotes (G:A) showed a higher incidence of cortical grey matter lesions compared to major allele homozygotes (G:G). rs5742909/CTLA4, heterozygotes (T:C) showed a higher proportion of remyelinated lesions compared to major allele homozygotes (C:C). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (generalized linear models).

Table 2. Six SNPs associated with MS lesion characteristics in autopsy tissue.

SNP	Chr:Location (hg19)	Gene	Position (UCSC, hg19)	*p*-value (FDR)	*p*-value (post-hoc test unadjusted)	Pathological outcome	Correlation with MS severity	cis eQTLs GTEx (FDR <0.05)
rs2234978	10:90771829	FAS	exon 7	0.020	CT vs CC (*p*=0,0003) TT vs CC (*p*=0,004)	Minor T allele associated with increased proportion of active lesions	Odds ratio 1.83 (TT vs CC) predicting time to EDSS6 [58]	FAS and ACTA-2
rs3130253	6:29634012	MOG	exon 3	0.020	AG vs GG (*p*=0,0001)	Minor A allele associated with increased proportion of active lesions		HLA-A
rs1064395	19:19361735	NCAN	exon 15	0.010	AG vs GG (*p*=0,0002)	Minor A allele associated with increased incidence of cortical grey matter lesions	NCAN significantly upregulated (FC 2.7) in the area around mixed active/inactive lesions compared to the area around inactive lesions [33]	CILP2, HAPLN, ZNF101, LPAR2, TSSK6, TM6SF2
rs11957313	5:169950394	KCNIP1	intron 1	0.047	AG vs GG (*p*=0,015) AA vs GG (*p*=0,026)	Minor A allele associated with decrease in proportion of active lesions	Brain parenchymal volume (Log p>5.0) [4]	C5orf58
rs8056098	16:11138812	CLEC16A	intron 15	0.047	AA vs GG (*p*=0,002) AA vs GA (*p*=0,0009)	Homozygotes for minor A allele show a lower proportion of mixed active/inactive lesions	Odds ratio 0.65 (MSSS<2.5 vs >7) [6]	NA
rs5742909	2:204732347	CTLA4	near '5	0.047	TC vs CC (*p*=0,0002)	Minor T allele associated with increase in remyelinated lesions		NA

rs2234978/FAS T allele carriers show an increased FAS RNA expression in brain tissues

To directly link the SNP genotypes to transcript abundance in MS brain tissue, we used qPCR to assess expression levels in standardly dissected superior temporal gyri from 40 MS cases. Gene expression levels were determined for the 10 significant single tissue eQTL genes. rs2234978/FAS showed a consistent effect on FAS gene expression, where heterozygotes for this variant (T:C) showed a higher FAS gene expression level compared to major allele homozygotes (C:C) (p=0.0001) (**Figure 3A**). Characterization of the tissue blocks showed that all sections contained both white and cortical grey matter. In these 40 standardly dissected sections three white matter lesions and 14 cortical grey matter lesions where present. In 22 cases meninges were present in the section. Log transformed relative expression values were analyzed using a linear regression model including genotype, lesion presence and meninges presence. The presence of a lesion showed no significant effect on FAS gene expression, however the presence of meninges in the tissue block was a significant factor in the linear model (p=0.005). Therefore we looked into the effect of the SNP in cases with and cases without meninges in the tissue block. In both groups there was significantly higher FAS expression in carriers of the minor T allele (**Suppl. Figure 3**). For the other nine genes we did not find a significant association between genotype and transcript level (**Suppl. Figure 4**).

To identify the major source of FAS in and around MS lesions, we performed immunohistochemistry for FAS on mixed active/inactive multiple sclerosis lesions. This showed that it is expressed by oligodendrocytes (**Figure 3B**) in the NAWM around a mixed active/inactive lesion, as shown by colocalization of FAS with NOGO-A (**Figure 3C**). FAS expression is also apparent in lymphocytes in the perivascular space (**Figure 3D**) as shown by colocalization of FAS with CD4 (**Figure 3E**). Since FAS expression was previously found to be highest in memory regulatory T cells ([53], **Suppl. Table 6**) we quantified FAS protein expression in freshly isolated lymphocyte populations from white matter brain samples and blood from three non-MS brain donors using flow-cytometry (**Suppl. Table 7, Suppl. Figure 5**). In lymphocytes derived from both blood (**Figure 3F**) and brain (**Figure 3G**),the percentages of FAS positive cells and mean fluorescence intensity of FAS shows that it is expressed by both CD4 and CD8 T cells. The highest FAS expression was observed in the rare events of CD4 T cells that were $CD127^{-}CD25^{+}$, supporting high FAS expression in a relatively small population enriched for regulatory T cells.

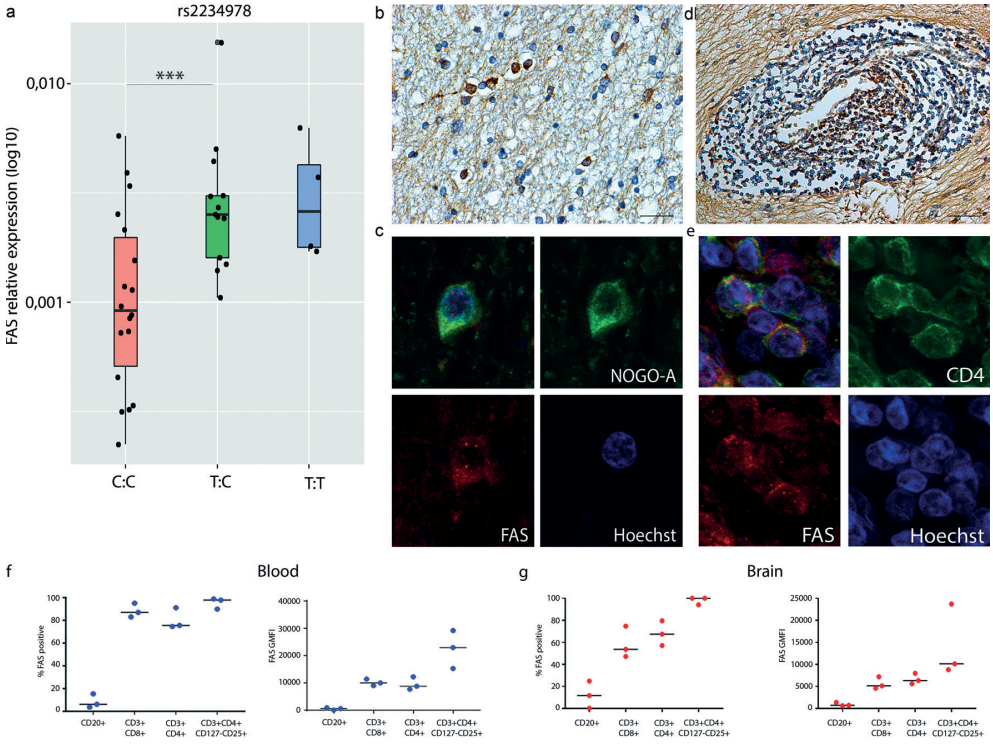

Figure 3. rs2234978/FAS and FAS expression in MS autopsy tissue.
A: relative expression of FAS with qPCR, heterozygotes showed an increased relative expression of FAS compared to major allele homozygotes C:C n=22 T:C n=14 T:T n=4 *** $p<0.001$. B: Immunohistochemistry for FAS on active and mixed active/inactive MS lesions showed FAS expression by oligodendrocytes around a mixed active/inactive lesions. (40x magnification, scalebar represents 250 µm) C. Colocalization of FAS with NOGO-A, showing it is expressed by oligodendrocytes around MS lesions. (63x magnification) D. FAS expression by lymphocytes in the perivascular space. (20x magnification, scalebar represents 500 µm) E: Colocalization of FAS with CD4, showing it is expressed by T cells in MS lesions. (63x magnification) F: FAS protein expression in lymphocytes derived from blood, showing the percentage of FAS positive cells and geometric mean fluorescence intensity (GMFI) for FAS on CD20, CD4, CD8 and $CD127^{-}CD25^{+}CD4^{+}$ T cells. FAS is expressed by CD4 and CD8 T cells and suggested to be increased in the $CD127^{-}CD25^{+}$ T cell population that is enriched for T-regulatory phenotype. G. FAS protein expression in lymphocytes derived from normal appearing white matter brain tissue, showing the percentage of FAS positive cells and GMFI for FAS on CD20, CD4, CD8 and $CD127^{-}CD25^{+}CD4^{+}$ T cells. FAS is expressed by CD4 and CD8 T cells and suggested to be increased in the few T cells that are enriched for T regulatory phenotype.

DISCUSSION

Here, we translate genotypic information into pathological mechanisms, by analyzing the Netherlands Brain Bank MS autopsy cohort consisting of 179 pathologically characterized MS brain donors in relation to genotyping results for 67 SNPs previously related to the clinical disease course or MRI measures[4,6,7,57] and 35 SNPs located in genes previously related to MS pathological characteristics. This analysis shows that six genetic variants show an effect on post-mortem MS lesion characteristics. rs2234978/FAS, rs11957313/KCNIP1 and rs3130253/MOG genotypes

affected the proportion of active lesions, while rs8056098/CLEC16A genotype had an effect on the proportion of mixed active/inactive lesions. rs1064395/NCAN genotype was associated with the incidence of cortical grey matter lesions and rs5742909/CTLA4 genotype was associated with the proportion of remyelinated lesions. Furthermore, SNP function assessment showed that rs2234978/FAS T allele carriers, which had a higher proportion of active lesions, also showed increased FAS gene expression levels in MS autopsy tissue.

rs2234978/FAS has previously been associated with clinical disease severity.[57] We were able to show that the rs2234978 T allele is related to an increased FAS expression in MS brain tissue, which is in line with the single tissue eQTL in brain nucleus accumbens in the GTEx database.[59] FAS expression in peri-lesional oligodendrocytes and T cells, particularly regulatory T cells, suggests two possibilities for the involvement of FAS in MS pathology. The first possible mechanism is that increased FAS expression in oligodendrocytes makes them more likely to undergo apoptosis. FAS is expressed by oligodendrocytes in the area around mixed active/inactive lesions suggesting that carriers of the T allele have a higher FAS expression in oligodendrocytes. FAS functions as an apoptosis receptor for oligodendrocytes,[2,14,64] therefore higher FAS expression may make oligodendrocytes more likely to undergo apoptosis and thus more vulnerable to the MS disease process. FAS receptor can be activated by membrane bound FAS-L which is expressed by T cells and in MS lesions by microglia, astrocytes and oligodendrocytes.[13,20,62] In spinal cord injury oligodendrocytes undergo FAS-mediated apoptosis along degenerating axons.[10] In mice with traumatic spinal cord injury blocking of FAS-mediated apoptosis resulted in reduced oligodendrocyte apoptosis, reduced microglial activation and reduced neuronal and axonal loss and improved functional outcome.[1,11,18,53,66,67] In the experimental autoimmune encephalomyelitis (EAE) mouse model it is shown that mice lacking FAS expression on oligodendrocytes are partially protected from EAE with a decrease in demyelination and a mild decrease in infiltration of lymphocytes.[34]

The second possible mechanism is that higher FAS expression in T-cells leads to a more pro-inflammatory T-cell population. Immunohistochemistry shows FAS expression by lymphocytes in mixed active/inactive lesions. FAS is mainly expressed by $CD4^+$ T cells and its expression is highest in Treg cells in both blood and brain, in line with previous findings.[25,53] It has been shown that Treg cells are present in MS lesions, with densities from 3 to >15 /mm^2, and are found more often in inactive compared to active MS lesions.[24] Treg cells are more vulnerable to FAS mediated apoptosis and exhibit a higher rate of apoptosis compared to other T cell populations *ex-vivo*.[24,25,46] Interestingly, FAS is also highly expressed by Th17 cells,[53] but in contrast to Tregs, activation of the FAS receptor promotes the stability of the Th17 phenotype and prevents their differentiation into Th1 cells.[43] Therefore, an increased overall FAS expression in T cells could result in the inhibition of the Treg cells and a more pro-inflammatory T cell response, leading in turn to a more severe MS disease course.[8,39] This suggests that inhibition of FAS mediated apoptosis within the central nervous system (CNS) is a potential target for protection of oligodendrocytes and inhibition of inflammatory disease activity in MS.[48]

Rs11957313/KCNIP1 is associated with the proportion of active lesions. Previously, rs11957313 was associated with brain parenchymal volume on MRI, however, the effect on RNA and protein expression remains unknown.[4] It is located in intron 1 of the KCNIP1 gene which encodes the potassium voltage gated channel interacting protein 1. The single tissue *cis* eQTL analysis shows an association with C5orf58 expression levels in cortical brain tissues. Unfortunately, as C5orf58 expression in CNS is very low and not detectable with qPCR, we were not able to validate this association.

rs8056098/CLEC16A minor allele was associated with a less severe disease course in the IMSGC GWAS,[6] and in our analysis the minor allele is associated with a lower proportion of mixed active/inactive lesions. A higher proportion of mixed active/inactive (chronic active) lesions in autopsy tissue has been repeatedly associated with a more severe and progressive disease course.[23,41] Our findings are consistent with the reported GWAS association (7) and suggests a link between genotype and disease severity via the propensity to form mixed active/inactive lesions. Recently, the function of CLEC16A in the mouse CNS has been described, showing that deficiency of CLEC16A protein impairs autolysosome function and neuronal survival.[51] How rs8056098 affects CLEC16A protein level or function in MS patients awaits further analysis. Currently, there is no data supporting a functional link between rs8056098 and CLEC16A expression levels or function.

This analysis shows that rs1064395/NCAN in exon 15 of the gene encoding Neurocan is associated with an increased incidence of cortical lesions in MS brain donors. Microarray analysis of mixed active/inactive and inactive MS lesions revealed NCAN to be specifically upregulated in the area around mixed active/inactive lesions compared to the area around inactive lesions, normal appearing white matter and white matter from healthy controls.[33] rs1064395 has previously been found to be associated with susceptibility to bipolar disease and schizophrenia in GWAS studies[12,44] and with cortical folding in schizophrenia patients.[54] In healthy individuals the minor allele is associated with poorer verbal memory performance.[50] The SNP is located in the 3' untranslated region of NCAN mRNA and so it is well placed to affect translation and localization of this transcript. How this SNP influences NCAN RNA and Neurocan protein expression or function and how it relates to neuronal functions remains to be experimentally determined. The Neurocan protein is involved in the regulation of peri-neuronal nets around (parvalbumin) neurons[58] and it is shown that peri-neuronal nets are decreased in MS cortical grey matter lesions compared to normal appearing grey matter without a reduction in the number of neurons.[31] This suggests that Neurocan and the stability of peri-neuronal nets could be associated with cortical demyelination in MS, and could possibly be influenced by the genotype at rs1064395.

Rs3130253/MOG minor allele is associated with an increased proportion of active lesions. rs3130253 is located in exon 3 of the MOG gene and causes a missense mutation resulting in an amino acid change. It has been shown that A allele carriers show relatively increased expression of MOG transcripts that contain exon 2.[36] Exon 2 codes for the extra-cellular, encephalitogenic IgV-like domain of the MOG protein on oligodendrocytes.[16] The immune system may ignore the

isoforms that lack exon 2 during development, and thus truncated MOG proteins likely play a role in maintenance of central and peripheral tolerance and/or in inflammatory and demyelinating disease.[16,35,36] The putative effect of the relative increased presence of MOG exon 2 on auto-immunity is a promising area for future research.

Finally, we show that rs5742909/CTLA4 is associated with a lower proportion of remyelinated lesions. rs5742909 minor allele is expected to increase the promotor activity of CTLA4, however it had no effect on CTLA4 transcript levels in brain tissue.[60,63] CTLA4 is a co-stimulatory molecule that is expressed by activated T cells that may downregulate T cell proliferation and activation during T cell-dependent immune responses. CTLA4 is abundantly expressed by T cells in human normal white matter.[55] In a cuprizone mouse model for demyelination and remyelination, it was shown that the increased infiltration of CD4 T cells impaired spontaneous remyelination.[5] These results therefore suggest that an increased inhibition of T cell activation relates to a higher proportion of remyelinated MS lesions. Furthermore, CTLA4 is suggested to be an important checkpoint in the development of active MS lesions, as administration of CTLA4 blocking antibodies for treatment of melanoma in an MS patient was linked to development of MS clinical symptoms and an increase in gadolinium enhancement of MS lesions on MRI.[30]

As far as we know this is the most comprehensive study[17,47,61,65] analyzing genotypes in relation to MS pathological characteristics. Our results illustrate that extensive and quantitative pathological characterization of autopsy tissue in sufficient numbers of MS brain donors enables the translation of genotypic variation with known effects on clinical outcome into pathological mechanisms, for example lesion activity. It is unlikely that these SNPs influence the incidence of gadolinium-enhancing lesions on MRI, since these are known to decline over time,[37,68] while actual lesion activity remains high even over long disease durations,[23,41] indicating a separate pathogenic mechanism for lesion activity in MS cases with long disease duration.

Analyzing the RNA and protein expression associated with the identified variants in different cell populations has great potential to lead to discoveries of targets for disease modifiers.[3,28] Modeling the SNPs in relevant human cells, for example stem cell-derived neurons or oligodendrocytes using gene editing tools like CRISPR-CAS9 will be of interest to elucidate the functional effect of these SNPs. Furthermore, RNA sequencing of isolated nuclei from frozen autopsy tissue is a promising approach to study the transcriptome of human cell-populations from the central nervous system of brain donors and its association with genotype.[38] Improving our understanding of the molecular mechanisms that underlie differences in clinical course will help us to identify biomarkers to improve the prognosis and development of precise therapies in MS patients.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data used for the analyses described in this manuscript were obtained from: GTEx Analysis Release V7 (dbGaP Accession phs000424.v7.p2) the GTEx Portal on 30-5-2018. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. SNP selection.

Available under supporting information at https://onlinelibrary.wiley.com/doi/full/10.1111/bpa.12760

Supplementary Table 2. SNP alleles, genotype numbers, MAF and Hardy-Weinberg equilibrium: results for Hardy Weinberg equilibrium analysis.

Available under supporting information at https://onlinelibrary.wiley.com/doi/full/10.1111/bpa.12760

Supplementary Table 3. SNPs in strong LD with pathology associated SNPs.

Available under supporting information at https://onlinelibrary.wiley.com/doi/full/10.1111/bpa.12760

Supplementary Table 4. Donor characteristics.

	Number	**Age** (years)	**Disease duration** (years)	**Post-mortem pH CSF**
Total	40	64.5 (13.15)	30.3 (13.6)	6.43 (0.25)
SP	20	65.8 (14.0)	34,5 (16,6)	6.4 (0.21)
PP	12	62.0 (14.45)	26,2 (7,8)	6.4 (0.28)
Relapsing	6	66.3 (10.3)	24,7 (9,1)	6.6 (0.34)
Male	20	62.0 (12.0)	31,3 (14,6)	6.43 (0.27)
Female	20	67.0 (14.1)	29,4 (12,9)	6.43 (0.24)

40 MS cases that were included in qPCR analysis. Mean ± SD is provided for age, disease duration and post-mortem pH of CSF.

Supplementary Table 5. Primer sequences and characteristics used for qPCR analysis.

Available under supporting information at https://onlinelibrary.wiley.com/doi/full/10.1111/bpa.12760

Supplementary Table 6. FAS in peripheral lymphocyte populations.

	Fas (protein copy numbers)
Intensity_B.naive_steady-state	0
Intensity_B.memory_steady-state	0,5
Intensity_B.plasma_steady-state	0,2
Intensity_T4.naive_steady-state	0,2
Intensity_T4.CM_steady-state	4,2
Intensity_T4.EM_steady-state	6,6
Intensity_T4.EMRA_steady-state	0,8
Intensity_nTregs_steady-state ($CD4^+$, CD25 low, CD45 RA low, IL7R low)	0,7
Intensity_mTregs_steady-state ($CD4^+$, CD25 high, CD45RA low, IL7R low)	14,9
Intensity_Th1_steady-state	2,1
Intensity_Th2_steady-state	3,9
Intensity_Th17_steady-state	7
Intensity_T8.naive_steady-state	0,1
Intensity_T8.CM_steady-state	4,3
Intensity_T8.EM_steady-state	4
Intensity_T8.EMRA_steady-state	1,7
Intensity_mDC_steady-state	0,6
Intensity_pDC_steady-state	0,3
Intensity_Eosinophil_steady-state	0,2
Intensity_Neutrophil_steady-state	1
Intensity_Basophil_steady-state	0,5
Intensity_MO.classical_steady-state	0,4
Intensity_MO.intermediate_steady-state	1
Intensity_MO.nonclassical_steady-state	0,8
Intensity_NK.bright_steady-state	0,1
Intensity_NK.dim_steady-state	0,6
Intensity_Thrombocyte_steady-state	0
Intensity_Erythrocyte_steady-state	0

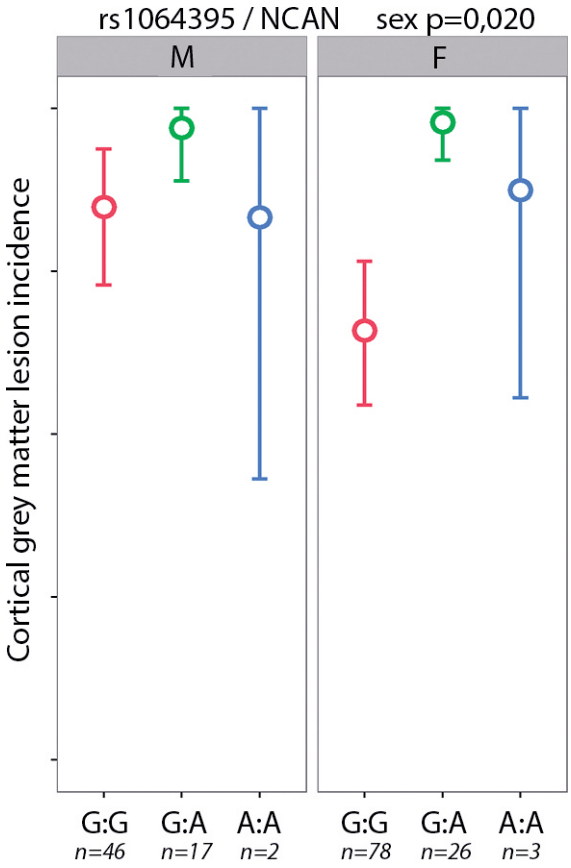
Mass spectrometry data extracted from www.immprot.org.

Supplementary Table 7. Donor and tissue characteristics: cases included in FACS analysis.

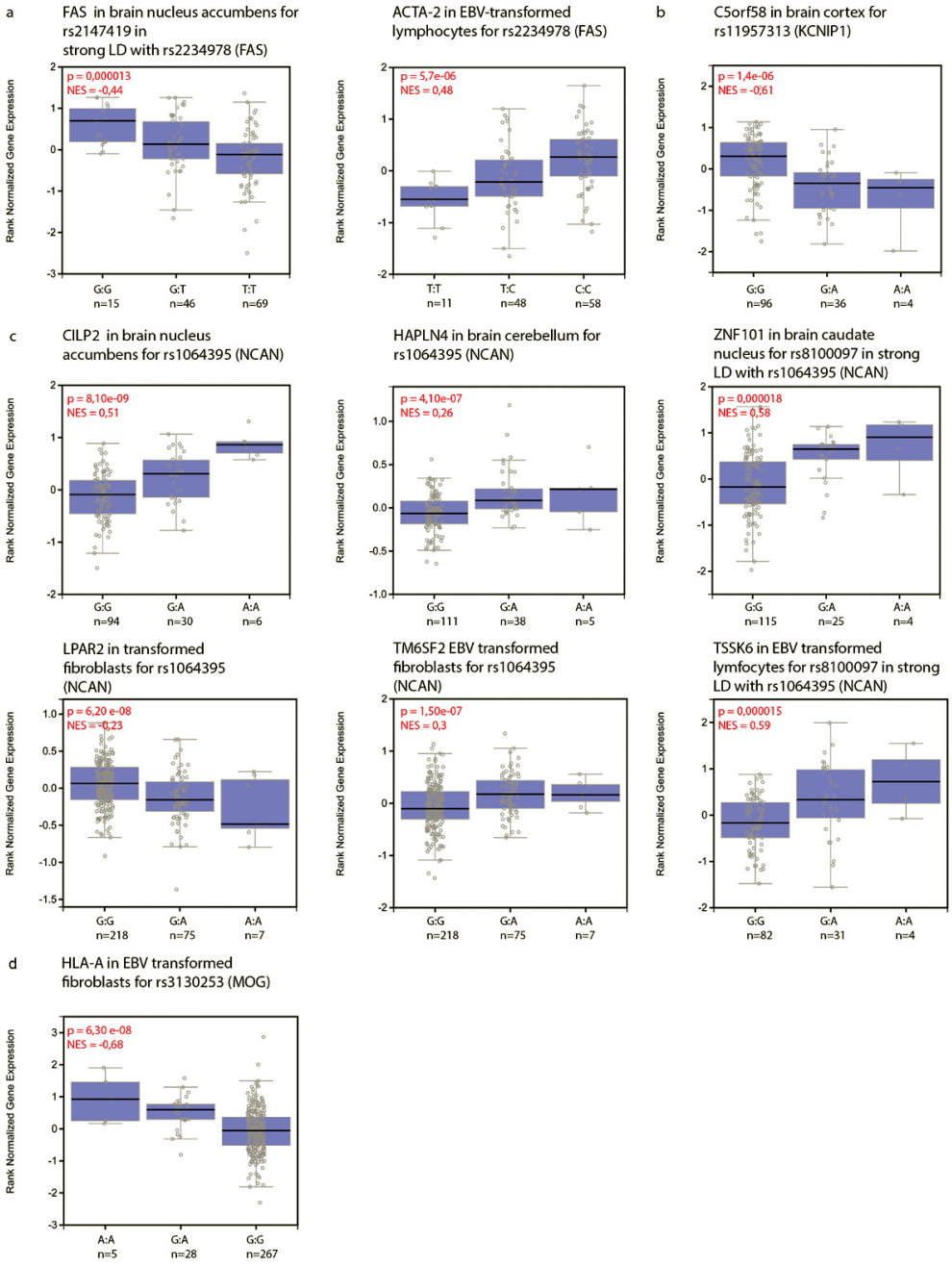
nhb	Tissue type	PA diagnosis	Cause of death	Age (years)	Gender
16-034	brain WM	MSA	Euthanasia	69	F
15-050	brain WM	Parkinson and Alzheimer	Cachexia and delirium	84	F
17-113	brain WM	Alzheimer	Euthanasia	63	M
14-034	pbmc	Lewybody dementia	Atrial fibrillation, Asystole	72	M
14-048	pbmc	Alzheimer	Old age	111	F
14-043	pbmc	Breast cancer	Metastasized mammacarcinoma	60	F

Supplementary Table 8. Results for all statistical tests performed.

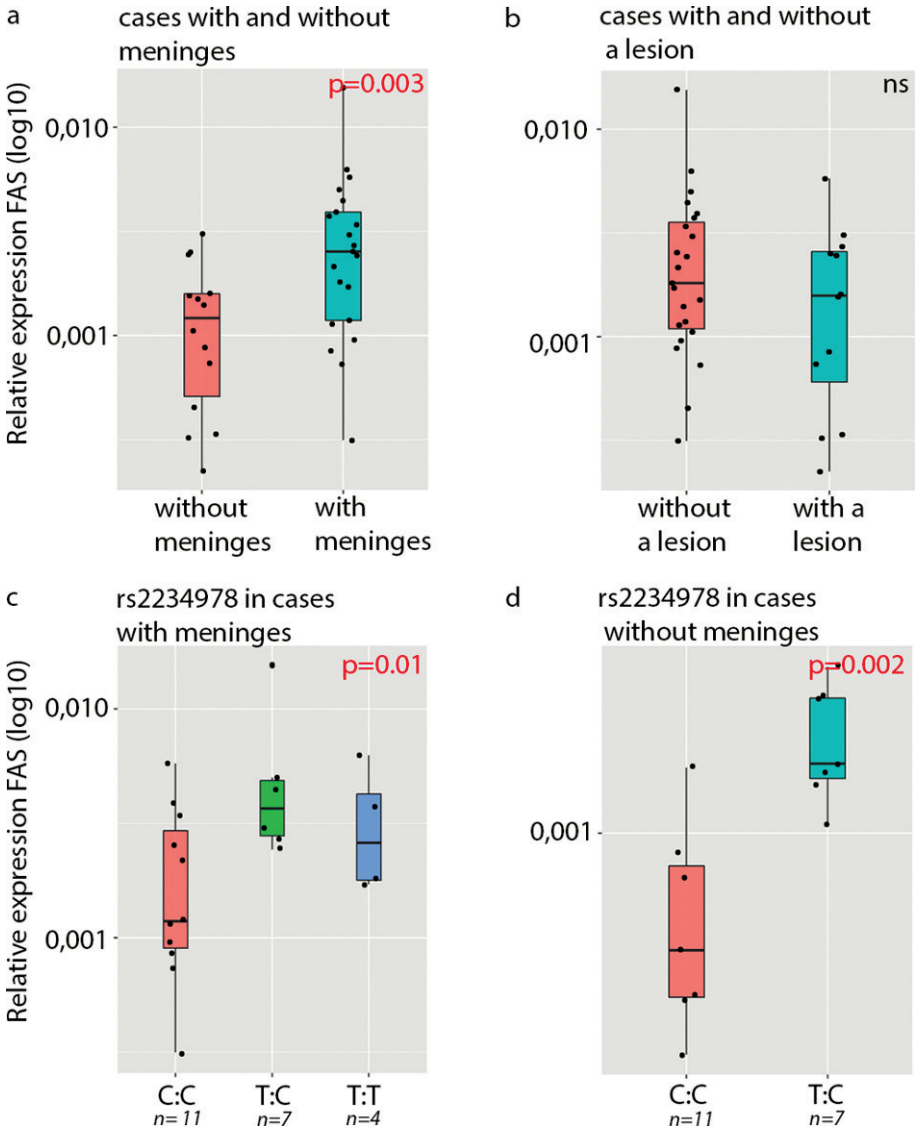
Available under supporting information at https://onlinelibrary.wiley.com/doi/full/10.1111/bpa.12760



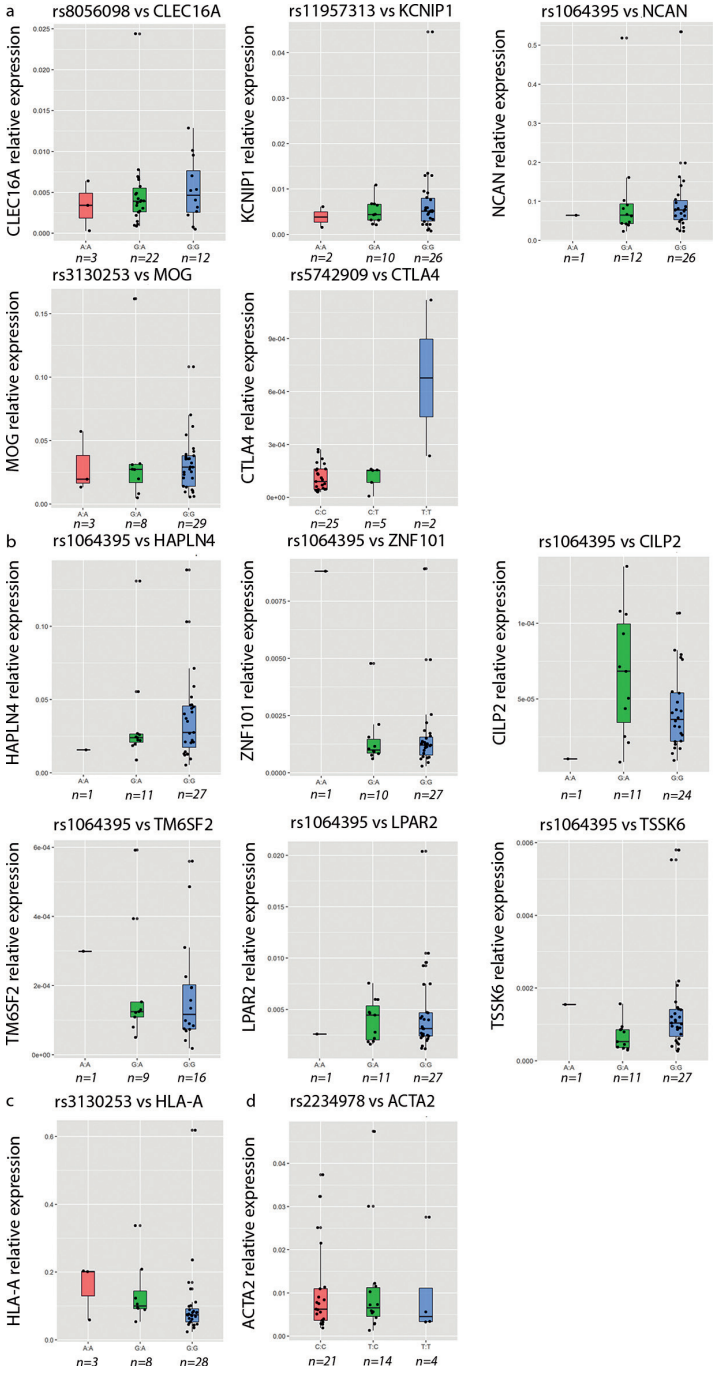
Supplementary Figure 1. rs1064395 in males and females.
Both sex and genotype of rs1064395 have an effect on incidence of cortical grey matter lesions. Males and carriers of the A allele for rs1064395 show a higher incidence of cortical grey matter lesions.



Supplementary Figure 2. Single tissue eQTLs of significant SNPs and their SNPs in strong LD in brain tissues and EBV transformed cells (lymphocytes and fibroblasts).
a: rs2234978/FAS single tissue eQTLs. b: rs11957313/KCNIP1 single tissue eQTL c: rs1064395/NCAN single tissue eQTLs d: rs3130253/MOG single tissue eQTL.

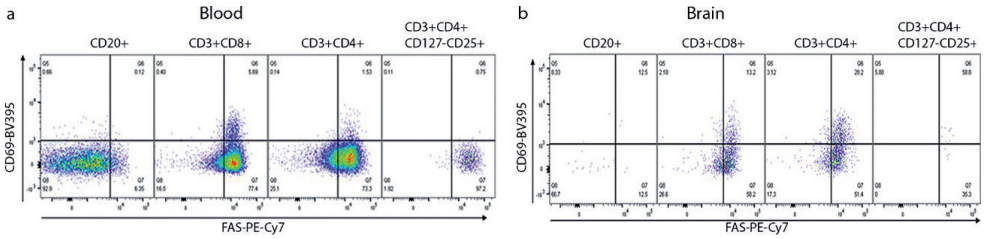


Supplementary Figure 3. rs2234978 and relative gene expression for FAS in subgroups.
a: FAS expression is significantly higher in cases that had meninges in the section (n=22) compared to cases without meninges (n=18). b: there is no difference in FAS expression in cases without (n=25) and with (n=15) an MS lesion in the section. c: rs2234978 T allele is associated with increased FAS expression in cases with meninges in the section. d: rs2234978 T allele is associated with increased FAS expression in cases without meninges in the section.



Supplementary Figure 4. Relative gene expression levels of eQTL genes that were not significantly related to the MS pathology-associated SNPs.

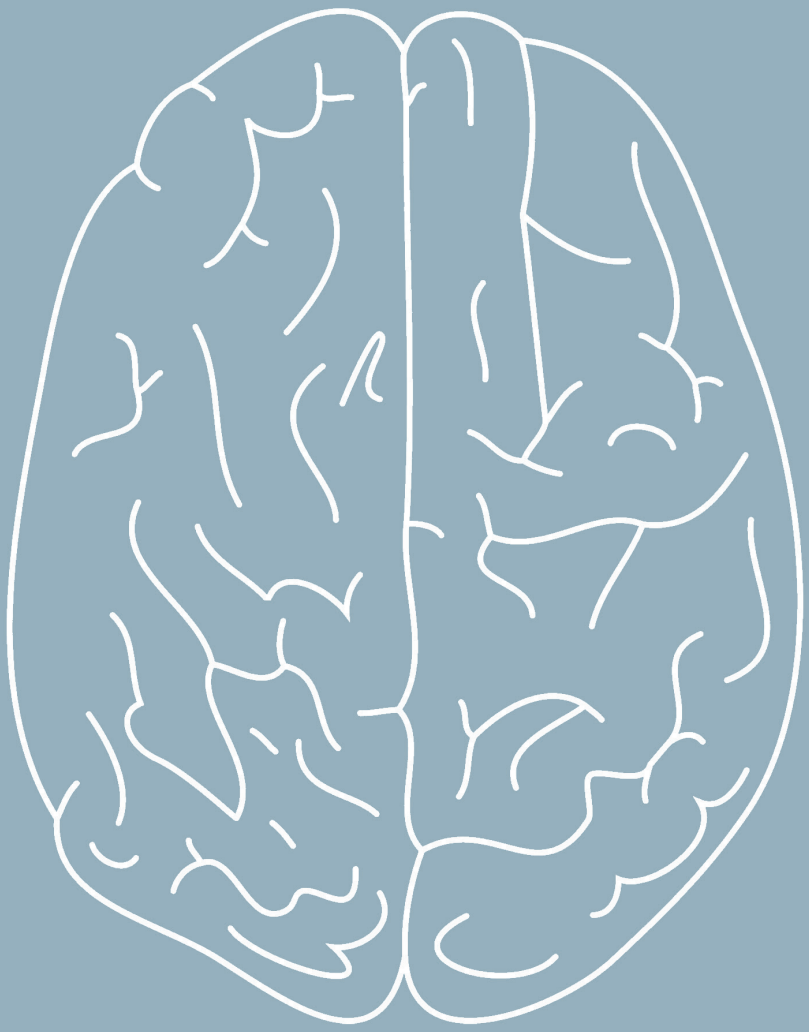
a.Relative expression levels of genes that the SNPs are located in that were not significantlycorrelated with the genotype. b. Relative expression levels of the eQTL gene for rs11957313. c.Relative expression levels of the eQTL genes for rs1064395. d. Relative expression of the eQTLgene for rs3130253. E. Relative expression of the eQTL gene for rs2234978.



Supplementary Figure 5. Flow-cytometric analysis of lymphocytes derived from peripheral blood and brain. a. Gating strategy for flow-cytometric analysis of blood samples. b. Gating strategy for flow-cytometric analysis of brain samples. Only rare events could be recorded in the population enriched for Treg cells ($CD3^+CD4^+CD127^-CD25^+$; n=7/4,604, n=2/425 and n=17/1,957 of $CD4^+$ T cells, respectively), as well as B cells ($CD20^+$; n=24, n=42 and n=501).

CHAPTER 9

Discussion



Part 1 of this chapter is an adjusted version of the review:
Perivascular tissue-resident memory T cells as therapeutic target in multiple sclerosis
Smolders J, **Fransen NL**, Hsiao C-C, Hamann J, Huitinga I

Expert Review of Neurotherapeutics. 2020; 20(8):835-848

PART 1 SUBSTANTIAL INFLAMMATORY LESION ACTIVITY IN ADVANCED MULTIPLE SCLEROSIS

Multiple sclerosis (MS) in an inflammatory, immune-mediated disease of the central nervous system (CNS). This view is supported by a vast body of genetic evidence, pointing towards T- and B-cell interactions as drivers of the disease,[1,2] and has led to the development of disease-modifying treatments (DMTs) that successfully silence relapses and magnetic resonance imaging (MRI) lesions in the early phases of MS.[3] DMTs may be responsible for the, in general, milder disease course noted in contemporary patient cohorts,[4] since relapse rate and MRI lesions in the early phases predict the accumulation of disability in the later phases of MS.[5] Oligoclonal immunoglobulin (Ig)G and high levels of soluble CD27 in the cerebrospinal fluid (CSF) indicate intrathecal adaptive immune activation and predict a more severe disease course.[5–7] Of note, the currently available DMTs fail to have a meaningful impact at late, progressive stages of the disease. Despite brain atrophy and cortical demyelination are the most prominent features of progressive MS pathology,[8] a contribution of ongoing focal white matter inflammation to disability progression in advanced disease has also been suggested.

Here, we discuss post-mortem studies of the natural disease course of MS that point toward the perivascular space (PVS) as a relevant hotspot of immune (re)activation in the context of white matter lesion development in progressive MS. Moreover, we elaborate on potential approaches to target T cells in the PVS for the benefit of people suffering from progressive MS.

1 DISTINCT WHITE MATTER AND LESION PROFILES IN EARLY MS

In the majority of people with MS, the onset of the disease is characterized by sub-acute, temporary exacerbations of clinical symptoms, reflecting focal dysfunction of the CNS.[4] These attacks are caused by inflammatory cells, which invade the CNS and cause focal inflammatory, demyelinated lesions. In the currently leading immunological concept of MS, exacerbations are initiated by presentation of unknown molecular structures by antigen-presenting cells (APCs) to T cells in the lymph nodes.[9] This process leads to T-cell activation and clonal expansion, and to the recruitment of T cells, B cells, and bone marrow-derived circulating monocytes towards the CNS. Waves of circulating, inflammatory cells migrate to the focally inflamed endothelium, cross this specialized endothelium of the blood brain barrier (BBB) at the level of post-capillary venules, and enter the PVS in close association with lesion formation. Here, the T cells are reactivated by resident APCs and move into the parenchyma to contribute to inflammatory, demyelinated lesions. Early in their development, these lesions are characterized by cellular infiltrates consisting of T cells, B cells, activated HLA-positive microglia, and (possibly) infiltrating monocyte-derived macrophages.[10,11] At later stages, these lesions are characterized be a demyelinated, hypocellular sclerotic core with an active rim of myeloid cells (**Figure 1**).[10] This lesion type is known by many names (smoldering, slowly-expanding, chronic active) but currently mostly referred to as mixed active/inactive.

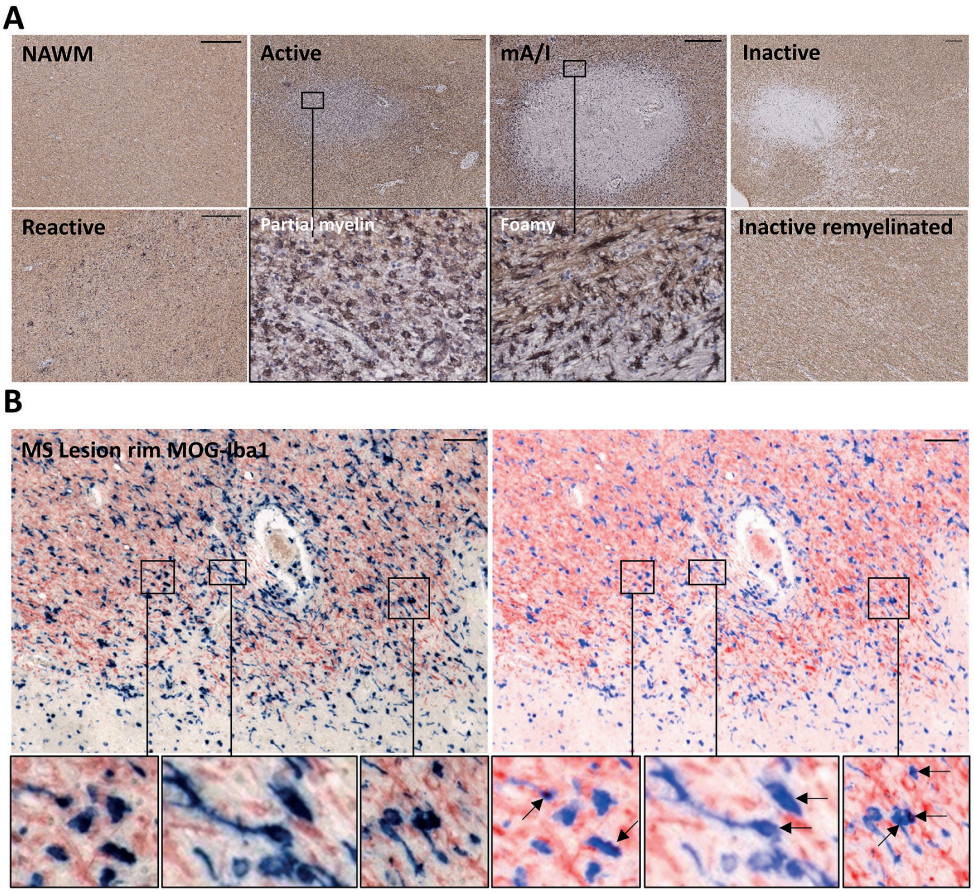


Figure 1. Characterization of postmortem MS white and gray matter lesions.
(A) Immunohistochemical staining of formalin-fixed paraffin-embedded postmortem white matter MS tissue for HLA-DR (black) and PLP (brown). Panels show normal-appearing white matter (NAWM; no demyelination, no infiltration of HLA-DR$^+$ cells), reactive site (no demyelination, infiltration of HLA-DR$^+$ cells), active lesion (demyelination, infiltration of HLA-DR$^+$ cells throughout the lesion), mixed active/inactive lesion (mA/I; demyelination, infiltration of HLA-DR$^+$ cells at the lesion rim), inactive lesion (demyelination, no infiltration of HLA-DR$^+$ cells), and inactive remyelinated/shadow plaque (partial demyelination/loose myelin, no infiltration of HLA-DR$^+$ cells). Bar = 500 µM. (B) Double staining for MOG (red) and Iba-1 (blue), showing the original (left) and optimized (right) figures. The arrows show internalized MOG-positive fragments by Iba-1 positive cells, indicating active myelin uptake. Bar = 50 µM.

Focal disruption of BBB integrity can be visualized in MS patients on MRI scans as gadolinium-enhancing T1 lesions. Current disease-modifying treatments in MS generally or more specifically inhibit critical components of this model, as take place in lymph nodes and circulation (i.e., outside the CNS).[3] For instance, teriflunomide disables clonal expansion of lymphocytes,[12] fingolimod prevents immune cells from leaving the lymph nodes,[13] and natalizumab inhibits attachment of immune cells to the endothelium.[14] Circulating T and B cells are depleted by drugs as cladribine,[15] alemtuzumab,[16] and ocrelizumab.[17] MS treatment with autologous hematopoietic stem cell

transplantation also depends on the depletion of circulating lymphocytes and their precursors in the host.[18] With these treatments, relapses and MRI lesions can be prevented.

The pathological characteristics at the earliest phases of MS have been investigated using diagnostic brain biopsies of patients with MS and autopsy brain material of MS patients who died shortly after disease onset. A point of caution in the interpretation of these studies lies in the fact that only a minor proportion of MS patients receives a diagnostic biopsy, and the representativeness of this sub-group for the pathological profile at onset in the entire MS population is uncertain. Luchinetti et al. characterized white matter lesions in a combined autopsy and biopsy cohort, consisting of patients with a short disease duration.[19] White matter lesions were in all donors characterized by T-cell infiltrates and myelin-containing microglia/macrophages, coinciding with distinct patters of demyelination, IgG accumulation, and activated complement deposition. In a longitudinal study, these different patterns were consistent within donors with multiple subsequent biopsies.[20] This observation led to the proposition of four distinct pathological patterns in early MS.[21] Type I lesions are defined as perivenous, radially expanding lesions, which contain T cells and macrophages, and display degeneration of myelin. Type II lesions are type I lesions with IgG and complement deposition at site of demyelination. In type III lesions, T-cell and macrophage activation coincides with small vessel vasculitis and degeneration of distal oligodendrocytes. Type IV lesions are similar to type III lesions but characterized by oligodendrocyte loss and less by inflammation. Notably, the four pathological patterns in early biopsy samples did not result in differences in clinical disease course. It is at present unclear whether these pathological patterns are also associated with a distinct phenotypic profile of infiltrating T cells. Furthermore, Breij et al. could not distinguish these different patterns in active MS lesions at later disease stages in an autopsy study but rather observed a homogenous pattern of demyelination with complement and IgG deposition in all donors. This suggests that in later phases of MS, ongoing demyelination is mediated by complement and IgG.[22]

2 ONGOING INFLAMMATION IN WHITE MATTER AND DEEP GREY MATTER LESIONS IN END-STAGE MS

In the later phases of MS, exacerbations of the disease are often lacking, and patients may experience a gradual deterioration of neurological symptoms.[4] In patients with longstanding relapsing–remitting MS, gadolinium-enhanced lesions become less prevalent when compared to people early in their disease.[23] In primary progressive MS, gadolinium-enhancing lesions were only found in early phases and markedly declined during 5-years follow-up.[24] These observations indicate that focal BBB leakiness, associated with local trafficking of leukocytes into the white matter and gadolinium-enhancing MRI lesions, is less prevalent at the later, progressive stages of MS. Nevertheless, post-mortem pathological studies showed in progressive MS altered immunostaining profiles, associated with a reduced BBB integrity, which was supported by the observation of fibrinogen-depositions in the adjacent white matter.[25,26] This leakiness apparently differs from the local disruption of the BBB associated with lymphocyte trafficking toward acute

white matter lesions in early, active MS,[27] since gadolinium-enhancing MRI lesions are sparse in advanced MS.

Therefore a possible link between inflammation and neurodegeneration in progressive MS has been largely debated and it has been suggested that neurodegeneration develops independently from inflammation in the later disease stages.[28–32] The analysis of MS brainstem lesions from a small number of progressive MS patients in **Chapter 2** established a link between neurodegenerative changes and innate and adaptive immune response in advanced progressive MS brainstem lesions. In the deep grey matter, parenchymal inflammation (HLA^+ microglia/macrophages) is extensive, supporting a role for inflammation in the pathology of MS lesions during the progressive phase of the disease. We also report substantial lymphocyte infiltration within the brain parenchyma of the brainstem in progressive MS patients. Interestingly, this is in contrast to cortical subpial demyelination where lymphocytes are rarely found to infiltrate the cortical grey matter lesions of progressive MS patients.[33,34] In the cortex, lymphocytes rather aggregate in the meningeal compartment from where they are thought to contribute to pathology by producing cytotoxic mediators that diffuse across the injured glial limitans into the underlying subpial cortex (reviewed in[35]). The difference in the localization of leukocytes and extent of microglia/macrophage activation in cortical versus deep grey matter lesions of progressive MS patients may reflect region-specific differences in the inflammatory response between these two sites.

In addition to lymphocytes, we also report substantial complement activation within the brainstem of progressive MS patients. Early complement activation components (C1q and C3d) are a common feature between MS and classic neurodegenerative diseases such as Alzheimers disease,[36] suggesting a convergence of neurodegenerative processes, although deposition of the terminal complement component MAC may constitute a specific feature of the inflammatory response with pathogenic significance in progressive MS. **Chapter 2** therefore suggests that although gadolinium enhancing MRI lesions are sparse in advanced MS, innate and adaptive immune response within the CNS are related to the ongoing demyelination and neurodegeneration in the brainstem of progressive MS patients in later disease stages.

Furthermore the pathology of white matter lesions in the most advanced end stages of MS has been the focus of extensive autopsy studies performed on post-mortem human MS brain samples. Several groups characterized the presence of inflammatory white matter lesions in MS. We reported in **Chapter 3** that 78% of n=182 MS brain donors of the Netherlands Brainbank (NBB) display active and/or mixed active/inactive white matter lesions at the time of death.[37] Of all white matter lesions studied, mixed active/inactive lesion were most prevalent (33%), followed by inactive lesions (27%) and active lesions (24%). In the NBB collection, shadow plaques, suggestive of remyelinated lesions, were encountered least prevalent (16%).[37] These lesions were significantly enriched in brain donors with a preserved relapsing–remitting disease course at autopsy. This finding is in line with the positive correlation of remyelinated area proportion with disease

duration reported by Patrikios et al. in an autopsy cohort of n=51 MS brain donors.[38] A longer disease duration between diagnosis and autopsy in post-mortem studies is a marker of a less severe disease course.[39] Frisher et al. analyzed samples of n=102 post-mortem MS brain donors of the Vienna and Mayo MS autopsy collections, consisting of both, acute (those died within 1 year after diagnosis) and chronic MS cases, which showed a slightly different distribution of lesion types as compared to the NBB collection. Of all white matter lesions studied, active plaques were most prevalent (35%), followed by inactive lesions (35%), mixed active/inactive lesions (15%), and shadow plaques (14%).[40] Where active lesions dominated the pathology in donors with a short MS duration, mixed active/inactive lesions were most prevalent in donors with a longer disease duration and a progressive disease course. Mixed active/inactive lesions can be considered as ongoing demyelinating or post-demyelinated lesions, based on the presence of myelin degradation products inside the microglia/macrophages.[10,37] The inverse correlation between remyelinating and mixed active/inactive lesions observed in NBB donors suggests that these lesion types may reflect two fundamentally distinct fates of active MS white matter lesion progression.[37] It remains to be consolidated whether ongoing active demyelination in the rim hampers remyelination or processes underlying remyelination suppress lesion activity.

It can be concluded that inflammatory disease activity in white matter lesions is still prevalent in advanced MS. The relative contribution of these active and mixed active/inactive lesions to clinical disability progression in MS can be debated. Many studies point towards cortical demyelination as a critical pathological process in progressive MS. Although cortical demyelination is already present early in MS,[41] it is far more extensive in progressive MS.[42] In primary progressive MS, Choi et al. reported a proportionally larger cortical area to be demyelinated, when compared to white matter.[43] Active cortical demyelination has been associated with the formation of follicle-like inflammatory structures in the overlying meninges.[43–45] These structures contain T-cell, B-cell, and plasma cell zones, which resembles tertiary lymphoid structures.[45,46] The presence of these follicle-like structures correlated with a more severe disease course, characterized by earlier onset of disease, faster accumulation of disability, and earlier death.[43,45] Progressive MS is also characterized by more diffuse instead of focal changes in the normal-appearing white matter.[42] However, the persisting relevance of focal white matter lesions in advances progressive MS is supported by the association of pathological findings with clinical characteristics. In the NBB tissue collection, donors with a high percentage of mixed active/inactive lesions showed a shorter time between first symptoms and walking with a stick or being wheelchair-bound and also displayed a shorter total disease duration.[37] Additionally, several prognostic factors associated with a faster accrual of disability during life were also associated with a higher proportion of active and mixed active/inactive lesions. Male MS brain donors showed a higher percentage of mixed active/inactive lesions in both the NBB and Vienna/Mayo cohorts.[37,40] MS brain donors with a progressive disease course showed a higher lesion load and a higher percentage of mixed active/inactive lesions when compared to donors without progressive disease. A similar association of mixed active/inactive lesions with progressive disease was observed in the Vienna/Mayo cohort.[40] Furthermore in **Chapter 8** we show genetic polymorphisms, which have been associated with a more detrimental

disease course of MS during life, also correlated with a higher proportion of either active or mixed active/inactive lesions. These include single nucleotide polymorphisms (SNPs) within genes, such as Fas, Kv channel-interacting *protein*-1 (KCN1P1), and C-type lectin domain-containing 16A (CLEC16A).[47]

The association of the inflammatory lesion activity in the mixed active/inactive lesion rim with disability progression and prognostic markers of disability progression supports its relevance for the disease process of MS. These observations corroborate the idea that mixed active/inactive white matter lesions are a relevant contributor to progressive MS.[40,48] Therefore, targeting this inflammatory response could be of therapeutic benefit for people with advanced MS. Acknowledging the clinical and pathological differences between early and end-stage MS can provide insight into the fundamentally different efficacy of current DMTs in modulating meaningful clinical endpoints. With the absence of gadolinium-enhancing lesions on MRI scans, suggesting absence of extensive local trafficking of infiltrating leukocytes into the PVS at sites of lesion formation in advanced MS, the role of lymphocytes also likely changes with the course of disease. We will focus on the role of T cells in advanced MS, as investigated recently in post-mortem human autopsy studies.

3 T-CELL PRESENCE IN NON-INFLAMED BRAIN WHITE MATTER

In the absence of inflammatory conditions, low numbers of T cells can be observed in post-mortem human white matter, as we show in **Chapter 4** (**Figure 2**).[49–52] Although substantial variation exists, $CD8^+$ T cells in general outnumber $CD4^+$ T cells.[49–52] Approximately three T cells/mm2 could be encountered in white matter of donors without brain diseases.[50] Under non-inflammatory conditions, most T cells in white matter are found in close association with the extra-luminal side of blood vessels.[49] Laminin staining revealed that the majority of T cells is located in the PVS,[50] and that T cells only occasionally exist in the parenchyma (**Figure 2**).

Brain white matter T cells show a phenotypic profile consistent with tissue resident-memory T (T_{RM}) cells. In contrast to central memory and effector memory T cells (T_{CM} and T_{EM} cells, respectively), T_{RM} cells arise locally in a multitude of tissues after a primary infection and have the cardinal hallmark that they do not recirculate.[53] In skin, lung, gut, and vagina, among other barrier tissues, T_{RM} cells are believed to serve as sentinels to mount a swift immune response after re-exposure to their antigen. [53,55] They are characterized by a core transcriptional and phenotypic profile, of which expression of CD69 and CD103 are important markers, among many others.[56] We optimized our approach to isolate viable primary human microglia from post-mortem rapid autopsy-acquired brain tissue for the isolation of viable brain T cells.[57] The clear phenotypic differences between T cells isolated from post-mortem rapid autopsy-acquired blood samples and brain tissue supported the applicability of this approach to study brain T-cell phenotypes (**Figure 3**).[50,58] Viable brain white matter T cells displayed a profile of surface markers and transcription factors resembling T_{RM} cells.[49,50] They express markers of memory cells (CD44, CD45RO, CD127), lack receptors for

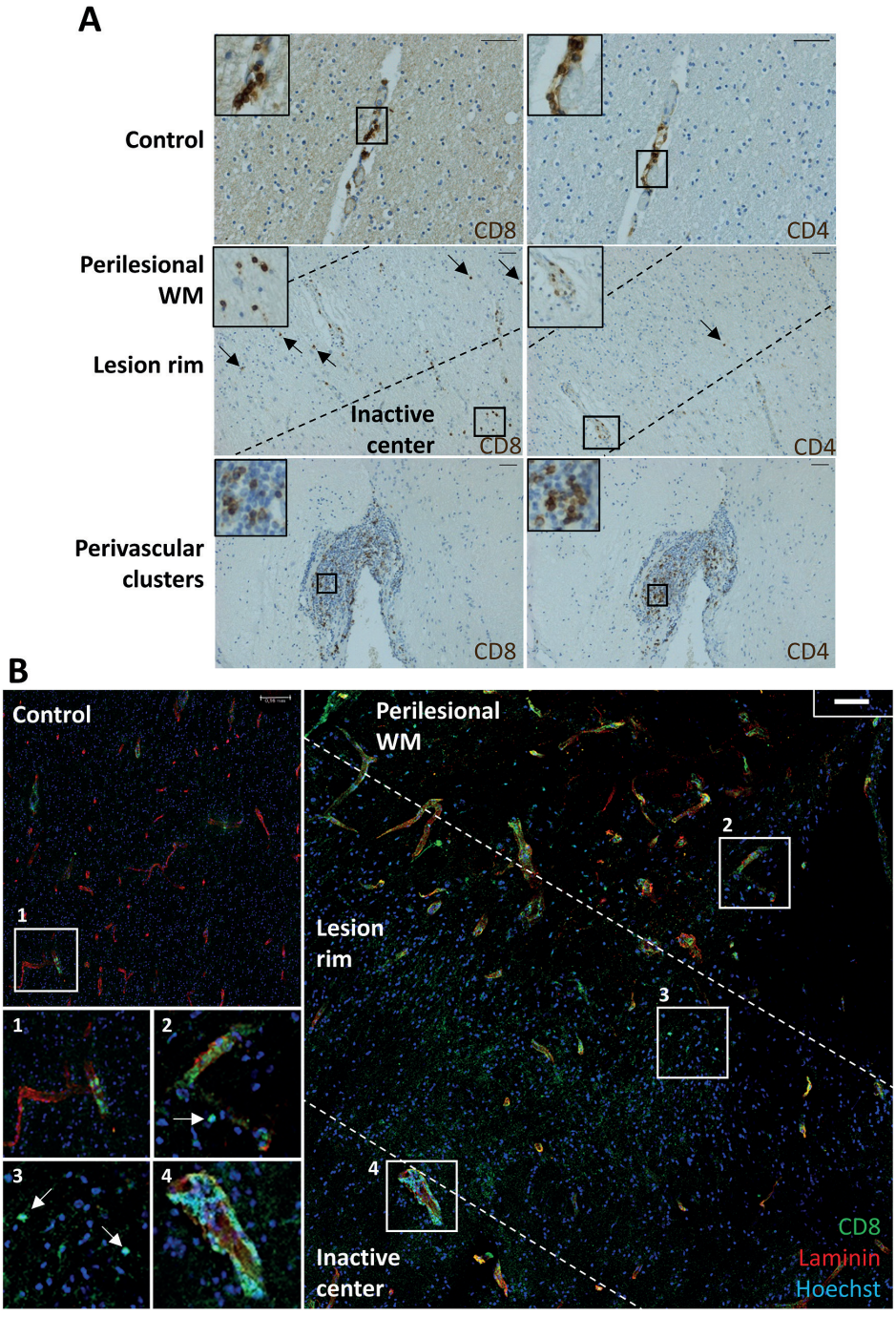


Figure 2. T-cell distribution in post-mortem white matter control and MS tissue.
(A) Staining for CD8 and CD4, showing perivascular distribution in control white matter, parenchymal localization of some $CD8^+$ and $CD4^+$ T cells in the rim of active lesions, and presence of both $CD8^+$ and $CD4^+$ T cells in perivascular MS clusters. (B) Double staining for CD8 (green) and laminin (red), confirming predominant localization of $CD8^+$ T cells in the PVS of a control donor. In a mixed active/inactive white matter lesion, $CD8^+$ T cells are observed in the parenchyma of the active rim and surrounding perilesional white matter. Bar = 50 µM.

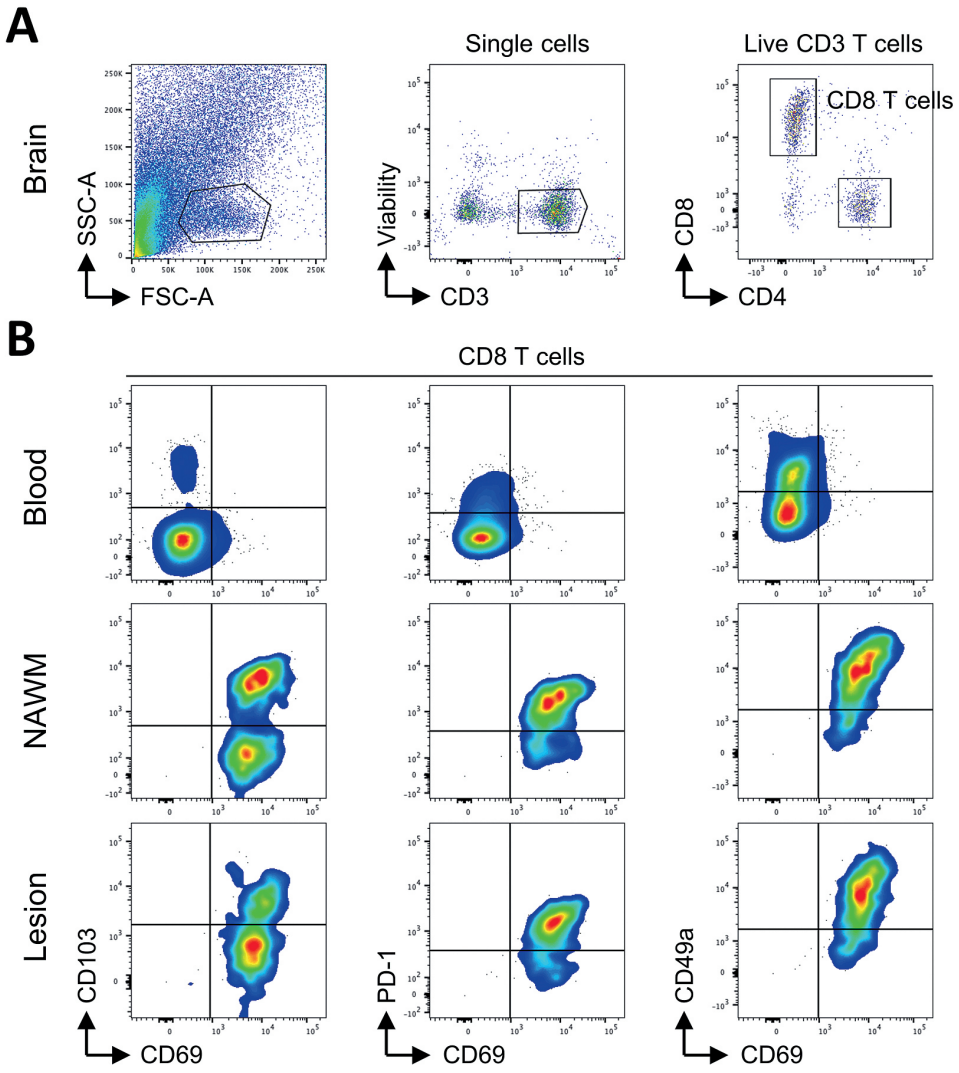


Figure 3. Flow-cytometric analysis of brain CD8$^+$ T_{RM} cells.
(A) Gating procedure based on forward scatter (FSC), side scatter (SSC), viability, and expression of CD3, CD4, and CD8. (B) Expression of T_{RM} cell-associated surface markers on CD8$^+$ T cells isolated from blood, MS normal-appearing white matter (NAWM), and an active MS lesion.[50,75]

lymph node homing (CCR7), and expose molecules associated with tissue residency (CD49A, CD69, CD103, CTLA-4, PD-1). Functionally, post-mortem human brain T_{RM} cells produced low levels of granzyme B but detectable amounts of granzyme K directly *ex situ* and made lots of interferon (IFN)γ and tumor necrosis factor (TNF) upon reactivation *in vitro*. Since T_{RM}-cell populations in other tissues have been described to arise after exposure to a wide range of viral or bacterial antigens,[53] we reasoned the common human brain T_{RM} cells to be most likely directed against

common neurotropic viruses. In experimental models of neurotropic virus infections, populations of specific T_{RM} cells are generated.[59–63] The dominant localization of human brain T_{RM} cells inside the PVS is a marked difference compared to the distribution of $CD8^+$ T_{RM} cells in other tissues, where these cells are scattered through the tissue. This tissue organization of T_{RM} cells in the human brain outside the context of acute neurotropic virus infection may be attributable to the unique characteristics of the PVS. Notably, although the PVS is a continuum with the subarachnoid space, the phenotype of T cells isolated from these compartments show substantial differences. In CSF acquired by lumbar puncture, $CD4^+$ T cells are more prevalent than $CD8^+$ T cells and show a contrasting T_{CM}-cell phenotype, including expression of CCR7.[64,65] The small population of CSF $CD8^+$ T cells was also reported to display a T_{CM}-cell phenotype.[65] These CSF T-cell populations have been argued to enter the CSF via the choroid plexus and meningeal vessels.[27] How and whether these CSF T-cell population relate to the development and maintenance of white matter PVS T_{RM}-cell populations is not known. Additionally, whether T cells in meninges and choroid plexus also display a T_{RM}-cell profile has to our knowledge not been extensively studied.

4 THE PVS AS A PHYSIOLOGICAL T_{RM}-CELL NICHE IN WHITE MATTER

The PVS is the only compartment in the human body, which is bordered by two basal membranes, an endothelial and a parenchymal basement membrane (EBM and PBM), respectively.[66] These basement membranes are made of extracellular matrix molecules, including laminin, fibronectin, and collagen type IV.[67] On the luminal side, specialized endothelium with tight junction covers the EBM to form the BBB. On the parenchymal side, astrocyte end-feet form the glia limitans, covering the PBM. The glia limitans forms with astrocytic tight junctions a secluded barrier between the PVS and the parenchyma.[68] The PVS plays a crucial role in the drainage of the suggested CNS flow of interstitial fluid,[69] which removes waste products from the parenchyma. Therefore, the PVS could be an excellent hub to screen for antigens. The PVS is populated by a variety of APCs, including specialized perivascular macrophages.[70] Although disputed, the presence of perivascular macrophages has been reported in the PVS of white matter venules.[71] The compartmentalization of T_{RM} cells in the PVS could be mediated by their signature surface markers. CD69 interferes with sphingosine 1-phospate receptor 1 (S1P1) to prevent tissue egress, CD49a is a receptor for collagen type IV, and the T_{RM}-associated molecule CD44 is a receptor for laminin.[72] Finally, a ligand for CD103 is E-cadherin, which has been described on activated $CD103^+$ lymphocytes, enabling cluster formation.[66,72] Interaction of these receptors with their ligands may mediate the homing and clustering of brain T_{RM} cells in the PVS. We assume them being under tight control by surrounding signals in the PVS, while awaiting potential reactivation. An interesting candidate for providing this local control of reactivation is the perivascular macrophage. The perivascular macrophage can present antigens yet can also express the inhibitory cytotoxic T lymphocyte-associated protein 4 (CTLA-4) ligand CD86, which may prevent activation of brain T_{RM} cells.[73] Moreover, activated astrocytes may present the inhibitory programmed death-1 (PD-1) ligand PD-L1 to the T_{RM} cells via their end-feet at the glia limitans.[50]

5 T CELLS IN MS NORMAL-APPEARING WHITE MATTER AND WHITE MATTER LESIONS

In post-mortem MS brain normal-appearing white matter, T cells are enriched as we show in **Chapter 5**.[74] On average 2-6 times as many $CD3^+$ T lymphocytes were encountered in MS normal-appearing white matter when compared to control white matter.[40,43,75] Like in control donors, these were more $CD8^+$ than $CD4^+$ T cells, and they were almost exclusively localized in the PVS (**Figure 2**). Perivascular cuffs of large clusters of lymphocytes, including T cells and B cells, are a known feature of neuroinflammatory disease, including MS.[76] Perivenular infiltrates, believed to contain infiltrating lymphocytes from the circulation, have been identified in white matter of both acute and chronic MS cases.[77,78] Despite advanced progressive MS not being associated with relapses or gadolinium-enhancing MRI lesions, perivascular cuffs were observed in some autopsy cohorts with advanced progressive MS.[42,75] Frischer et al. observed perivascular cuffs only in cases with active progressive disease.[79] In the NBB tissue collection, donors with perivascular cuffs in the brainstem had a higher brain stem lesion load and an overall higher proportion of mixed active/inactive lesions.[75] These observations suggest that presence of perivascular cuffing can be regarded as a detrimental phenomenon in advanced progressive MS, a clinical phenotypic entity not associated with attacks of infiltrating lymphocytes from the circulation.

The association of T cells with different white matter lesion types has been quantified both in the NBB and Vienna/ Mayo post-mortem MS-tissue collections, which show a comparable profile.[75,79] When compared to normal-appearing white matter, active MS lesions showed the most pronounced enrichment of T cells, followed by the mixed active/inactive lesion. Interestingly, there was no enrichment of T cells in inactive lesions. This enrichment comprised both $CD4^+$ and $CD8^+$ T cells, in which $CD8^+$ T cells were most prevalent.[51,80–82] Interestingly, the ratio of CD8/CD4 T cells was remarkably consistent within a donor between regions investigated.[75] Where brain T cells were located almost exclusively in the PVS in normal-appearing white matter, they infiltrated the parenchyma in both active and mixed active/inactive lesions (**Figure 2**).[51,75] This was, however, not the case in inactive lesions. Altogether, these observation show that white matter lesion activity is associated with both T-cell number and distribution. Besides association with inflammatory lesions, a positive correlation between $CD8^+$ T cells and APP-positive axons as marker of axonal damage has been reported.[79,83,84] This was not only the case in relapsing and secondary progressive cases but also in primary progressive cases.[79]

6 MS WHITE MATTER LESIONAL T CELLS HAVE A T_{RM}-CELL PROFILE

The phenotypic characteristics of T cells in MS white matter lesions in advanced MS have been analyzed by immunohistochemistry and by flow cytometry after rapid post-mortem autopsies.[49–51,85] Several studies support a phenotypic profile consistent with T_{RM} cells, although there are several contrasting observations. Lesional T cells stained in autopsy cases lacked the

T_{EM} cell-associated recirculation marker S1P1.[51,75] Machado-Santos et al. reported absence of the lymph node homing receptor CCR7 on lesional T cells.[51] CD69 expression has been described by van Nierop et al. using immunostaining[85] and was confirmed by our group on all lesional T cells using flow cytometry,[75] yet was not found with immunohistochemistry by Machado-Santos et al.[51] Immunostaining for CD103 was not observed on lesional T cells by van Nierop et al. but has been reported by Machado-Santos et al. and our group.[51,75,85] In our flow-cytometric studies, a sub-population of lesional T_{RM} cells expressed CD103.[75] Whether these inconsistencies between studies are contributable to technical issues or donor and tissue selection remains to be clarified. Expression of the T_{RM}-cell-associated markers CD49a and PD-1 has been observed in lesional T cells with immunohistochemistry and flow cytometry.[51,75] Among the chemokine receptors expressed by these cells were CCR5, CXCR3, and CXCR6, which are all T_{RM}-cell phenotypic markers, possibly mediating homing into the parenchyma.[51,75,86] Previously, we showed the ligand for CXCR6, CCL16, to be upregulated by macrophages in the rim of mixed active/inactive lesions.[87] In the mouse experimental autoimmune encephalomyelitis model of neuroinflammation, the CCR5 and CXCR3 ligands CCL5 (RANTES), CXCL9, CXCL10, CXCL11, and CXCL12 were also expressed by resident macrophages.[88] Importantly, we were unable to identify clusters of cells lacking T_{RM}-cell characteristics among $CD8^+$ T-cell fractions isolated from MS white matter lesions.[75] When summarizing these characteristics, identification of white matter lesional T cells in autopsy tissue as T_{RM} cells appears valid. Recently, Bell et al. stained in n=33 MS autopsy samples meningeal follicle-like structures for T cell-phenotypic markers.[89] Besides variable fractions of $CXCR5^+$ T-follicular helper cells and $CD27^+$ $CD8^-$ memory T cells, they observed meningeal follicle-like structures to be populated by $CD69^+$ $CD4^+$ T_{RM}-like cells. Further characterization of these T cells should reveal whether they also express other markers consistent with a T_{RM}-cell phenotype, and if and how they contribute to the cortical pathology of MS.

An important question is whether white matter T_{RM} cells are contributing to inflammation and demyelination in MS white matter lesions. In other tissues, re-encounter of T_{RM} cells with their antigen results in robust proliferation, cytokine release, and production of lytic enzymes.[55] With immunohistochemistry, Machado-Santos et al. observed low proportions of cells positive for the proliferation marker proliferating nuclear antigen (PCNA) in relapsing–remitting (median 1.45%) and progressive (median 0.5%) MS cases.[51] With flow cytometry, Ki-67 expression was higher in $CD8^+$ T_{RM} cells isolated from MS lesions, compared to control white matter.[75] Immunohistochemical staining for Ki-67 revealed positive cells in the perivascular cuff in active lesions. These findings suggest antigen presentation and proliferation of T_{RM} cells in the context of mixed active/inactive lesion formation, but its extent is uncertain. An important site of this reactivation could be perivascular cuffs, where CD103-positive T cells were observed in close association with HLA-DR positive cells.[75] These HLA-DR-positive cells double stained both with CD20 (B cells) and CD163 (perivascular macrophages). An increased number of $CD163^+$ $HLA\text{-}DR^+$ perivascular macrophages has been reported in active MS white matter lesions, in close association with perivascular T cells.[73,90] B cells have a well-characterized capacity of antigen uptake and MHC-dependent presentation,[91] and could hereby serve an important role in the reactivation of brain T_{RM} cells.

The effector functions of white matter lesional T cells are uncertain. Although an increased rate of parenchymal infiltration suggests cellular cytotoxicity of small numbers of lesional $CD8^+$ T cells towards other parenchymal cells,[75] diffusion of soluble molecules produced by the proportionally larger fraction of perivascular activated $CD8^+$ T cells has been proposed by Machado-Santos et al. as an effector mechanism.[51] Mixed results have been published on the role of granzyme B as lytic mediator in white matter lesions. Van Nierop et al. quantified immunohistochemical stainings for granzyme B in mixed active/inactive white matter lesions and reported perivascular and parenchymal T cells to express granzyme B.[85] The majority of cells displayed a punctate pattern of granzyme B immunostaining, with a fraction of cells showing evidence of granzyme B polarization. Machado-Santos et al. found with immunostainings a median average of 4.2% (range 0-30%) of $CD8^+$ T cells to express granzyme B, while this was in chronic MS cases only observed in 1.7% (range 0-27%).[51] Salou et al., reported infiltration of granzyme B-positive $CD8^+$ T cells in white matter lesions, but showed no quantification.[92] Applying immunohistochemistry, we observed in active MS lesions a very low median number of 0.017 (IQR 0.012-0.026) granzyme B-positive cells/mm2.[75] Additionally, flow-cytometric analysis of isolated $CD8^+$ T_{RM} cells showed no enrichment for granzyme B in white matter MS lesions, compared to normal-appearing white matter and control donors. These inconsistencies between studies warrant further investigation.

We showed lesional $CD8^+$ T_{RM} cells to upregulate the adhesion family G protein-coupled receptor GPR56,[75] which on circulating lymphocytes indicates cytotoxic capacity.[93] It is uncertain whether non-circulating GPR56-positive $CD8^+$ T_{RM} cells bear cytolytic activity in the PVS and parenchyma. Human brain $CD8^+$ T cells expressed in our studies almost no *perforin*,[49,50] although this lytic mediator is important in the control of neurotropic virus infections by brain T_{RM} cells in animal models.[59,60] *Perforin* and granzymes synergize to mediate apoptosis of target cells. Notably, Magliozzi et al. reported immunostaining of meningeal $CD8^+$ T cells for granzyme B, perforin, and the degranulation marker CD107 in association with Ig-positive cells in n=5 MS cases.[94] Konjevic Sabolek et al. reported immunostaining for perforin in white matter lesional $CD8^+$ T cells of several cases with acute but also progressive MS.[95] Brain $CD4^+$ and $CD8^+$ T_{RM} cells did express granzyme K in our earlier studies.[49,50] Expression of granzyme K by lesional T cells remains to be shown, but a possible relevance of this lytic mediator is suggested by the expanded fraction of granzyme K-positive $CCR5^+$ $CD4^+$ T cells in the circulation of MS patients.[96] In sum, conflicting evidence exists regarding lytic molecule production by white matter $CD8^+$ T cells. Interestingly, Van Nierop et al. showed white matter lesion $CD8^+$ T cells to express high levels of Fas ligand (FasL, CD95L), which may lead to Fas (CD95)-mediated target cell lysis.[85] Furthermore, production of cytokines is well possible, since human brain $CD4^+$ and $CD8^+$ T_{RM} cells rapidly make IFNγ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF upon stimulation *in vitro*.[50] Production of IFNγ by brain T_{RM} cells is a critical component in the control of neurotropic virus infections.[60] However, cytokine production by white matter lesional T cells *in situ* has not yet been investigated.

7 WHITE MATTER T_{RM} CELLS AS POTENTIAL TARGETS FOR MS THERAPIES

The events leading to the establishment of T_{RM} cell-containing perivascular cuffs in MS are not known. These populations of T_{RM} cells most likely evolve from the circulating populations of T cells invading the perivascular space in early MS, and can possibly already be established at the earliest phases of MS.[9] Notably, the study by Machado-Santos et al. included also some donors with a fairly short disease duration,[51] suggesting that population of the PVS by T_{RM} cells could be starting at an early stage of MS. We observed CD103-positive T cells in infiltrates of early MS biopsies, albeit relatively less frequently when compared to autopsy material of advanced MS cases.[75] Since a high relapse rate and gadolinium-enhancing lesions are risk factors for developing progressive disease,[5] a timely intervention on these endpoints could potentially reduce T_{RM}-cell formation in the course of MS and hereby their possible contribution to progressive disease. This is also suggested by the lower point estimate of secondary progressive MS in the DMT era,[4] and the efficacy of early treatment with ocrelizumab in delaying disability progression in primary progressive MS.[97] Therefore, early treatment with DMTs could theoretically prevent the establishment or maintenance of perivascular T_{RM}-cell cuffs in the course of MS. It is unlikely that current DMTs affect the mobilization of perivascular T_{RM} cells from the PVS into the parenchyma in progressive MS. Regarding the limited penetrance of these compounds through the BBB, their effects on events in the PVS and parenchyma are presumably limited. Although fingolimod reaches the PVS,[98] the absence of S1P1-receptor expression on T_{RM} cells[51,75] and the migration of parenchyma-invading lymphocytes away from the sphingosine phosphate gradient[99] makes a relevant functional interference of this drug with T_{RM} cell-migratory behavior unlikely. Metz et al. observed only very few $CD8^+$ T cells in the post-mortem CNS of patients treated with autologous hematopoietic stem cell transplantation, suggesting at least some depletion by this treatment regimen.[100] Of note, besides a major role for local homeostatic proliferation, recruitment of memory T cells from the circulation to contribute to secondary T_{RM} cells has been described.[55,101] Via interference with this replenishment, DMTs could potentially reduce the sustainability of the PVS T_{RM}-cell pool. Since reactivation, proliferation, and mobilization of brain T_{RM} cells can be a critical component in the maintenance of active and mixed active/inactive lesion in progressive MS, drugs interfering with these processes could be of benefit for patients with progressive disease. We just start to learn the exact phenotype of these cells and identify potential markers, which could be therapeutic targets.

In recent years, the therapeutic arsenal for T cells has been expanded by drugs that target molecules involved in activation, inhibition, and migration. In oncology, a major development has been the use of immune checkpoint inhibitors to enhance cytotoxicity. Several lines of evidence suggest that drugs that target the PD-1–PDL-1 and the CD28–CTLA-4 pathway also modulate the behavior of T cells in the CNS. The development of inflammatory CNS lesions as side effect is part of this evidence. Treatment with the CTLA-4 inhibitor ipilimumab has been associated with the

occurrence of inflammatory demyelinated white matter lesions with T-cell infiltrates.[102–104] During treatment with the PD-1 inhibitor nivolumab, white matter T-cell infiltration with demyelination and macrophage activation has been described.[105] Also treatment with the PD-1 inhibitor pembrolizumab resulted in inflammatory demyelinating lesion of the CNS.[106] Potentially beneficial activation of CNS T cells has also been described. In a proportion of patients with a progressive multifocal leukoencephalopathy (PML) due to reactivation of the JC polyomavirus, treatment with pembrolizumab boosted JC-specific T-cell responses together with a down-regulation of PD-1.[107] Additionally, despite not modulating total tumor-infiltrating cells quantitatively, treatment with neo-adjuvant pembrolizumab therapy resulted in potentially beneficial T-cell phenotypic changes in patients with recurrent glioblastoma.[108] Small molecules and viral vector-induced ligands boosting rather than inhibiting check points could reach and modulate T cells within the PVS in a beneficial way to suppress their reactivation in the CNS.

Several chemokine receptors, which are highly expressed by human brain T_{RM} cells and are part of their core phenotypic profile, have been targeted in the context of inflammatory diseases. $CD103^+$ T_{RM} cells highly express CCR5,[50] which can also act as a receptor for infection of $CD4^+$ T cells by the R5-tropic human immunodeficiency virus (HIV). Maviroc is a drug, which blocks the CCR5 receptor and hereby prevents the virus from infecting T cells. In patients suffering from an immune reconstitution inflammatory syndrome (IRIS) after cessation of natalizumab due to PML, some case reports suggest an attenuation of the detrimental influx of inflammatory T cells in the CNS.[109–111] Small-molecule inhibitors for the CXCR6–CXCL16 pathway could potentially attenuate migration of reactivated T_{RM} cells into the parenchyma. Antibodies directed against CXCL6 are available but may not reach the PVS and lesions.[112] CXCR3 is a core phenotypic T_{RM}-cell marker, which is also expressed at high levels on brain T_{RM} cells.[50,56] In the murine skin, lack of CXCR3 expression in $CD8^+$ T cells was associated with a reduced T_{RM}-cell formation.[113] In an adoptive transfer but not an actively immunized experimental autoimmune encephalomyelitis model, treatment with anti-CXCR3 inhibited T-cell infiltration into the CNS and reduced disease severity.[114] CXCR3 has several ligands; CXCL4 is expressed by microglia,[115] and CXCL9, CXCL10, and CXCL11 have been associated with the infiltration of the CNS by T cells in various inflammatory diseases including MS.[116] Targeting these chemokine receptors or their ligands with small molecules could hypothetically be of benefit for progressive MS.

8 CHALLENGES FOR THE UPCOMING YEARS

Recent post-mortem neuropathological studies made a case for mixed active/ inactive lesions, fueled by reactivation of populations of T_{RM} cells in the PVS, as contributors to the disease process in advanced/progressive MS (**Figure 4**). The identification of T_{RM}-cell recruitment from the PVS offers possibilities to better understand the role of T cells in advanced MS, but also to develop new approaches to target the contribution of these cells in the disease process of progressive MS. There are however several questions that do still require clarification.

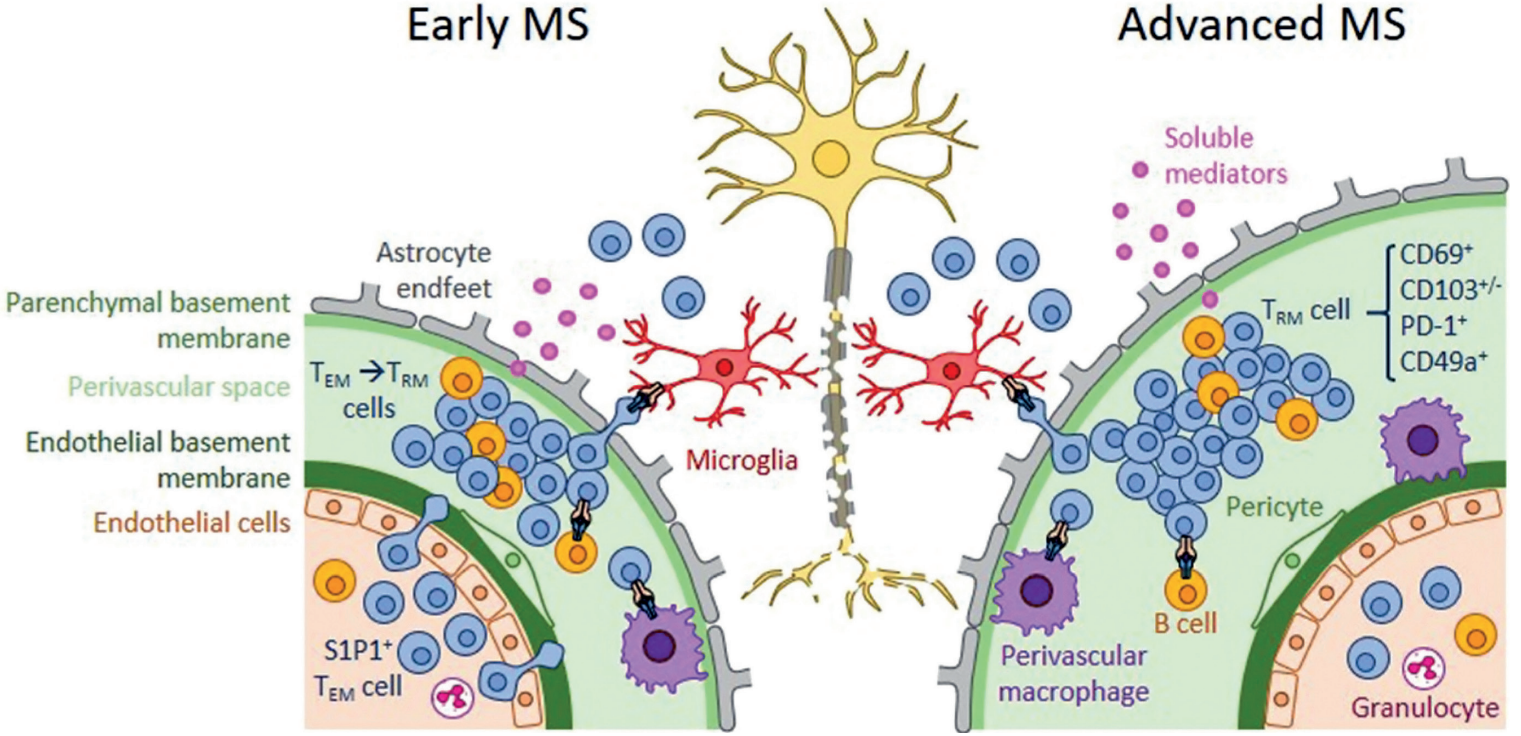


Figure 4. Concept of compartmentalized immune activation in advanced MS white matter lesions.
In early MS, shown to the left, activated T_{EM} cells and effector T cells cross the endothelium of the blood brain barrier at the postcapillary venules and enter the perivascular space (PVS), forming perivascular infiltrates in acute lesions. These infiltrating T cells may give rise to a local T_{RM}-cell population. The extent to which T_{RM} cells contribute to acute infiltrates in early MS is incompletely understood. In mixed active/inactive lesions of advanced MS, shown to the right, T-cell trafficking is not evident, and perivascular cuffs are populated by T_{RM} cells. Perivascular T_{RM} cells are reactivated by APCs and contribute to inflammatory lesion formation, either locally in the PVS or upon entering the parenchyma, through producing soluble effector molecules and/or displaying cellular cytotoxicity.

An urgent question is the identification of the antigen(s) against which T-cell responses in MS and specifically the T_{RM}-cell response is mounted. In other tissues, T_{RM} cells have been mostly studied in and associated with virus infections.[53,54] Therefore, a viral antigen appears tempting. A vast body of literature associates MS with Epstein-Barr virus (EBV) infection.[117] Accumulation of EBV-infected B cells and EBV-directed $CD8^+$ T cells has been described in MS CSF[118–120] and in MS lesions,[85,94,121–123] although the reproducibility of these findings has also been debated.[124–126] Other viruses have also been associated with MS, including human herpesvirus (HHV)-6.[127] Moreover expression of endogenous retrovirus sequences has been described in MS lesions,[128] which may also elicit a $CD8^+$ T-cell response.[129] Alternatively, a potential role of autoantigen-directed T_{RM} cells in autoimmune diseases has not been explored extensively yet.

Since brain $CD4^+$ and $CD8^+$ T_{RM} cells are physiological residents of the normal human PVS,[49,50] therapeutic strategies interfering with their functional profile may disrupt physiological functions of these cells. In MS, the importance of physiological T-cell trafficking to the CNS became eminent with the development of PML in natalizumab-treated patients.[130] Therefore, a role of physiological T_{RM}-cell pools in immune surveillance of the CNS can be anticipated. The risks of interfering directly with this surveillance are not known. Although JC virus has been propagated to be retained in an inactive state in the kidneys,[131] post-mortem human studies also revealed JC virus genetic fragments in brains of 28-68% of asymptomatic cases.[132–134] The latter observations suggests JC virus to latently infect the human CNS, flaring up in the case of PML.

Lastly, there is a timeframe gap of knowledge in the immunopathology of MS. Thanks to the availability of biopsy material, the pathology of the earliest phases of MS has been extensively studied. Post-mortem autopsy studies have provided much insight in the pathology of MS at its end-stage. Differences between these extreme groups can be identified, and the first group is likely to evolve into the latter. However, what happens in the intermediate years or even decades is uncertain and has been highlighted as 'black box' in MS-pathology research.[135] The dynamics of findings in human circulating lymphocytes must be interpreted in correlation with the natural history of MS and phenotypic characteristics of cells observed within the PVS. Circulating cell fractions associated with MS-disease activity must ultimately give rise to the T_{RM}-cell populations as they are encountered in MS. We have limited data on the presence of T_{RM} cells in white matter lesions at the early stages of MS, as well as their association with pathological patterns of early MS. Cells with T_{RM} cell-related characteristics have been observed in the blood and CSF of people with MS, as indicated by the enhanced presence of circulating $CD4^+$ T cells expressing high levels of CCR5 and granzyme K.[96] Additionally, clonally expanded $CD8^+$ T cells with T_{RM}-cell characteristics could be isolated from the CSF of twins with prodromal MS.[136]

9 CONCLUSIONS

Our understanding of the pathology of MS has enormously benefited in recent years from studies of large tissue collections. These initiatives allowed to capture common elements as well as heterogeneous components of the pathology of MS. They also warrant a reflection of gratitude

to all MS patients who donated CNS tissue for research to better understand the disease they suffer(ed) from. Pathological data on demyelination and myeloid cell activation point towards the mixed active/inactive lesion as a detrimental phenomenon in advanced progressive MS. Recent immunohistochemical and flow-cytometric studies revealed brain T_{RM} cells not only to be physiological residents in the human brain PVS but also to be numerically and spatially associated with mixed active/inactive MS lesions. Phenotypic changes of T_{RM} cells in correlation with these lesions suggests an active role of $CD8^+$ T_{RM} cells in lesion formation and/or maintenance. Further understanding of the functional dynamics of brain T_{RM} cells may offer intriguing new avenues to target mixed active/inactive lesion formation in advanced MS, for which current DMTs show in general a disappointing efficacy.

10 EXPERT OPINION

The treatment of MS saw many important advances over the last decades, with an exponential growth of the number of DMTs registered. Except for interferon beta (IFNβ), glatiramer, and natalizumab, current DMTs have originally been developed within other fields in medicine. These drugs mostly target lymphocyte activation or migration. At present, T_{RM} cells are a subject of study in many organs. Although a role in the control of (viral) infections is best consolidated,[53,54] a contribution of T_{RM} cells to local inflammatory reactions in autoimmune diseases has not been extensively explored. Certainly, T_{RM} cells will receive attention in inflammatory diseases in other organs with the aim to affect their behavior. These approaches likely will elude new classes of treatments, targeting specifically local inflammatory cells and mechanisms. An important CNS-specific bottle-neck will be the development of drugs that cross the BBB and reach the PVS. Not only the activation, mobilization, and inflammatory potential of T_{RM} cells themselves may be a target of therapy but also the crosstalk with other inflammatory players in the PVS and brain parenchyma. In the PVS, perivascular macrophages and B cells can present antigens, provide co-stimulatory/inhibitory signals, and/or make cytokines controlling the activation and recruitment of T_{RM} cells. Likely, myelin-laden microglia/macrophages in mixed active/inactive lesions are particularly important. They not only may provide signals critical for T_{RM}-cell activation, but also may receive signals from T cells amplifying their phagocytic potential. The dynamics of microglia morphology and phenotype in relation to demyelinating lesion formation is only poorly understood.[11,137]

A challenge for therapies directly targeting brain T_{RM} cells will be to preserve their physiological roles. Attenuating their inflammatory potential without compromising too much normal immune surveillance may suppress mixed active/inactive lesion formation without reactivation of latent neurotropic viruses. Therefore, it is important to comprehensively unravel the phenotype and functional programs of T_{RM} cells associated with MS lesions. In the end, modulating T_{RM}-cell activation and migration into the CNS parenchyma may suppress a component of disease activity but likely will not cure MS. However, disclosure of critical antigens and the cells presenting them may bring the field closer to the cause of MS. As discussed above, neurotropic viruses as well as the lymphotropic virus EBV are likely candidates.

PART 2 HETEROGENEITY OF THE IMMUNOPATHOLOGY IN ADVANCED MULTIPLE SCLEROSIS

MS is a heterogeneous disease regarding clinical disease course, radiological appearance of lesions and response to immunomodulatory therapies.[138] The analysis of pathological heterogeneity in an MS autopsy cohort enabled us to identify pathophysiological mechanisms which potentially contribute to the heterogeneity in the clinical disease course of MS.

1 HETEROGENEITY OF THE HUMORAL IMMUNE RESPONSE IN ADVANCED MS

Interestingly, variability between MS patients is observed in the involvement of humoral immunity, especially in effector B cell functions in the disease. At time of diagnosis, 10% of MS patients show absence of oligoclonal bands (OCBs) consisting of intrathecally produced immunoglobulin (Ig)Gs.[139] This absence of OCBs is associated with a decreased number of lesions on magnetic resonance imaging (MRI) at baseline and a more benign disease course in follow-up compared to MS patients that do show OCBs.[139,140] Also pathologically, a large heterogeneity in the number of B cells and the presence of IgG depositions in MS lesions has been described over the past decades. In both early MS biopsy lesions as well as the chronic autopsy lesions, the presence[19,141,142] and absence[19,143] of IgG deposits in MS lesions has been described. In the analysis of biopsy samples it has been suggested by Lucchinetti et al. that different patterns of IgG deposition between MS cases represent different etiologies of the disease. MS cases with IgG deposits were argued to have an antibody mediated disease, potentially with antibodies directed against unknown CNS antigens, while alternative disease mechanisms were postulated in cases without IgG deposits.[144] However, it has been debated whether these differences between MS patients in the biopsy samples represent true etiological differences, or rather reflect the temporal development of MS lesions.[10] Furthermore a correlation of the different pathological patterns of MS in the biopsy samples with distinct clinical profiles has not been described.

Therefore in **Chapter 6** we set out to investigate whether differences in the involvement of in the humoral immune response could be detected in the later disease stages of MS. We show that the number of B cells in MS autopsy lesions is highly heterogenous between MS cases. Interestingly in 34% of the inflammatory active MS lesions in autopsy tissue, we identified no B cells and we showed that presence of B cells correlates between different locations (MO and subcortical white matter) and compartments (parenchyma, perivascular space, meninges) in an individual donor. This consistency suggests that limited presence of B cells is a donor characteristic. The donors without B cells in the investigated regions showed a better clinical and pathological profile. Furthermore in a subgroup of MS cases without B cells in the investigated regions, a lower intrathecal IgG production and a more frequent absence of OCBs was found. Possibly, we selected an extreme subgroup of MS cases at one side of a continuum with a genetic profile that restricts involvement of B cells in MS lesion pathogenesis.[145]

However the incidence of CSF-unique OCBs we observed in 60% of MS autopsy cases is markedly lower compared to clinical MS cohorts, where 90% showed OCBs at diagnosis.[139] Therefore, since we identified B cells in 92% of the early MS biopsy lesions, and the MS cases with limited B-cell presence in autopsy tissue had a longer disease duration and older age, B cell involvement in white matter lesion activity might be extinguishing over time. Differences in involvement of the humoral immune response, in both autopsy and biopsy tissue, may alternatively represent the temporal development of MS lesions. We provided some support for this hypothesis, by observing in a clinical cohort the absence of OCBs in 27.4% of patients after a disease duration of 11.7 ± 8.5 (mean ± SD) years. In six of these patients without OCBs, their presence at diagnosis could be validated. Accordingly, Frischer et al. reported higher numbers of perivascular B cells in donors with relapsing and progressive disease, when compared to inactive disease.[79] This suggests that humoral immune response is regressing over time, at least in a subgroup of MS patients. However, this remains to be confirmed in a prospective clinical study analyzing IgG index and OCBs in MS patients over time for a longer period. This could help to determine if indeed IgG index and the presence OCBs in CSF are clinically relevant biomarkers for the white matter inflammatory disease activity in advanced and progressive MS. Especially regarding cessation of immunomodulatory therapies in advanced MS, this could be a clinically useful hypothesis to pursue.

2 SEX DIFFERENCES IN CORTICAL MS PATHOLOGY

Sex differences in MS clinical disease course have been well characterized. Female MS patients more often show a relapsing-remitting and a more benign disease course compared to males.[146] Interestingly, males develop MS at an older age and they faster reach a more severe disability score.[147] Male MS patients show a higher incidence of cognitive decline compared to females.[148,149] MRI studies revealed more destructive white matter lesions and more often cortical grey matter lesions in males compared to females.[150,151] Indeed, we report in **Chapter 2** that males showed more ongoing inflammatory and demyelinating lesion activity in the white matter[40,152] and a higher incidence of cortical grey matter lesions compared to females.[152]

The hypothesis that sex steroids are the basis for these clinical and pathological differences between males and females is supported by the observation that MS relapses decrease during pregnancy and increase again post-partum, when estrogen and progesterone levels rapidly decrease.[147] A history of multiple pregnancies is also associated with a decreased risk of a first demyelinating event.[153] Not only sex steroids produced in peripheral tissues target the CNS in MS, but also steroids produced within the CNS, "neurosteroids", might influence MS lesion pathology.[154] There is a large body of *in-vitro* and *in-vivo* evidence suggesting that neurosteroids and especially progesterone and androgens and their metabolites show inhibition of demyelination and promotion of remyelination, anti-inflammatory and neuroprotective effects in models for MS.[154–156] Furthermore peripherally produced progesterone shows effects on the peripheral immune system; both macrophages and lymphocytes show inhibition of pro-inflammatory cytokines and interferon response after progesterone treatment *in vitro*.[155]

We recently demonstrated an altered expression of progesterone synthetic enzymes and their receptors in MS white matter lesions. In female MS lesions, progesterone signaling was up-regulated while this was not the case in male MS lesions.[157] Unpublished gas-spectrometry data from mixed active/inactive lesions shows that there are increased allopregnanolone and progesterone levels in MS lesions from females while this is not increased in males (Mason and Luchetti et al in prep). These consistent findings suggest that decreased progesterone and allopregnanolone signaling in male white matter lesions might facilitate the ongoing inflammatory and demyelinating lesion activity and thereby the higher probability of clinical disease progression in male MS patients.

However, the protective effect of the increased progesterone and allopregnanolone signaling in female MS white matter lesions and the relation with a decreased incidence of cortical grey matter lesions remains poorly understood. In **Chapter 7** we confirm in a standardly dissected cortical region that male MS patients show a higher susceptibility to develop specifically leukocortical lesions compared to females. Additionally, we show an increased gene expression of synthetic enzymes for the progestogen allopregnanolone and the androgen 3-α-DIOL in the normal appearing cortex of female MS patients, while this is not increased in males. Which suggests a neuroprotective effect of allopregnanolone and 3-α-DIOL in the normal appearing MS brain tissue.

Interestingly, both allopregnanolone and 3-α-DIOL are positive agonists for the GABA-A receptor on neurons.[158,159] Therefore a neuroprotective effect of increased allopregnanolone and 3-α-DIOL synthesis in the normal appearing cortical grey matter may be mediated by the ability to attenuate excitotoxicity which is associated with brain injury.[160,161] Increased expression of glutamate receptors and transporters has been shown in MS cortical grey matter compared to controls.[162–164] Since oligodendrocytes are most vulnerable to excessive glutamate, it has been suggested that increased re-uptake of glutamate is a potential protective mechanism for demyelination in MS.[165,166] Since allopregnanolone and 3-α-DIOL are expected to alter neurotransmitter expression and release,[154] we analyzed gene expression of GABA and glutamate synthetic enzymes and glutamate re-uptake transporters. However, we did not see a significant difference between males and females in the GABA and glutamate synthesis and glutamate re-uptake genes. Interestingly we showed that progestogen and androgen synthetic enzymes AKR1C2, HSD3B1, CYP17A1 and STS are positively correlated with glutamate re-uptake genes GLAST and GLT1. Indicating that allopregnanolone and 3-α-DIOL synthesis can potentially impact on the glutamate re-uptake and thereby mediating the excitotoxicity[160,161] and potentially preventing cortical demyelination in females.

In **Part 1** of the thesis we show that reactivation of $CD8^+$ T cells in MS white matter lesions is associated with ongoing inflammatory and demyelinating lesion activity in progressive MS.[37,51,75,1675151] Furthermore it has been shown that also in cortical demyelinated lesions increased numbers of $CD8^+$ T cells are present, suggesting $CD8^+$ T cell reactivation is related to cortical demyelination.[94,168] Interestingly, MS males showed in normal appearing grey matter a higher expression of CD8 and interferon gamma (IFNG), while this was not increased in the female

normal appearing cortex. This could suggest that in male cortex the CD8 T cell interferon response is potentially more (re-) activated compared to females. It has been shown that allopregnanolone effects $CD8^+$ T cell activity by binding to the GABA-A surface receptor on T cells.[169] GABA-A agonists have been reported to inhibit antigen specific T cell proliferation,[170] suggesting that allopregnanolone potentially inhibits cytotoxic $CD8^+$ T cell responses. It remains to be determined whether altered allopregnanolone and 3-α-DIOL contributes to the difference in $CD8^+$ T cell and IFNG expression in the NAGM between males and females. We did not see a significant negative correlation between the synthetic enzymes and CD8 expression, indicating that other factors then sex steroids alone (for example differences in local inflammatory environment) are impacting on the $CD8^+$ T cell response in MS cortical grey matter.

We show that allopregnanolone and 3-α-DIOL synthesis is less induced in the MS normal appearing cortex in males compared to females, potentially impacting on a decreased glutamate re-uptake and an increased $CD8^+$ T cell and interferon response in males compared to females. These events could culminate in an increased susceptibility of the male MS cortex for the development of leukocortical demyelinated lesions. These results together with our earlier findings in mixed active/inactive lesions,[171] suggests that supplementation with progesterone or allopregnanolone in MS patients might be effective in preventing ongoing inflammatory lesion activity and cortical demyelination and thereby effecting the progression of the disease. There is one clinical trial reported with progesterone treatment in MS which was targeted to prevent post-partum relapses (POPARTMUS), where 12 weeks treatment with 10 mg nomegestrol acetate was compared with placebo, however it is reported that this showed no reduction of post-partum relapses, however the analysis have never been published.[172,173] Interestingly the cochrane review on several clinical trials for the administration of progesterone in traumatic brain injury concluded progesterone treatment improved neurological outcome after traumatic brain injury, but larger clinical trials are needed for a definite conclusion.[174] Further clinical investigation of the effect of progesterone and allopregnanolone treatment in MS patients is therefore still warranted.

3 GENETIC FACTORS ASSOCIATED WITH MS LESION CHARACTERISTICS IN AUTOPSY TISSUE HELP TO FOCUS ON DISEASE RELEVANT PATHOGENIC MECHANISMS

Sex differences in (neuro)steroid synthesis and signaling in and outside the CNS is a relevant mechanism in the analysis of heterogeneity in MS lesion pathology. However this does not fully explain the large heterogeneity in MS clinical disease course and lesion pathology between MS cases, and other relevant pathogenic mechanisms underlying this heterogeneity remain largely unknown.[40,152] Over the past decades several common genetic variants have been associated with clinical outcome of MS in candidate gene and genome-wide association studies (GWAS).[175–178] On their own the identified common genetic variants show a minor effect on the clinical outcome and therefore they have no clinical predictive utility, but nevertheless they possess an important translational potential.[179–182] The genes and associated biological pathways implicated in clinical

outcome by genetic association may represent targets for interventions that potentially affect these pathways more extensively than the naturally occurring genetic variants.[181,183]

However, the genes and biological pathways associated with the identified variants remain largely unknown due to the inability to translate genotype into disease-relevant mechanisms.[180,182,183] In **Chapter 8** we translate genotypic information into pathogenic mechanisms by using the semi-quantitative pathological characterization of MS lesion pathology in the MS autopsy cohort together with genetics and gene expression analysis of autopsy tissue samples. We analyzed the MS lesion characteristics in the NBB MS autopsy cohort in relation to genotyping results for 67 SNPs previously related to the clinical disease course or MRI measures in MS[175–178] and 35 SNPs located in genes previously related to MS pathological characteristics. The analysis showed that six genetic variants are significantly related to post-mortem MS lesion characteristics. For rs2234978/FAS T allele carriers we were able to confirm that they show increased FAS expression in brain tissues[184] and we showed for the first time that they have a higher percentage of active MS lesions. Interestingly it was previously shown that they have a more severe clinical MS disability progression.[177] Since FAS, which functions as an apoptosis receptor,[185–187] is expressed by T cells and peri-lesional oligodendrocytes this suggests two possibilities for the involvement of FAS in MS pathology. The first possible mechanism is that increased FAS expression in rs2234978 T allele carries in oligodendrocytes around mixed active/inactive lesions make them more likely to undergo apoptosis. In the experimental autoimmune encephalomyelitis (EAE) mouse model it is shown that mice lacking FAS expression on oligodendrocytes are partially protected from EAE with a decrease in demyelination and a mild decrease in infiltration of lymphocytes.[188] The second possible mechanism is that higher FAS expression in T-cells leads to a more pro-inflammatory T-cell population. Immunohistochemistry shows FAS expression by lymphocytes in mixed active/inactive lesions. FAS is mainly expressed by $CD4^+$ T cells and its expression is highest in regulatory T (Treg) cells in both blood and brain, in line with previous findings.[189,190] Presence of Treg cells has been reported in CSF samples from MS cases and they are shown to be present in MS lesions, more often in inactive compared to active MS lesions.[191] Intra-cerebral Treg cells are more vulnerable to FAS mediated apoptosis and exhibit a higher rate of apoptosis compared to other T cell populations *ex-vivo*.[190–192] Therefore, an increased overall FAS expression in T cells could result in the inhibition of the Treg cells, leading in turn to a more severe MS disease course.[193,194] Therefore the identified genetic association with FAS gene expression in brain autopsy tissue and the percentage of active MS lesions suggests that inhibition of FAS mediated apoptosis within the central nervous system (CNS) is a potential target for the protection of oligodendrocytes and inhibition of inflammatory disease activity in MS.[195]

Although we did not identify associations with brain gene expression levels for the other SNPs associated with MS lesions characteristics, for some of them functional effects on gene expression have been previously described. This can provide guidance for future studies into the functional effects of these SNPs and the pathogenic mechanisms that they relate to.

Since the rs8056098/CLEC16A minor allele was previously associated with a less severe disease course in the IMSGC GWAS,[178] and in our analysis the minor allele is associated with a lower proportion of mixed active/inactive lesions this is a highly interesting genetic variant for future studies. As shown in **Chapter 2** a higher proportion of mixed active/inactive lesions in autopsy tissue has been repeatedly associated with a more severe and progressive disease course.[40,152] Our findings are consistent with the reported GWAS association and suggests a link between genotype and disease severity via the propensity to form or expand mixed active/inactive lesions. The functional effects of rs8056098 are poorly understood. Therefore, the question remains how rs8056098 (which is located in intron 15 of the CLEC16A gene) influences the function of the CLEC16A protein. The GTEx-data showed that rs8056098 A-allele homozygotes show significantly lower CLEC16A mRNA expression levels in thyroid tissue samples.[184] This observation suggests that rs8056098 impacts on CLEC16A gene expression levels.

The function of CLEC16A in the brain, however, is poorly understood, in the mouse CNS it has been recently described that deficiency of CLEC16A protein impairs autolysosome function and neuronal survival.[196] Therefore, an altered CLEC16A function could have implications in neurodegenerative disease processes potentially effecting MS disease progression. Since the CLEC16A gene is most strongly associated with several autoimmune diseases[197,198] the function of CLEC16A is mostly studied in the peripheral immune system. CLEC16A is part of the C-type lectin protein family that are involved in the recognition of pathogens. They target antigens of the endosomal pathway required for HLA-II-mediated antigen-presentation to T cells.[199] The short carbohydrate domain of CLEC16A, is predicted to be inactive suggesting a non-classical C-type lectin function.[200] In macrophages it is shown that CLEC16A serves as a direct regulator of the HLA-II antigen-presentation pathway.[200] In addition, it has been suggested that CLEC16A is also linked to the antigen-presentation pathway in B-cells.[201,202] In early MS, T cells are triggered by memory B cells[203] in secondary lymph nodes to infiltrate the central nervous system (CNS). However, we showed in part 1 of the thesis, that advanced and progressive MS is characterized by ongoing microglial activation and reactivation of brain specific T_{RM} cells.[50,51,167] In progressive MS lesions, we encountered reactivated T_{RM} cells in close contact with B cells and perivascular macrophages, which suggests that antigen-presenting cells reactivate T_{RM} cells within the CNS of progressive MS patients. Notably, B-cells that reside in the brain have been shown to express CXCR3, which characterizes populations with increased APC and transmigration capacities.[204] An important role for B-cells in the advanced MS disease-activity is supported by the effect of anti-CD20 directed therapies on disease progression.[205] In **Chapter 6** we show that MS autopsy cases without infiltrating B-cells show a more favourable pathological and clinical profile. Interestingly, preliminary analysis shows that these cases more often had the A:A genotype of rs8056098 (**Figure 5**).

These observations supports the view that rs8056098 potentially contributes to homing or retention of B-cells in the perivascular space and meninges in advanced MS, and suggest that CLEC16A mediates effective antigen-presentation by human APCs, including B-cells to T-cells as also demonstrated *in vitro* by Rijvers et al.[202] Altogether, this work raises the hypothesis that

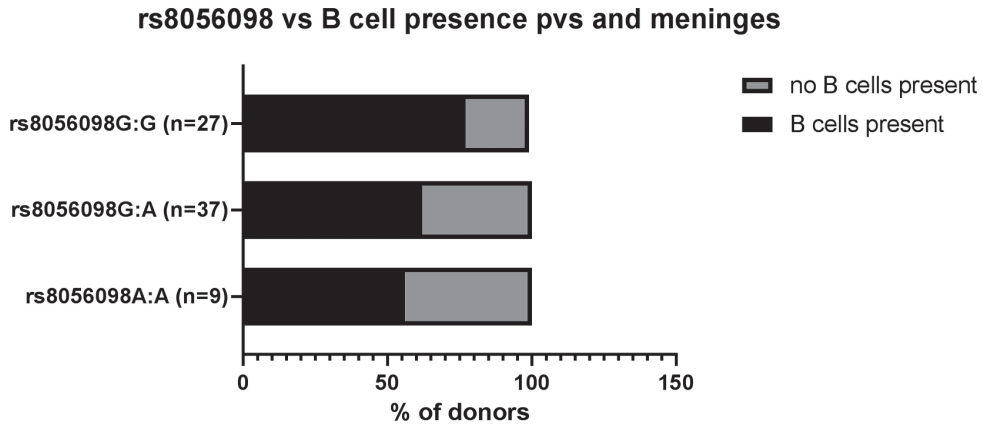


Figure 5.
MS cases that are A:A homozygote for rs8056098 more often show the absence of B-cells in the perivascular space and meninges in the standardly dissected medulla oblongata.

the protective effect of rs8056098 on disease progression can be explained by reduced antigen presentation and transmigration capacities of B-cells, resulting in impaired T_{RM} cell reactivation and impaired progression of MS lesion pathology. (**Figure 6**) This would be an interesting hypothesis for future experimental studies to explore the function of rs8056098 and the contribution of antigen presentation on progressive MS lesion pathology.

In summary, **Chapter 8** illustrates that the extensive analysis of the heterogeneity in MS lesion pathology in a brain autopsy cohort in combination with genetics and gene expression enabled us to identify pathogenic mechanisms but also to generate hypotheses on relevant pathogenic mechanisms involved in MS lesion pathogenesis. These hypotheses would need further analysis to identify potential new targets for therapy. For example, analyzing the RNA and protein expression associated with the identified variants in different cell populations would be promising to identify new targets for disease modifying treatments[182,183] RNA sequencing of isolated nuclei from frozen autopsy tissue is a promising approach to study the transcriptome of different human cell-populations from the central nervous system in association with genotype. This isolation of nuclei from brain autopsy tissue enables the analysis of the function of these SNPs in RNA expression in human brain cells.[206] Furthermore, modeling the SNPs in relevant human cells, for example stem cell-derived neurons or oligodendrocytes using gene editing tools like CRISPR-CAS9 will be of interest to elucidate the functional cellular effects of these SNPs. This will improve our understanding of the molecular mechanisms that underlie the differences in clinical course and hopefully help to identify new targets for therapies in MS.

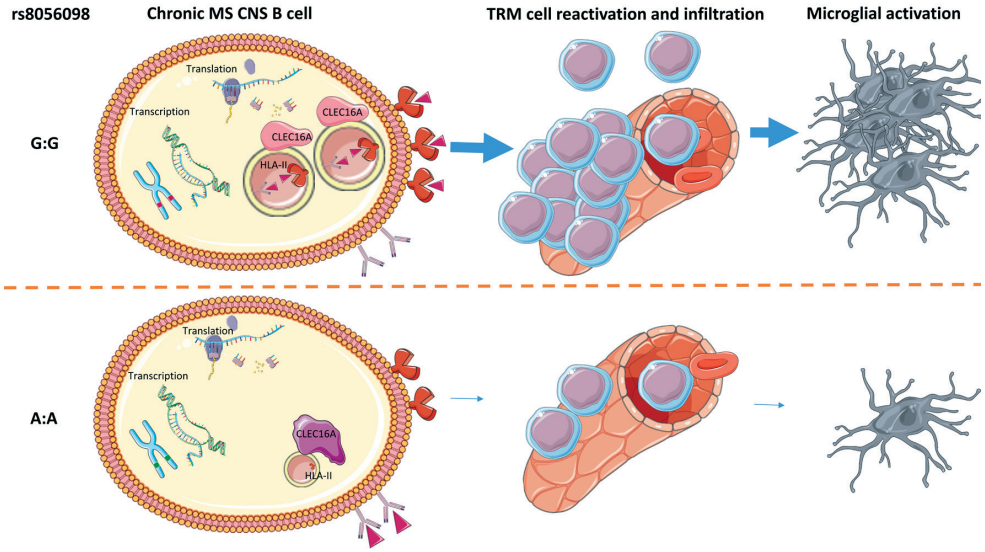


Figure 6.
Illustrating the hypothesis that rs8056098 mediates altered functionality of CLEC16A leading to impaired antigen presentation by B cells, which leads to impaired T_{RM} cell reactivation and infiltration and therefore impaired microglial activation in progressive MS lesion pathology.

4 FUTURE PERSPECTIVES FOR THE STUDY OF HETEROGENEITY OF MS LESION PATHOLOGY IN ADVANCED MS AUTOPSY COHORTS

This thesis illustrates different possibilities of analyzing the heterogeneity of MS lesion pathology in an MS autopsy cohort leading to the identification of relevant pathogenic mechanisms that are related to the MS clinical disease course.

The heterogeneity in humoral immune response in both biopsy and autopsy cases should deserve more attention in future analysis. In advanced MS we identified a large group of patients that showed little involvement of B cells and plasma cells and these cases had a longer disease duration. This could implicate that in these cases the B cell response (both the IgG opsonization of myelin and the antigen presentation functions to T_{RM} cells) is less pronounced and therefore their MS lesion pathology is less aggressive resulting in a longer disease duration. Alternatively, their humoral immune response could regress over time and therefore we find little B cells in cases with a longer disease duration. This remains an open question that will not be answered with the analysis of autopsy tissue. The increased absence of oligoclonal bands in CSF during follow-up compared to inclusion in a clinical MS cohort, indicates that in MS patients the humoral immune response indeed is plausible to show regression over time. This suggests indeed the differences in humoral immune response in MS autopsy and biopsy lesions might represents the temporal development of MS lesion pathology. However, potentially, in some MS cases the humoral immune response regresses

more quickly compared to others. Therefore, it would be interesting to prospectively analyze the disease course in MS patients with and without disappearing OCB's in the CSF. Potentially the MS patients with disappearing OCB's would develop a more benign disease course compared to the patients with a persisted humoral immune response in the CSF. This potential subgroup of patients could be characterized by specific genetic or environmental factors which would be interesting to further analyze. Preliminary analysis suggested that the MS cases without B cells in autopsy tissue more often had the rs8056098/CLEC16A genotype compared to the autopsy cases with B cells, which would suggest that an impaired function of CLEC16A and impaired efficiency of antigen presentation by antigen presenting cells like B cells would lead to limited progression of MS lesion pathology and a less severe disease course. Identification of MS autopsy cases with a genetically altered CLEC16A function would enable us to study the effect on antigen presentation inside the CNS analyzing the MS autopsy lesions. Identification of these patients in a clinical cohort would provide opportunities to analyze the effect on antigen presentation functions in the peripheral immune cells. This combined approach would provide insight in the cellular mechanisms of antigen presentation relevant for MS lesion pathogenesis. This would potentially lead us to new drug targets that impact on the (re)activation of T_{RM} cells in MS lesions.

The analysis of the heterogeneity in humoral immune response in the autopsy cohort showed that a subgroup of patients show pronounced presence of B cells with pronounced perivascular and meningeal clustering of B cells. This probably represents an extreme end of the spectrum of MS pathology. However, since in a small group of patients these perivascular lymphocytic infiltrates are pronounced we would like to hypothesize that in these cases the demyelinating disease is more antibody mediated compared to the cases without these infiltrates. We showed that these cases are negative for the auto-antibodies for MOG and AQP4, the analysis of potentially unknown CNS derived antigens in CSF or plasma from these autopsy cases, would be interesting for the discovery of potential new subgroups of MS patients with a more specific antibody mediated demyelinating disease.

Furthermore the relation between the inflammatory response in advanced MS and the neurodegenerative disease process in advanced MS brains needs to be better explored in the autopsy cohort. In **Chapter 1** we show in the brainstem from a selection of MS autopsy cases that the neuronal and axonal damage is most pronounced in mixed active/inactive lesions compared to the other lesion subtypes. The extend of neuronal and axonal pathology is also correlated with the number of T and B cells. This suggests that in advanced MS, in line we previous studies analyzing different locations in the brain, the innate and adaptive immune response are related to the extent of neuronal and axonal damage. However, the heterogeneity in axonal and neuronal pathology in autopsy cohorts has been poorly described. Based on previous studies and our own brainstem study in relatively small groups of MS autopsy cases, heterogeneity in the neurodegenerative component of the disease could be expected. However, retrieving a donor specific measure for neurodegeneration derived from standardized locations in the brain in the entire autopsy cohort would be interesting for the analysis of the heterogeneity and the relation with clinical parameters

and with donor specific measures of innate and adaptive immune response. Furthermore, it would provide an opportunity to correlate these measures with CSF neurofilament light chain levels. Although neurofilament light chain is a popular biomarker in the neurological clinical practice and it is assumed that this measures the extend of neurodegeneration in MS patients,[207] this has not been proven neuropathologically. Future analysis of neurodegeneration in relation with neurofilament light chain levels in the CSF would therefore help to better understand these clinically used measures.

These examples, illustrate the enormous possibilities of a clinically and pathologically well-characterized autopsy cohort for biomarker discovery and validation and for the identification of disease relevant pathogenic mechanisms and drug target discovery. The digitalization of MS sections available at the Netherlands Brain Bank would provide a next step in the characterization of MS lesion pathology. The different characterization systems of MS lesions have been debated for decades and a unified consensus on MS lesion pathology and a definition of MS lesion characteristics is only recently provided by Kuhlmann et al.[10] Digitalization of the sections would allow more collaboration between MS pathology expert and ensure a standardized characterization of MS lesion pathology across laboratories. This would enable an improvement in the brain donor specific measures for MS lesion activity and the correlation with clinical parameters.

Digitalization of the histological sections from MS brain autopsy cohorts worldwide and a standardized characterization of MS lesions across laboratories, would also provide the opportunity to combine autopsy cohorts and enable a well-powered GWAS analysis with MS pathological characteristics. This will provide insights in the genetic variants involved in MS lesion pathogenesis, which will lead to a better understanding of the cellular and molecular mechanisms that are relevant for MS patients.

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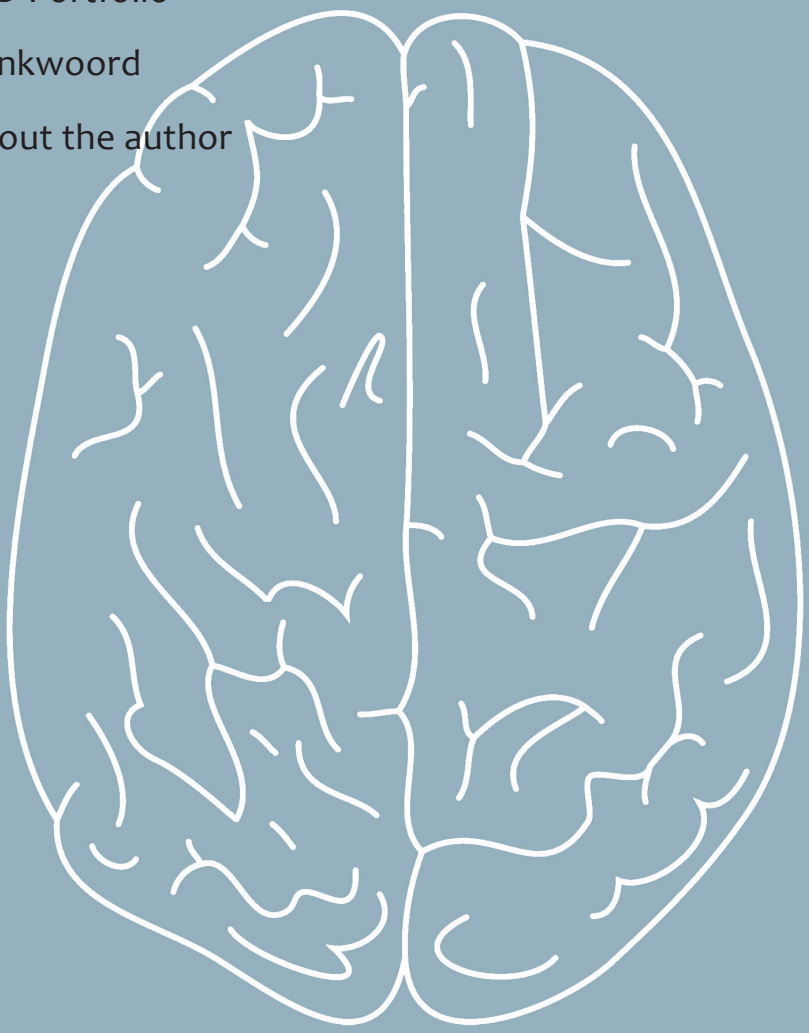
APPENDICES 10

- Summary
- Nederlandse samenvatting
- Author contributions
- List of publications
- PhD Portfolio
- Dankwoord
- About the author



SUMMARY

HETEROGENEITY OF THE IMMUNOPATHOLOGY IN ADVANCED MULTIPLE SCLEROSIS

An autopsy cohort analysis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system which mostly presents in young adults. The disease often starts with a relapsing-remitting phase where patients show acute onset of neurological symptoms with spontaneous remission. Over time most MS patients accumulate disability and enter a progressive disease phase. The current available immunomodulatory therapies for MS directed at peripheral T or B cells show an effect on the relapse rate in the early disease phase however they do not halt the progression of the disease in advanced stages. This led to the concept that circulating immune cells contribute to the onset and early phase of the disease, while they are not involved in disease progression in advanced MS. Therefore, it is thought that disease progression might arise from neurodegenerative disease processes rather than inflammation. In **Part 1** of this thesis we aimed to characterize the immune cells that are involved in advanced and progressive MS lesion pathology in autopsy tissue. We analyzed immune cells in post-mortem MS brain tissue from the MS autopsy cohort of the Netherlands Brain Bank of 182 MS brain donors with advanced MS. We show that there is substantial inflammatory lesion activity in advanced MS and that brain specific tissue resident memory T cells contribute to the inflammatory lesion activity in advanced MS.

While most MS patients accumulate disability over time, the rate of disability progression is highly variable between MS patients. Furthermore there are sex differences in the clinical disease course of MS. However, the molecular and cellular mechanisms that underly these differences in disability progression remain poorly understood. In **Part 2** of this thesis we aimed to identify pathophysiological mechanisms that contribute to the heterogeneity of the immunopathology in MS. We analyzed MS lesion characteristics in the MS autopsy cohort of the Netherlands Brain Bank in relation with clinical disease course, sex, and genetic factors. We show that the heterogeneity of the immunopathology in an advanced MS autopsy cohort is associated with the variable involvement of B cells and plasma cells, sex differences in neuro-steroid synthesis and genetic factors.

PART 1 SUBSTANTIAL INFLAMMATORY LESION ACTIVITY IN ADVANCED MULTIPLE SCLEROSIS

In **Part 1** we show in advanced and progressive MS lesions increased complement deposition and an increased number of T cells, B cells and plasma cells as compared to neurodegenerative brain diseases. Thus, in the progressive late phase of MS there is substantial inflammatory lesion activity which also correlates with the rate of clinical disability progression. T cells isolated from fresh

brain autopsy tissue from both non-neurological controls and from advanced MS lesions show a tissue resident memory (T_{RM}) profile and they usually reside in the perivascular space. T_{RM} cells in advanced MS lesions are re-activated and they infiltrate the brain parenchyma. In MS lesions of MS brain donors with advanced MS no infiltration of non-T_{RM} cells is detected. This indicates that $CD8^+$ T_{RM} cells are involved in the ongoing inflammatory and demyelinating lesion activity which is seen in advanced MS.

In **Chapter 2** we show that in progressive MS autopsy cases 74% of the MS lesions in the brainstem are inflammatory active or mixed active/inactive. The mixed active/inactive lesions show an increased number of $CD8^+$ and $CD4^+$ T cells and $CD20^+$ B cells, and increased acute axonal damage, mitochondrial stress, axonal loss and synapse loss compared to the normal appearing MS brainstem. In MS brainstem lesions and in the brainstem of patients with a neurodegenerative disease there is C1q deposited on synapses and C3d on both synapses and axons. Depositions of the terminal complement component, MAC are seen specifically in MS lesions, inside microglia/macrophages and deposited on astrocytes. This suggests that the inflammatory lesion activity contributes to ongoing demyelination and axonal damage in the progressive MS brainstem.

In **Chapter 3** we performed a histological characterization of 182 MS autopsy cases from the Netherlands Brain Bank, from which 3188 sections containing 7562 MS lesions were analyzed. The MS cases had an average disease duration of 28.6 ± 13.3 years and 57% of the MS lesions were active or mixed active/inactive. Furthermore in 78% of the MS cases a mixed active/inactive lesion was present. The percentage of mixed active/inactive lesions and the brainstem lesion load correlated with the rate of disability progression during life. Secondary progressive and primary progressive MS cases showed a higher lesion load and a higher percentage of mixed active/inactive lesions and a lower percentage of remyelinated areas compared to the relapsing remitting cases. Males showed a higher percentage of mixed active/inactive lesions and a higher incidence of cortical grey matter lesions compared to females. This shows that in advanced MS there is substantial inflammatory lesion activity that correlates with the rate of disability progression during live.

In **Chapter 4** we phenotyped viable $CD8^+$ and $CD4^+$ T cells derived from post-mortem brain tissue from non-MS brain donors using flow-cytometry and we assessed the location in the brain using immunofluorescence and confocal imaging. T cells isolated from brain tissue show a phenotypic and transcription factor profile consistent with T_{RM} cells. Brain T_{RM} cells can be subsetted in $CD103^-CD69^+$ and $CD103^+CD69^+$cells. CD103 expression in brain $CD8^+$ T cells correlates with reduced expression of differentiation markers, increased expression of tissue-homing chemokine receptors, intermediate and low expression of the transcription factors T-bet and eomes, increased expression of PD-1 and CTLA-4, and low expression of cytolytic enzymes with preserved polyfunctionality upon activation. Brain $CD4^+$ T cells also display T_{RM} cell associated markers but they have low CD103 expression. We conclude that the human brain is surveilled by T_{RM} cells, and argue that these cells provide protection against neurotropic virus reactivation whilst being under tight control of key immune checkpoint molecules.

In **Chapter 5** we analyzed T cells in relation to the inflammatory lesion activity in advanced MS. For this study, we performed histological analysis of T cells and perivascular T cell clustering in the Netherlands Brain Bank MS autopsy cohort. Furthermore we analyzed viable $CD8^+$ T-cells, isolated from fresh autopsy tissues from subcortical MS white matter lesions, MS normal-appearing white matter and control white matter, by flow cytometry. In active and mixed active/inactive lesions, the number of T-cells was increased compared to the normal-appearing white matter. Active and mixed active/inactive lesions were enriched for both $CD4^+$ and $CD8^+$ T-cells, the latter being more abundant in all lesion types. Perivascular clustering of T-cells was found in cases with a progressive disease course and correlated with a higher percentage of mixed active/inactive lesions and a higher brainstem lesion load compared to cases without perivascular clusters. In mixed active/inactive lesions, $CD8^+$ T-cells were more frequently encountered in the brain parenchyma compared to the normal appearing white matter. $CD8^+$ T-cells from mixed active/inactive lesions showed a tissue-resident memory phenotype with expression of CD69, CD103, CD44, CD49a, and PD-1 and absence of S1P1. They upregulated markers for homing (CXCR6), re-activation (Ki-67), and cytotoxicity (GPR56), yet lacked the cytolytic enzyme granzyme B. We show that in advanced MS cases, inflammatory lesion activity and demyelinated lesion load is associated with an increased number of T-cells clustering in the perivascular space. These are $CD8^+$ tissue-resident memory T-cells, which show signs of re-activation and infiltration of the brain parenchyma.

PART 2 HETEROGENEITY OF THE IMMUNOPATHOLOGY IN ADVANCED MULTIPLE SCLEROSIS

In **Part 2** we studied the heterogeneity and sex differences in the immunopathology of MS. We used different approached analyzing the immunopathology in the MS autopsy cohort aiming to identify pathophysiological mechanisms that are related to differences in clinical disease course in MS patients.

In **Chapter 6** we analyzed the clinical and pathological profile of MS autopsy cases in association with the extent of B cell and plasma cell infiltration. We performed a systematic characterization of the presence of $CD20^+$ B cells and $CD138^+$ plasma cells in brainstem and white matter lesions from the NBB MS autopsy cohort and in early MS biopsy lesions. B cells were mostly found in the perivascular space and the meninges and enriched in active and mixed active/inactive MS lesions. Interestingly in 34% of active and 71% of mixed active/inactive lesions, B cells were absent. Absence of B cells and plasma cells in brainstem and white matter lesions was associated with a longer disease duration, less frequent a secondary progressive disease course and a lower proportion of mixed active/inactive lesions. We selected the extreme cases from the autopsy cohort and showed that the IgG ratio was lower and CSF OCBs were more often absent in MS cases without B cells and plasma cells. In a clinical MS cohort, the numbers of patients without OCBs in CSF were increased after an average disease duration of 11,3 years. Absence of B cells is associated with a favorable clinical and pathological profile. This finding may reflect extremes of a continuum of genetic or

environmental constitution, but also a regression of WM humoral immunopathology in the natural course of advanced MS.

In **Chapter 7** we aimed to identify pathophysiological mechanisms that are involved in the sex differences observed in the development of cortical MS lesions. In Chapter 2 we describe that in post-mortem autopsy tissue males show a higher incidence of cortical grey matter lesions and previously sex differences in progesterone signaling have been described in white matter MS lesions. Therefore we performed an analysis on the sex differences in progestogen and androgen synthesis and signaling and their potential neuroprotective effects in the cortical grey matter. We selected a standardly dissected cortical region and used frozen tissue from males and females, from both MS and controls for a quantitative PCR analysis. In the standardly dissected superior temporal gyrus males more often showed a leukocortical lesion compared to females. The neurosteroidogenic enzymes STS, AKR1C1, AKR1C2 showed increased expression in female normal appearing cortex while this was not increased in males compared to the non-neurological controls. CD8 and IFNG mRNA expression was increased in male normal appearing cortex while this was not increased in females compared to non-neurological controls. This suggests in female MS normal appearing cortical grey matter allopregnanolone and 3aDIOL synthesis is induced, but not in males, while CD8 and interferon gamma expression is increased in males compared to females. This may contribute to their increased susceptibility for the development of leukocortical MS lesions.

In **Chapter 8** we aimed to translate genotypes previously associated with the rate of clinical disability progression in MS into disease relevant pathogenic mechanisms involved in MS lesion pathogenesis. We genotyped 179 MS brain donors from the Netherlands Brain Bank MS autopsy cohort for 102 SNPs, selected based on their reported associations with clinical outcome in previous GWAS studies or their associations with MS pathology associated genes. After correction for multiple testing, three SNPs previously linked to disability progression in MS showed a significant association with either the proportion of active lesions (rs2234978/FAS and rs11957313/KCNIP1) or the proportion of mixed active/inactive lesions (rs8056098/CLEC16A). Three SNPs linked to MS pathology-associated genes showed a significant association with either proportion of active lesions (rs3130253/MOG), incidence of cortical grey matter lesions (rs1064395/NCAN) or the proportion of remyelinated lesions (rs5742909/CTLA4). In addition, rs2234978/FAS T-allele carriers showed increased FAS gene expression levels in perivascular T cells and perilesional oligodendrocytes, cell types that have been implicated in MS lesion formation. Here we show that by combining the pathological characterization of an MS autopsy cohort with genetics, we start to translate genotypes linked to disability progression in MS into cellular mechanisms involved in MS lesion pathogenesis that could potentially help explain the differences in clinical disease course.

We conclude that advanced progressive MS is characterized by substantial inflammatory lesion activity which is correlated with the rate of clinical disability progression. This suggests that inflammatory lesion activity is involved in the clinical disease progression in advanced MS. Brain T_{RM} cells, that under non-inflammatory conditions reside in the perivascular space, are reactivated

and invade the brain parenchyma in advanced progressive MS white matter lesions. These observations suggest that resident brain immune cells contribute to the ongoing inflammatory lesion activity in advanced MS. Finally, by analyzing the heterogeneity of the immunopathology of MS in an autopsy cohort in relation with the clinical disease course, sex and genetic factors, we identified pathophysiological mechanisms that potentially contribute to the heterogeneity in the clinical disease course of MS patients.

NEDERLANDSE SAMENVATTING

HETEROGENITEIT IN DE IMMUNOPATHOLOGIE VAN VERGEVORDERDE MULTIPLE SCLEROSIS

Een autopsie cohortanalyse

Multiple sclerosis is een chronische inflammatoire ziekte van het centrale zenuwstelsel die vooral voorkomt bij jong volwassenen. De ziekte begint vaak met een relapsing-remitting fase, waarbij patiënten aanvallen van neurologische symptomen hebben die spontaan weer verdwijnen. In de loop van de tijd ontstaan er blijvende neurologische symptomen die geleidelijk verergeren, patiënten komen dan in de progressieve fase van de ziekte. De huidige immunomodulerende therapieën voor MS, die gericht zijn op de circulerende T- en B-cellen, laten vooral een effect zien op het aantal aanvallen in de vroege fase van de ziekte maar ze stoppen niet de progressie van de ziekte in het vergevorderde stadium. Dit heeft geleid tot het concept dat circulerende immuuncellen bijdragen aan het ontstaan van de ziekte in de vroege fase, maar dat deze niet betrokken zijn bij de progressie van de ziekte bij vergevorderde MS. Daarom is de heersende gedachte dat progressie van de ziekte vooral gedreven wordt door neurodegeneratieve pathologische processen en niet zozeer door inflammatie.

In **Deel 1** van deze thesis hebben we ons tot doel gesteld om in autopsiehersenweefsel verschillende cellen van het immuunsysteem die betrokken zijn bij vergevorderde en progressieve MS pathologie te karakteriseren. We hebben deze cellen geanalyseerd in het post-mortem verkregen hersenweefsel van het autopsiecohort van de Nederlandse Hersenbank met in totaal 182 MS hersendonoren met vergevorderde MS. We laten zien dat er substantiële inflammatoire activiteit is in de MS laesies van vergevorderde MS en dat hersenspecifieke weefselresidente T-geheugencellen bijdragen aan de voortdurende inflammatoire activiteit in de MS laesies bij vergevorderde MS.

Bij de meeste MS patiënten verergeren de neurologische symptomen geleidelijk over de tijd, maar de snelheid waarmee en mate waarin de symptomen progressief worden varieert zeer tussen patiënten. Daarnaast zijn er duidelijke geslachtsverschillen in het klinische beloop van de ziekte. Echter, de moleculaire en cellulaire mechanismen die ten grondslag liggen aan deze verschillen tussen MS patiënten zijn grotendeels onbekend. In **Deel 2** van dit proefschrift hebben we ons tot doel gesteld om pathofysiologische mechanismen te identificeren die bijdragen aan de heterogeniteit in de immunopathologie van MS. We hebben de karakteristieken van MS laesies in het autopsiecohort van de Nederlandse Hersenbank gerelateerd aan het klinische beloop van de ziekte, geslachtsverschillen en genetische factoren. We laten zien dat de heterogeniteit in de immunopathologie van vergevorderde MS in een autopsiecohort is geassocieerd met wisselende betrokkenheid van B- en plasmacellen, geslachtsverschillen in de synthese van neurosteroïden en genetische factoren.

DEEL 1 SUBSTANTIËLE INFLAMMATOIRE LAESIE-ACTIVITEIT IN VERGEVORDERDE MS

In **Deel 1** laten we zien dat in vergevorderde en progressieve MS laesies er meer complement deposities zijn en een verhoogd aantal T-cellen, B-cellen en plasmacellen vergeleken met neurodegeneratieve hersenziekten. In de progressieve late fase van MS is er dus substantiële inflammatoire laesie-activiteit die ook correleert met de mate van klinische verergering van de neurologische symptomen. T-cellen die geïsoleerd zijn uit vers hersenweefsel van zowel niet-neurologische controles als van vergevorderde MS hersendonoren hebben een weefselresident geheugen (T_{RM}) profiel en verblijven voornamelijk in de perivasculaire ruimte. De T_{RM}-cellen in vergevorderde MS laesies zijn gereactiveerd en infiltreren in het hersenparenchym. In MS laesies van hersendonoren met vergevorderde MS is er geen infiltratie van niet-T_{RM}-cellen gedetecteerd. Hiermee laten we zien dat $CD8^+$ T_{RM}-cellen betrokken zijn bij de voortdurende inflammatoire en demyeliniserende laesie-activiteit die gezien wordt in vergevorderde MS.

In **Hoofdstuk 2** laten we zien dat in progressieve MS autopsiecasussen 74% van de MS laesies in de hersenstam inflammatoir actief of gemixt actief/inactief zijn. In de gemixt actieve/inactieve laesies zien we een verhoogd aantal $CD8^+$ en $CD4^+$ T-cellen en $CD20^+$ B-cellen en een verhoogde mate van acute axonale schade, mitochondriale stress en axonaal verlies vergeleken met de normaal uitziende MS hersenstam. In de MS hersenstam-laesies en in de hersenstam van donoren met neurodegeneratieve ziekten zijn er deposities van C1q op synapsen en C3d op zowel synapsen als axonen. Specifiek in de MS laesies worden er deposities van het terminale complement component MAC gezien zowel in microglia/macrophagen als op astrocyten. Dit suggereert dat de inflammatoire laesie-activiteit bijdraagt aan de voortdurende demyelinisatie en axonale schade van de hersenstam in progressieve MS.

In **Hoofdstuk 3** hebben we een histologische karakterisatie van 182 MS autopsiecasussen van de Nederlandse Hersenbank uitgevoerd, waarbij er 3188 coupes met in totaal 7562 MS laesies zijn geanalyseerd. De MS casussen hadden een gemiddelde ziekteduur van 28,6 ± 13.3 jaar en 57% van de MS laesies waren actief of gemixt actief/inactief. Daarnaast had 78% van de MS-casussen een gemixt actieve/inactieve MS laesie. Het percentage van gemixt actieve/inactieve laesies en het totale aantal laesies in de hersenstam correleerde met de snelheid van progressie van de neurologische symptomen tijdens het leven. Secundair progressieve en primair progressieve MS casussen lieten een hoger aantal laesies en een hoger percentage van gemixt actieve/inactieve laesies zien en een lager percentage geremyeliniseerde gebieden, vergeleken met de relapsing-remitting casussen. Mannen hadden een hoger percentage van gemixt actieve/inactieve laesies en een hogere incidentie van corticale grijze stof laesies vergeleken met vrouwen. Dit laat zien dat in vergevorderde MS er substantiële inflammatoire laesie-activiteit is die correleert met het klinische beloop van de ziekte.

In **Hoofdstuk 4** hebben we de levende $CD8^+$ en $CD4^+$ T-cellen die verkregen zijn uit post-mortem hersenweefsel van niet-MS hersendonoren gefenotypeerd door middel van flow-cytometrie. We hebben de locatie van deze cellen in het brein geanalyseerd door middel van confocale laserfluorescentie microscopie. T-cellen die geïsoleerd zijn uit het hersenweefsel laten een fenotypisch- en transcriptiefactor-profiel zien dat past bij T_{RM}-cellen. Hersen T_{RM}-cellen kunnen worden onderverdeeld in $CD103^-CD69^+$ en $CD103^+CD69^+$ cellen. CD103 expressie in hersen $CD8^+$ T-cellen correleerde met verminderde expressie van differentiatiemarkers, een verhoogde expressie van tissue-homing chemokine receptoren en een gemiddelde en lage expressie van de transcriptie factoren T-bet en eomes, een verhoogde expressie van PD-1 en CTLA-4 en een lage expressie van cytolytische enzymen met een behouden polyfunctionaliteit na activatie. Hersen $CD4^+$ T-cellen laten ook de T_{RM}-cel-geassocieerde markers zien, maar ze hebben een lage expressie van CD103. We concluderen dat het humane brein wordt gesurveilleerd door T_{RM}-cellen en beredeneren dat deze cellen zorgen voor bescherming tegen neurotrope virus re-activatie terwijl ze onder strakke controle staan van de immuun checkpoint-moleculen.

In **Hoofdstuk 5** hebben we T-cellen bestudeerd in relatie met de inflammatoire laesie-activiteit in vergevorderde MS. Voor deze studie hebben we een histologische analyse uitgevoerd van T-cellen en perivasculaire clusters van T-cellen in het autopsiecohort van de Nederlandse Hersenbank. Daarnaast hebben we levende $CD8^+$ T-cellen geïsoleerd uit vers autopsieweefsel van subcorticale witte stof MS laesies, normaal uitziende witte stof van MS en witte stof van controle hersendonoren. Deze hebben we bekeken door middel van flow-cytometrie. In actieve en gemixt actieve/inactieve laesies was het aantal T-cellen verhoogd vergeleken met de normaal uitziende witte stof. Actieve en gemixt actieve/inactieve laesies waren verrijkt met zowel $CD4^+$ als $CD8^+$ T-cellen, waarbij de laatste het meest aanwezig waren in alle MS laesie-subtypes. Perivasculaire clustering van T-cellen werd gevonden in de casussen met een progressief ziektebeloop en dit correleerde met een hoger percentage gemixt actieve/inactieve laesies en een hoger aantal laesies in de hersenstam vergeleken met casussen zonder perivasculaire clusters. In gemixt actieve/inactieve laesies werden de $CD8^+$ T-cellen meer gevonden in het hersenparenchym vergeleken met de normaal uitziende witte stof. $CD8^+$ T-cellen van gemixt actieve/inactieve laesies lieten een weefselresident geheugen fenotype zien met expressie van CD69, CD103, CD44, CD49a en PD1 en afwezigheid van S1P1. Ze lieten verhoging zien van homing-markers (CXCR6), re-activatie (Ki67), en cytotoxiciteit (GPR56), maar hadden geen granzyme-B. In casussen met vergevorderde MS is de inflammatoire laesie-activiteit en het aantal gedemyeliniseerde laesies geassocieerd met een verhoogd aantal T-cellen die clusteren in de perivasculaire ruimte. Dit zijn $CD8^+$ weefselresidente T-geheugencellen, die tekenen van re-activatie laten zien en ook infiltreren in het hersenparenchym.

DEEL 2 HETEROGENITEIT IN DE IMMUNOPATHOLOGIE VAN VERGEVORDERDE MS

In **Deel 2** hebben we de heterogeniteit en geslachtsverschillen in de immunopathologie van MS bestudeerd. We hebben verschillende benaderingen gebruikt om de immunopathologie van MS te analyseren met als doel het identificeren van pathofysiologische mechanismen die een rol spelen bij de verschillen in het klinische beloop tussen MS patiënten.

In **Hoofdstuk 6** hebben we het klinische en pathologische profiel van MS autopsiecasussen gerelateerd aan de mate van B- en plasmacel infiltratie. We hebben een systematische karakterisatie uitgevoerd van de aanwezigheid van $CD20^+$ B-cellen en $CD138^+$ plasmacellen in de hersenstam en witte stof MS laesies in zowel het MS autopsiecohort van de Nederlandse Hersenbank als in vroege MS laesies die zijn verkregen uit hersenbiopten. B-cellen werden vooral gezien in de perivasculaire ruimte en de meninges en waren verrijkt in de actieve en gemixt actieve/inactieve MS laesies. In 34% van de actieve en in 71% van de gemixt actieve/inactieve MS laesies waren er geen B cellen aanwezig. De afwezigheid van B- en plasmacellen in de hersenstam en witte stof laesies was geassocieerd met een langere ziekte duur, het minder vaak voorkomen van een secundair progressief beloop en een lager percentage van gemixt actieve/inactieve laesies. We hebben in het autopsiecohort extreme casussen geselecteerd en laten daarmee zien dat de IgG ratio lager was en dat de liquor oligoclonale banden vaker afwezig waren in de MS casussen zonder B- en plasmacellen. In een klinisch MS cohort was het aantal MS patiënten zonder oligoclonale banden in de liquor verhoogd na een gemiddelde ziekteduur van 11,3 jaar. De afwezigheid van B-cellen is geassocieerd met een gunstiger klinisch en pathologisch profiel. Deze bevindingen wijzen op extreme uitersten van een continuüm van genetische- en omgevingsfactoren die de immunopathologie beïnvloeden, maar kunnen ook wijzen op regressie van witte stof humorale immunopathologie in het natuurlijk beloop van vergevorderde MS.

In **Hoofdstuk 7** stelden we onszelf tot doel om pathofysiologische mechanismen te identificeren die betrokken zijn bij de geslachtsverschillen in de ontwikkeling van corticale MS laesies. In hoofdstuk 2 beschreven we dat in post-mortem autopsieweefsel mannen een hogere incidentie van corticale grijze stof laesies hebben. Eerder werden er al geslachtsverschillen in progesteronsignalering beschreven in witte stof MS laesies. Daarom hebben we geanalyseerd of geslachtsverschillen in de synthese en signalering van progestagenen en androgenen steroïden een mogelijk neuroprotectief effect hebben in de corticale grijze stof. We hebben een standaard uitgenomen corticale grijze stof regio geselecteerd en hebben vriesweefsel van mannen en vrouwen van zowel MS als controles met kwantitatieve PCR geanalyseerd. In de standaard uitgenomen superieure temporale gyrus hadden mannen vaker een leukocorticale laesie vergeleken met vrouwen. De neuro-steroidogenen enzymen STS, AKR1C1 en AKR1C2 laten een verhoogde expressie zien in de normaal uitziende cortex van vrouwen met MS terwijl dit niet verhoogd was in mannen met MS vergeleken met niet-neurologische controles. CD8 en IFNG mRNA expressie was verhoogd in

normaal uitziende cortex van mannen met MS terwijl dit niet verhoogd was in normaal uitziende cortex van vrouwen met MS vergeleken met niet neurologische controles. Dit suggereert dat in de normale corticale grijze stof van vrouwen met MS allopregnanolon- en 3alphaDIOL-synthese is geïnduceerd maar niet in de normale corticale grijze stof van mannen met MS, terwijl CD8 en interferon gamma expressie verhoogd is in mannen met MS maar niet in vrouwen met MS. Dit kan bijdragen aan de verhoogde gevoeligheid van mannen voor het ontwikkelen van leukocorticale MS laesies.

In **Hoofdstuk 8** stelden we onszelf tot doel om genotypes die eerder geassocieerd zijn met de snelheid van klinische progressie in MS te vertalen naar pathogene mechanismen die betrokken zijn bij de pathogenese van MS laesies. We hebben 179 MS hersendonoren van het MS autopsiecohort van de Nederlandse Hersenbank gegenotypeerd voor 102 SNPs die geselecteerd zijn op basis van hun associatie met klinische uitkomsten in eerdere GWAS studies of hun associatie met MS-pathologie-geassocieerde genen. Na correctie voor multiple-testing zijn er drie SNPs die eerder gelinkt zijn aan de progressie van neurologische uitval in MS significant geassocieerd met het percentage actieve laesies of het percentage van gemixt actieve/inactieve laesies. Drie SNPs die gelinkt zijn aan MS-pathologie-geassocieerde genen lieten een significante associatie zien met het percentage actieve laesies, de incidentie van corticale grijze stof laesies of het percentage geremyeliniseerde gebieden. Daarbij lieten de rs2234978 T-alleldragers verhoogde genexpressie levels van FAS zien in zowel peri-vasculaire T-cellen als peri-lesionale oligodendrocyten, beiden celtypes die betrokken zijn bij de MS laesie formatie. Hier laten we zien dat door de combinatie van de pathologische karakterisatie van het MS autopsiecohort met genetica we een begin kunnen maken met het vertalen van genotypes gelinkt aan de klinische progressie van MS naar cellulaire mechanismen betrokken in MS laesie pathogenese.

We concluderen dat vergevorderde progressieve MS wordt gekarakteriseerd door substantiële inflammatoire laesie-activiteit die correleert met de klinische progressie van de ziekte. Dit suggereert dat inflammatoire laesie-activiteit is betrokken bij de klinische progressie van de ziekte in vergevorderde MS. Hersenspecifieke weefselresidente T-geheugencellen die onder niet-inflammatoire condities verblijven in de perivasculaire ruimte worden in MS witte stof laesies gereactiveerd en infiltreren in het hersenparenchym bij vergevorderde en progressieve MS. Deze observaties suggereren dat residente immuuncellen in het brein, en dus niet zo zeer circulerende immuuncellen, bijdragen aan de voortdurende inflammatoire laesie-activiteit in vergevorderde MS. Tot slot hebben we de heterogeniteit in de immunopathologie van MS in een autopsiecohort geanalyseerd in relatie met het klinische beloop, het geslacht en genetische factoren. Daarmee hebben we pathofysiologische mechanismen geïdentificeerd die potentieel bijdragen aan de heterogeniteit en geslachtsverschillen in het klinische beloop van MS patiënten.

AUTHOR CONTRIBUTIONS

Chapter 2

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Nina L. Fransen	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; acquisition of data; analysis and interpretation of data; drafting/ revision of the manuscript
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Chapter 3

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Nina L. Fransen *	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept or design; acquisition of data; analysis and interpretation of data; drafting/ revision of the manuscript
Corbert G. van Eden	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept or design; acquisition of data; interpretation of data
Valeria Ramaglia	Department of Immunology, University of Toronto, Toronto, Canada Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept or design; acquisition of data; interpretation of data; revision of the manuscript
Matthew Mason #	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Statistical analysis and interpretation of data; drafting/ revision of the manuscript
Inge Huitinga #	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands; Erasmus Medical Center, Rotterdam, The Netherlands	Study concept and design; acquisition of data; interpretation of data; revision of the manuscript

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Matthew R.J. Mason	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Analysis and interpretation of data
Jörg Hamann *	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands Department of Experimental Immunology, Amsterdam Infection & Immunity Institute, Amsterdam UMC, Amsterdam, The Netherlands	Study concept and design; analysis and interpretation of data; revision of the manuscript
Joost Smolders *	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands ErasMS, Erasmus Medical Center, Rotterdam, The Netherlands	Study concept and design; acquisition of data; analysis and interpretation of data; drafting/ revision of the manuscript
Inge Huitinga *	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; analysis and interpretation of data; revision of the manuscript

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Chapter 6

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Jörg Hamann, Ph.D.	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands Department of Experimental Immunology, Amsterdam Infection & Immunity Institute, Amsterdam UMC, Amsterdam, The Netherlands	Analysis and interpretation of data; drafting/revision of the manuscript
Inge Huitinga, Ph.D.	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands	Study concept and design; analysis and interpretation of data; drafting/revision of the manuscript
Joost Smolders, M.D., Ph.D.	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands ErasMS, Erasmus Medical Center, Rotterdam, The Netherlands	Study concept and design; analysis and interpretation of data; drafting/revision of the manuscript

Chapter 7

Name	Location	Contribution
Nina L. Fransen	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; acquisition of data; analysis and interpretation of data; drafting/ revision of the manuscript
Sabina Luchetti	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; interpretation of data; revision of the manuscript
Matthew Mason	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Analysis and interpretation of data; revision of the manuscript
Inge Huitinga	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; interpretation of data; revision of the manuscript

Name	Location	Contribution
Nina L. Fransen	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; acquisition of data; analysis and interpretation of data; drafting/ revision of the manuscript
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Sabina Luchetti	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Acquisition of data
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Jörg Hamann	Department of Experimental Immunology, Amsterdam Infection & Immunity Institute, Amsterdam UMC, Amsterdam, The Netherlands Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study design; analysis and interpretation of data
Matthew R.J. MAson	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; statistical analysis; interpretation of data; revision of the manuscript
Inge Huitinga	Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands	Study concept and design; acquisition of data; analysis and interpretation of data; revision of the manuscript

LIST OF PUBLICATIONS

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Progressive multiple sclerosis patients show substantial inflammatory lesion activity that correlates with clinical disease severity and sex: a retrospective autopsy cohort analysis
Acta Neuropathol. 2018 Apr;135(4):511-528
Luchetti S*****, **Fransen NL***, van Eden CG, Ramaglia V, Mason M, Huitinga I

Tissue resident memory T cells populate the human brain
Nat Commun. 2018 Nov 2;9(1):4593
Smolders J*****, Heutink KM*****, **Fransen NL***, Remmerswaal EBM, Hombrink P, Ten Berge IJM, van Lier RAW, Huitinga I, Hamann J

Post-mortem multiple sclerosis lesion pathology is influenced by single nucleotide polymorphisms
Brain Pathol. 2020 Jan;30(1):106-119
Fransen NL, Crusius JBA, Smolders J, Mizee MR, van Eden CG, Luchetti S, Remmerswaal EBM, Hamann J, Mason MRJ, Huitinga I

Tissue resident memory T cells invade the brain parenchyma in multiple sclerosis white matter lesions
Brain. 2020 June; 143(6): Pages 1714–1730
Fransen NL, Hsiao C, van der Poel M, Engelenburg HJ, Verdaasdonk K, Vincenten MCJ, Remmerswaal EBM, Kuhlmann T, Mason MRJ, Hamann J*****, Smolders J*****, Huitinga I*****

Reply: Tissue- resident $CD8^+$ memory T cells in multiple sclerosis
Brain. 2021 Jan; 144(1): e8
Smolders J, **Fransen NL**, Huitinga I, Hamann J

Absence of B cells in brainstem and white matter lesions associates with a less severe disease and absence of oligoclonal bands in multiple sclerosis
Neurol Neuroimmunol Neuroinflamm; 2021 Mar; 8 (2): e955
Fransen NL, De Jong B, Vincenten MCJ, Heß K, Kuhlmann T, Hamann J, Huitinga I*****, Smolders J*****

Perivascular tissue-resident memory T cells as therapeutic target in multiple sclerosis
Expert Review of Neurotherapeutics, 2020, 20:8, 835-848
Smolders J, **Fransen NL**, Hsiao C-C, Hamann J., Huitinga I

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Neurofilament light chain CSF levels correlate with inflammatory lesion activity and acute axonal damage in multiple sclerosis
Under revision
Van den Bosch AMR*, **Fransen NL***, Mason MRJ, Rozemuller A, Teunissen C, Smolders J, Huitinga I

White matter lesions in multiple sclerosis are enriched for $CD20^{dim}$ $CD8^{+}$ tissue-resident memory T cells
Eur J Immunol. 2020 Sep 19
Hsiao C, **Fransen NL**, Huitinga I, Hamann J, Smolders J

An inflammatory landscape for pre-operative neurologic deficits in glioblastoma
Front Genet. 2019 Jun 4;10:488
Katrib A, Jeong HH, **Fransen NL**, Henzel KS, Miller JA

Temporal dynamics of hippocampal neurogenesis in chronic neurodegeneration
Brain. 2014 Aug;137(Pt 8):2312-28
Gomez-Nicola D, Suzzi S, Vargas-Caballero M, **Fransen NL**, Al-Malki H, Cebrian-Silla A, Garcia-Verdugo JM, Riecken K, Fehse B, Perry VH

Regulation of microglial proliferation in chronic neurodegeneration
J Neurosci 2013 Feb 6;33(6):2481-93
Gomez-Nicola D, **Fransen NL**, Suzzi S, Perry VH

Association of vascular factors with apathy in community dwelling elderly individuals
Arch Gen Psychiatry. 2012 Jun;69(6):636-42
Ligthart SA, Richard E, **Fransen NL**, Eurlings LS, Beem L, Eikelenboom P, van Gool WA, Moll van Charante EP

Erfelijke prionziekten in Nederland: klinisch beeld, incidentie en risicodragers
Tijdschrift voor Neurologie en Neurochirurgie. 2012 Jun; 113(3):115-121
Fransen NL, Jansen C, Rozemuller AJM, van Duijn CM, van Gool WA

PhD PORTFOLIO

1. PhD training	Year	Workload (ECTS)
General courses		
• Introduction course ONWAR	2015	0,9
• Project Management LUMC	2015	1.5
• Grant Writing	2019	1.8
Specific courses		
• Practical Biostatics Course E-Learning AMC	2016	1.4
• Computing in R	2016	0,4
• Functional neuroanatomy (ONWAR)	2017	1.5
• Summer School Systems Genetics of Neurodegeneration, Frauenchiemsee, Bavaria, Germany	2017	1.9
• Teaching Courses ECTRIMS London and Paris	2016, 2017	0.2
• International Congress of Neuroimmunology/ISNI Jerusalem Teaching course	2016	0.3
• International Congress of Neuropathology Tokyo Educational Course	2018	0,3
Seminars, workshops and master classes		
• Annual ONWAR meetings	2015–2019	3,6
• Swammerdam lectures ONWAR	2015–2019	0,7
Oral presentations		
• ECTRIMS Stockholm: Post-mortem MS lesion pathology is influenced by single nucleotide polymorphisms	2019	0,5
• ECTRIMS Stockholm: T_{RM} cells in advanced MS white matter lesions	2019	0,5
• MS research day's: Post-mortem MS lesion pathology is influenced by single nucleotide polymorphisms	2019	0,5
• MSIF Global Networking Meeting Amsterdam: the Netherlands Brainbank for MS	2018	0,5
Poster presentations		
• ECTRIMS London: Post-mortem MS lesion pathology is influenced by single nucleotide polymorphisms	2016	0,5
• International congress of Neuroimmunology Jerusalem: Post-mortem MS lesion pathology is influenced by single nucleotide polymorphisms	2016	0,5
• ECTRIMS Paris: The analysis of MS lesion pathology in the progressive Netherlands Brain Bank autopsy cohort	2017	0,5
• The Lancet Summit Barcelona: perivascular cuffing of T cells is related to chronic MS lesion activity in progressive MS cases: a retrospective autopsy cohort analysis	2018	0,5
• International congress of Neuropathology Tokyo: The analysis of MS lesion pathology in the progressive Netherlands Brain Bank autopsy cohort	2018	0,5
• ECTRIMS Stockholm: Immunopathology of MS brainstem	2019	0,5
(Inter)national conferences		
• MS Research days	2015–2019	1.0
• ECTRIMS 2016, 2017, 2019	2016–2019	3.0
• International congress of Neuroimmunology, Jeruzalem	2016	1.0
• The Lancet Summit: Inflammation and Immunity in disorders of the brain and mind, Barcelona	2018	1.0
• International congress of Neuropathology, Tokyo	2018	1.0

PhD portfolio (continued)

1. PhD training	Year	Workload (ECTS)
Other		
• Visiting Neuropathology department, University of Göttingen: collaboration with Wolfgang Brück	2016	5
• Visiting Institute for Neuropathology, University of Münster: collaboration with Tanja Kuhlmann	2019	0.6
2. Teaching		
Lecturing		
• Lecture at auto-immunity symposium for honours program pharmaceutical sciences University of Utrecht; Trm cells in MS lesion pathology	2019	0,5
• Lecture Master Neuroscience UvA 'From cell to Behaviour'; The analysis of MS lesion pathology in the progressive Netherlands Brain Bank autopsy cohort	2019	0,5
• Lecture at Kenniscafe: wetenschappers over de 2020's organisation by de Balie, KNAW and de Volkskrant	2019	0,5
Supervising		
• Wendelien Bergmans (master internship)	2015	2
• Chaimae Chomrikh (master thesis)	2015	1
• Myrna Brandt (master thesis)	2016	0.6
• Louise Pierneef (master internship)	2016	2
• Soraya van Eten (bachelor internship)	2017	2
• Berfin Gulave (master thesis)	2017	1
• Jeen Engelenburg (bachelor internship)	2018	2
• Sophie Jacobs (master thesis)	2018	1
• Kim Verdaasdonk (bachelor internship)	2019	2
• Kristina Salontaij (master internship)	2019	2
3. Parameters of Esteem		
• Selected for summer school systems genetics in neurodegeneration – travel and accommodation grant	2017	
• ECTRIMS Paris travel grant	2017	
• Best poster presentation International congress of Neuropathology, Tokyo	2018	
• ECTRIMS Stockholm travel grant	2019	
• Best oral presentation ECTRIMS Stockholm Young Investigator Award	2019	

DANKWOORD

Er zijn veel mensen die ik wil bedanken voor hun bijdrage aan mijn promotietraject en aan de totstandkoming van dit proefschrift.

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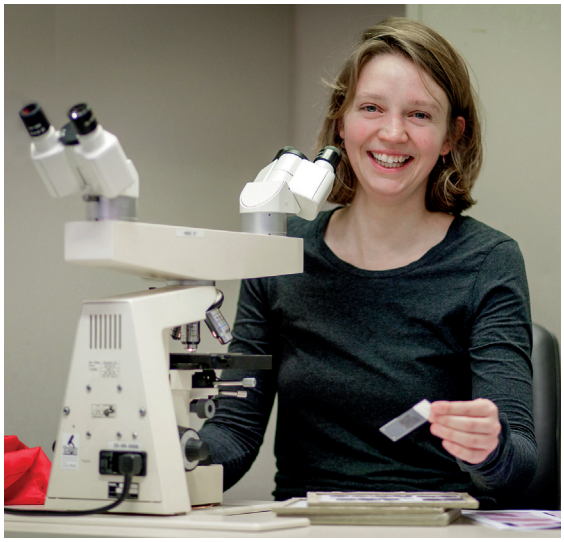
ABOUT THE AUTHOR

Nina Louise Fransen was born in Amsterdam on September 2nd in 1989. After completing secondary school at the Veluws College in Apeldoorn in 2007, she started studying Medicine at the University of Amsterdam. As part of the University of Amsterdam's honours programme, Nina conducted several research projects on neurodegeneration under supervision of prof. W.A. van Gool. She did a research internship of 6 months in the neuroimmunology group of prof. V.H. Perry, supervised by dr. D. Gomez at the University of Southampton, performing experiments to study microglial cell proliferation. She received her medical degree in 2014 and started working as a ANIOS neurology at the Onze Lieve Vrouwe Gasthuis in Amsterdam under supervision of prof. P. Portegies.

In 2015, she started her PhD project at the Netherlands Institute for Neuroscience in the neuroimmunology group under supervision of prof. I. Huitinga and dr. J. Smolders. She visited the department of neuropathology in the University Medical Center Göttingen in 2016 where she performed experiments to study axonal degeneration in MS and where she examined the MS biopsy lesion collection. In 2017 she was selected to participate in the residential summer school systems genetics in neurodegeneration, at Frauenchiemsee in Germany, were she learned to analyze online available genetic data. In 2018, she won the Best Poster Award at the International Conference for Neuropathology (ICN) in Tokyo and in 2019 she received the Best Oral Young Investigator Award for her presentation at the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) in Stockholm. Nina is currently a clinical resident in pathology at the University Medical Centre in Utrecht under the supervision of prof. P. van Diest and prof. M. van Dijk.



Nina lives in Amsterdam with Olivier Frinking and their two children, Kiera (2016) and Kalle (2020).