CHAPTER 9

General discussion
Preactive MS lesions – myth or mystery?

Demyelinating lesions, associated with inflammation, oligodendrocyte injury and axonal damage, within the central nervous system (CNS) are the hallmark of multiple sclerosis (MS). The anatomical location in the CNS of these lesions contribute to the range of clinical symptoms characteristic of MS. The lesions continually form and regress during the disease course and while activity is reported to diminish with disease progression, active lesions are still observed in patients with long-standing disease.

The temporal order of MS lesion development is considered to start with the active lesion, associated with activated macrophages and microglia containing myelin debris indicative of ongoing myelin phagocytosis and destruction of myelin. As the myelin is progressively removed from the centre of the lesion, activated macrophages/microglia accumulate at the rim of the demyelinated zone and the lesion is referred to as chronic active. Finally the macrophage/microglia activation and numbers of cells at the rim disappear and an inactive lesion remains. That the demyelinated areas undergo remyelination especially in the early stages of the disease implies healing capacities within the CNS. However remyelination regularly fails or is insufficient to fully repair the damage, especially during disease progression, and thus clinical symptoms accumulate and persist.

Despite this proposed sequence of events in MS lesion development it is unclear how MS lesions are initiated and what happens between this ‘trigger’ and the presence of active lesions. Finding the driving mechanisms of lesion formation would provide us novel clues for therapeutic approaches to halt damage in MS.

The use of a magnetic resonance imaging (MRI) guided sampling protocol during brain autopsies of people with MS has revealed signals on the MRI in normal appearing white matter. Closer microscopic examination of those areas shows clusters of activated microglia in normal appearing tissue. Several imaging approaches, e.g. MRI, magnetisation transfer imaging and positron emission tomography (PET), have provided compelling support and evidence for changes in NAWM, which precede the appearance of full blown inflammatory MS lesions. This has led to the hypothesis that these abnormalities represent a phase of pathological alterations that antecedes destructive demyelination. Thus these so-called preactive lesions are evident candidates to study lesion development. Interestingly, most preactive lesions will not develop into full-blown active lesions. This strongly suggests that some intrinsic regulation exists that stops lesion progression at this early stage.

In this thesis we examined the composition of preactive lesions with the aim to unravel mechanisms underlying their formation and resolution. In addition, we explored pathways that could lead to the transition of preactive lesions into destructive active lesions. Here we will summarise and discuss our results, to give an update on the knowledge we have gained and to place our findings in perspective.
To what extent are preactive lesions specific to multiple sclerosis?

The key feature of preactive lesions is the activation and clustering of microglia. However, microglia activation is associated with a wide variety of CNS disorders and cluster formation is not unique for MS\(^4\).\(^{10} \)\(^{12}\). Moreover, microglia nodules are even a diagnostic tool for a variety of infectious diseases of the CNS\(^4\).\(^{13}\). Obviously this provokes the question whether the microglia clusters that characterise preactive lesions are specific for MS.

Microglial nodules are observed in many types of neurotropic viral infections but are generally described as loose aggregations of cells that mainly consist of microglia but also various haematogenous inflammatory cells, including T-cells\(^14\). Signs of infection, i.e. the presence of the pathogenic agent, are often detectable within the nodules\(^13\) (Figure 1A). In contrast, the clusters of activated microglia in MS have not been associated with the occurrence of etiological agents so far nor with the presence of lymphocytes\(^4\). Occasionally some foamy macrophages are observed, but in the majority of preactive lesions microglia are the only inflammatory cells present. The presence of foamy macrophages may well represent a secondary event that we have termed ‘transitional’ lesions: a step between preactive lesions and full-blown active MS lesions.

In this thesis examination of the cellular components of preactive lesions reveals that a cluster generally consists of 10-20 nuclei (average 16) of which 80% stain positive for the microglial marker HLA-DR (chapter 5). Morphologically the clusters show a round or oval shape and in some preactive lesions the microglia appear to have migrated towards a central point since a halo around the lesion is devoid of HLA-DR positive microglia\(^15\). Oligodendrocytes within the preactive lesions express the stress protein HSPB5, suggesting a role for stressed oligodendrocytes in these
clusters\(^{16}\) (Figure 1C-D). However, family members of HSPB5, also involved in stress responses, are not upregulated in oligodendrocytes in the preactive lesions, although they are produced by astrocytes in active white matter MS lesions (chapter 3). In contrast, in the genetic white matter disorder x-linked adrenoleukodystrophy (X-ALD) an increased expression in astrocytes is already noticeable in the preactive phase where there are no signs of demyelination yet (unpublished data). In X-ALD demyelination is, similar to MS, accompanied by prominent inflammatory infiltrates and microglia activation\(^{17,18}\). Like MS, early pathological changes precede the detectable demyelination, e.g. stress responses in astrocytes and diffuse activation of microglia (unpublished data)\(^{18}\). However, clustering of the microglia is not observed in this preactive phase. Thus microglia nodules are not a generalised phenomenon in inflammatory demyelinating disorders.

Although neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease, have not been widely considered as classical neuroimmunological disorders, as has been the case with MS, studies from the last decades have revealed that microglia activation is an early event in their pathogenesis\(^{11,12}\). While in Parkinson’s disease microglia are diffusely activated in the affected area, microglia form clusters around extracellular deposits of fibrillar A\(\beta\) in Alzheimer’s disease (AD)\(^{19}\) (Figure 1B). Thus microglia nodules are also observed in neurodegenerative diseases. However, neurofibrillary aggregates and insoluble protein inclusions are not associated with MS preactive lesions, implying that microglia cluster formation seen in neurodegenerative diseases substantially differs from preactive lesions.

The description of preactive lesions is based on studies performed on post-mortem brain material from people with MS. Yet post-mortem studies reveal a snap shot in time, and do not provide univocal information on the course of pathological processes. For this reason we examined the presence of preactive lesions in the autoimmune animal model of MS namely experimental autoimmune encephalomyelitis (EAE), to evaluate whether preactive lesion formation is associated with a specific disease phase or pathological feature e.g. oligodendrocyte stress and axonal damage (chapter 2,7). The clinical course of EAE varies from an acute disease to a progressive form, depending on the species and strain of animals used\(^{20}\). Moreover, in chapter 2 we showed that the clinical course of EAE is even dependent on the age of the animals at disease induction. While 2 week old Biozzi ABH antibody high (ABH) mice did not develop EAE after immunisation with spinal cord homogenate in complete Freund’s adjuvant, 8-12 week old mice exhibit relapsing-remitting EAE (RREAE) followed by a secondary progressive disease (SPAE) after approximately 3 months. In contrast, induction of EAE in old mice (12 months) resulted in a progressive course from disease onset (PEAE). In these three different manifestations of EAE in Biozzi ABH mice we examined the presence of microglia clusters (preactive lesions)\(^{21}\). In addition we studied post mortem brains from rhesus monkeys immunised with MOG that induces a hyperacute monophasic EAE resembling acute Marburg’s MS (chapter 7)\(^{22,23}\). The acute EAE in rhesus monkeys is characterised by haemorrhagic-necrotic lesions with massive infiltration of neutrophils and T-cells\(^{23}\). In contrast, the SPEAE model induced in 8-12 week old Biozzi ABH mice exhibits T-cell dependent relapses, but also progressive neurodegeneration independent of adaptive immune responses\(^{21}\). We did not observe microglia clusters (preactive lesions) in the
hyperacute EAE in rhesus monkeys while microglia clusters were observed in RREAE, SPEAE as well as the PEAE in old mice. This suggests that preactive lesions accompany the factors associated with relapses as well as disease progression. Some of the clusters are associated with axonal damage, however not all of them. In MS, biopsies from rapidly expanding lesions revealed microglia nodules in close association with degenerated and damaged axons\textsuperscript{24}. However, in post-mortem material from patients with long standing disease this association was not observed by van Horssen et al.\textsuperscript{15}, neither by us in this thesis. The differences observed may be explained by the moment of examination during the disease. Furthermore, the rapidly expanding lesion might be accompanied by furious damage and as a consequence more axonal damage. In the chronic EAE model, however, there was no clear correlation between the stage of the disease and axonal damage or oligodendrocyte stress. Yet, we should keep in mind that MS is an exclusive human disease and EAE only resembles parts of its pathogenesis.

In summary, microglia nodules are not specific for MS and can be seen in various human neurological disorders of the CNS. However, the clusters described as preactive lesions are unique to MS; small aggregates of HLA-DR positive microglia associated with stressed oligodendrocytes, in an area with no signs of myelin damage, leukocyte infiltration, clear marks of pathogenic agents, neurofibrillary deposits or misfolded proteins. Microglia clusters that resemble preactive lesions are observed in Biozzi ABH mice with RREAE followed by SPEAE as well as PEAE observed old mice, but not in rhesus monkeys with a quickly progressing form of EAE.

**How do stressed oligodendrocytes trigger preactive lesions?**

The observation that exclusively oligodendrocytes express the stress protein HSPB5 in preactive lesions suggests that stressed oligodendrocytes play a central role in preactive lesions\textsuperscript{25}. Yet, this is a chicken and egg situation; do the stressed oligodendrocytes activate the microglia or might the activated microglia stress the oligodendrocytes? Yet, accumulating data support the notion that oligodendrocytes are able to trigger the innate immune system.

Oligodendrocytes, the myelin producing cells within the CNS, were originally thought of as immunologically inert. Due to their high metabolic rate and high iron levels oligodendrocytes are extremely vulnerable to homeostatic disturbances and particularly susceptible to oxidative stress\textsuperscript{26,27}. Thus, they were generally considered as bystander victims during immune responses such as in cases where TNF is present\textsuperscript{28,29}. However, lately this view has been changed due to the increasing evidence that oligodendrocytes express receptors for immune related molecules, and moreover they are capable of expressing immunomodulatory molecules. In chapter 4 we review data supporting the idea that oligodendrocytes actively participate in immune responses. In addition we describe various ways of cross-talk between microglia and oligodendrocytes and the consequences of this communication. Via the release of a plethora of cytokines, chemokines and growth factors microglia can determine the fate of oligodendrocytes. On the other hand, by the secretion of immunomodulatory molecules as well as exosomes oligodendrocytes could attract and activate microglia and influence their phenotype. Exosomes are small membrane vesicles that are secreted and can be
internalised by neighbouring cells. In this way they can deliver their content, e.g. proteins, lipids or RNA, to the recipient cell\textsuperscript{30}. Exosomes secreted by oligodendrocytes are specifically and efficiently taken up by microglia both \textit{in vitro} and \textit{in vivo}\textsuperscript{31}. This implies that oligodendroglial exosomes can actively participate in the activation of microglia.

**Stressed oligodendrocytes trigger microglia activation**

That oligodendrocytes might contribute to the activation of microglia in preactive lesions is evidenced by the expression of HSPB5, known to trigger an immune regulatory response in microglia \textit{in vitro}\textsuperscript{16}. HSPB5 acts via toll-like receptor (TLR) 2 and the essential co-receptor CD14. Both receptors are expressed by microglia in preactive lesions\textsuperscript{32}. Furthermore, various markers that were identified by transcript profiling of cultured human microglia activated with HSPB5, could be detected by immunohistochemistry in the preactive MS lesions\textsuperscript{32}. Although these data suggest that oligodendrocytes trigger microglia activation in preactive lesions, the assumption is based on indirect evidence. Yet, unpublished data from our lab reveal that human primary microglia become activated following exposure to medium derived from stressed oligodendrocytes. Whether this is due to HSPB5 released into the media, however, is currently unknown (Figure 2).

As summarised in chapter 4 a wide variety of factors has been shown to induce oligodendrocyte stress. MO3.13 cells, a human oligodendrocytes cell line\textsuperscript{33}, were exposed for 1 h to three stressors suggested to be involved in MS, either glutamate\textsuperscript{34}, IFNγ and TNF as an inflammatory trigger\textsuperscript{35}, or H\textsubscript{2}O\textsubscript{2} as an oxidative agent\textsuperscript{36}. To exclude that stimulus application affects cell viability, MTT assay was performed to determine metabolic activity (data not shown). After stressing, the oligodendrocytes were rinsed to remove the stress agents and allowed to recover (3 h). The culture medium was collected, filtered and added to primary human microglia cultures. RNA was collected after 4 h of incubation and transcript levels of PTX3, TNF, CCR7 and CXCL10, all reported to be increased in microglia stimulated with HSPB5\textsuperscript{32}, measured by real-time polymerase chain reaction (RT-PCR). Although sample sizes were small, increases in transcript levels for all mediators tested could be observed (Figure 2). Transcript levels of CXCL10 were remarkably high in microglia stimulated with medium from oligodendrocytes exposed to inflammatory factors, compared to glutamate excitotoxicity or oxidative stress. The chemokine CXCL10, also known as IFN\gamma inducible protein-10 (IP-10), mediates immune responses via binding to its receptor CXCR3\textsuperscript{37}. CXCL10 is highly expressed in classically polarised pro-inflammatory microglia (IFN\gamma + LPS), but not in anti-inflammatory microglia stimulated with IL-4 (chapter 5). This suggests that microglia respond in a more pro-inflammatory way to oligodendrocytes subjected to inflammatory stress. However, compared to classical pro-inflammatory (M1) microglia, transcript levels of CCR7 were relatively low (data not shown), implying that stress responses of oligodendrocytes do not induce a classical M1 microglia. The upregulation of CXCL10 but no other mediators upregulated after HSPB5 stimulation, suggests that the phenotype induced in microglia by stressed oligodendrocytes is not exclusively driven by HSPB5. Although CXCL10 was identified as an IFN-inducible gene, its expression can be regulated by a variety of agents including TNF, IL-1β and CXCL10, but also viral and bacterial agents\textsuperscript{38-40}. Moyon et al.\textsuperscript{41} described increased transcript levels of IL-1β in adult oligodendrocyte progenitors after cuprizone treatment, however whether mature
oligodendrocytes are also capable of expressing IL-1β is unknown. Moreover, immunohistochemistry studies of post-mortem brain material with anti-IL-1β revealed specific staining in microglia and some astrocytes, but not oligodendrocytes (chapter 7). Clarner et al.40 showed that stimulation of microglia with recombinant CXCL10 resulted in the upregulation of pro-inflammatory factors including CXCL10. Thus by a positive feedback loop extracellular CXCL10 can induce production of CXCL10 in microglia, at least at transcript level. Besides, Balanov et al.42 revealed that primary rat oligodendrocytes stimulated with IFNγ produce several chemokines among which CXCL10. Furthermore, stimulation of OLN93 cells, a cell line with characteristics of late stage rat oligodendrocytes, with TNF showed the release of CCL2 and CXCL10, whereas oxidative stress or glutamate did not (unpublished data). These data are suggestive for a similar response in the human oligodendrocyte cell line and the production of chemokines, like CXCL10, could induce the specific response in microglia.

In summary, studies on HSPB5 in preactive lesions indicate that oligodendrocytes could activate microglia, our preliminary data confirm this and show that depending on the stress pathway activated in oligodendrocytes microglia respond differently. However, in this pilot study a human oligodendrocyte (tumour) cell line was examined and this may well respond differently from primary human oligodendrocytes and indeed oligodendrocytes in MS, therefor further research with primary oligodendrocytes is needed. Moreover, many more questions remain to be answered, e.g. ‘what factors are produced by the stressed oligodendrocytes?’ and ‘what condition mimics best what happens in preactive lesions?’.
**Activated oligodendrocytes attract microglia**

The appearance of preactive lesions suggests that besides microglia activation, microglia attraction and migration to a central point is necessary for their formation. The halo-like area devoid of microglia that surrounds some preactive lesions suggests that migration and aggregation are more likely than microglia proliferation to cause clustering (Figure 1C)\(^5\).

Chemokines are soluble proteins that belong to the cytokine family. Their name derives from their ability to induce directed cell migration, including cells that are involved in inflammatory processes\(^4^3\). The knowledge that oligodendrocytes are able to produce chemokines has led to the notion that oligodendrocytes could attract microglia\(^2^7,^4^2\).

In support of this Nicholas et al.\(^4^4\) showed that dying rat oligodendrocytes, as a result of eliminating essential survival factors, e.g. insulin and serum, from the culture medium express chemotactic factors that attract microglia *in vitro*. The observed migration of microglia was dose-dependent and not caused by cellular debris\(^4^4\). In line with these results, unpublished data from our lab revealed that increased numbers of microglia migrate towards medium from stressed oligodendrocytes (Figure 3). In this study MO3.13 cells were stressed with glutamate or H\(_2\)O\(_2\) for 1 h, after which the cells were washed to remove the applied stimulus and cultured for an additional 3 h. The medium was collected, filtered to remove any cells or cell debris and used in a chemotaxis assay. Primary human microglia were cultured in the upper chamber of a transwell Boyden chamber system (pore size 8μm; Corning; Figure 3A) inserted into the lower chamber containing the supernatants from stressed oligodendrocytes. After a 24 h incubation period, migrated microglia from the upper chamber were fixed, stained with toluidine blue and counted (5 fields per transwell were counted by 3 individual observers).

Results were compared to medium from unstimulated oligodendrocytes, microglia medium containing 10% FCS (maximal migration control\(^4^5\)) and serum free microglia medium, to determine unspecific cell migration (Figure 3B). While migration of microglia towards medium from unstimulated oligodendrocytes was similar to unspecific cell migration, the numbers of microglia that migrated to media from glutamate and H\(_2\)O\(_2\) stressed oligodendrocytes were comparable to the 10% FCS medium, (positive control; Figure 3B). These pilot data imply that stressed oligodendrocytes produce chemotactic factors that attract microglia. We anticipate that oligodendrocytes will not respond in a uniform manner, but depending on the stimulus and intensity of the stressor they rather selectively regulate microglia. However, the activation state of microglia could influence the survival outcome of the oligodendrocytes. Nicholas et al.\(^4^4\) showed that IFNy-activated microglia migrate more towards oligodendrocyte medium than non-stimulated microglia and that these microglia also induced a contact-dependent oligodendrocyte death. This complex interplay between microglia and oligodendrocytes may thus be a key event in preactive lesion formation.
What are possible triggers of oligodendrocyte stress in multiple sclerosis?

That stressed oligodendrocytes both attract and activate microglia, suggests that the first step in preactive lesion formation is the initiation of oligodendrocyte stress. Clearly this raises the question what factors induce this stress. The prominent place of oligodendrocyte and myelin dysfunction in many neuropathological conditions, indicates the wide variety of factors that can affect oligodendrocytes (chapter 4). Inflammation

For a long time the general view of oligodendrocyte damage in MS was attributed to the fulminant influx of inflammatory cells, like T- and B-cells. While inflammation could cause oligodendrocyte stress and death in active MS lesions, the absence of leukocytes disproves this in preactive lesions. However, inflammatory mediators, e.g. TNF and IFNγ, within the cerebrospinal fluid (CSF) have been suggested to induce microglia activation and demyelination of parenchyma in close contact with CSF. Thus the inflammatory cytokines derived from the CSF could contribute to oligodendrocyte stress, without the leukocytes being present. Although the focal appearance of preactive lesions in the parenchyma not associated with blood vessels or the perivascular zone contradicts this. It is more likely that local changes in the environment trigger oligodendrocyte stress.
Oxidative stress
Transcript profiling of NAWM revealed upregulation of genes known to be upregulated under hypoxic conditions, viz. the transcript factor HIF1α, CREB and members of the PI3K/Akt signalling pathway. Oligodendrocytes are highly sensitive to hypoxia induced injury, due to high intracellular iron stores and relatively low levels of anti-oxidative enzymes. Hypoxia is indissolubly related to oxidative stress. Due to a fall of oxygen tension cells are reliant on the production of oxidative energy accompanied by the expression of reactive oxygen species (ROS) i.e. oxidative stress. Accumulating evidence point towards the contribution of oxidative stress in the pathogenesis of MS. Inflammatory processes in MS play a major role in the production of free radicals and oxidative related tissue damage. However, the increased expression of transcription factors involved in the protection against oxidative stress in NAWM suggests that the MS brain is already subjected to oxidative stress before inflammation occurs. Moreover, the expression of HSPB5 could support the idea that oxidative stress contributes to the activated state of oligodendrocytes, since HSPB5 is upregulated to protect the cell from oxidative injury. The absence of HSPB5 in a chemically induced hypoxia model in rats worsened tissue damage. This protective effect of HSPB5 seems to be related to the HIF1α and PI3K/Akt signalling pathways, both of which are triggered in the NAWM. In chapter 3 we show that HSPB5 is the only small heat shock protein associated with preactive lesions. The supposition that oxidative stress is involved in early changes could explain the lack of HSPB6 and HSPB8, since the expression of these two small heat shock proteins was not sensitive to exposure to oxidative stress. However, this cannot throw light on the absence of HSPB1 in preactive lesions, since both HSPB1 and HSPB5 showed comparable responses in neuronal rat cultures. Although the latter two small heat shock proteins show many similarities not all cell types are able to produce both of them. Literature lacks information on HSPB1 expression in oligodendrocytes and it might be that oligodendrocytes are just not able to produce this small heat shock protein. On the other hand, the selective expression of HSPB5 could also imply that another stimulus stresses the oligodendrocytes in preactive lesions.

Antibody responses to oligodendrocytes
Damage to oligodendrocytes can be induced by pathogenic antibody responses directed against oligodendrocytic or myelin proteins. The presence of antibody responses towards myelin particles in MS patients suggests a role for antibodies in the pathogenesis of MS. Furthermore, anti-myelin antibodies are pathogenic and contribute to damage in EAE. Of particular interest are the antibodies directed against myelin oligodendrocyte glycoprotein (MOG). Not only are anti-MOG antibodies detected in the CSF of people with MS but MOG is CNS specific explaining the specificity of oligodendrocytes but not Schwann cell damage in MS. Moreover, HSPB5 is upregulated in oligodendrocytes following application of anti-MOG antibodies in vitro. In contrast, HSPB1 expression was not affected by the antibody response. Immunohistochemical analysis of the demyelinated areas in the anti-MOG mediated in vitro model reveals similar expression patterns of HSPB5 as observed in active MS lesions, viz. in oligodendrocytes and astrocytes. Furthermore, autoantibodies directed against MOG have been described in demyelinating MS lesions. However, whether they are associated with preactive lesions is currently unknown. Yet, a recent study by Kakalaceva et
al.\textsuperscript{68} indicates that infectious mononucleosis, symptomatic primary infection with Epstein-Barr virus (EBV), could lead to the generation of MOG-specific autoantibodies. Combining these data with the possibility of MOG-antibodies to induce oligodendrocyte stress, could provide a direct link for EBV in the pathogenesis of MS.

**A joint effort of factors**
The complex pathogenesis of MS implies that probably not just one single stimulus induces the observed oligodendrocyte stress, but multifarious factors contribute. Goldbaum et al.\textsuperscript{69} showed that the expression of HSPB5 increased when oligodendrocytes were exposed to oxidative stress followed by heat stress, compared to either stress alone. In contrast thereto HSPB1 decreased\textsuperscript{69}. Besides mutated small heat shock proteins can alter the function of another member of the family\textsuperscript{60}. Recently genetic variants for HSP32, have been associated with the risk to develop MS\textsuperscript{70}. Like HSPB5, HSP32 is upregulated under oxidative stress as well as after anti-MOG induced demyelination\textsuperscript{65,69}. Genetic variants could influence the stress responses in the cell and increase the susceptibility of oligodendrocytes to stressors.

To summarise, the pathological hallmarks of MS provide many factors that could stress oligodendrocytes, e.g. inflammation, neuronal and axonal degeneration and oxidative stress. Signs of protective mechanisms directed against oxidative stress already present in the NAWM, before visible inflammation, suggest that this contributes to the stress responses of oligodendrocytes seen in preactive lesions. Genetic variants might increase vulnerability to these stress events.

**Can microglia phenotyping predict whether a preactive lesion will develop into an active multiple sclerosis lesion?**

Preactive lesions are widely-considered to be the first step in MS lesion formation, yet given the numbers of these early lesions compared to active lesions it is proposed that not all preactive lesions progress and some must resolve. This thus implies a level of intrinsic control and regulation. We suggest that microglia are the key regulators in this process. Microglia are considered the tissue-resident macrophages of the CNS, that fulfil the main immunological functions in the immune privileged CNS. By the production of a wide range of mediators microglia play a key role in maintaining homeostasis\textsuperscript{71}.

**M1 versus M2 microglia phenotypes**
Influenced by the environment microglia, like macrophages, adopt differential phenotypes\textsuperscript{72,73}. The two most polarised phenotypes are the classically activated pro-inflammatory M1 and the alternatively activated anti-inflammatory M2 phenotype, so called to mimic the T-helper cell nomenclature\textsuperscript{74}. M1 microglia are considered to be neurotoxic and oligodendrocyte damaging, while M2 microglia fulfil immunosuppressive functions and promote repair\textsuperscript{75–77}. This duality suggests that M1 microglia dominate during active demyelination while M2 microglia are involved in tissue restoration and repair phases. In line with this preactive lesions that resolve would be predicted to consist of M2 microglia while a switch to M1 microglia could indicate active lesion development. To investigate this hypothesis
we systematically characterised microglia in preactive lesions (chapter 5). In vitro activation with IFNγ and LPS (M1 polarisation) resulted in distinctive upregulation of the receptors CD40, CD74, CD86 and CCR7 together with the pro-inflammatory cytokines IL1-β, TNF and CXCL10. By contrast mannose receptor (MR) and the anti-inflammatory chemokine CCL22 were significantly increased when microglia were stimulated with IL-4 (M2 polarisation). Immunohistochemical characterisation of microglia in preactive lesions revealed that microglia express the receptors CD40, CD74 and CD86, while only one fifth of the microglia were positive for CCR7 and only a few (2%) for MR. By contrast, CCL22 is abundantly present on microglia in preactive lesions. Yet, TNF, which is explicitly expressed after M1 polarisation, has been associated with microglia in preactive lesions as well. Thus our data show that microglia in preactive lesions do not adopt a classical M1 or M2 phenotype but reveal an intermediate phenotype.

The HSPB5 induced microglia phenotype
HSPB5 activates human microglia and macrophages in vitro into an immunoregulatory phenotype indicating that immune–regulatory profiles of macrophages and microglia can be induced by factors other than IL-4. The studies by Bsibsi et al. revealed that HSPB5 stimulated microglia upregulate a panel of IFN-I inducible genes and immune suppressive mediators. One of the most prominent transcripts in this HSPB5 induced microglia phenotype is pentraxin 3 (PTX3). Pentraxin 3 is a member of the pentraxin superfamily, which are key components of the humoral arm of innate immunity. As well as antibody-like functions PTX3 plays a regulatory role in inflammatory responses. In chapter 6 we studied the expression of PTX3 in the pathology of MS and revealed that PTX3 is already expressed by microglia in preactive lesions. This is in line with the expression of type I- IFN inducible proteins, e.g. IFIT1, IFIT2, IFIT3 and RIG-I, and suggests that HSPB5, at least partly, contributes to the microglia phenotype found in preactive lesions.

IL-1β expression in preactive lesions
The above mentioned markers, both the classical M1-M2 as well as the HSPB5 induced ones, showed a homogenous expression pattern in all preactive lesions. By contrast only a selection of the preactive lesions showed microglia that were IL-1β positive (chapter 7). This clearly raises the question whether IL-1β is the discriminating factor between preactive lesions that resolve and the ones that develop into active lesions. The cytokine IL-1β is an important mediator in inflammatory responses. The secretory mechanism of bioactive IL-1β is controlled by a cytosolic protein complex called the inflammasome, however the exact pathways are intricate and still not very well understood. Activation of pattern recognition receptors, including TLRs, results in the production of the precursor protein pro-IL-1β which needs to be cleaved to become active IL-1β. This could explain that IL-1β transcript levels were upregulated after stimulation with the TLR4 agonist LPS, inducing a pro-inflammatory microglia phenotype but also after activation with HSPB5 that triggers immune regulatory responses after binding to TLR2 (chapter 5). However, one should take into account that gene patterns of pro-IL-1β and mature IL-1β have a big overlap and transcript studies frequently do not discriminate, therefore discrepancies between IL-1β transcript and protein levels are often observed.
The expression of active IL-1β requires two steps, first the production of pro-IL-1β and secondly the activation of the inflammasome pathway that triggers caspase-1 to cleave pro-IL-1β\textsuperscript{85,86}. The presence of HSPB5 in preactive lesions implies that those microglia accumulate high levels of cytosolic pro-IL-1β\textsuperscript{32}. An additional stimulus could lead to the selective expression of active IL-1β seen in some preactive lesions. IL-1β possesses important inflammatory properties that lead to the attraction of inflammatory cells into the tissue\textsuperscript{87}, suggesting that IL-1β could play an important role in the progression into active demyelination. However, it is also possible that the presence of IL-1β reflects a transient response to cellular stress and indicates that IL-1β positive preactive lesions are in a different moment of development compared to the ones in which IL-1β is absent. Comparative proteomic and genomic analysis could shed light on the mechanisms that result in the production of IL-1β in some preactive lesions but not in others.

In conclusion, microglia in preactive lesions reveal an unique phenotype that does not reflect a classical M1 or M2 phenotype, but rather reflect activation by environmental factors like HSPB5. Characterising the expression patterns of microglia could crystallise the exact phenotype of the microglia in preactive lesions and could provide information on the different stimuli that could contribute to their activation as well as the mechanisms that control and reverse the process of preactive lesions formation. In figure 4 we give an overview of the receptors and cytokines known to be expressed by microglia in preactive lesions.

![Figure 4. Microglia characterisation in preactive lesions](image)

**Figure 4. Microglia characterisation in preactive lesions** Microglia do not reveal a classical pro-inflammatory (M1) or anti-inflammatory (M2) phenotype in preactive lesions. From the data reported in this thesis (*) and other reports (van Horssen et al.\textsuperscript{15}, Sing et al.\textsuperscript{24}, Bsibsi et al.\textsuperscript{32}) the receptors and factors expressed by microglia in preactive lesions are presented. While most of them are generally expressed by microglia, CCR7 and IL-1β (in red) are both selectively produced in a some preactive lesions.
What is the switch between preactive and active lesion formation?

Prevention of damage is one of the main goals for therapeutic strategies in MS. Knowing the trigger that could switch preactive lesions into active demyelinating lesions would be the key to finding new therapeutic approaches to halt lesion formation in MS.

In chapter 8 we show that IFNγ stimulation is able to change the protective response to HSPB5 into an pro-inflammatory reaction. Primary human microglia were stimulated with IFNγ 24 h prior to the addition of HSPB5. Changes in transcript levels were evaluated 4 h after the addition of HSPB5 and protein levels were determined after 24 h in the supernatant. This illustrated the unmistakable pro-inflammatory effect of IFNγ priming, while the anti-inflammatory cytokine IL-10 was on the wane, the pro-inflammatory TNF, IL-12 and IL-1β significantly increased. Moreover, combined activation with both IFNγ and HSPB5 led to a significantly increased expression of reactive oxygen and nitrogen species, both associated with a pro-inflammatory activation state of microglia. A small-scale microarray transcript analysis of IFNγ/HSPB5 stimulated microglia revealed selective markers for combined activation, viz. the guanylate-binding proteins GBP1, GBP4, GBP5 and the ubiquitin like protein F-adjacent transcript 10 (FAT-10). These markers were prominently expressed by HLA-DR positive cells in perivascular infiltrates in demyelinating lesions, but not by microglia in preactive lesions. This suggests that IFNγ regulated re-programming of the otherwise HSPB5-induced immunoregulatory phenotype plays a role in the observed damage in active demyelinating lesions and implies that IFNγ priming could contribute to the switch from preactive to active lesions.

The primary cell types that express IFNγ are natural killer cells and T-cells, however both are absent in preactive lesions. This clearly raises questions about the sources of IFNγ in preactive lesions as well as whether the IFNγ/HSPB5 induced phenotype in active lesions is rather a result of T-cell influx and thus not involved in the switch from preactive to active lesions.

One could hypothesise that the above mentioned selective IL-1β production in some preactive lesions results in an increased blood-brain barrier permeability and the recruitment of Th1 and Tc1 cells and consequently the production of IFNγ by these T-cells. HSPB5 could actively play a role in the stimulation of the T-cells, since this small heat shock proteins serves as an immunodominant antigen to human T-cells.

On the other hand, studies over the last decades have suggested the possibility that IFNγ production within the CNS is independent of T-cells and thus that glial cells, predominantly microglia, are able to express IFNγ. Stimulation of murine microglia with IL-12 and IL-18 resulted in the expression of IFNγ in vitro. This could imply that microglia are triggered by surrounding cells, e.g. astrocytes and oligodendrocytes, to produce IFNγ that results in IFNγ priming of neighbouring microglia.

The phenomenon of microglia priming following IFNγ activation has been extensively studied, however other factors have been described to induce priming as well, such as M-CSF, GM-CSF, CXCL10 and CCL2. However, conflicting data on CCL2 have been published since Hinojosa et al. described that CCL2 is not able to directly activate primary murine microglia in vitro. In contrast, CXCL10 is able to induce a pro-inflammatory microglia phenotype in...
Short term exposure to cuprizone in mice resulted in the expression of CXCL10 in both oligodendrocytes and astrocytes\textsuperscript{40} and suggests that oligodendrocytes as well as astrocytes can produce CXCL10 that primes microglia in preactive lesions. However, whether CXCL10 is able to re-programme microglia in the same way as IFN\textgamma is yet unknown.

In summary, combined stimulation of IFN\textgamma and HSPB5 reveals a pro-inflammatory microglia phenotype \textit{in vitro} in contrast to HSPB5 alone. Markers of this IFN\textgamma/HSPB5 induced phenotype can be observed in active lesions, but not in preactive lesions. This indicates that IFN\textgamma is clearly involved in active demyelination, whether this is a result of T-cell influx or IFN\textgamma re-programming results in the attraction of leukocytes that damage myelin remains to be elucidated.

**Preactive lesions: the key to therapeutic approaches?**

While many therapeutic approaches reduce inflammation in the CNS and thus reduce relapses currently available therapies for MS patients do not halt the progression of the disease. Characterisation of mechanisms underlying preactive lesions will lead to new targets that could manipulate preactive lesion formation and stop the progression into active demyelinating lesions. Here we will discuss potential therapeutic strategies based on the current knowledge about preactive lesions (Figure 5).

Accumulating evidence indicates that oxidative stress is early involved in the pathogenesis of MS and could contribute to the oligodendrocyte stress seen in preactive lesions\textsuperscript{50,55,56,100,101}. Given that oxidative stress is accompanied by the production of ROS, antioxidant compounds could play a crucial role in the prevention of oxidative induced oligodendrocyte stress and thus stop preactive lesion formation in its earliest phase. Various antioxidant therapies have shown beneficial effects in EAE, \textit{in the initiation of disease as well as in progression}\textsuperscript{100,102–104}. Moreover, a synthetic analogue of Coenzyme Q10, a natural antioxidant, idebenone is being tested in a phase II clinical trial in primary progressive MS. However, idebenone has failed to prevent or ameliorate chronic EAE and this research group predicts no beneficial effects in MS patients\textsuperscript{105}. Although oral administration of idebenone results in detectable levels in the CSF of the treated mice it is questionable whether the amounts used were large enough to have functional implications, a drawback of many exogenous anti-oxidants\textsuperscript{105}. Future studies should uncover steps to activate the production of endogenous anti-oxidants\textsuperscript{102}. A critical pathway involved in the production of anti-oxidants is the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant response element (ARE) pathway, which can be activated by various factors including the FDA approved drug dimethyl fumarate\textsuperscript{106}. This growing interest in the anti-oxidative therapeutic approaches might be very important in preventing the initiation of preactive lesion formation.

In response to stress conditions, such as oxidative stress, viral infection and endoplasmic reticulum (ER) stress, cells activate a protective innate mechanism called the integrated stress response (ISR)\textsuperscript{107,108}. This pathway is activated when the eukaryotic translation initiation factor eIF2\textalpha is phosphorylated and results in transcriptional changes and protein synthesis attenuation trying to protect the cell from stress\textsuperscript{107,108}. Recent data from preclinical studies suggest an important role
for ISR activation in the survival of oligodendrocytes during neuroinflammatory conditions like MS\textsuperscript{108,109}. This highly protective role of ISR implies that therapies modulating this pathway could protect oligodendrocytes from stress in MS. Encouraging data from preclinical \textit{in vitro} and \textit{in vivo} studies with a selective inhibitor of phosphorylated eIF2α, guanabenz, have resulted in the initiation of a phase I clinical trial\textsuperscript{108,110,111}. In addition, a guanabenz derivate has already been developed, to overcome side-effects of guanabenz due to its effects on the α\textsubscript{2}-adrenoceptor\textsuperscript{112}. These recent developments offer promising options for new therapeutic strategies.

As a result of the activated stress pathways oligodendrocytes produce HSPB5 in preactive lesions. We know now that HSPB5 serves a key role in preactive lesion formation and is not just a response to stress. Research over the last years has pointed out the broad neuroprotective and immunoregulatory functions of this heat shock protein\textsuperscript{25,32,113,114}. Moreover, HSPB5 has revealed therapeutic qualities in several disease models\textsuperscript{115–117}. However, large amounts of HSPB5 could induce contradictory effects by the activation of T-cell responses, since HSPB5 can act as an autoantigen when presented to T-cells\textsuperscript{91}. Therefore the dose of HSPB5 for therapeutic purposes should be below the threshold of T-cell activation.
A 48-week randomized placebo-controlled double-blind phase IIa trial revealed that low doses of intravenous HSPB5 led to a significant decline in MS lesion activity on MRI images but not the clinical score\textsuperscript{118}. However, definitive conclusions about the clinical efficacy of HSPB5 cannot be made since this study had a short duration and sample size was small\textsuperscript{118}. These results provide the first indication for therapeutic benefits of HSPB5, but additional research is needed. Given the protective effects of HSPB5 we investigated in chapter 6 whether PTX3 could contribute to this. PTX3 is one of the strongest induced proteins in the immunoregulatory HSPB5 induced microglia phenotype\textsuperscript{32}. Accumulating evidence shows the tissue protective and immunoregulatory functions of PTX3\textsuperscript{119–125}. However, in contrast to the promising data on HSPB5 treatment in both EAE as well as MS, PTX3 was not able to alter the clinical course of EAE and thus is not a suitable target for MS treatment (chapter 6).

The prominent role of activated microglia in preactive lesions and the multifaceted phenotypes they can adapt, both beneficial and detrimental, makes them interesting therapeutic targets. Modifying the activation state of microglia into an immunoregulatory and reparative phenotype could help to restore preactive lesions and prevent active lesion formation. The FDA approved medicine glatiramer acetate (GA) for example is able to reduce microglia activation and induce anti-inflammatory responses, which are thought to play a significant role in the immunoregulatory functions of this drug\textsuperscript{126–128}. As an alternative therapeutic approach mesenchymal stem cells (MSC) are currently being investigated, since cross-talk between MSC and microglia resulted in alternatively activated microglia\textsuperscript{126,129}. However manipulating microglia activation states could be very delicate since they are extremely influenced by the environmental factors and priming could result in re-programming of initially protective stimuli, as we have shown in chapter 8 with combined IFNγ/HSPB5 activation. Modulating specific pro-inflammatory factors might be more convenient, like IFNγ and IL-1β. By the selective expression of IL-1β in some preactive lesions one could hypothesise that IL-1β is the trigger for the switch to active lesion formation and thus preventing IL-1β production could halt demyelination. The therapeutic effect of the FDA approved drug IFNβ is, at least partly, assigned to the inhibition of inflammasome activation\textsuperscript{130}. However the exact mode of action of IFNβ in the suppression of the inflammasome complex is unknown. Recently more specific inhibitors of inflammasome components, mainly the NOD-like receptor protein 3 (NLRP3), have been described\textsuperscript{131}. NLRP3 is currently the most comprehensively described inflammasome and is suggested to be involved in the development of MS\textsuperscript{132}. The injection of a specific NLRP3 inhibitor intraperitoneally in EAE mice resulted in a delayed onset of the disease and attenuated severity\textsuperscript{131}. These preliminary data imply that targeting the inflammasome complex has beneficial effects in the treatment of MS.

In summary, mechanisms involved in preactive lesions as well as the switch to active lesion formation provide various promising pathways that can be manipulated by therapeutic intervention. However, many of these ideas require further research to elucidate their exact role in MS pathogenesis and thus their benefit in MS treatment.
Concluding remarks

The objective of this thesis was to understand more about preactive lesion formation, progression and resolution with the ultimate goal of identifying new therapeutic strategies to promote their resolution and prevent the devastating inflammation and demyelination characteristic of active MS lesions. By means of a multidisciplinary approach we tried to characterise the various aspects of preactive lesions, our main findings are summarised in Box1. We hypothesised that stressed oligodendrocytes are the trigger for microglia activation, by the expression of HSPB5. We showed that HSPB5 is the only member of the small heat shock protein family that is expressed by oligodendrocytes in preactive lesions. This selective upregulation could shed light on the triggers that stress the oligodendrocytes in preactive MS lesions.

That HSPB5 activates microglia in vitro, and the accumulating evidence that oligodendrocytes are able to produce immunomodulatory factors that can trigger microglia responses, points to the central role for oligodendrocytes in early MS lesion formation. Preliminary studies on the activation and attraction of microglia by stressed oligodendrocytes described above strengthen this idea. These data look promising and provide a base for future research. Questions about the exact stimuli that stress oligodendrocytes in a way that resembles the responses in preactive

**Box 1 Conclusions of the studies performed in this thesis**

- Microglia clusters resembling preactive lesions are observed in young Biozzi ABH mice that show a relapsing-remitting clinical course followed by secondary progressive disease, as well as old mice that show a progressive course from disease onset (Chapter 2)

- The small heat shock protein HSPB5 is expressed by oligodendrocytes in preactive lesions, while other small heat shock family members, e.g. HSPB1, HSPB6, HSPB8 and HSPB11 are not (Chapter 3)

- Oligodendrocytes produce immunomodulatory factors known to modulate immune responses in the CNS (Chapter 4)

- Microglia in preactive lesions do not adapt a classical pro-inflammatory M1 or anti-inflammatory M2 phenotype but reveal an intermediate activation state (Chapter 5)

- The immunoregulatory protein pentraxin-3 (PTX3) is strongly induced in human microglia activated with HSPB5. Microglia in preactive MS lesions highly express PTX3, however PTX3 does not play a role in regulating inflammatory pathways during early stages of experimentally induced neuroinflammation (Chapter 6)

- The selective expression of IL-1β in some preactive MS lesions implies an important role in the progression to active lesion formation (Chapter 7)

- IFN-γ induced re-programming of an otherwise protective response to HSPB5 is selectively associated with active demyelination in MS (Chapter 8)
lesions and what factors are produced by stressed oligodendrocytes may well point to new ideas about the cause of MS. Expanding the study of the cross-talk between oligodendrocytes and microglia may provide further information on the phenotype of microglia in preactive lesions. Due to their dual activation state, both beneficial and detrimental, microglia are thought to have a key function in the transformation from preactive to active lesions. We hypothesised that HSPB5 activates an immunoregulatory phenotype in an attempt to restore homeostasis and that a second trigger switches the microglia phenotype into a pro-inflammatory activation state in active demyelination. Our studies showed, however, that microglia do not adapt the classical M1 and M2 phenotypes but they show an intermediate activation state, probably induced by the factors expressed by stressed oligodendrocytes.

IFNγ re-programming of the otherwise protective HSPB5 induced responses in microglia is the distinct difference between preactive and active lesions. Markers of combined activation are expressed in active lesions but not preactive lesions, suggesting that IFNγ is clearly involved in inflammatory demyelination. Whether IFNγ is the only mediator to switch preactive lesions into active lesions is unknown. One could hypothesise that IFNγ re-programming occurs after the influx of T-cells, since they are the major source of IFNγ and thus an extra trigger for the switch would be necessary. The selective induction of IL-1β in a minority of the preactive lesions implies that the expression of this interleukin could lead to T-cell attraction and activation, and thus the initiation of an active lesion. Laser capture dissection of IL-1β positive and IL-1β negative preactive lesions for further genomic and proteomic analysis could reveal important distinctive pathways involved. The finding that preactive lesions are present in secondary progressive EAE provides an in vivo model to manipulate the involved pathways and follow up the effects on preactive lesion formation and progression.

In conclusion, in this thesis we expanded the knowledge of preactive MS lesions and showed that underlying mechanisms reveal potential promising leads for new therapeutic approaches to halt lesion formation in MS.

References

60. Simon, S. *et al.* Analysis of the dominant effects mediated by wild type or R120G mutant of αB-crystallin (HspB5) towards Hsp27 (HspB1). *PLoS ONE* 8, e70545 (2013).
GENERAL DISCUSSION