Neurodegeneration and macrophages; a beneficial or harmful role for macrophages and microglia in neuronal damage during multiple sclerosis

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Abstract

Axonal loss and neuro-inflammation are two early hallmarks of multiple sclerosis (MS), which is a chronic neurodegenerative disease of the central nervous system (CNS). Removing myelin and neuronal debris is a general role for microglia to maintain CNS homeostasis and to promote remyelination. However, during neuro-inflammation in MS two phenotypes of activated macrophages (M1 and M2) are present in active and chronic active MS lesions. They originate from bone marrow or the yolk sac and are named bone marrow-derived macrophages or resident microglia respectively. Several studies suggest a dual role for macrophages in MS, when they are activated they might play a neurotoxic or a neuroprotective role. A phenotypic switch of macrophages induces M1 macrophages in active MS lesions, which leads to an enhanced level of pro-inflammatory cytokines. Macrophages produce neurotrophic factors to communicate with neurons, which is essential to promote neuronal survival. Some of the current therapies for MS are based upon regulation of M1 and M2 macrophages, to reduce neuronal loss.

We propose that macrophages play both a harmful and beneficial role in neuroregeneration in which clearance of debris and secretion of macrophage-derived cytokines results in neuronal protection. Conversely, ROS, NO and pro-inflammatory cytokines secreted by M1 macrophages promote neuronal damage. During MS the clearance of debris and the production of cytokines that promote neuronal survival might be inhibited.

Keywords: multiple sclerosis, neuronal damage, M1 and M2 macrophages, neuro-inflammation, neuroprotective, neurotoxic

Layman summary

Clinical signs of multiple sclerosis are loss of motor ability, which is reversible during early stages of MS. Other symptoms are sensory disability in limbs and face, visual loss and disturbance of balance. The clinical course of MS consists of two phases, during the early phase neuronal damage is reversible and a high level of neuro-inflammation is observed. During MS progression neuronal damage is irreversible and neuro-inflammation is reduced.

MS pathology

Inflammation, damaged myelin sheaths and neuronal damage in the CNS are features of MS lesions. CNS inflammation is characterized by infiltration of large numbers of immune cells, like lymphocytes and macrophages, which are normally present in the CNS in very low amounts. Lymphocytes belong to the adaptive immune system, which are involved in killing pathogens and infected cells, and their activation results in immunological memory. They are activated by the innate immune system through macrophages, which phagocytose pathogens or pathogen infected cells and activate the adaptive immune system. During MS both macrophages and lymphocytes cross the blood-brain barrier (CNS blood vessel wall) to enter the CNS. Besides macrophages deriving from the peripheral immune system, another population of macrophages is already present in the CNS, resident microglia. They explore the CNS environment to clear damaged cells maintaining a stable condition of the CNS (homeostasis). Therefore, when no immune cells are present resident microglia can promote neuronal survival.

This review aims to investigate the role of macrophages in neuronal damage and loss in MS lesions. To answer this question we will focus on the switch between two phenotypes of macrophages (M1 and M2).

Neuronal and myelin damage

Besides neuro-inflammation, neuronal damage and damaged myelin sheaths are two other characteristics of MS. Neuronal injury in which axons (projection of the neuron that transmits signals to other neurons) are damaged results in disrupt transport of proteins through the axon, which leads to functional loss of neurons. Myelin sheaths are also damaged, which contribute to the loss of function of neurons. Myelin insulates the axon and is important for fast and efficient neuronal transmission in which electric signals are transported to other neurons.

Role of activated macrophages in MS lesions

Several lesion types are distinguished in MS, based upon the amount of macrophages and the level of inflammation and neuronal damage. Active MS lesions are observed during early stages of MS and are

characterized by axonal damage and inflammation caused by enhanced infiltration of macrophages and lymphocytes. Phagocytes present in this lesion type are resident microglia (permanent present in the CNS) and blood-derived macrophages that cross the blood-brain barrier, also called bone marrow-derived macrophages. During MS both types of phagocytes are activated by different cytokines (signalling molecules secreted by immune cells) and the type of activation factor defines which macrophage phenotype is activated. As a result, classically activated (M1) or alternatively activated (M2) macrophages are found in MS lesions. M1 macrophages play a harmful role, because they secrete pro-inflammatory cytokines that induce the inflammatory response, which results in neuronal loss. Conversely, M2 macrophages secrete anti-inflammatory cytokines that reduce the inflammatory response and protect neurons.

Neuronal-macrophage crosstalk

Neurons communicate with resident microglia and bone marrow-derived macrophages through receptor-ligand interactions (ligand secreted by neurons communicate with receptor expressed on the surface of macrophages) or neurotrophic factors. Neurotrophic factors promote neuronal survival and regeneration. Several studies demonstrate an inhibition of macrophage receptors or neurotrophic factors leads to a disruption of the crosstalk between neurons and macrophages. This results in activation of M1 macrophages, which promote neuronal loss.

Phenotypic macrophage switch

In active MS lesions M1 macrophages are more abundantly present, indicating a role for M1 macrophages in neuronal damage and suggesting that a phenotypic switch from resting macrophages to M1 macrophages takes place. Additionally, enhanced levels of pro-inflammatory cytokines are secreted by M1 macrophages that might result in neuronal damage. However, we suggest M1 macrophages also play a beneficial role in promoting neuroregeneration by clearing damaged cells and myelin.

Therapies for MS

Many MS therapies are based upon regulation of inflammation. These therapies include regulation of monocyte infiltration (precursor cells of bone marrow-derived macrophages), modulation of the inflammatory responses and/or promoting remyelination at the site of injury. Binding of monocytes to blood vessel walls is inhibited by Tysabri and thereby this therapy reduces infiltration of monocytes into the CNS. Furthermore, current MS therapies are based on inhibition of pro-inflammatory cytokines by classically activated macrophages, which reduce the inflammation response in the CNS. Reducing pro-inflammatory cytokines might be beneficial for neuronal survival.

Besides secretion of inflammatory cytokines, activated macrophages also secrete growth factors that induce oligodendrocyte development. Oligodendrocytes produce myelin and promote remyelination

by which the myelin sheath around axons is restored. Oligodendrocytes are damaged during MS, but M2 macrophages secrete growth factors that induce oligodendrocyte development, which results in remyelination. During remyelination myelin sheaths are restored and thereby axonal outgrowth is promoted.

To conclude, we suggest that M1 macrophage-derived pro-inflammatory cytokines play both a harmful and a beneficial role in neuronal survival. Anti-inflammatory cytokines secreted by M2 macrophages play a beneficial role in neuronal survival.
Besides macrophages, astrocytes might also play a dual role in neuroregeneration. Researchers need to focus on the role of resident microglia and bone marrow-derived macrophages in clearing myelin and neuronal debris, which promotes remyelination and might prevent axonal loss.

Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative and inflammatory disease of the CNS [1]. Among young adults MS is the most common cause for neurologic disability [34]. Prevalence of MS is highest in Europe, the US and Australia and New Zealand (about 0.1%) and can therefore be characterized as a disease of Western countries [67], MS is most common among woman (3:1) [34]. The etiology of MS is not clear, however both genetic and environmental factors have an effect on disease manifestation [9]. The first clinical symptoms of MS are characterized by episodes of motor disability, which are reversible during early stages of MS. Other symptoms that are most common are sensory disability in limbs and face, visual loss and disturbance of balance [67]. The impact of the disease is high during late stages when disease progression is slow and motor disability is irreversible [60]. Severe and permanent clinical signs of MS are observed during progression of the disease. However, axonal damage is already observed in early stages of MS, when clinical signs are reversible. Apparently permanent neurological disability is not caused by axonal damage in early stages.

Pathology of MS is characterized by the presence of large numbers of immune cells present in active and chronic active lesions [61]. A correlation between enhanced infiltration of immune cells and axonal damage is observed during early stages of MS [19, 37]. Therefore, therapy based on modulation of the immune system is most effective during early stages [17, 21]. Both resident microglia and bone marrow-derived macrophages play a role in maintaining CNS homeostasis, which is disturbed in MS [48] and causes axonal damage during early stages of the disease [12, 44].
In this review we will focus on bone marrow-derived macrophages and microglia in the CNS and their two opposite roles during MS [1, 12, 61]. A phenotypic switch to M1 macrophages and overactivation of this phenotype is observed in MS lesions. The question still remains how overactivation of a specific macrophage phenotype can cause neurodegeneration, depending on the local environment and interaction with other cells in the CNS [12].

The aim of this review is to investigate the role of bone marrow-derived macrophages and resident microglia in axonal damage in MS lesions. To answer this question we will focus on the switch between two phenotypes of macrophages (M1 and M2) and which factors are involved in neuronal loss. In addition, we will discuss how future and current therapies might cause a shift in macrophage activation leading to a more protective phenotype to reduce axonal loss.

Chapter 1 **Pathogenesis of multiple sclerosis**

Multiple sclerosis (MS) is a chronic neurodegenerative and inflammatory disease of the central nervous system (CNS). Two hallmarks of the disease are demyelination and axonal loss, which are detected in grey and white matter of the brain and also in the spinal cord [1, 9]. Ferguson et al (1997) suggested an association between axonal loss and inflammation; for demyelination such an association with inflammation was already described in MS [19, 21]. During early onset of MS axonal loss and an increase in immune cell infiltration was observed in MS lesions [24, 26, 37]. This review will highlight the relation between axonal loss and inflammation during development of MS.

MS lesion types

Different types of lesions, like reactive, active, chronic active and chronic inactive lesions are observed in the grey and white matter of MS patients [54]. Reactive lesions contain microglia that express M1 markers, but no demyelination is observed in contrast to the other three lesion types. Active lesions are characterized by axonal damage and inflammation caused by enhanced infiltration of foamy macrophages and lymphocytes [24]. Active lesions are frequently observed during early stages of the disease [38]. Features of chronic active lesions are low numbers of M1 macrophages in the centre surrounded by foamy macrophages at the rim [24]. Foamy macrophages present in active and chronic active lesions show uptake of myelin lipids or products of myelin breakdown [26, 61]. These macrophages produce anti-inflammatory cytokines and originate from resident microglia or bone marrow-derived monocytes [3]. The last type of lesion is called chronic inactive lesion and is characterized by gliosis and a reduced amount of infiltrated macrophages and lymphocytes [24].

Clinical course of MS

Four types of clinical courses are related to progression of MS, which often starts with the relapsing-remitting course (RRMS). This course is characterized by episodes of neurological disability caused by inflammation leading to axonal damage (figure 1). Subsequently, such episode is followed by neurological recovery, because axonal loss could be compensated by other neurons causing reorganization in for example the cortex [17, 60]. Within approximately 25 years the majority of MS patients develop a secondary-progressive course (SPMS) in which persistent neurological disability is the main feature [10, 17, figure 1]. The SPMS phase is called secondary because it is a follow up phase of RRMS. Patients that reach this phase show no full recovery of axonal damage and loss, because axonal loss cannot be compensated by other neurons in the CNS. Furthermore, inflammation is not the main cause of axonal loss anymore. Both phases of MS (RR and SP) show acute axonal damage, which is higher during early phases of the disease compared to later phases.

A minor percentage of patients will not develop SPMS but instead show another form of progressive MS named primary-progressive MS (PPMS) or progressive-relapsing course (PRMS). In PPMS

neurological ability declines gradually from onset of disease on, and no periods of recovery occur [38]. PRMS shows acute attacks with partial or full recovery, however progression of neurological functions declines [17].

MS pathogenesis

Inflammation of the CNS starts with immune cell infiltration, like T and B cells and macrophages leading to remyelination, gliosis, formation of glial scars and axonal loss [38]. Acute axonal damage is already observed during early stages of MS [37] and is a characteristic of chronic active and active lesions; little axonal damage is observed in remyelinated lesions. During MS progression axonal damage continuous and shows no difference in acute axonal damage over time [20, 37]. Studies on post-mortem tissue of MS patients with progressive disease shows for the first time a correlation between axonal loss in motor tracts in the spinal cord and disability of movements [60]. Furthermore, a correlation was found between the amount of macrophages and the extent of damaged axons in active MS lesions [19].

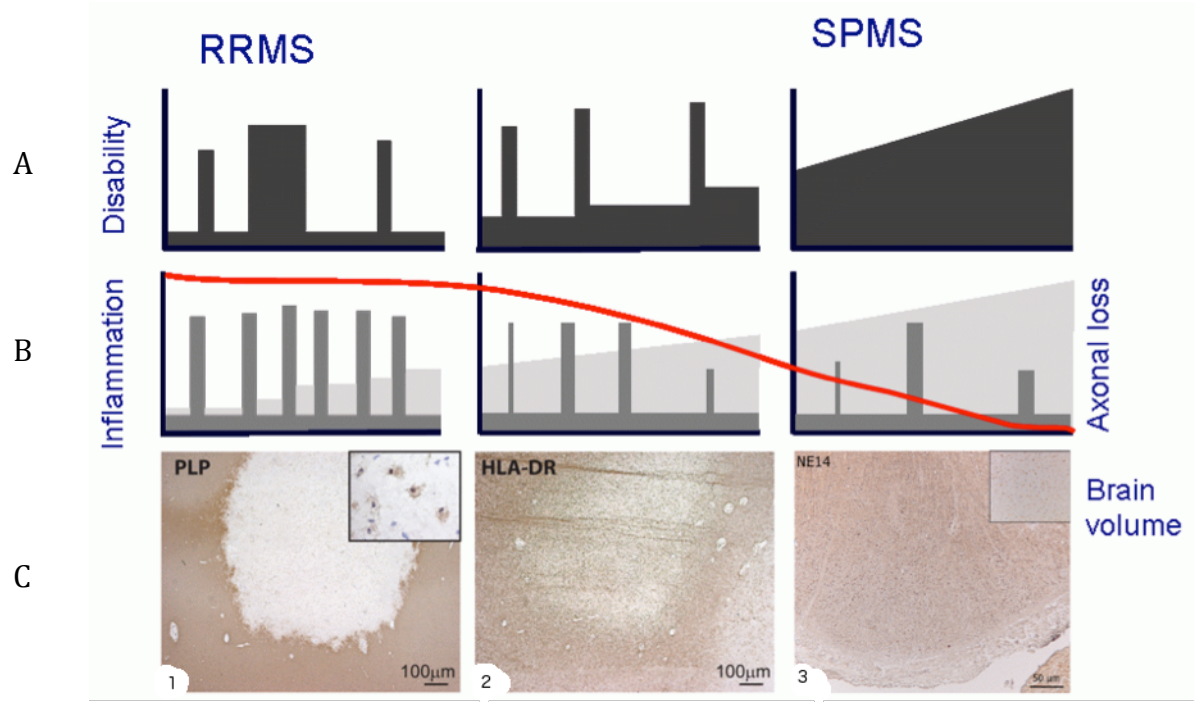

Figure 1 Clinical course of MS, demonstrating neurological disability and inflammation during stages of MS.
A) Early stages of MS (RRMS) are characterized by episodes of neurological disability (reversible), during MS progression (SPMS) increased neurological disability (irreversible) is observed. B) RRMS is associated with axonal loss (grey line) and high inflammation (grey beams). During SPMS axonal loss (grey line) is increased and inflammation is decreased (grey beams). The red line shows decreased brain volume during MS progression and indicates increased brain atrophy. C.1) Certain macrophages in an active MS lesion have taken up proteolipid proteins (PLP, insert), indicating they phagocytose myelin debris, C.2) shows that all cells expressing HLA-DR are macrophages indicating that high numbers of macrophages are located at the lesion site. C.3) The number of neurons in the lesion, marked by NE14 (neurofilament), is decreased during late stage of MS, indicating neurological disability.

Three different mechanisms were proposed, which might explain the development of progressive MS pathogenesis [38]. One mechanism suggests that in the beginning the main feature of MS is inflammation, however after a couple of years this chronic inflammation is not responsible for neuronal degeneration anymore. A second proposed mechanism explains that MS is characterized by inflammation, which results in neuronal damage. However, current therapies are not able to treat the inflammation response during progressive stage of MS. The last proposed mechanism describes that inflammation can regulate neurodegeneration during early stages of the disease. This mechanism suggests that MS is primarily caused by neurodegeneration [38]. Taken together all mechanisms are based upon inflammation and axonal damage during early development of MS.

Markers for inflammation and neurodegeneration

Multiple markers are used to monitor neurodegeneration in MS. In MS patients a correlation between atrophy of the brain and inflammation was detected with MRI and spectroscopy. Here, atrophy was a marker used to determine neurodegeneration and gadolinium marked inflammation levels that correlates with active lesions [21]. Pro-inflammatory cytokines are markers, which are often used to detect inflammation in cerebral spinal fluid (CSF). A couple of examples of these markers are interleukins (ILs), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which are elevated in MS and are secreted by microglia and macrophages [65]. The blood-brain barrier forms a barrier between the bloodstream and the brain preventing MS markers to leave the CNS. Therefore, markers in the bloodstream do not correlate with brain pathology and CSF is used to detect inflammatory markers in the CNS [65]. Markers for progressive MS (late stage) are actin and tubulin, which are fragments of the cytoskeleton. CSF from MS patients shows enhanced concentrations of both actin and tubulin [65].

Animal models for MS

Animal models are essential to unravel the pathogenesis of MS and to design novel therapies. Experimental autoimmune encephalomyelitis (EAE) is a model frequently used to study pathological and clinical aspects of MS [49]. Around 85 years ago, this model was introduced for the first time. To induce EAE, animals (mostly rodents) are immunized with an antigenic myelin protein, for example myelin basic protein (MBP) or myelin oligodendroglial glycoprotein (MOG) in combination with Freund's adjuvant including lipopolysaccharide (LPS). As a result, autoreactive T lymphocytes are generated which, after transmigration across the blood brain barrier, cause inflammatory lesions in the CNS; most of them were found in the spinal cord white matter. Furthermore, axonal loss was detected in the spinal cord of mice that reached the end-stage of the disease. However, this model showed virtually no demyelination, which is besides axonal loss another main feature of MS [49, 54]. Paralyzed hind limbs are clinical signs observed in EAE animal models and they show spontaneous recovery of their paralyzed limbs, therefore animals recovered from EAE are immune for EAE re-

induction, the reason for this is unclear [54]. In this review we will focus on axonal loss and inflammation in MS and the EAE model will be a good model to study these features, since EAE is accompanied by inflammation and axonal loss in the CNS.

MS therapies

Current therapies for MS treatment are based upon modulation of the immune system and/or prevention or reduction of neuronal loss during early stages of MS [17, 21, 33, 51]. Currently, several therapies are available to treat MS and about eight of them were approved by the US food and drug administration (FDA) [60]. Most common first-line therapies to treat MS are interferon-β (interferon-β1a and interferon-β1b) and glatiramer acetate (Copaxone), which are used during early stages of MS. Both therapies regulate macrophage activation leading to modulation of cytokine production in which secretion of pro-inflammatory cytokines is decreased [47, 64]. Furthermore, other therapies are based upon promoting neuronal survival and reducing neuronal loss.

Axonal damage is one of the hallmarks already observed during early stages of MS, the relapsing-remitting phase (RRMS) [37]. Axonal damage is correlated with increased macrophage and lymphocyte recruitment to active and chronic active lesions. Current treatment is based on immune modulation during early phases of MS. However, during late stages this treatment is not effective anymore and progression of the disease cannot be reduced. We suggest axonal loss is too extensive, therefore axonal loss cannot be compensated by other neurons in the CNS and proper remyelination cannot take place. To determine in which stage treatment is still effective, focusing on prevention of major axonal loss, studies need to demonstrate in which stage axonal loss is reversible.

Furthermore, to design a therapy aimed at reducing axonal damage, the role of immune cells in this process needs to be studied. In the next chapter the role of macrophages, like microglia, during steady-state and pathology of the CNS will be discussed. Different phenotypes of macrophages are present in the CNS during MS and they can play two roles, a neuronal protective or neuronal toxic role.

Chapter 2 **Macrophage/microglia activation in multiple sclerosis**

Several articles discuss the role of macrophages and microglia during MS pathology. Here, we focus on the activation of macrophages and the factors they secrete after activation and the consequences for lesion development and axonal loss in MS.

Two types of macrophages

Phagocytes of the CNS form a heterogeneous population, which consist of two types of phagocytes, resident microglia and bone marrow-derived macrophages [12, 22, 48, 61]. Macrophages belong to the innate immune system and appear in all types of tissue. An important function of macrophages is phagocytosis. Macrophages respond to exogenous and endogenous signals and can migrate to the signal origin in the tissue [44]. Microglia are resident phagocytes of the CNS, they are constitutively present in the CNS [23, 29]. Microglia are only present in the CNS and using their branched processes they search the environment for exogenous and endogenous signals, which can be distinguished into pathogen-associated molecular patterns (PAMPs) or (DAMPs). Thereby, they are playing a role in surveillance of the CNS environment to maintain CNS homeostasis in which they show a resting phenotype and express a low amount of several receptors involved in activation of microglia [12, 52].
Human microglia and bone marrow-derived macrophages regulate homeostasis by clearing debris of damaged cells and myelin, independently of immune cell activation [16, 28]. Both cell types are also required for apoptosis, promotion of axonal growth and differentiation of neurons during steady-state condition of the CNS [12]. Neurotrophic factors can regulate the steady-state phenotype of macrophages in the CNS and are involved in neuronal development. Examples of neurotrophic factors are insulin-growth factor (IGF) and brain-derived neurotrophic factor (BDNF) and they are secreted by neurons [59, 63]. Neurons also express receptors that can down regulate differentiation of macrophages [48].

Resident microglia

Microglia are CNS specific phagocytes that have been studied intensively and can be distinguished from other types of macrophages by their morphology; microglia are characterized by branched processes. Furthermore, the density of microglia differs over brain regions, in the substantia nigra for example a high density of microglia was observed compared to the cerebral cortex [13, 35].
The origin of resident microglia has long been debated, but recent studies show that they originate from myeloid progenitor cells in the yolk sac and enter the CNS during embryonic development. Microglia represent a distinct population of macrophages, they are long living cells and are not replaced by monocytes deriving from the bone marrow [45].

Bone marrow-derived macrophages in the CNS

Another population of phagocytes enter the CNS during or after CNS development, the so-called bone marrow-derived macrophages [12]. In contrast to microglia originating from the yolk sac, bone marrow-derived macrophages in the CNS originate from hematopoietic stem cells present in bone marrow [22]. These cells develop into monocytes, which will circulate in the bloodstream. As reaction on CNS injury, these monocytes will migrate to the CNS where they differentiate into macrophages and together with microglia react on CNS injury [50]. Differentiation of monocytes into macrophages is regulated by growth factors and differentiation factors [23].

Several studies have been performed to discriminate between microglia and infiltrated bone marrow-derived macrophages in the CNS [61]. Using a CCR2 knock-in mouse, bone marrow-derived macrophages are red fluorescent, therefore a clear distinction can be made between bone marrow-derived macrophages and microglia based on their fluorescence [51].

Macrophages/microglia activation

Macrophages use Toll-like (TLR) and NOD-like (NLR) receptors to detect "danger" signals, like pathogens or damaged cells, resulting in activated macrophages and changing of their phenotype [44, 53]. In the CNS, after activation by a "danger" signal, bone marrow-derived macrophages and microglia proliferate and will migrate to the site of injury to secrete cytokines and chemokines [12]. Lipopolysaccharide (LPS) is an example of a pathogen recognized by macrophages. LPS is present in bacterial membranes and is recognized by TLR-4 located on the plasma membrane of mammalian macrophages [56]. *In vitro* studies showed secretion of pro-inflammatory cytokines after stimulation of human microglia, which are classical activated (M1) macrophages, with bacterial endotoxin LPS [8]. Furthermore, injection with LPS caused enhanced axonal damage in EAE animal models [43, 48]. Various other signals can influence the activation status of macrophages [29].

M1 and M2 macrophages

In this review we will discuss two macrophage phenotypes, which play an opposite role in MS lesions leading to neuronal damage or neuronal survival (figure 2). After stimulation with LPS and/or IFN-γ macrophages will differentiate into type 1 (M1), this pathway is called the classical activation pathway. After classical activation, M1 macrophages produce pro-inflammatory cytokines TNF-α, IL-1β and IL-6, which promote neuronal toxicity [12, 48, 61]. M1 macrophages also produce growth factors to promote moderate axonal growth [32]. Classically activated, type 1, macrophages also secrete reactive oxygen species (ROS), nitric oxide (NO) and matrix metalloproteinases (MMP) [12, 53]. Production of ROS and NO results in neuronal oxidative stress and MMPs are proteins that are involved in increased transmigration of leukocytes to the CNS in MS [5, 10, 43]. All these factors play a role in defence of the host, but can also contribute to neuronal damage [12, 44]. Research on

macrophage activation is mainly focused on *in vitro* situations, but *in vivo* it is much more complex to distinguish between different types of macrophages, because not one specific marker can be linked to one macrophage phenotype [61].

In contrast, alternative activated macrophages (M2) play a neuroprotective role by secreting anti-inflammatory cytokines and neurotrophic factors, which are necessary to regulate the immune response. Also, these cells play a role in axonal outgrowth promoting long-distance outgrowth of axons [32]. M2 macrophages are activated by IL-4 or IL-13 [12, 32, 48, 61]. After activation they secrete anti-inflammatory cytokines IL-10, IL-6 and TGF-β to promote tissue repair [12].

The role of pro- and anti-inflammatory cytokines secreted by M1 and M2 macrophages during MS will be described in more detail in the next chapter.

Markers to distinguish between M1 and M2 macrophages in MS lesions

It is difficult to distinguish between classical activated (M1) and alternative activated (M2) macrophages in MS lesions, because macrophages in MS lesions express a variety of markers and no specific marker can be linked to one macrophage phenotype *in vitro*. Furthermore, markers expressed on human microglia/macrophages do not match with markers on microglia/macrophages derived from mice. De Groot et al (2001) [24] described markers expressed on macrophages in different MS lesions; active, chronic active and chronic inactive lesions. Active lesions show CD68, CD45 and HLA-DR (human MHC class II receptor) markers on both bone marrow-derived macrophages and resident microglia. Chronic active lesions are characterized by CD68 expression, which is a marker for M1 and M2 macrophages and is present in both bone marrow-derived macrophages and resident microglia. Vogel et al (2013) demonstrated CD40 expression is the most distinctive markers to characterize M1 macrophages in active and chronic active lesions. M2 macrophages can be distinguished based on the expression of the mannose receptor (MR) in active lesions alone [61].

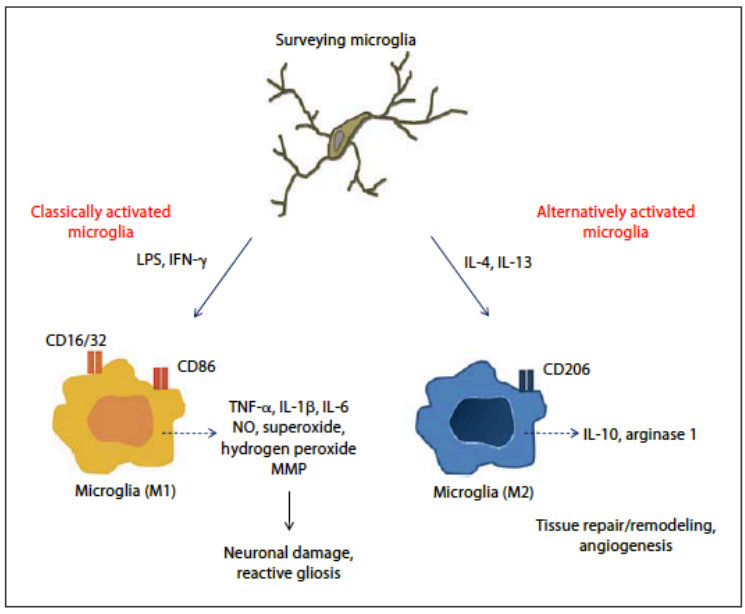

Figure 2 Two pathways of microglia/macrophage activation. Classically activated microglia or macrophages are activated by LPS or IFN-γ and differentiate into a M1 phenotype. This phenotype produces pro-inflammatory cytokines and promotes neurotoxicity. The M2 phenotype of microglia/macrophages is activated by IL-4 or IL-13 and these microglia/macrophages play a neuronal protective role and secrete anti-inflammatory cytokines and neurotrophic factors [12].

Distinction between resident microglia and bone marrow-derived macrophages in MS lesions

An EAE lesion in mice contains both resident microglia and bone marrow-derived macrophages, the majority are microglia [42]. In human MS lesions it is difficult to distinguish between resident microglia and bone marrow-derived macrophages. Melief et al (2012) used CD11 and CD45 markers to distinguish between microglia and macrophages in human MS lesions, but these markers are only useful when flow cytometry is performed to distinguish between microglia and macrophages.

The identification of microglia can be based upon the morphology, resting microglia have branched cell processes and express low surface molecules. But microglia change their morphology during activation into an amoeboid morphology [12] and express high levels of MHC II after activation [24].

Macrophages/microglia activation in MS lesions

The two discussed phenotypes of macrophages are found in active MS lesions in which M1 macrophages are abundantly present [11, 61]. The majority of macrophages found in active lesions are foamy macrophages, which show uptake of myelin lipids and secrete anti-inflammatory cytokines. Vogel et al (2013) demonstrated expression of markers for both M1 and M2 macrophages on a majority (around 70%) of foamy macrophages. Therefore, they suggest that these macrophages represent an intermediate activation phase in active lesions. Active and chronic active lesions are characterized by axonal damage, indicating a role for M1 macrophages in axonal loss. In contrast, lesions with no or limited amount of axonal injury show an increase in IL-10 expression, indicating enhanced infiltration of alternative activated macrophages (M2) [43]. Initiation of EAE in animal models was regulated by classical activated macrophages (M1), however M2 macrophages were highly expressed during recovery of EAE [54]. Taken together, these results indicate M1 macrophages and foamy macrophages might promote active lesions and M2 macrophages promote neuronal recovery and may eventually stop lesion expansion.

In summary, two phenotypes of activated macrophages are present in the CNS, the classical activated macrophages (M1) and the alternative activated macrophages (M2). They are activated by different cytokines or bacterial endotoxins and the type of activation factor defines the activation phenotype. Active MS lesions contain enhanced numbers of macrophages with an M1 phenotype. Both M1 and M2 macrophages play a role in neuronal outgrowth, but have an opposite role in MS pathogenesis. M1 macrophages secrete pro-inflammatory cytokines that may cause neuronal damage. In contrast, M2 macrophages produce anti-inflammatory cytokines that play a role in neuronal repair. Evidence for the hypothesis that M1 macrophages are involved in axonal damage was demonstrated *in vitro*. In active lesions the number of activated M1 macrophages is also increased and they secrete pro-inflammatory cytokines. Therefore, factors secreted by M1 macrophages might be harmful during MS development. The question remains how M1 and M2 phenotypes contribute to the development of MS focussing on

axonal damage and axonal loss. The next chapter will describe which factors and cell types are involved in the activation of M1 and M2 phenotypes resulting in axonal damage.

Chapter 3 The role of macrophages/microglia during initiation of axonal damage and neuroprotection in MS

An important hallmark for early stages of MS is axonal damage [17, 20], which was demonstrated in autopsy material of MS patients showing axonal loss of approximately 70% in the spinal cord [2]. During early stages of MS axonal loss is reversible due to compensation capacity of other axons in the CNS [17]. Active MS lesions are characterized by acute axonal damage, in chronic active lesions a lower amount of acute axonal damage is observed [18].

As a result of axonal damage Wallerian degeneration takes place in which transection of the distal part of axons is induced. Degeneration of the myelin sheath and gliosis are consequences of Wallerian degeneration. Dziedzic et al (2010) observed Wallerian degeneration in early lesions of MS and showed that axonal transection is a feature of Wallerian degeneration, which indicates irreversible axonal loss and is correlated with an increased amount of immune cells in MS lesions [18].

Macrophage-neuron interaction in the CNS

Both neurons and astrocytes can regulate phenotype activation of macrophages via receptors or neurotrophic factors. It has been demonstrated that crosstalk between macrophages and neurons is regulated by receptor-ligand interaction [12, 48]. Several studies demonstrated an interaction between CD200, expressed on neuronal membranes and CD200 receptor, expressed on bone marrow-derived macrophages and microglia. This interaction between neurons and macrophages down regulates the immune response by inhibition of pro-inflammatory cytokines [40]. A disruption in neuronal and macrophage crosstalk mediated by CD200-CD200R interaction is found in human MS plaques [40]. Another receptor involved in crosstalk between neurons and microglia is CX3CR1, which is expressed on microglia and is activated by its ligand CX3CL1, expressed by neurons. CX3CR1-CX3CL1 interaction promotes neuronal survival and suppresses the immune response [33]. These data show the importance of interactions between neurons and macrophages to promote neuronal survival and to modulate the immune responses, thereby maintaining CNS homeostasis.

Not only receptor-ligand interactions are involved in neuronal protection, also neurotrophic factors play a role in neuronal survival and regeneration. Examples of neurotrophic factors are brain-derived neurotrophic factors (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT3), which are secreted by neurons, oligodendrocytes, macrophages/microglia and astrocytes. They promote neuronal survival and regeneration and inhibit pro-inflammatory cytokine release [15].

Neurons also secrete cytokines, like the pro-inflammatory cytokine IL-6. Despite the role of IL-6 as a pro-inflammatory cytokine it also plays a beneficial role in neuronal survival. IL-6 is secreted after neuronal damage and induces proliferation of microglia leading to regeneration of motor neurons [57].

Crosstalk between neurons and macrophages maintains the CNS homeostasis through phenotypic regulation of macrophages, which is regulated via receptor-ligand interactions or neurotrophic factors. Disrupted crosstalk results in neuronal damage and loss, suggesting that neurons control macrophage activation and neuronal survival. Besides receptor-ligand interactions and neurotrophic factors, neurons also produce cytokines that promote neuronal survival.

Macrophage infiltration into the CNS and macrophage activation

Axonal damage is correlated with increased numbers of resident microglia and bone marrow-derived macrophages in the CNS. Besides microglia, the resident innate immune cells of the CNS, bone marrow-derived macrophages enter the CNS via the bloodstream. They need to pass the blood-brain barrier (BBB), in particular the cerebrovascular endothelium, to infiltrate the CNS. The general mechanism of leukocyte infiltration through the endothelium involves attraction of leukocytes through chemokines, secreted at the site of injury. Then, leukocytes interact with selectins on the endothelium causing rolling of leukocytes along the endothelium. Subsequently, leukocytes are triggered, they bind to integrins and migrate through the endothelium to the site of injury [6]. In case of monocyte infiltration, monocytes interact with the cerebrovascular endothelium by binding to receptors to infiltrate the CNS. Therefore, this interaction is necessary for the last step of monocyte infiltration into the CNS.

Once monocytes are arrived in the CNS they migrate to the site of injury and differentiate in bone marrow-derived macrophages. Furthermore, cytokines and "danger" signals activate both resident microglia and bone marrow-derived macrophages, which differentiate into M1 or M2 macrophages. In addition, macrophages and microglia are also activated after loss of neuron-macrophage interaction. In MS patients M1 macrophages dominate the active lesion site [61]. Cunningham (2013) proposed that macrophages in the CNS undergo a phenotypic switch during brain disease, they can change from deactivated macrophages (M2c) to classical activated macrophages (M1) in active lesions [11]. In contrast, MS lesions characterized by remyelination show a dominant M2 phenotype suggesting a phenotypic switch from M1 to M2 macrophages to promote remyelination in remyelinating lesions [42].

To conclude, bone marrow-derived macrophages enter the CNS via the blood-brain barrier, which results in the development of MS lesions. Both bone marrow-derived macrophages and resident microglia are activated by cytokines and "danger" signals in the CNS that results into a phenotypic switch from M2c macrophages into M1 macrophages in MS lesions.

Macrophages and axonal damage

Axonal damage can be observed in active and chronic active lesions with several markers, like amyloid precursor protein (APP), N-acetyl aspartate (NAA), altered distribution of ion channels and non-phosphorylated neurofilaments [2, 20]. APP protein is transported in the axon by fast transport

and accumulation of this protein is detected in active lesions, indicating a defect in axonal transport caused by acute axonal damage [2, 19]. Another marker used to detect early axonal damage is NAA, which is present in axons. It has been suggested that this excitatory amino acid plays a role in synthesis of proteins, metabolism of neurotransmitters and prevents osmotic stress to take place in neurons. A decreased expression level of NAA was observed in MS lesions [2]. The third marker for axonal damage we will discuss here is a redistribution of ion channels in neurons, leading to disturbed ion channel homeostasis due to demyelination of these axons. Subsequently, an influx of high Ca^{2+} levels is observed, leading to axonal loss because high Ca^{2+} levels are toxic for cells [35]. The last marker to demonstate axonal loss is the detection of non-phosphorylated neurofilaments. Normally these non-phosphorylated neurofilaments are present in dendrites, during axonal loss this marker is observed in the entire damaged axon [2].

An important characteristic of MS is neuronal damage in which activated macrophages are involved. Many inflammatory mediators are secreted by activated macrophages in active MS lesions [2], therefore we will describe the role of these mediators in neuronal damage and loss. Pro-inflammatory cytokines are secreted by classical activated macrophages (M1) and promote neuronal damage *in vitro* [32]. An example of a pro-inflammatory cytokine is TNF-α, which can promote myelin damage and oligodendrocytic death resulting in demyelination in an animal model in which neuronal inflammation was induced after LPS injection [14]. However, the role for TNF-α during neurodegeneration in MS is not clear, so the exact role of pro-inflammatory cytokines in MS pathology need to be studied in more detail. Other mediators like free radicals promote neuronal damage, for example ROS and NO, which are secreted by activated macrophages and induce oxidative stress in neurons resulting in axonal damage [43, 14]. Furthermore, other inflammatory mediators secreted by classically activated macrophages are the neurotransmitter glutamate and protease MMP. Increased levels of both mediators promote neuronal and axonal damage, MMPs also promote myelin degradation [5]. Enhanced expression of MMP-9 has been found in tissue of RRMS patients. MMP-9 alters the permeability of the blood-brain barrier leading to enhanced infiltration of leukocytes [10]. The mediators described above are secreted by classical activated macrophages (M1) after LPS or IFN-γ stimulation. Therefore, M1 macrophages are involved in neuronal damage and toxicity.

To conclude, mediators secreted by classical activated macrophages induce inflammation and results in oligodendrocytic death and oxidative stress in neurons. Finally, inflammation leads to neuronal damage, which decreases the success of neuronal regeneration and remyelination [27].

Macrophages and neuroprotection

An increased amount of M1 macrophages was observed in active MS lesions, suggesting macrophages switch to an M1 phenotype [11, 61]. To investigate the role of M1 and M2 macrophages in MS pathology their interactions with neurons need to be studied.

As described above, interactions between neurons and macrophages is modulated by for example the CD200 and CD200 receptor. This interaction between neurons and macrophages down regulates the immune response by inhibition of pro-inflammatory cytokines [40]. In contrast, Meuth et al (2007) demonstrated an enhanced infiltration of immune cells caused by disrupt interaction between CD200 and CD200R *in vitro* and in an EAE model. Finally, disruption of CD200-CD200R interaction leads to axonal damage mediated by macrophages [40]. A disruption in neuronal and macrophage crosstalk mediated by CD200-CD200R interaction is also found in human MS plaques [40]. Another receptor involved in crosstalk between neurons and microglia is CX3CR1, which is expressed on microglia and is activated by its ligand CX3CL1, expressed on neurons. After LPS injection CX3CR1 deficient mice show increased neuronal cell death, suggesting an important role for CX3CR1-CX3CL1 interaction promoting neuronal survival [7]. To conclude, interactions between neurons and macrophages promote neuronal survival and when this interaction is disrupted neuronal damage and eventually neuronal death is observed.

Not only receptor-ligand interactions are involved in neuronal protection, also neurotrophic factors play a role in neuronal survival and regeneration. Neurotrophic factors, like BDNF, NGF and NT3 promote neuronal survival and regeneration and inhibit pro-inflammatory cytokine release. An increased level of astrocyte and macrophage-derived BDNF was observed after spinal cord injury. This study suggested that neurotrophic factors play a role in neuronal survival and regeneration [15].

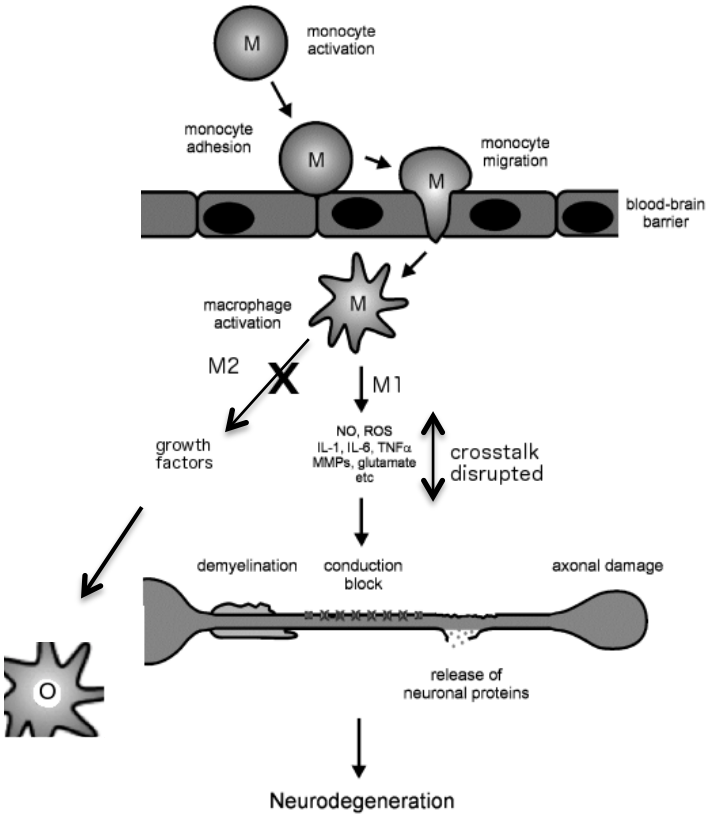

Figure 3 Different mechanisms that cause neurodegeneration. M1 macrophages secrete pro-inflammatory cytokines that damage oligodendrocytes and neurons, resulting in demyelination and conduction block. Crosstalk between neurons and macrophages is disrupted and growth factor secretion by M2 macrophages is inhibited. Macrophage (M) and oligodendrocyte (O) [25].

Besides neurotrophic factors and receptor-ligand interactions, cytokines play also a role in neuronal survival, like IL-6 and IL-10. Despite the role of IL-6 as a pro-inflammatory cytokine it also plays a beneficial role in neuronal survival. IL-6 is secreted by neurons or macrophages after neuronal damage and induces proliferation of microglia leading to regeneration of motor neurons [43]. The anti-inflammatory cytokine IL-10 regulates inflammatory responses and also promotes survival of microglia and neurons [58]. Both IL-6 and IL-10 are produced by alternative activated macrophages (M2) resulting in reduced features of EAE pathology, therefore the M2 phenotype promotes neuronal survival.

Neuronal outgrowth is a characteristic of neuronal regeneration and is promoted by several growth factors, like insulin-like growth factor 1 (IGF-1) and transforming growth factor β1 (TGF-β1) [5]. Both M1 and M2 macrophages stimulate axonal growth, but axonal outgrowth is highly promoted by M2 macrophages [32]. Not only axonal growth, also oligodendrocyte differentiation is promoted by macrophage-derived growth factors [5]. Both axonal and oligodendrocyte growth factors are expressed by alternatively activated macrophages (M2), however during axonal damage and demyelination secretion of these growth factors might be inhibited because the number of M2 macrophages is decreased.

Taken together, crosstalk between neurons and macrophages through receptors, neurotrophic factors or cytokines promote neuronal regeneration. Additionally, oligodendrocytic growth factors secreted by M2 macrophages promote remyelination, which leads to neuroregeneration.

To summarize, alternative activated macrophages (M2) express several receptors that interact with neurons to promote neuronal survival. Also, neurotrophic factors secreted by M2 macrophages are involved in neuronal survival and neuronal outgrowth. When these factors are inhibited or neuronal-macrophage crosstalk is disrupted neuronal regeneration and remyelination is disturbed. In contrast to M2 macrophages, classical activated macrophages (M1) secrete several mediators that promote inflammation and neuronal toxicity.

The function of activated macrophages, whether they play a harmful or beneficial role, depends on several factors, like the CNS microenvironment, stress and “danger” signals, the timing of acute axonal damage and the interaction with other cell types [45]. The functions of M1 and M2 macrophages during M pathology can be used to design efficient therapies to treat axonal loss in MS patients. The last chapter will discuss different therapies for MS to reduce or prevent neuronal loss, like regulation of monocyte infiltration, modulation of the inflammatory response and inducing the remyelination process at the site of injury.

Chapter 4 **Macrophage/microglia related therapies for MS**

Current therapies to treat MS are based upon modulation of the inflammatory response and/or reduction of permanent neurological disability during early stages of MS [17, 21, 33, 45]. In this review we focus on the reduction or prevention of neuronal damage and loss, which is based on modulating neuro-inflammation.

General effect of MS therapies

Rawji and Yong (2013) reviewed several studies about the secretion of several factors by bone marrow-derived macrophages and resident microglia, which can promote neuro-inflammation. Therefore, successful therapies might be based upon targeting these two types of activated macrophages [50]. However, no beneficial treatment to reduce neuro-inflammation showed successful reduction of neuronal damage [21, 52]. In contrast to early stages of MS (RRMS), several studies suggest that neuronal damage occurs independently from inflammation during progressive stage of MS [17, 21]. Therefore, activated macrophages play no essential role anymore during late stages of MS. In this chapter we will focus on therapies to treat axonal damage in early stages of MS, thereby focusing on inflammation mediated by activated macrophages.

The therapies described below are based upon regulation of activated macrophages, M1 and M2 types. These therapies include regulation of monocyte infiltration, modulation of the inflammatory response and/or promoting remyelination at the site of injury.

EAE animal model for MS therapies

As described in chapter 1, features of the EAE animal model are inflammation and axonal loss in the CNS, therefore it is widely used to investigate MS pathology and to design therapeutic strategies. This model shows an increased level of inflammatiory mediators and demonstrates a role for M1 macrophages during development of EAE. During recovery phase of EAE high numbers of M2 macrophages are observed, suggesting a switch has been made from M1 phenotype to M2 phenotype [41]. The EAE model is useful to test therapies based on modulation of activated macrophage phenotypes. However, the EAE model shows no signs of demyelination, so to investigate the role of macrophages in demyelination and remyelination it is useful to use another animal model. In this chapter we will discuss therapies that are focused on modulation of the inflammatory response and/or promotion of neuroregeneration, therefore most studies use the EAE animal model to test potential therapies.

Role of macrophage/microglia during CNS homeostasis and neuro-inflammation

Bone marrow-derived macrophages and resident microglia are key players in neuro-inflammation and axonal damage. As reviewed in Czeh et al (2011) and Geissmann et al (2010) these macrophages show a resting phenotype during steady state of the CNS in which they clear death cells and secrete growth factors, thereby maintaining CNS homeostasis [12, 22]. During MS, the activation of macrophages is altered due to dangerous signals in the environment and results in disturbed homeostasis. If activation signals for macrophages are not regulated properly, Mosser and Edwards (2008) proposed that activated macrophages cause neurodegeneration [44]. Effective therapies need to maintain CNS homeostasis, this can be obtained by preventing macrophage activation in the CNS. However, after tissue injury or when pathogens are present in the CNS activated macrophages are needed to regulate the inflammation response.

Several inflammatory mediators are secreted by activated macrophages and have a pro- or anti-inflammatory role depending on the type of macrophage (M1 or M2). Blocking pro-inflammatory cytokine production by for instance inhibition of M1 activation can be used as therapeutic target. Additionally, blocking infiltration of monocytes and lymphocytes into the CNS may also be a therapeutic target (figure 4, table 1).

Monocyte infiltration via the blood-brain barrier

CNS inflammation starts with infiltration of bone marrow-derived monocytes, therefore these monocytes need to cross the blood-brain barrier and secrete inflammatory mediators. Several therapies can inhibit infiltration of bone marrow-derived monocytes across the blood-brain barrier, targeting the receptors involved in adhesion of monocytes to the endothelium [14, 27, figure 4, table 1].

Natalizumab (Tysabri) is an example of a drug that prevents monocyte infiltration and is based upon blocking VLA-4 binding to VCAM-1 on the cerebrovascular endothelium or blocking CD81 binding to VLA-4 (figure 3). Both mechanisms show beneficial therapeutic effects in EAE models and MS patients [14].

Regulation of the inflammation response

The therapeutic drugs discussed here are based upon modulation of the immune system through regulation of cytokine production by macrophages (figure 4, table 1). However, modulation of the immune system alone is not sufficient to promote neuronal survival, which is essential to prevent neurological disability.

Methylprednisolone, a glucocorticoid drug, reduces the inflammatory response via decrease of pro-inflammatory cytokines in MS patients. Additionally, methylprednisolone induces apoptosis of leukocytes in which T helpercells ($CD4^+$) show the highest rate of apoptosis [39].

A widely used therapeutic drug is interferon-β, which is a cytokine that modulates the immune response. Interferon-β reduces oxidative stress, secretion of cytokines and decreases activation of microglia, resulting in inhibition of axonal damage [47]. Several types of interferon-β, like interferon-β1a (Avonex and Rebif) and interferon-β1b (Betaseron) are used as therapeutic drug.

Glatiramer acetate (Copaxone) is an example of a drug that is designed to increase TH_2 cell production of anti-inflammatory cytokines and growth factors. This therapeutic drug reduces the activation of monocytes by LPS *in vitro* and *in vivo* [64], which might reduce the secretion of inflammatory cytokines.

Minocycline is an example of a therapeutic drug that is used for several neurodegenerative diseases and its main role is to inhibit inflammation through reducing the activation status of macrophages [5, 27, 33, figure 3]. Furthermore, minocycline reduces the production of MMPs changing the permeability of the blood-brain barrier [5].

TNF-α is secreted by classically activated macrophages (M1) and antibodies against TNF-α are effective to treat rheumatoid arthritis. It might also be effective as therapeutic target for MS therapy, but in MS TNF-α has two opposite roles, a neurotoxic or neuroprotective role [56]. After blocking the p55 TNF receptor, the role of TNF-α as pro-inflammatory cytokine is blocked and TNF-α maintains its neuroprotective role in EAE animals [31]. However, as reviewed in Kaltsonoudis et al (2014) clinical studies in MS patients treated with TNF-α antagonist showed increased demyelination indicating that TNF-α antagonists are not effective to treat MS [30].

Recently, a new drug, mapracorat, was designed that can down regulate the inflammatory response via regulation of cytokine production. The MAPK pathway is negatively regulated by MPK-1 and after LPS stimulation the glucocorticoid receptor agonist mapracorat stimulates the expression of MPK-1 in macrophages, which results in reduced secretion of pro-inflammatory cytokines. Mapracorat is a potential therapeutic target to treat eye and skin inflammation, but it can also be effective to treat MS due to its anti-inflammatory characteristics [62].

Efficient MS therapies are based upon inhibition of pro-inflammatory cytokines or classical activated macrophages. Furthermore, therapies may also target activation of monocytes and T lymphocytes.

Promoting remyelination and neuroregeneration

Besides secretion of inflammatory mediators, activated macrophages also secrete growth factors, like IGF-1 and TGF-β involved in remyelination. Growth factors promote both axonal growth and oligodendrocyte differentiation, which is necessary to induce remyelination. Oligodendrocytes play an important role in restoring the myelin sheath after demyelination. Intact myelin is crucial to promote axonal survival and to induce neuroregeneration [5, 42]. Macrophages play a role in neuroregeneration by clearing myelin debris [28] and secreting neurotrophic factors and growth factors. This function of

macrophages might be useful as efficient therapeutic target to induce remyelination (figure 4, table 1). Furthermore, crosstalk between macrophages and neurons is important for efficient neuroregeneration. Neurotrophic factors secreted by neurons and macrophages are involved in crosstalk and are potential therapeutic targets to induce neuronal survival [7, 14, 40].

Miron et al (2013) demonstrated a role for M2 macrophages in promoting oligodendrocyte differentiation. An example of a factor involved in remyelination is activin-A, which can promote oligodendrocyte differentiation [42]. Activin-A is an anti-inflammatory cytokine and a member of the TGF-β family produced by M2 macrophages. This cytokine is a potential therapeutic target, because it is essential for remyelination *in vitro* and *in vivo* and might promote neuroregeneration.

Ion channels are other potential therapeutic targets involved neuronal survival. An example is flecainide, which is a potential neuroprotective agent to treat MS. In an animal model for chronic-relapsing EAE flecainide already shows reduced neurodegeneration via sodium channel blockade [1].

Efficient therapeutic drugs based on neuronal regeneration consist of ideally neurotrophic growth factors, which promote oligodendrocyte differentiation and neuronal survival. Additionally, blocking neuronal ion channels can reduce neuronal degeneration and is a potential therapeutic agent to treat MS.

MS treatment is based upon regulation of cytokine production and/or promoting neuronal survival. Several therapies approved by the FDA are combinational therapies, based on modulating several mechanisms involved in neurodegeneration and inflammation. Some therapies lead to reduced infiltration of monocytes, however this results in fewer leukocytes present in the CNS to remove pathogens and results in a less efficient immune response. Therefore, combinational therapies based on modulation of cytokine secretion and oligodendrocyte differentiation might be more effective. Additionally, timing of therapeutic intervention is essential to treat MS efficiently. Therapies to prevent major axonal loss needs to be induced before axonal loss is irreversible.

To design an efficient therapy for MS, researchers need to focus on several aspects, like the consequences after modulation of the inflammatory response. Also, the consequences after neurotrophic growth factor increase is important to study, because these factors might regulate differentiation of other cells besides oligodendrocytes. To conclude, more detailed research including the role of immune cells and growth factors during early stages of MS is essential to treat MS.

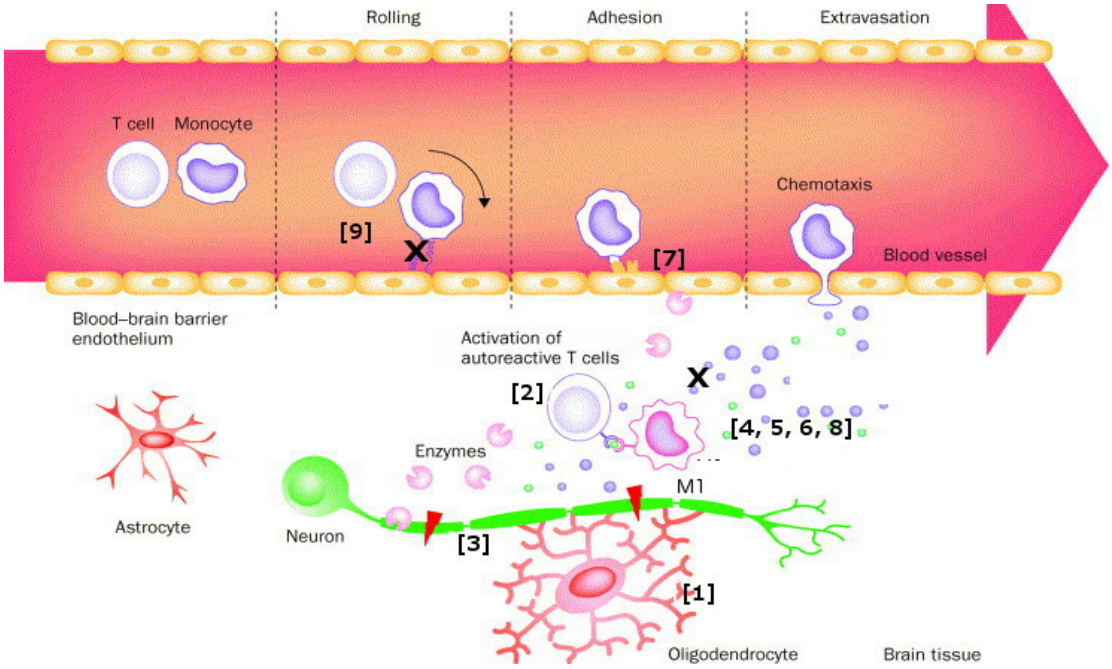

Figure 4 Therapies to treat MS [1-9; see table 1] are involved in monocyte migration [32, 44], production of pro-inflammatory cytokines [4, 36, 52, 53], oligodendrocyte differentiation [38], ion channel blockade [61] or activation of M2 macrophages [2].

Therapy		Effect	Results
Activin-A	[42]	Remyelination	Promotes oligodendrocyte differentiation
Copaxon	[64]	Inflammation	Reduces monocyte activation and increases amount of TH_2 cells secreting anti-inflammatory cytokines
Flecainide	[1]	Neuronal survival	Blocks neuronal sodium channels
Interferon-β	[47]	Inflammation	Reduces oxidative stress and cytokine secretion
Mapracorat	[62]	Inflammation	Down regulates cytokine production via MPK-1
Methylprednisolone	[39]	Inflammation	Reduces pro-inflammatory cytokines and induces apoptosis of leukocytes
Minocycline	[27, 36]	Blood-brain barrier Inflammation	Reduces monocyte infiltration into CNS Reducing activation status of macrophages
TNF-α	[31]	Inflammation	Blocks role of TNF-α as pro-inflammatory cytokine
Tysabri	[14]	Blood-brain barrier	Blocks binding of monocytes to cerebrovascular endothelium

Table 1 Therapies used to treat MS during early stages, all based on modulation of the role of macrophages in MS lesions

Discussion

This review aims to investigate the role of bone marrow-derived macrophages and resident microglia during axonal damage in MS lesions. To answer this question we focussed on the role of M1 and M2 macrophages during neuronal damage or survival and the factors involved in neuronal loss. In addition, we will discuss the involvement of macrophages and microglia in MS therapies and the role of astrocytes in neuroregeneration.

General role for macrophages/microglia

Removing neuronal and myelin debris is generally performed by resident microglia and is essential to maintain CNS homeostasis. This does not induce activation of the innate immune system, therefore no infiltration of immune cells is observed during CNS homeostasis. Phagocytosis of debris does not lead to neuronal damage and promotes remyelination. Intact neurons communicate with bone marrow-derived macrophages and resident microglia and inhibit their activation, which results in inhibition of pro-inflammatory cytokine secretion. Besides direct neuron-macrophage communication, neurons and macrophages also secrete neurotrophic growth factors, like insulin-like growth factors (IGF). Neurotrophic factors promote neuronal survival and regeneration and inhibit pro-inflammatory cytokine release *in vitro* and *in vivo*. During chronic inflammation in the CNS, when pro-inflammatory cytokine levels are increased, reduced IGF production by microglia was observed leading to neurodegeneration [15, 40, 59].

The role of macrophages/microglia in axonal damage and loss in MS lesions

Several studies suggest that axonal damage and loss initiates activation of resident microglia [46]. Bone marrow-derived monocytes are not recruited after axonal damage, therefore they play no role in removing neuronal and myelin debris in the CNS after neuronal injury. After axonal damage, clearance of debris and promotion of oligodendrocyte differentiation are essential aspects of microglia activity [42]. Furthermore, microglia that clear debris produce anti-inflammatory cytokines and play a beneficial role in neuroregeneration. The pro-inflammatory cytokine level is not increased after neuronal damage [3, 46, 55], indicating that M1 macrophages are not activated after neuronal loss. In addition, it is unlikely that axons are attacked by macrophages directly, because most axons survive demyelination. During MS progression the inflammatory response is low, however axonal loss is still observed [17, figure 1]. These observations suggest that macrophages do not promote neuronal damage.

Several studies demonstrated an increased number of macrophages during lesion development. A switch from M2 macrophages to M1 macrophages was observed in active lesions in EAE animal models and in active MS lesions [61, 11, 54]. Enhanced levels of pro-inflammatory cytokines and ROS are secreted by M1 macrophages, which might result in neuronal and oligodendrocyte damage.

Several studies show that overactivation of macrophages results in an enhanced level of neurotoxic macrophages (M1), which secrete pro-inflammatory cytokines [63]. It has been suggested that overactivation is a result of disturbed crosstalk between macrophages and neurons [63].
A phenotypic switch leads to an increased amount of M1 macrophages in MS lesions, however neuronal damage alone does not activate M1 macrophages, which indicates systemic inflammation is necessary to induce M1 macrophage activation. Several studies propose that M1 macrophage-derived cytokines promote neuronal damage. Nevertheless, we suggest a dual role for pro-inflammatory cytokines produced by M1 macrophages and microglia in neuroregeneration. These pro-inflammatory cytokines might regulate both neuronal survival and neuronal toxicity.

Macrophages/microglia in axonal repair and neuroprotection

An increased amount of anti-inflammatory cytokines was observed directly after CNS injury, suggesting that axonal damage activates alternative activated macrophages (M2) [46]. However, when neuronal injury was followed by peripheral LPS injection an increased level of pro-inflammatory cytokines was detected in C57Bl/6 mice, suggesting an increase in M1 macrophages in the CNS [46]. Several studies proposed a neurotoxic role for pro-inflammatory cytokines, however we suggest these cytokines might also have a neuroprotective role. After Wallerian degeneration an increase in the growth factor TNF-α was observed [46]. TNF-α has neurotoxic and neuroprotective properties depending on the microenvironment and the brain region where it is expressed [56]. Another example of a pro-inflammatory cytokine with beneficial effects on neuronal survival is IL-6. After neuronal damage, this cytokine is secreted by neurons inducing proliferation of microglia, which leads to regeneration of motor neurons [57]. Besides pro-inflammatory cytokines, M1 macrophages also produce ROS and NO, which results in neuronal damage caused by oxidative stress [43].
We suggest a beneficial and detrimental role for M1 macrophages during neuroregeneration, because ROS, NO and pro-inflammatory cytokines play a neurotoxic role and other pro-inflammatory cytokines might have a neuroprotective role.

The role of alternatively activated (M2) macrophages in neuronal repair

Increased numbers of M2 macrophages are present in human MS lesions and may play a role in spontaneous recovery from EAE in animal models [42, 54]. Furthermore, M2 macrophages promote differentiation of oligodendrocytes, which promotes neuronal survival [42]. M2 macrophages promote neuronal repair by secretion of neurotrophic growth factors and anti-inflammatory cytokines, which down regulate the immune response.
IL-4 or IL-13 cytokines activate differentiation of bone marrow-derived macrophages and/or resident microglia into alternative activated macrophages (M2) [12, 48, 61]. Microglia and macrophages that remove myelin and neuronal debris may display an alternative activation status (M2), because they secrete anti-inflammatory cytokines. Furthermore, M2 macrophages secrete activin-a that promotes

neuroregeneration [51]. Both observations suggest M2 macrophages have a beneficial effect on neuronal survival.

Macrophages and microglia as targets for MS therapy

Therapies based on reducing pro-inflammatory cytokines via regulation of classical activated macrophages are partly effective in MS, because we suggest that macrophages are not directly involved in neuronal damage and pro-inflammatory cytokines secreted by macrophages are both harmful and beneficial for neurons. Moreover, therapies based upon reducing monocyte infiltration into the CNS are also partly effective in MS. We suggest that these therapies inhibit the migration of M1 and M2 macrophages to the CNS, therefore not only the production of harmful cytokines is reduced, but the amount of cytokines that are beneficial for neuronal survival is also decreased.

Current therapies for MS are based upon macrophage activation and macrophage-derived cytokines. We propose that M1 macrophages play a harmful and beneficial role in neuronal survival, which is regulated by pro-inflammatory cytokines. Additionally, we suggest a dual role for astrocytes in neuroregeneration. They recruit microglia to the site of injury to clear debris and promote regeneration of oligodendrocytes, therefore playing a beneficial role in neuroregeneration [15, 55]. Besides their beneficial role, astrocytes also secrete harmful cytokines involved in neurodegeneration [46, 55].

To conclude, therapies to treat MS need to focus on the role of resident microglia and bone marrow-derived macrophages in clearing myelin and neuronal debris, which promotes remyelination and might prevent axonal loss. Furthermore, the role of astrocytes during neuronal damage in MS need to be studied in more detail to unravel the association between macrophages or microglia and astrocytes.

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