The role of differently activated macrophages in axonal damage during multiple sclerosis

Vereyken, E.J.F.

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CHAPTER

1

GENERAL INTRODUCTION

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Macrophages and axonal damage and repair in multiple sclerosis
Vereyken E.J.F., Dijkstra C.D., Teunissen C.E.

MULTIPLE SCLEROSIS

Clinical symptoms and diagnosis in vitro

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The prevalence of MS is approximately 2 million people worldwide, with an incidence of about 1:1000 in Europe and Northern America, and women are affected more often compared to men, at a ratio of approximately 2:1. It is the most common cause of neurological disability among young adults with an onset generally between 20 and 40 years of age [1]. MS is characterized by multiple sclerotic lesions affecting areas such as the cerebellum, cerebrum (periventricular white matter), optic nerve, brainstem and spinal cord [2]. The clinical symptoms of MS are very heterogeneous depending on lesion location, size and number. The symptoms include motor function disturbances, such as muscle weakness, tremor and paralysis, and progressive sensory malfunction, for instance impaired vision. The main criterium for the diagnosis of MS is the occurrence of two (or more) independent episodes of clinical symptoms consistent with focal demyelination separated in space (part of the CNS) and time (more than one occasion) [3]. Magnetic resonance imaging (MRI) techniques have become very important for the verification of the diagnosis [4-6], for the detection of the number and size of the lesions and to differentiate between ongoing inflammation and blood-brain-barrier leakage [7-10]. In the cerebrospinal fluid (CSF) of MS patients abnormalities can be detected, such as the presence of elevated immunoglobulin G (IgG) levels and the identification of two or more unique oligoclonal bands [11]. In approximately 90% of MS patients oligoclonal immunoglobulin bands are detected [12]. These oligoclonal bands are also detected in other neurological diseases, although not as consistently or as persistently as in MS. In patients with clinically isolated syndrome the presence of oligoclonal bands is predictive for the progression to clinically definite MS [13]. In MS patients the absence of oligoclonal bands has been associated with a benign disease course, while high levels of oligoclonal bands have been correlated with a severe disease, suggesting the oligoclonal bands may be clinically relevant [11].

Four major subtypes of MS with different progression and relapse characteristics have been recognized: the relapsing-remitting (RR-MS), the secondary-progressive (SP-MS), primary progressive (PP-MS) and progressive-relapsing (PR-MS) subtype [14]. Approximately 70% of the cases start with the RR subtype, which is characterized by clinical attacks that are followed by a clinically silent period with almost complete recovery. After a period of 15 to 20 years most cases of the RR-MS subtype develop progressive neurological deterioration without apparent remission, the SP-MS subtype. About 15-20% of MS patients show a progressive disease course without relapses and remissions from the beginning, the PP-MS subtype. Finally, less than 5% suffer from PR-MS, characterized by progressive neurological impairment with occasional relapses.

Pathology

The major neuropathological hallmarks of MS are multiple focal inflammatory

demyelinating lesions spread throughout the CNS. These lesions are associated with perivascular infiltrates containing macrophages and lymphocytes. Other features of MS plaques are axonal damage and loss, oligodendrocyte death and astrogliosis [15], which is hypertrophy and an abnormal increase in the number of astrocytes. Lesions are classified based on the degree of myelin loss, the presence of inflammatory cells and HLA-DR expression on leukocytes and microglial cells. Four different stages in MS lesions have been identified: (p)reactive, active, chronic active and chronic inactive lesions [16;17].

In (p)reactive lesions no demyelination is apparent. Clusters of activated microglia can be observed with increased expression of HLA-DR expression and occasionally perivascular leukocyte infiltrations can be seen.

Active lesions are characterized by areas of demyelination containing macrophages, activated microglia and activated hypertrophic astrocytes. Activated astrocytes fill up the lesion area and form a gliotic scar. T-cells and some B-cells can be found, mostly in the perivascular space. The macrophages and microglia in the lesions contain myelin degradation products, such as myelin proteins and lipids, giving them a foamy appearance. The presence of myelin proteins in these macrophages reflect ongoing demyelination taking place [18;19]. Oligodendrocyte death occurs in these lesions, often via apoptotic mechanisms [20;21]. This apoptosis of oligodendrocytes may be a disease initiating event, since it precedes leukocyte infiltration [22;23].

Chronic active lesions are defined by a hypocellular demyelinated centre surrounded by a hypercellular rim with high numbers of foamy macrophages and reactive astrocytes. In these lesions oligodendrocyte numbers are reduced and lymphocytes are present in perivascular spaces.

In chronic inactive lesions almost no cellular infiltrates are present. They are hypocellular, demyelinated and contain widened extracellular spaces and gliotic scar tissue. In the CNS parenchyma and perivascular spaces relatively small numbers of macrophages and lymphocytes still remain. No myelin proteins can be detected in the macrophages.

Next to demyelination, remyelination also occurs [24]. Remyelination can be restricted to the lesion edge, but can also extend throughout the lesions which are then called shadow-plaques [25-29]. Oligodendrocyte precursor cells, after maturation into mature oligodendrocytes, generate thin myelin sheaths and could therefore contribute to recovery in MS patients. Remyelination in MS is limited. The cause of remyelination failure in MS is unknown, but several mechanisms have been proposed, such as restricted oligodendrocyte precursor cell migration, maturation and a growth inhibitory environment [24;30-32].

AXONAL DAMAGE IN MS

Historically, MS has been viewed as a primary demyelinating disease with relative axonal sparing, although early papers did describe axonal damage and loss [33]. However, the view that axonal damage is important in MS pathology is now being widely accepted. The earliest studies on MS pathology already described

axonal damage and loss [15;16;34], although, this fact was obscured by the much more evident demyelination. Demyelination has long been considered the main cause of disability in MS. However, more recent reports suggest that axonal injury is the main correlate of irreversible clinical disability in MS patients [35;36] and experimental autoimmune encephalomyelitis (EAE), an animal model for MS [37]. Early axonal damage may be either compensated for and/or repaired, but the continuous progression of axonal loss could ultimately lead to irreversible clinical dysfunction. A current hypothesis poses that the transition from the RR to the SP subtype takes place once the loss of a critical number of axons is exceeded [33].

An indication that axonal damage might be important in MS pathology was that axonal transections are common in MS lesions, even in the periplaque white matter [38;39]. It was most extensive in areas of active demyelination and inflammation [40]. Even in the earliest stages of the disease widespread axonal damage was observed [41]. In chronic MS patients, axonal density was significantly decreased, in both normal appearing white matter (NAWM) and lesions in the cervical spinal cord, compared to controls [39;42]. The decrease in axonal density was more extensive inside most lesions compared to the adjacent NAWM [33]. A marker for axonal injury is the amyloid precursor protein (APP) [43;44]. APP, a transmembrane protein that has been suggested to be involved in Notch signaling [45], is transported axonally by means of the fast anterograde component [46]. During acute injury anterograde axonal transport is interrupted causing APP to accumulate. In lesions with active demyelination APP accumulation was found in so-called “bulbs”, suggesting that axonal damage is a feature of early pathology and possibly associated with inflammation [47]. In inactive lesions significant, though low-level axonal damage was observed associated with residual inflammation [48].

Another marker for axonal damage is non-phosphorylated neurofilament (NP-NF) [49]. Caliber changes, axonal transections and terminal ovoids have been observed in MS lesions using this method. Discontinuous NP-NF staining suggests that axons undergo Wallerian degeneration, degeneration distal from the sites of transsection in MS patients [49;50]. Also, in EAE alterations in the distribution of dendritic and synaptic proteins have been described, suggesting a loss of neuronal contacts in this model [51]. Finally, redistribution of sodium channels and sodium/calcium exchanger NCX on demyelinated axons was shown to associate with APP accumulation providing another marker for axonal damage [52;53].

Magnetic resonance imaging (MRI) and spectroscopy (MRS) have been used to visualize neuronal damage in living patients. MRI parameters in the brain of RR- and SP-MS patients were found to correlate with Expanded Disability Status Scale (EDSS) scores, while in PP-MS spinal cord abnormalities, spinal cord cross section and the number of segments showing diffuse abnormalities, correlated better with clinical symptoms [54]. Next to specific lesions, brain atrophy was found to occur in MS patients [55;56]. Brain atrophy was found to be a better predictor than number of inflammatory lesions for disability in MS patients [57;58]. The findings of cerebellar cortical atrophy in EAE mice parallel findings in humans [59]. These findings suggest that brain atrophy leads to functional impairment.

A more specific marker for axonal damage in MS is N-acetylaspartate (NAA),

which can be detected *in vivo* in the brain using MRS [35;60]. NAA, a mitochondrial amino acid, is primarily localized in neurons and neuronal processes, which means that a reduction in NAA signal reflects axonal injury and/or neuronal loss [61]. In both RR-MS and SP-MS patient groups a decrease in NAA:Creatinine ratio in both NAWM and lesions was observed compared to controls, with the decrease in lesion areas being higher compared to NAWM. The NAA:Creatinine ratio was significantly lower in the NAWM of SP-MS compared to RR-MS patients [62;63]. A strong indication that NAA levels measured by MRS might really reflect neuroaxonal integrity was the fact that a relationship was observed between the decrease in NAA levels measured by MRS in MS lesions and the decrease in axonal density in corresponding biopsy specimens [38;64]. A correlation was found between the reduction in NAA levels, motor conduction times and functional disability in 12 MS patients [65]. Furthermore, in RR-MS patients clinical disability as observed with EDSS correlated with brain NAA:Creatinine ratio [62;66]. The SP-MS patients did not show this correlation with EDSS, which might be due to lower disease activity and the small number of patients.

Changes in cerebrospinal fluid (CSF) concentrations of axon specific markers also point to a role of axonal damage in MS pathology and progression. In CSF of MS patients the NAA concentration was found to correlate with EDSS, a lower brain volume and a higher lesion load [67]. In the CSF of SP-MS patients decreased levels of NAA and increased levels of neurofilament heavy (NF H) were observed. Neurofilament light (NF L) levels were increased already in clinically isolated syndrome (CIS) patients, especially with those who converted to clinically definite MS, and correlated with the number of gadolinium enhancing lesions and relapses, suggesting that this marker has prognostic value [68]. The autoantibody index for the NF L chain has been found to correlate with atrophy [69]. Another biomarker for axonal damage in the CSF is tau.

All these data confirm the importance of axonal damage in MS pathology, which can already be observed early in the disease course. The mechanisms causing this damage are largely unknown. Several mechanisms have been proposed. First, demyelination has been may be involved in axonal damage. Myelin forms a physical barrier that protects axons from damaging agents. Furthermore myelin and oligodendrocytes seem to generate survival signals. In mice that do not form proper myelin, due to deficiency of myelin associated glycoprotein (MAG), 2´,3´-cyclic nucleotide 3´-phosphodiesterase (CNPase) or proteolipid protein (PLP), axons were found to show chronic atrophy and signs of degeneration [70-74]. Demyelination also leads to redistribution of sodium channels, in order to maintain proper signal propagation. This redistribution leads to enhanced energy metabolism and increased intracellular calcium concentrations, causing toxicity [75]. In MS lesions axons and astrocytes showed increased activity of complex IV and numbers of mitochondria, associated with an upregulation of the mitochondrial heat shock protein 70, indicative of oxidative stress in the mitochondria [76]. Furthermore, mitochondrial dysfunction due to oxidative stress, mediated by reactive oxygen species (ROS) produced by mitochondria themselves, has been observed early in EAE lesions, suggesting another mechanism for neuronal degeneration [77]. Another

hypothesis is that infiltrating macrophages might play a crucial role in axonal damage.

MACROPHAGES

Macrophages (meaning “big eaters”) are phagocytic cells that play a vital role in innate immunity, the first line of defense against pathogens. Cells of the innate immune system such as macrophages are able, to some extent, to discriminate between “self” and “non-self” antigens (reviewed by Janeway [78;79]). Via a limited number of germline-encoded pattern recognition receptors, macrophages recognize highly conserved structures from bacteria, viruses and fungi. Several different families of pattern recognition receptors have been identified, for example macrophage scavenger receptors and Toll-like receptor family [80;81]. After recognition, the binding of the receptor with its ligand on the pathogen, macrophages usually engulf the pathogen, a process called phagocytosis. This process results in the containment of microbes in the phagosome, which fuses with lysosomal vesicles containing a multitude of microbicidal products. Both oxygen-dependent, called the respiratory burst, and oxygen-independent microbicidal mechanisms exist. The respiratory burst uses an enzymatic complex called nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase. Upon stimulation active NADPH oxidase forms. This active complex transfers two electrons from NADPH to two molecules of oxygen to form superoxide anion [82;83]. From this superoxide anion other ROS, such as hydrogen peroxide, are formed [83]. Oxygen-independent microbicidal mechanisms include acidification of the phagolysosome, nutrient depletion and antimicrobial proteins or peptides.

Macrophages differentiate from circulating monocytes. Once a monocyte migrates into a specific tissue, during steady-state or inflammation, they develop into macrophages. Macrophages are present in virtually all tissues and usually specialize according to the tissue they are in, for instance osteoclasts (in bone), Kupffer cells (in liver) and microglia (in the CNS) [84].

Next to their role in innate immunity they have an important function in tissue homeostasis, since they are crucial for the clearance of apoptotic cells and the remodeling and repair of tissues after inflammation [85;86]. Phagocytosis of apoptotic cells does not induce the expression of inflammatory mediators in unstimulated macrophages [87].

During an infection macrophages also clear cellular debris of necrotic cells that contain endogenous danger signals, such as heat-shock proteins and nuclear proteins [88]. The detection of these danger signals alters the physiology of the macrophages, including expression of cell surface proteins, cytokines and pro-inflammatory mediators, increasing immune function of macrophages. However, macrophages can respond to many signals in the microenvironment of tissues and not all increase immune function.

Subtypes of macrophages

Macrophages are highly plastic cells able to respond to a variety of

environmental cues changing their phenotype and physiology in response to these signals, resulting in different subtypes of macrophages. These different subtypes of macrophages have different functions in the immune response, homeostasis and tissue repair [89-91].

Based on activation pathways several subtypes of macrophages have been described [92;93]. The two most studied subtypes are: 1) the classically activated macrophages (CA, also called M1), induced by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS); 2) the alternatively activated macrophages (AA, also called M2), stimulated by IL-4 and/or glucocorticoids. In 1992 Stein et al. introduced the concept of alternatively activated macrophages [94]. In contrast to the classically activated macrophages, macrophages stimulated with interleukin-4 (IL-4) increased the expression of mannose receptor (MR). Another study showed that Th1 cytokines (e.g. IFN-γ) and Th2 cytokines (e.g. IL-4) induced two distinct functional states. Exposure of macrophages to Th2 cytokines led to an upregulation of certain phagocytic receptors and arginase, which reduced ability to kill intracellular pathogens, while Th1 cytokines led to induction of inducible nitric oxide synthase (iNOS) in macrophages [95].

CA macrophages are cytotoxic and secrete high amounts of oxygen and nitrogen radicals in order to kill pathogens [96]. CA macrophages also produce pro-inflammatory cytokines [97]. In mice, CA macrophages are characterized by their production of nitric oxide (NO) [92;98;99]. Human macrophages derived from circulating monocytes do not generally produce NO [90], therefore other markers should be used to discriminate between CA and AA macrophages, such as MR, E-cadherin [100], CD40 [101] and Fc-gamma receptor I (FcγRI). Markers for the different types of macrophages are presented in Table 1. CA macrophages are essential for host defense [84;102] and tumor killing. The pro-inflammatory mediators produced by CA macrophages can cause extensive damage to the host.

AA macrophages seem to play a role in immune suppression and tissue repair, due to production of anti-inflammatory cytokines and extracellular matrix components and in addition failure to produce NO [92]. The most common used distinctive marker for AA macrophages, in mice, is the higher expression and activity of arginase [92]. Due to the activation of arginase, arginine is converted to ornithine, a precursor for polyamines and collagen, which contributes to the production of extracellular matrix [103-106]. The polyamines produced can influence production of cytokines and suppress clonal expansion of lymphocytes, thereby having a regulatory effect on the immune response [107].

Functional differences can be observed between CA and AA macrophages. As mentioned above, due to the production of ROS, CA macrophages are efficient in the killing of bacteria, while AA macrophages do not produce ROS and are therefore less efficient in killing bacteria [84]. Furthermore, CA macrophages are efficient antigen presenting cells, while AA macrophages are not [92]. CA macrophages are also more efficient in activating T-cell proliferation compared to AA macrophages [92]. AA macrophages are involved in scar formation, since they enhance fibrogenesis, while CA macrophages do not. AA macrophages stimulate proliferation and activation of fibroblasts, by expression and release of potent fibrogenic growth

MARKER	CA-MΦ	AA-MΦ	SPECIES	REF
ENZYMES				
iNOS mRNA expression, activity, NO release	↑	-	Mouse	91, 204
Arginase mRNA expression actvity	-	↑	Mouse	91
SPHK1 mRNA	-	-	Mouse	91
12,15-lipoxygenase	↓	↑	Human, mouse	205
MEMBRANE RECEPTOR EXPRESSION				
CD163 mRNA and protein expression	-	↑	Human, mouse	91, 206
Mannose receptor expression	-	↑	Mouse	93
β-glucan receptor (Dectin-1)	-	↑	Mouse	207
MGL1/2 mRNA and protein expression	-	↑	Human, mouse	208
FcγRI	↑	↓	Human	209
LIGHT MRNA	-	-	Mouse	91
E-CADHERIN	-	↑	Human, mouse	99, 210
FACTOR XIIIA	-	↑	Human	211
CCR7	↑	↓	Human	212
ANTIGEN PRESENTATION				
MHC class II protein expression	↑	↓	Human, mouse	91
CD86 protein expression	↑	↓	Human, mouse	91
MS1-HMWP	↓	↑	Human	89
CD40	↑	-	Human	100
CYTOKINE				
IL-12 release IL-12 mRNA	↑ ↑↑	- -	Human mouse	91, 213, 214
IL-10 mRNA and release	-	-	Mouse	91, 214, 215
Ratio IL-10/IL-12	↓	-	Mouse	214, 215
IL-23 release	↑	-	Human	216
IL-6	↑	↓	Mouse	214
TNF	↑	↓	Human	217
IL-1Ra/IL-1 decoy receptor release	-	↑	Mouse	89
CHEMOKINE				
AMAC-1 release	-	↑	Human	218
MIP-1α mRNA	↑	-	Human	89, 218
MDC (CCL22) mRNA expression	-	↑	Human	219
TARC (CCL17)	↓	↑	Human, mouse	220, 221
SECRETORY PROTEINS				
FIZZ1 mRNA	-	↑	Mouse	222
YM1/2 mRNA	-	↑	Mouse	222

Table 1: Markers for the different macrophage subtypes. ↑ an increase in expression/activity, – no change in expression/activity. ↓ : a decrease in expression/activity. ? : unknown.

factors, like transforming growth factor-beta (TGF-β) and platelet derived growth factor (PDGF) [108]. The angiogenic potential of AA macrophages is higher compared to CA macrophages [109]. Due to the production of growth factors and stimulation of angiogenesis, AA macrophages are considered tumor promoting. *In vitro* CA macrophages were shown to be cytotoxic to tumor cells but not to normal cells [110].

Until now, little research has been done about the presence and function of these different subsets of macrophages in MS.

MACROPHAGES IN AXONAL DAMAGE

It is generally accepted that macrophages/microglia are involved in the pathogenesis of MS and EAE. Some indirect, though very convincing, evidence has been found supporting a role for macrophages in axonal damage. First of all, a close association has been found between inflammation and neurodegeneration in all lesional and disease stages of MS [111]. Another important finding is the correlation between the location of axonal damage and cellular infiltrates, the locations with the most axonal loss also showed signs of inflammation and macrophage presence [40;48;112]. Furthermore a correlation between the number of infiltrating macrophages and the extent of axonal damage was observed in active and chronic active MS lesions [47;49;112]. Elimination of infiltrating macrophages or resident microglia in the CNS has a suppressive effect on the clinical signs of EAE and reduced CNS inflammation [113;114]. Conversely, activating macrophages by blocking the inhibitory signal of CD200/CD200R between neurons and macrophages, led to a significant enhancement of the clinical signs of EAE [115;116]. In MS lesions a decrease in expression of macrophage inhibitory molecules, CD200 and CD47, was found indicating that release of inhibition of macrophages could play a role in MS [116]. Macrophage and microglial activation is associated with an upregulation of a plethora of inflammatory mediators that could mediate the acute damage seen in the axons even early in the disease. In the following paragraphs these mediators with their possible mechanisms will be shortly reviewed.

Oxygen radicals

During inflammation, as occurs in MS lesions, ROS, such as superoxide and NO, are released in high concentrations. Many studies have shown increased concentrations of markers for oxidative stress, such as oxidized proteins, lipids and DNA, in the CNS of EAE animals and MS patients and sera of MS patients [117-122]. ROS production by activated peripheral blood mononuclear cells is increased in MS patients during a relapse [123;124]. A higher production of NO has also been found in MS lesions, reflected by the expression of iNOS in macrophages/microglia and astrocytes [125;126]. In MS lesions, nitrotyrosine, a marker for peroxynitrite formation, was observed [126;127] and in EAE it was found to correlate with disease severity [128]. In CSF, serum and urine of MS patients increased levels of the metabolites of NO production have been found [129;130].

ROS play an important role in the pathogenesis of MS and EAE. A strong indication for the role of ROS in EAE was given by the fact that treatment with ROS

scavengers and antioxidants reduced inflammation and axonal damage in acute and chronic EAE [131-134]. ROS can also indirectly induce toxic effects through their ability to inhibit glutamate transporters and thereby inducing excitotoxicity [113;135]. A few possible ways by which ROS induce axonal damage and neuronal death have been proposed. Peroxynitrite can damage both myelin and axons [136]. NO can induce a reversible conduction block in axons exposed to low frequency stimulation [137]. When axons, that are conducting impulses at physiological frequencies, are exposed to levels of NO which are likely to occur at sites of inflammation, they degenerate [138;139]. Furthermore, by inducing oxidative stress in mitochondria, ROS and NO could lead to axonal damage [140-142]. Axons are metabolically very active, which makes them especially sensitive to the effects of extracellular ROS from macrophages. Mitochondrial dysfunction, due to the oxidative stress, leads to energy deficiency and can thereby lead to impairment of axonal transport, accumulation of APP and ultimately neuronal death [122]. In both EAE and MS mitochondrial function has been shown to be impaired [76;143] and to correlate with the presence of macrophages/microglia and production of ROS [144]. Reduced energy levels can also cause increased sodium leakage into the axon and thereby reversal of the operation of the sodium-calcium exchanger, axonal swelling and increased intracellular calcium concentrations, leading to the induction of apoptosis [145]. Treatment with sodium and calcium entry blockers protected axons from this oxidative induced mitochondrial damage [142;146;147].

Cytokines

Cytokines, low-molecular weight immunoregulatory proteins, can have both pro- and anti-inflammatory properties. They can be produced by a variety of cells in a variety of tissues. During MS a plethora of cytokines is produced [148]. Both pro- and anti-inflammatory cytokines are upregulated, seemingly simultaneously as they are all detected in serum, CSF, and cultured mononuclear cells of MS patients and in lesions in EAE in marmosets. A correlation has been observed between the levels of certain cytokines and disease activity, since tumor necrosis factor-alpha (TNF-α) and IFN-γ were correlated with clinical relapses and TGF-β, IL-12p40 and IL-10 are related to remission phase [149-152]. Pro-inflammatory cytokines are thought to be involved in disease pathogenesis, while anti-inflammatory cytokines could be important for disease resolution [148]. Interestingly, specific subpopulations of macrophages/microglia express different cytokines [91]. These subpopulations might correspond to the different activational subtypes of macrophages, i.e. alternatively or classically activated (as described above).

Cytokines have many different functions and in a complex disorder like MS it is not always clear whether they are beneficial or detrimental. For example TNF-α has been shown to be toxic to oligodendrocytes *in vitro* and therefore could induce demyelination. After induction of Wallerian degeneration, a process in which TNF-α has been shown to be involved [153], the number of preserved axons was higher in TNF-α deficient mice compared to wild-types [154]. Further, moderate overexpression of TNF-α leads to demyelination and axonal damage, similar to that observed in EAE and MS [155]. Moreover, TNF-α mRNA expression has been positively correlated

with the demyelinating activity and negatively correlated with oligodendrocyte integrity in periplaque white matter in MS biopsies [156]. TNF also has protective effects like its elimination of autoreactive T cells via TNF receptor 1 [157]. Finally, treatment with anti- TNF-α antibodies was beneficial in EAE. However, treatment in humans with an antibody directed to TNF-α enhanced the disease [113;158;159]. Another indication that cytokines might be differentially involved in MS and EAE came from studies using IFN-γ. Treatment with anti-IFN-γ led to more severe EAE, while administration of IFN-γ ameliorated the disease [160;161]. In humans the opposite occurred, since treatment with IFN-γ worsened MS [162]. Similar divergent effects have been described for IL-6. No demyelination and inflammation were found in EAE induced in mice deficient for IL-6, however absence of IL-6 immunoreactivity correlated with oligodendrocyte loss in inactive demyelinated MS lesions. IL-6 might be involved in remyelination through its effects on oligodendrocyte survival, migration and differentiation [163].

Glutamate

Glutamate is the most common excitatory neurotransmitter in the CNS. However, excessive concentrations of glutamate lead to overstimulation of the glutamate receptors and finally cell death, through influx of calcium. This type of cell death is called excitotoxicity. In EAE excitotoxicity was reported to be of importance since AMPA/kainate receptor antagonists were shown to ameliorate the clinical score, which corresponded pathologically to a reduction in the loss of oligodendrocytes and axonal damage [164-166]. Excitotoxicity is thought to play a role in MS since increased concentrations of glutamate have been observed in CSF of MS patients [167;168] and this increase was found to be associated with the severity and course of the disease [169]. Alterations in glutamate homeostasis, e.g. patterns of expression of glutamate receptors, transporters and glutamate-metabolizing enzymes, have been found in both MS and EAE and were associated with demyelination and axonal damage [170-175]. In lesions of MS patients increased expression of glutaminase has been observed in activated macrophages and microglia near damaged neurons [170]. Macrophages, microglia and astrocytes are sources of excess glutamate in EAE and MS. Furthermore, pro-inflammatory mediators, like IL-1β and TNF-α are suggested to decrease glutamate clearance by astrocytes, increasing extracellular glutamate and enhancing excitotoxicity [176].

Proteases

Macrophages also release matrix metalloproteinases (MMPs), in order to degrade the extracellular matrix to facilitate migration in the CNS. In MS lesions MMPs are present and they may directly cause axonal transsections [177-182]. Macrophage-mediated axonal retraction, as visible in spinal cord injury, has been found to be due to the expression of MMP9 [183]. Another protease that plays a role during MS is tissue plasminogen activator (tPA). It is increased in both MS and EAE [184-187]. Microglia and macrophages secrete tPA. Furthermore, tPA is involved in monocyte migration over the blood brain barrier, by the break down of tight junctions between endothelial cells [187]. *In vitro*, tPA is able to induce neuronal apoptosis [188].

Delayed demyelination and axonal degeneration has been found after induction of EAE in tPA knock out mice [184].

MACROPHAGES IN AXONAL REPAIR

Although the studies mentioned above all indicate that macrophages play a detrimental role, the truth is probably not as black and white. Several studies have shown that macrophages could also play beneficial roles during CNS repair. A first indication, suggesting that macrophages actually stimulate regeneration in axons, came from retinal ganglion cell regeneration. An early study found that macrophages appeared to mediate the pro-regenerative effects of lens injury after nerve crush, since macrophage infiltration corresponded with an upregulation in growth-associated protein (GAP)-43 expression levels. Moreover, intraocular zymosan injection, which results in massive macrophage infiltration, led to increased GAP-43 expression and axonal regeneration in absence of lens injury. *In vitro*, zymosan stimulated macrophage conditioned medium was able to enhance axon regeneration, with the axon-promoting effects being mediated by molecules smaller than 30 kD. The molecule responsible for the pro-regenerative effects of activated macrophages was oncomodulin and affected not only retinal ganglion cells but also dorsal root ganglion cells [189]. Moreover, macrophages recruited to the site of nerve crush express the axon guidance molecule EphB3, while injured retinal ganglion cell axons express EphrinB3, a receptor for EphB3. EphB3 stimulated retinal ganglion cell outgrowth toward the source of EphB3 *in vitro,* whereas in heterozygous and homozygous null mutant adult animals a significant decrease in axon outgrowth in the injured nerve could be observed [190].

In the field of spinal cord injury (SCI), divergent effects of macrophages have been reported as well. After SCI, implantation of macrophages that were pre-exposed *ex vivo* to peripheral nerve fragments induced repair and partial recovery of functionality [191]. During SCI myeloid cells, including macrophages, appeared to be essential for repair since they create a growth-permissive environment in which axonal regeneration can take place [192]. Shechter et al. [193] have found that at the margins of a lesion infiltrating macrophages mediate an immunoregulatory role by secreting the anti-inflammatory cytokine IL-10, which contributes to recovery after SCI. The release of IL-10 indicates that these beneficial macrophages could have an AA macrophage phenotype, since AA macrophages secrete high levels of IL-10 [92]. Finally, Kigerl et al. [194] have identified both CA and AA macrophages during SCI in the mouse. They found that the CA macrophage response persisted, while AA macrophage response was transient, which could lead to the stunted functional recovery seen in these animals. *In vitro* these authors showed that CA macrophages are neurotoxic, while AA macrophages were not and could actually induce neuronal outgrowth even on a growth inhibiting substrate.

Recent evidence also points to a beneficial role of macrophages during repair in MS. Depletion of macrophages has been shown to reduce remyelination in demyelinating models [195;196], due to reduced oligodendrocyte progenitor recruitment and differentiation and altered growth factor expression [196]. Conversely, enrichment of whole brain aggregate cultures with macrophages promotes the capacity for

initial myelination and remyelination following demyelination [197]. Moreover, through the removal of myelin debris and stimulation of oligodendrocyte progenitor migration, proliferation and differentiation, macrophages can promote remyelination. Activated macrophages/microglia are also a source of a variety of growth factors, neurotrophins and their receptors during EAE and in MS lesions [198-200].

Macrophages could contribute to the resolution of the inflammation in MS thereby inhibiting further injury to the axons. It was found that myelin-laden foamy macrophages in active lesions expressed anti-inflammatory molecules, while pro-inflammatory molecules were not expressed. *In vitro*, myelin ingestion induced foamy macrophage morphology and expression of anti-inflammatory molecules and inhibited the response to pro-inflammatory stimuli. This indicated a strong immunosuppressive function for foamy macrophages [201]. These foamy macrophages display functions and activities that might put them in the category of AA macrophages, since they produced anti-inflammatory cytokines and showed suppressed LPS activation *in vitro*. Another indication that foamy macrophages might be AA macrophages was that they express CD163, a marker for AA macrophages, although the expression of mannose receptor, another marker for AA macrophages, is low in these cells [202]. Furthermore, perivascular macrophages, which are located at the blood brain barrier, have an AA macrophage phenotype, since they do express both CD163 and mannose receptor. This could be important since their location at the blood brain barrier means they occupy a strategic position to control innate and adaptive immune responses in the brain. The AA macrophage phenotype might be responsible for a lower inflammation rate at the blood brain barrier.

Another indication that macrophages are involved in axonal repair was found in the fact that activated macrophages are present in the areas of increased GAP-43 expression. Levels of GAP-43, a marker for axonal growth and synaptogenesis, were decreased inside lesions, while being upregulated around lesions. Although no correlation was found between the intensity of GAP-43 staining and macrophage presence, macrophages were consistently observed in areas of increased GAP-43 expression. Macrophages could be the source of neurotrophic factors that increase GAP-43 expression [203]. However, the authors did not investigate the phenotype of the macrophages present in the areas of GAP-43 expression in comparison to the macrophages present inside the lesions.

AIM OF THE THESIS

As discussed above, activated macrophages can have both beneficial and detrimental effects in the development of MS lesions. These divergent effects of macrophages could be due to the fact that different subtypes or activation phenotypes of macrophages exist (hypothesis depicted in Figure 1). In the CNS and specifically in MS relatively little research has been done focusing on the divergent effects of the different subtypes of macrophages. In this thesis the aim was to determine the effects of differently activated macrophages on axonal damage and repair.

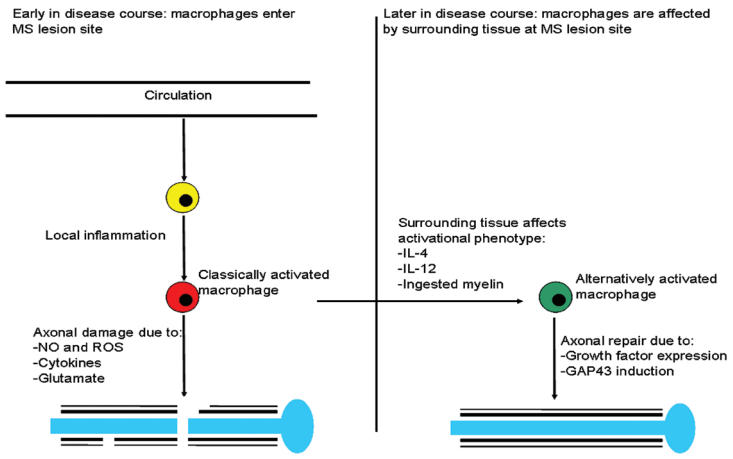

Figure 1: Hypothesis of macrophage activation in MS lesions. As the macrophages enter the lesion site, they become classically activated due to the local inflammation. These classically activated macrophages induce axonal damage by secreting neurotoxic substances such as NO, pro- inflammatory cytokines and glutamate. The macrophages at the lesion are present in the CNS tissue for longer periods of time. Slowly the surrounding tissue starts to affect the activational phenotype of the macrophages. Due to IL-12 and IL-4 secreted by astrocytes and ingestion of myelin, the macrophages take on an alternatively activated phenotype. These macrophages are involved in axonal repair due to the expression of growth factors, induction of GAP-43 expression in neurons and secretion of anti-inflammatory cytokines.

Outline

The main focus of this thesis was to investigate the role of phenotypically different macrophages on various phases of axonal damage during MS. Whole brain spheroid cultures provide a valuable model in which to study the effects of macrophages on neuronal damage during de- and remyelination. In the spheroid cultures all CNS cells are present in a three dimensional conformation and multilayered myelin is formed. Axons are myelinated in spheroids, in contrast to MS lesions where the myelin sheath is damaged. Therefore, in order to mimic the demyelinated state in the spheroid model we aimed to develop a model to induce demyelination in the spheroids (**chapter 2**).

Little research has been performed on differently activated macrophages in the CNS. First we determined the migratory characteristics of differently activated macrophages in the context of the CNS. We studied the migration of AA and CA macrophages towards conditioned medium from different CNS cell types and the motility and adhesion of AA and CA macrophages (**chapter 3**).

Next, we addressed the question how the AA phenotype is induced in MS lesions. Boven et al. 201 had suggested that in MS lesions foamy macrophages have a AA phenotype, since they expressed little pro-inflammatory cytokines and iNOS. *In vitro*, myelin ingestion was found to inhibit the LPS response. Liver X receptor (LXR) is involved in both lipid metabolism and immune modulation. We hypothesized that activation of liver X receptor (LXR) by myelin ingestion could lead to the AA

phenotype and reduction in LPS response due to the blocking effect of LXR on nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB). In **chapter 4** we investigated whether knock-down of LXR could inhibit the repression of the LPS response seen after myelin ingestion. However, during this study we observed differences between myelin preparations in cytokine induction. One preparation induced the expression of IL-10 and TNF-α and LPS insensitivity, while another did not. We questioned whether LPS contamination in one of the preparations was responsible for induction of both the high anti-inflammatory cytokine levels and the LPS insensitivity (**chapter 5**).

Chapters 6 and **7** focus on the effects of differently activated murine macrophages on neurons. In MS lesions the most prominent function of macrophages is the phagocytosis of myelin. However, due to axonal damage, neuronal debris will also be present in lesions. Therefore we aimed to determine whether the phagocytosis and degradation of neurons and neuronal fragments differs between the different subtypes of macrophages (**chapter 6**). We next investigated whether the differently activated macrophages could have divergent effects on neuronal cultures. We exposed neuronal cultures directly to differently activated macrophages or their conditioned medium. We hypothesized that CA macrophages would be damaging to neurons, due to the release of cytotoxic agents and the phagocytosis of neurons, and that AA macrophages could be neuroprotective (**chapter 7**).

In **Chapter 8** we focus on the presence of CA and AA macrophages in MS lesions. We determined whether these divergent phenotypes actually occurred in MS lesions (chapter 8). Indications that differently activated macrophages were present in MS lesions had been found, but untill now nobody had ever performed a systematic study of markers for both CA and AA macrophages in MS lesions. We selected a panel of markers based on literature that should be differently expressed on human AA versus CA macrophages and determined their expression in lesions.

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