Heterogeneity of the immunopathology in advanced multiple sclerosis

An autopsy cohort analysis

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Discussion

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PART 1 SUBSTANTIAL INFLAMMATORY LESION ACTIVITY IN ADVANCED MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is 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.
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). For instance, teriflunomide disables clonal expansion of lymphocytes, fingolimod prevents immune cells from leaving the lymph nodes, and natalizumab inhibits attachment of immune cells to the endothelium. Circulating T and B cells are depleted by drugs as cladribine, alemtuzumab, and ocrelizumab. MS treatment with autologous hematopoietic stem cell
transplantation also depends on the depletion of circulating lymphocytes and their precursors in the host.\textsuperscript{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.\textsuperscript{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.\textsuperscript{20} This observation led to the proposition of four distinct pathological patterns in early MS.\textsuperscript{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.\textsuperscript{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.\textsuperscript{4} In patients with longstanding relapsing–remitting MS, gadolinium-enhanced lesions become less prevalent when compared to people early in their disease.\textsuperscript{23} In primary progressive MS, gadolinium-enhancing lesions were only found in early phases and markedly declined during 5-years follow-up.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{28–32} The analysis of MS brainstem lesions from a small number of progressive MS patients in \textbf{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\textsuperscript{+} 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.\textsuperscript{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\textsuperscript{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 Alzheimer’s disease,\textsuperscript{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. \textbf{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 \textbf{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.\textsuperscript{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\%).\textsuperscript{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.\textsuperscript{38} A longer disease duration between diagnosis and autopsy in post-mortem studies is a marker of a less severe disease course.\textsuperscript{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\%).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{41} it is far more extensive in progressive MS.\textsuperscript{42} In primary progressive MS, Choi et al. reported a proportionally larger cortical area to be demyelinated, when compared to white matter.\textsuperscript{43} Active cortical demyelination has been associated with the formation of follicle-like inflammatory structures in the overlying meninges.\textsuperscript{43–45} These structures contain T-cell, B-cell, and plasma cell zones, which resembles tertiary lymphoid structures.\textsuperscript{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.\textsuperscript{43,45} Progressive MS is also characterized by more diffuse instead of focal changes in the normal-appearing white matter.\textsuperscript{42} However, the persisting relevance of focal white matter lesions in advanced 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.
lymph node homing (CCR7), and express molecules associated with tissue residency (CD49A, CD69, CD103, CTLA-4, PD-1). Functionally, post-mortem human brain T\textsubscript{RM} cells produced low levels of granzyme B but detectable amounts of granzyme K directly \textit{ex situ} and made lots of interferon (IFN)\textsubscript{γ} and tumor necrosis factor (TNF) upon reactivation \textit{in vitro}. Since T\textsubscript{RM}-cell populations in other tissues have been described to arise after exposure to a wide range of viral or bacterial antigens,\textsuperscript{53} we reasoned the common human brain T\textsubscript{RM} cells to be most likely directed against

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\caption{Flow-cytometric analysis of brain CD8\textsuperscript{+} T\textsubscript{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\textsubscript{RM} cell-associated surface markers on CD8\textsuperscript{+} T cells isolated from blood, MS normal-appearing white matter (NAWM), and an active MS lesion.\textsuperscript{50,75}}
\end{figure}
common neurotropic viruses. In experimental models of neurotropic virus infections, populations of specific T<sub>RM</sub> cells are generated. The dominant localization of human brain T<sub>RM</sub> cells inside the PVS is a marked difference compared to the distribution of CD8<sup>+</sup> T<sub>RM</sub> cells in other tissues, where these cells are scattered through the tissue. This tissue organization of T<sub>RM</sub> 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<sup>+</sup> T cells are more prevalent than CD8<sup>+</sup> T cells and show a contrasting T<sub>CM</sub>-cell phenotype, including expression of CCR7. The small population of CSF CD8<sup>+</sup> T cells was also reported to display a T<sub>CM</sub>-cell phenotype. These CSF T-cell populations have been argued to enter the CSF via the choroid plexus and meningeal vessels. How and whether these CSF T-cell population relate to the development and maintenance of white matter PVS T<sub>RM</sub>-cell populations is not known. Additionally, whether T cells in meninges and choroid plexus also display a TRM<sup>-</sup>-cell profile has to our knowledge not been extensively studied.

4 THE PVS AS A PHYSIOLOGICAL T<sub>RM</sub>-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. These basement membranes are made of extracellular matrix molecules, including laminin, fibronectin, and collagen type IV. 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. The PVS plays a crucial role in the drainage of the suggested CNS flow of interstitial fluid, 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. Although disputed, the presence of perivascular macrophages has been reported in the PVS of white matter venules. The compartmentalization of T<sub>RM</sub> cells in the PVS could be mediated by their signature surface markers. CD69 interferes with sphingosine 1-phosphate receptor 1 (S1P<sub>1</sub>) to prevent tissue egress, CD49a is a receptor for collagen type IV, and the T<sub>RM</sub>-associated molecule CD44 is a receptor for laminin. Finally, a ligand for CD103 is E-cadherin, which has been described on activated CD103<sup>+</sup> lymphocytes, enabling cluster formation. Interaction of these receptors with their ligands may mediate the homing and clustering of brain T<sub>RM</sub> 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<sub>RM</sub> cells. Moreover, activated astrocytes may present the inhibitory programmed death-1 (PD-1) ligand PD-L1 to the T<sub>RM</sub> cells via their end-feet at the glia limitans.
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. On average 2-6 times as many CD3⁺ T lymphocytes were encountered in MS normal-appearing white matter when compared to control white matter. 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. Perivenular infiltrates, believed to contain infiltrating lymphocytes from the circulation, have been identified in white matter of both acute and chronic MS cases. 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. Frischer et al. observed perivascular cuffs only in cases with active progressive disease. 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. 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. 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. Interestingly, the ratio of CD8/CD4 T cells was remarkably consistent within a donor between regions investigated. 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). 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. This was not only the case in relapsing and secondary progressive cases but also in primary progressive cases.

6 MS WHITE MATTER LESIONAL T CELLS HAVE A T\textsubscript{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. Several studies support a phenotypic profile consistent with T\textsubscript{Rm} cells, although there are several contrasting observations. Lesional T cells stained in autopsy cases lacked the
**T**

**EM** cell-associated recirculation marker S1P. Machado-Santos et al. reported absence of the lymph node homing receptor CCR7 on lesional T cells. CD69 expression has been described by van Nierop et al. using immunostaining and was confirmed by our group on all lesional T cells using flow cytometry, yet was not found with immunohistochemistry by Machado-Santos et al. 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. In our flow-cytometric studies, a sub-population of lesional T RM cells expressed CD103. 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. 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. Previously, we showed the ligand for CXCR6, CCL16, to be upregulated by macrophages in the rim of mixed active/inactive lesions. 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. 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. 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. 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. 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. With flow cytometry, Ki-67 expression was higher in CD8\(^+\) T RM cells isolated from MS white lesions, compared to control white matter. 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. These HLA-DR-positive cells double stained both with CD20 (B cells) and CD163 (perivascular macrophages). An increased number of CD163\(^+\) HLA-DR\(^+\) perivascular macrophages has been reported in active MS white matter lesions, in close association with perivascular T cells. B cells have a well-characterized capacity of antigen uptake and MHC-dependent presentation, 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, 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. 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. 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%). Salou et al., reported infiltration of granzyme B-positive CD8+ T cells in white matter lesions, but showed no quantification. 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. Additionally, flow-cytometric analysis of isolated CD8+ TRM 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+ TRM cells to upregulate the adhesion family G protein-coupled receptor GPR56, which on circulating lymphocytes indicates cytotoxic capacity. It is uncertain whether non-circulating GPR56-positive CD8+ TRM cells bear cytolytic activity in the PVS and parenchyma. Human brain CD8+ T cells expressed in our studies almost no perforin, although this lytic mediator is important in the control of neurotropic virus infections by brain TRM cells in animal models. 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. Konjevic Sabolek et al. reported immunostaining for perforin in white matter lesional CD8+ T cells of several cases with acute but also progressive MS. Brain CD4+ and CD8+ TRM cells did express granzyme K in our earlier studies. 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. 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. Furthermore, production of cytokines is well possible, since human brain CD4+ and CD8+ TRM cells rapidly make IFNy, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF upon stimulation in vitro. Production of IFNy by brain TRM cells is a critical component in the control of neurotropic virus infections. However, cytokine production by white matter lesional T cells in situ has not yet been investigated.
WHITE MATTER T<sub>RM</sub> CELLS AS POTENTIAL TARGETS FOR MS THERAPIES

The events leading to the establishment of T<sub>RM</sub> cell-containing perivascular cuffs in MS are not known. These populations of T<sub>RM</sub> 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. Notably, the study by Machado-Santos et al. included also some donors with a fairly short disease duration, suggesting that population of the PVS by T<sub>RM</sub> 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.

Since a high relapse rate and gadolinium-enhancing lesions are risk factors for developing progressive disease, a timely intervention on these endpoints could potentially reduce T<sub>RM</sub>-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, and the efficacy of early treatment with ocrelizumab in delaying disability progression in primary progressive MS. Therefore, early treatment with DMTs could theoretically prevent the establishment or maintenance of perivascular T<sub>RM</sub>-cell cuffs in the course of MS. It is unlikely that current DMTs affect the mobilization of perivascular T<sub>RM</sub> 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, the absence of S1P<sub>1</sub>-receptor expression on T<sub>RM</sub> cells and the migration of parenchyma-invading lymphocytes away from the sphingosine phosphate gradient makes a relevant functional interference of this drug with T<sub>RM</sub> cell-migratory behavior unlikely. Metz et al. observed only very few CD8<sup>+</sup> 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. Of note, besides a major role for local homeostatic proliferation, recruitment of memory T cells from the circulation to contribute to secondary T<sub>RM</sub> cells has been described. Via interference with this replenishment, DMTs could potentially reduce the sustainability of the PVS T<sub>RM</sub>-cell pool. Since reactivation, proliferation, and mobilization of brain T<sub>RM</sub> 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. During treatment with the PD-1 inhibitor nivolumab, white matter T-cell infiltration with demyelination and macrophage activation has been described. Also treatment with the PD-1 inhibitor pembrolizumab resulted in inflammatory demyelinating lesion of the CNS. 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. 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. 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, 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. 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. CXCR3 is a core phenotypic T_{RM}-cell marker, which is also expressed at high levels on brain T_{RM} cells. In the murine skin, lack of CXCR3 expression in CD8\(^+\) T cells was associated with a reduced T_{RM}-cell formation. 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. CXCR3 has several ligands; CXCL4 is expressed by microglia, and CXCL9, CXCL10, and CXCL11 have been associated with the infiltration of the CNS by T cells in various inflammatory diseases including MS. 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 TRM-cell response is mounted. In other tissues, TRM cells have been mostly studied in and associated with virus infections.\textsuperscript{53,54} Therefore, a viral antigen appears tempting. A vast body of literature associates MS with Epstein-Barr virus (EBV) infection.\textsuperscript{117} Accumulation of EBV-infected B cells and EBV-directed CD8\textsuperscript{+} T cells has been described in MS CSF\textsuperscript{118–120} and in MS lesions,\textsuperscript{85,94,121–123} although the reproducibility of these findings has also been debated.\textsuperscript{124–126} Other viruses have also been associated with MS, including human herpesvirus (HHV)-6.\textsuperscript{127} Moreover, expression of endogenous retrovirus sequences has been described in MS lesions,\textsuperscript{85} which may also elicit a CD8\textsuperscript{+} T-cell response.\textsuperscript{129} Alternatively, a potential role of autoantigen-directed TRM cells in autoimmune diseases has not been explored extensively yet.

Since brain CD4\textsuperscript{+} and CD8\textsuperscript{+} TRM cells are physiological residents of the normal human PVS,\textsuperscript{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.\textsuperscript{130} Therefore, a role of physiological TRM-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,\textsuperscript{131} post-mortem human studies also revealed JC virus genetic fragments in brains of 28-68\% of asymptomatic cases.\textsuperscript{132–134} The latter observations suggest 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.\textsuperscript{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 TRM-cell populations as they are encountered in MS. We have limited data on the presence of TRM cells in white matter lesions at the early stages of MS, as well as their association with pathological patterns of early MS. Cells with TRM cell-related characteristics have been observed in the blood and CSF of people with MS, as indicated by the enhanced presence of circulating CD4\textsuperscript{+} T cells expressing high levels of CCR5 and granzyme K.\textsuperscript{96} Additionally, clonally expanded CD8\textsuperscript{+} T cells with TRM-cell characteristics could be isolated from the CSF of twins with prodromal MS.\textsuperscript{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<sub>RM</sub> 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<sub>RM</sub> cells in correlation with these lesions suggests an active role of CD8<sup>+</sup> T<sub>RM</sub> cells in lesion formation and/or maintenance. Further understanding of the functional dynamics of brain T<sub>RM</sub> 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<sub>RM</sub> cells are a subject of study in many organs. Although a role in the control of (viral) infections is best consolidated,<sup>53,54</sup> a contribution of T<sub>RM</sub> cells to local inflammatory reactions in autoimmune diseases has not been extensively explored. Certainly, T<sub>RM</sub> 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<sub>RM</sub> 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<sub>RM</sub> cells. Likely, myelin-laden microglia/macrophages in mixed active/inactive lesions are particularly important. They not only may provide signals critical for T<sub>RM</sub>-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.<sup>11,37</sup>

A challenge for therapies directly targeting brain T<sub>RM</sub> 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<sub>RM</sub> cells associated with MS lesions. In the end, modulating T<sub>RM</sub>-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.
MS is a heterogeneous disease regarding clinical disease course, radiological appearance of lesions and response to immunomodulatory therapies.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{19,141,142} and absence\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \textbf{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.\textsuperscript{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. 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. 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. Interestingly, males develop MS at an older age and they faster reach a more severe disability score. Male MS patients show a higher incidence of cognitive decline compared to females. MRI studies revealed more destructive white matter lesions and more often cortical grey matter lesions in males compared to females. Indeed, we report in Chapter 2 that males showed more ongoing inflammatory and demyelinating lesion activity in the white matter and a higher incidence of cortical grey matter lesions compared to females.

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. A history of multiple pregnancies is also associated with a decreased risk of a first demyelinating event. 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. 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. 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.
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. 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. 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. Increased expression of glutamate receptors and transporters has been shown in MS cortical grey matter compared to controls. 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. Since allopregnanolone and 3-α-DIOL are expected to alter neurotransmitter expression and release, 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 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. 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. 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. GABA-A agonists have been reported to inhibit antigen specific T cell proliferation, 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, suggests that supplementation with progesterone or allopregnanolone in MS patients might be effective in preventing ongoing inflammatory lesion activity and cortical demyelination and thereby efficacying 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. 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. 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. 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). 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. 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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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\textsuperscript{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.\textsuperscript{177} Since FAS, which functions as an apoptosis receptor,\textsuperscript{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.\textsuperscript{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\textsuperscript{+} T cells and its expression is highest in regulatory T (Treg) cells in both blood and brain, in line with previous findings.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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.\textsuperscript{199} The short carbohydrate domain of CLEC16A, is predicted to be inactive suggesting a non-classical C-type lectin function.\textsuperscript{200} In macrophages it is shown that CLEC16A serves as a direct regulator of the HLA-II antigen-presentation pathway.\textsuperscript{200} In addition, it has been suggested that CLEC16A is also linked to the antigen-presentation pathway in B-cells.\textsuperscript{201,202} In early MS, T cells are triggered by memory B cells\textsuperscript{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\textsubscript{RM} cells.\textsuperscript{50,51,167} In progressive MS lesions, we encountered reactivated T\textsubscript{RM} cells in close contact with B cells and perivascular macrophages, which suggests that antigen-presenting cells reactivate T\textsubscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{202} Altogether, this work raises the hypothesis that
the protective effect of rs8056098 on disease progression can be explained by reduced antigen presentation and transmigration capacities of B-cells, resulting in impaired TRM 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. 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. 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 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.
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 TRM 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, 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. 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.
REFERENCES


9


