Imbalanced immunity in multiple sclerosis

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Introduction

1

Restoring immune suppression in the multiple sclerosis brain

Scope of the thesis

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Abstract

The inflammatory demyelinating disease multiple sclerosis (MS) is a disabling disease of unknown etiology that often affects adults at young age. The current therapies, mostly aimed to target T cells, can reduce the development of new lesions in the central nervous system (CNS) and partially prevent clinical disease activity. However, no available therapy can cure the disease or even halt its progression. Recent evidence indicates that myelin phagocytosis by infiltrated and disproportionally activated macrophages and microglia is not just a hallmark of MS, but rather a key determinant of lesion development and disease progression. In this review, we will therefore focus on these phagocytic cells, as a primary target for therapeutic approaches to stop the progression of MS. We will first review current therapeutic strategies, which are mostly discussed in literature in terms of their effective inhibition of T cells. However, we argue that many of these treatments also influence the myeloid compartment. The severe side effects of some of these therapies as well as their insufficient effectiveness, necessitates the search for novel therapeutic targets. We postulate that new therapeutic strategies should directly and specifically aim at manipulation of the activation and phagocytic capacity of macrophages and microglia. We will discuss three candidate targets with high potential, namely the complement receptor CR3, CD47-SIRPa interaction as well as CD200-CD200R interaction. Blocking the actions of CR3 could inhibit complement-mediated myelin phagocytosis, as well as the migration of myeloid cells into the CNS. CD47 and CD200 are known to inhibit macrophage/microglia activation through binding to their receptors SIRPa and CD200R, expressed on these cells. Triggering these receptors may thus dampen the inflammatory response. We conclude that the CD200-CD200R interaction is the most specific and hence probably best-suited target to suppress excessive macrophage and microglia activation, and to restore immune suppression in the CNS of patients with MS.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) affecting over 2.5 million individuals worldwide. MS debuts generally between the age of 20 and 30 and is one of the most disabling neurodegenerative diseases in young adults. The pathological hallmark of MS is white matter demyelination but there are other features that vary between patients, such as axonal and neuronal damage, subpial demyelination, perivascular cuffing of leukocytes, loss of oligodendrocytes as well as anatomical preference of lesions. The heterogeneic neuropathology suggests that it is unlikely that a single pathogenic mechanism underlies MS etiology.[1] Therapies that have been developed so far are aimed at multiple mechanisms such as broad immune suppression or inhibition of migration of immune cells. Some of these therapies reduce the clinical disease activity and the progression of lesion load as determined by magnetic resonance imaging (MRI), but so far no therapy is available that can cure MS or even halt its progression. The search for more effective approaches is therefore warranted.

Evidence is growing that activation of macrophages and microglia is pivotal in the development and expansion of MS lesions.[2-5] It has been observed that clusters of activated microglia are present even before demyelination is evident.[6] Furthermore, infiltrated macrophages and activated microglia are the predominant cell types present in expanding MS lesions[7] and they are actively involved in myelin phagocytosis.[4,8] As long as the cause of MS is unknown, one should therefore particularly target these myeloid cells to dampen inflammation and demyelination in the brain in order to halt progression of MS.

The aim of this review is to focus on the myeloid cell as the common pathological denominator in MS, which will be discussed in the context of current therapeutic strategies. In this respect, we will further discuss the possibilities of the brain's intrinsic macrophage/microglia suppressive mechanisms as a novel therapeutic approach in MS.

Macrophages and microglia: key players in MS

Macrophage populations in the CNS

The main type of immune cells that is found in the CNS belongs to the myeloid lineage and thus to the innate immune system. Myeloid progenitor cells give rise to blood monocytes that in turn can differentiate into macrophages. Macrophages (literally meaning 'big eaters') are phagocytes, providing a first line of defense

against infiltrating pathogens. They express high levels of major histocompatibility complex (MHC) class II molecules and are therefore professional antigen presenting cells as well.[9] After migration into tissues, they can further differentiate into resident macrophages, such as microglia in the CNS. Microglia form the largest population of immune cells in the CNS and are believed to provide a defense against infection and injury.[10] Morphologically they are quiescent ramified cells but despite their inactive phenotype, they continually survey their environment.[11] In contrast to macrophages, however, microglia express low levels of MHC class II, and do not exert substantial antigen presenting properties. Yet upon activation, these cells change their morphology and protein expression profile and are able to phagocytose for example pathogens, apoptotic cell debris, or myelin (as seen in MS).[12]

Other types of CNS macrophages are named after their location, at the interface of the CNS and the peripheral immune system: the perivascular, meningeal and choroid plexus macrophages. The perivascular macrophage is found in the Virchow-Robin space between the endothelial vessel lining and the glia limitans. Although many functions have been attributed to these cells,[13] it mainly involves scavenging of pathogens and other substances derived from the CNS or the circulation,[14] as well as antigen presentation.[15] With increased numbers in the CNS of MS patients and in animals with experimental autoimmune encephalomyelitis (EAE) as well as increased expression of adhesion molecules and chemokines, perivascular macrophages are most likely involved in attracting circulating monocytes/macrophages during MS.[16-18] However, it is unclear whether these cells remain at their location or also invade the brain parenchyma. Meningeal and choroid plexus macrophages possibly remove debris from the cerebrospinal fluid they encounter,[19,20] but it is unclear if and how these macrophages contribute to MS.

The final macrophage population is not present in the healthy CNS, but is frequently found in the CNS of MS patients. These are monocyte-derived macrophages that infiltrate the CNS.[21] In concert with activated microglia, these cells are mainly associated with myelin phagocytosis. The following discussion concerns microglia and macrophages, which here includes perivascular macrophages.

Activation of macrophages and microglia

Depending on the activation signals, macrophages can turn on a classical or alternative activation program.[22,23] Interferon-γ (IFN-γ) or lipopolysaccharide (LPS) stimulation induces classically activated macrophages (also called M1 cells) by signal transduction via mitogen activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK)1/2 and p38.[24] M1 cells are typically pro-inflammatory. They express high levels of nitric oxide (NO), cytokines like tumor ne-

crosis factor (TNF), interleukin (IL)-1, -6, -12 and -23 and opsonin receptors, like Fcγ receptors and complement type 3 receptor (CR3), that are necessary for complement- or antibody-mediated phagocytosis. M1 cells can promote T-helper 1 (Th1) responses and thus mediate the elimination of pathogens via inflammation and phagocytosis.[23] In contrast, IL-4, IL-13 or glucocorticoid hormones and their synthetic derivatives like dexamethasone induce alternatively activated macrophages (M2).[22,23] Although the exact intracellular pathways involved in this process are unknown, it is suggested that the anti-inflammatory phenotype of M2 cells results from down-regulation of nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)1.[23] M2 cells express high amounts of IL-10 and non-opsonin-dependent receptors such as the mannose receptor (MR), and are thought to mediate scavenging of debris, tissue remodeling and repair. M1/M2 polarization likely represents the extremes of a continuum, enclosing many intermediate phenotypes that further fine-tune the different responses.

Although normally quiescent cells, microglia are the first cells to react to any kind of brain insult or injury.[25] They become activated through processes similar to M1 cells, which are initiated by the activation of MAPK pathways.[26] Subsequently, microglia lose their ramifications, upregulate MHC class II molecules and secrete inflammatory substances like NO, IL-1, IL-6 and TNF. Similar to macrophages, activated microglia up-regulate surface receptors like Fcγ receptors and CR3 with which they can participate in antibody and complement mediated phagocytosis.[27]

In patients with active MS lesions, macrophages act mainly pro-inflammatory by secretion of NO, IL-1β and TNF,[28] corresponding with the M1 phenotype. However, it has been reported that foamy macrophages, that result from myelin ingestion, switch their phenotype and start to express markers consistent with M2-like cells.[29,30] This change probably contributes to processes such as gliosis formation by astrocytes. Microglia can also display highly diverse reactions that are associated with both protective and deleterious effects in the CNS.[31] It can therefore be speculated that also microglia activation may be polarized depending on the activation signal, but evidence for this is lacking.

It is still heavily debated what causes the (classical) activation of macrophages and microglia in MS, as is the question whether this activation is followed by, or a consequence of T cell activation, oligodendrocyte apoptosis, or other pathological events. However, macrophages and microglia are considered key players in lesion development and thus in disease progression for a number of reasons. Already in 1952, it was observed that clusters of microglia were present in the MS brain even before cellular infiltrates or demyelination was visible.[32] This was confirmed by many others and has been linked to the earliest stages of lesion development.[6,33,34]

As mentioned above, macrophages and microglia predominate in expanding MS lesions and greatly outnumber lymphocytes.[7] They localize in close proximity to damaged axons[35] and have recently been indicated to phagocytose myelin by a common mechanism mediated by complement and immunoglobulin deposition.[4] Macrophages and microglia furthermore secrete many inflammatory cytokines, reactive oxygen species and chemokines,[26] thereby enhancing inflammation. Interestingly, these data suggest that the disproportional activation of macrophages and microglia is a key event in triggering a cascade of events that ultimately leads to multiple demyelinated areas in the CNS. Therefore, treatments that interfere with the activation of these cells, as well as the process of phagocytosis, would be an excellent approach to halt lesion development and further disease progression.

In fact, it is surprising that none of the currently available or experimental therapies is specifically aimed to halt the actions of macrophages and microglia. Most therapeutic targets were originally developed in EAE and were aimed at limiting T cell activities and migration. Although T cells are required for the induction phase of EAE, their critical role in MS is still elusive.[3] It is therefore not surprising that experimental MS therapeutic approaches aimed at, for example, eliminating $CD4^+$ T cells, seemed beneficial in the treatment of EAE,[36,37] but appeared disappointing in the treatment of MS.[38,39] Notably, despite being induced by T cells, the myeloid cell compartment is essential during the effector phase of EAE.[40-44] We here propose to focus on the role of macrophages, which include perivascular macrophages, and microglia in MS. Interestingly, many experimental therapies, although primarily aimed at T cell activation and function, have effects on demyelinating macrophages and microglia. Hence, we will first highlight these effects of current therapeutic approaches, followed by suggestions for alternatives to more specifically target these cells.

Current therapeutic approaches and their effects on macrophages/microglia

Glucocorticoids

Glucocorticoids (GCs) are known for their broad anti-inflammatory properties.[45] Short courses of high-dose intravenously administered synthetic GCs (i.e. methylprednisolone) are nowadays frequently used to reduce the duration and severity of acute MS relapses.[46] However, the chronic use of GCs is unwanted as it usually causes severe side effects. In addition, despite their frequent use to treat MS re-

lapses, they have no proven beneficial effect on long-term disease progression.[46] The mechanisms by which GCs exert their therapeutic effects are not completely understood, but include altered transcription via GC response elements present in the promoter and enhancer regions of many genes, and interference with other factors such as NF-κB, STAT5 or phosphoinositide 3-kinase (PI3K).[47] GCs are very likely to affect macrophage activities as the glucocorticoid receptor is highly expressed in many macrophage populations, including microglia.[48] The outcome of GC treatment is, amongst others, decreased production and release of pro-inflammatory cytokines like TNF, IL-1β and IL-6 in macrophages and T cells. Also anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) are induced, that subsequently act to suppress these cells.[49] This change in cytokine profile can induce a shift from a Th1 into a Th2 response. However, GCs are also described to induce polarization of macrophages towards an M2 phenotype[23] with similar effects on cytokine production. Thus, direct effects of GCs on macrophages may involve the implicated shift from a TH1 to TH2 response. However, as macrophage polarization by GCs has mostly been studied *in vitro*, this has yet to be proven as an underlying mechanism. Other effects of GCs include preservation of the blood-brain barrier integrity, reduction of perivascular oedema, down-modulation of expression of chemokines, chemokine receptors, adhesion molecules and integrins, that can lead to decreased leukocyte migration.[47] It is however debated whether these are all direct effects of GCs, or whether they result more indirectly for example from the M2/Th2 skewing. Since GCs can freely pass the blood-brain barrier, they can affect cells of the CNS directly, including microglia. Although data on the effects of GCs on microglia are sparse, it has been described that GCs down-regulate TNF produced by activated microglia, prevent phosphorylation of p38 MAPK and decrease the expression of MHC class II molecules.[50,51] Thus, given the fact that macrophages and microglia highly express the glucocorticoid receptor and GCs have profound anti-inflammatory effects on these cells, the effects of GCs on MS relapses may be accounted for by direct effects on macrophage and microglia.

IFN-β

IFN-β is a type I interferon, which is used in the treatment of relapsing-remitting MS and can reduce the frequency of clinical exacerbations.[52] Still, IFN-β does not block lesion development, and can therefore only delay but not halt disease progression. Moreover, a large proportion of treated patients does not respond to the therapy due to the development of autoantibodies against IFN-β.[53] IFN-β has multiple effects on the immune system and due to its non-specific actions, is associated with multiple adverse events such as skin reactions, flu-like symptoms, fatigue and leu-

kopenia.[54] Its therapeutic effects were mostly studied in relation to T cell activation, where it affects many pathways, as shown by microarrays experiments.[55] In addition, it may mediate a shift from a Th1 towards a Th2 response by reducing pro-inflammatory cytokines and induction of anti-inflammatory cytokines.[56-58] Furthermore, the adhesion and migration of T cells, but also monocytes, into the CNS can be diminished because IFN-β inhibits the expression of adhesion molecules on vascular endothelium as well.[59] Despite all efforts, the exact mechanisms by which IFN-β is beneficial in MS are still unresolved. Interesting in the context of this review are the data obtained from studies of endogenous IFN-β, produced by many cell types including fibroblasts, dendritic cells and macrophages. In IFN-β knockout mice, the susceptibility and severity of EAE was significantly increased as compared to their wild type littermates. Whereas in the absence of IFN-β no changes were observed in Th1/Th2 balance or in T cell responses, macrophages and microglia were highly activated.[60] These findings suggest that the protective effect of (endogenous) IFN-β is based on inhibition of macrophages and microglia. This concept was recently corroborated by Prinz *et al.*, who showed aggravation of EAE in mice deficient for type I IFN receptor.[61] In a series of elegant experiments using conditional knockouts for this IFN receptor in different cellular compartments, they showed that there was no effect on the induction and progression of EAE due to absence of the receptor on either nestin-expressing CNS cells (mostly astrocytes) or T cells and B cells. However, upon deletion in myeloid cells, symptoms of EAE were increased and correlated with increased numbers of infiltrated macrophages and activated microglia that further showed enhanced myelin phagocytosing capacity and increased chemokine expression. These reports not only elucidate the action of endogenous IFN-β, but also shed new light on the possible mechanisms by which IFN-β treatment is beneficial in MS patients as IFN-β may thus directly influence macrophage and microglia activities. Although in man several reports have claimed effects on T cells, treatment might thus very well primarily affect the innate immune response.

Glatiramer Acetate

As one of the oldest animal models for a human disease, EAE resulted from 'paralytic' accidents following Pasteur rabies vaccination in human subjects.[62,63] It turned out that these accidents were induced by contaminants of the rabbit CNS tissue that the vaccine was prepared in.[64,65] Thus, traditionally EAE was induced using myelin extracts from different animals like rabbits and guinea pigs, but nowadays the induction of EAE is usually established by inoculating animals with purified myelin components such as myelin basic protein (MBP), or peptide derivatives of these

proteins. In an attempt to further down-scale the complexity of the immunogen, a synthetic derivate of MBP was developed. Copolymer 1 (Cop-1), later known as glatiramer acetate, is a synthetic random polymer of 4 amino acids in a ratio similar to that found in MBP.[66] However, instead of inducing EAE, glatiramer acetate prevented the development and suppressed established EAE induced by spinal cord homogenates or other encephalitogenic peptides.[67] Several clinical trials have shown positive effects of glatiramer acetate on mean relapse rate and proportion of relapse-free patients, but the effect on disability appeared small.[68-70] In the mean time, many studies were conducted to identify the mechanisms of its beneficial effect. Although still not entirely elucidated, two main modes of action were suggested.[66] First, T cells reacting to glatiramer acetate shift from a Th1 towards a Th2 response,[71,72] which is beneficial in MS as shown for most other MS therapeutics. In addition, glatiramer acetate is suggested to have neuroprotective actions, by inducing production of brain derived neurotrophic factor (BDNF) by different T cell lines.[73,74] However, clinical evidence on actions of T cell derived BDNF in MS patients is not available. Second, glatiramer acetate can bind MHC class II of antigen presenting cells such as dendritic cells and macrophages.[75,76] *In vitro* studies show that as the affinity of glatiramer acetate is higher than that of MBP, it can compete with binding of MBP to MHC class II molecules, thereby preventing the activation of MBP-reactive T cells.[77] No data are currently available on effects of glatiramer acetate on other macrophage activities.

Mitoxantrone

Mitoxantrone can be beneficial in relapsing-remitting and secondary progressive MS, but major drawbacks are its adverse effects including an increased risk on developing leukemia, and the restricted cumulative dose a patient may receive during life to avoid cardiac toxicity.[78] As anti-neoplastic agent interfering with DNA synthesis and consequently cell division, mitoxantrone is used for many years to treat malignancies as breast and prostate cancer, and leukemia. Its potential usefulness in the treatment of MS was considered when mitoxantrone appeared to have immune suppressive effects as well. It was shown to block myelin degradation by macrophages[79] and therapeutic treatment of EAE with this compound reduced clinical and histopathological symptoms of the disease.[80-82] In MS, clinical trials have shown that treatment with mitoxantrone reduced gadolinium-enhanced lesions on MRI as well as relapse rate and disability progression.[83-85] On the cellular level, mitoxantrone caused a marked reduction in the proliferation of B cells, as well as a reduced ability of T cells to induce an immune response.[86,87] Remarkably, absence of macrophages entirely abrogated these effects, as shown in EAE,[87] indi-

cating that it is the macrophage that is primarily affected by mitoxantrone which subsequently has suppressive influences on T and B cell activities. These results implicate that mitoxantrone may exert its therapeutic effects in part by affecting macrophages.

Anti-VLA-4

During any inflammatory disease, leukocyte infiltration into the inflamed tissue starts with the process of tethering, rolling and firm adhesion to the endothelial cells of the microvasculature.[88] In MS, immune cells transmigrate through the endothelium and can enter the brain parenchyma if they are able to pass the glia limitans, which is the final component forming the blood-brain barrier.[89] Many molecules are involved in this cascade of events that have consequently been proposed as targets to interfere with leukocyte invasion in the MS brain.

One of the most encouraging therapies currently available is a monoclonal antibody against the 'Very Late Antigen-4' (VLA-4). The integrin VLA-4, a heterodimeric molecule composed of an α4 and β1 chain, is expressed on most leukocytes, and binds to the vascular cell adhesion molecule (VCAM)-1 on vascular endothelial cells. This interaction is a key event in the arrest of leukocytes on the endothelium. The humanized monoclonal antibody against VLA-4, natalizumab, successfully reduced relapse rates and disease progression in MS patients.[90] Simultaneously however, several reports claimed adverse effects of VLA-4 antagonists. Natalizumab was temporarily withdrawn from the market when three patients developed progressive multifocal leukoencephalopathy (PML), an often fatal disease caused by the JC virus, usually in immuno-compromised patients. Two of these patients were also treated with IFN-β and the third patient received additional therapy for Crohn's disease.[91-93] It is still unclear if the intense immune suppression allows the virus to replicate and spread, or whether the treatment directly influences virus activity.[94] After a review of safety information and no further cases of PML, the drug was returned to the market in 2006. Recently, two further incidents of PML during treatment for MS were reported (http://www.fda.gov/cder/drug/InfoSheets/HCP/natalizumab2008HCP.htm). Both patients were on monotherapy for more than one year. Despite these new cases, the drug will stay available for the treatment of MS. Although anti-VLA-4 is considered one of the most successful therapies in MS so far, and having less severe side effects than glucocorticoids or IFN-β, also this therapy is not able to stop progression of MS.

In contrast to broad immunosuppressants as glucocorticoids, IFN-β and mitoxantrone, natalizumab was specifically designed to limit T cell migration. The reason for this was that in the early 1990s, the first evidence was provided that

interaction between VLA-4 and VCAM-1 was a crucial migratory mechanism in MS, as VCAM-1 was highly expressed in MS and EAE,[95,96] and antibodies blocking this interaction prevented leukocyte adhesion and infiltration into the CNS and inhibited the induction of EAE.[97-100] Recently it was shown that circulating monocytes and B cells express significantly higher levels of VLA-4 compared to T cells.[101] It is therefore not surprising that natalizumab significantly reduced not only the number of T cells, but also of macrophages and B cells in the cerebrospinal fluid in treated patients.[102] So despite being specifically aimed at T cells, natalizumab may additionally influence migration of other cell types, including that of macrophages.

Macrophage/microglia: targeted immune suppression

CR3

Apart from monocyte/macrophage infiltration in the CNS, the activation and subsequent myelin phagocytosis by macrophages and microglia are crucial in MS lesion development as mentioned earlier. Molecules specifically involved in these processes would be interesting targets for MS therapy. An appropriate candidate is the complement receptor 3 (CR3), which is constitutively expressed on both macrophages and microglia.[103] Like VLA-4, CR3 is an integrin, also known as Mac-1. CR3 consists of CD11b (αM) and CD18 (β2) chains, and binds the intercellular adhesion molecule ICAM-1 on endothelial cells and the C3b component of complement. Through these interactions CR3 is involved in monocyte/macrophage adhesion, transmigration and phagocytosis predominantly of targets opsonized by complement.[104-106] CR3 expression levels are increased on activated microglia, as seen in the facial nerve transsection model.[107] Blocking CR3 appears highly efficient in EAE as antibodies against CR3 inhibited both leukocyte adhesion via interaction with ICAM-1 as well as phagocytosing activity of macrophages, thereby preventing the disease from progressing.[108,109] Furthermore, activation of microglia could also be blocked by CR3 inhibition, which was also correlated with suppression of EAE.[110] These data strongly suggest that the inhibition of this molecule could be attractive as MS therapeutic (Fig. 1), but so far this target has not been explored for a clinical trial.

Intrinsic immune suppressive mechanisms

The CNS comprises several intrinsic mechanisms that tightly regulate the activities of microglia. This is important, since an uncontrolled inflammatory reaction could be detrimental to a tissue that is notoriously known for its poor regenerating capacity. Normally, quiescent microglia remain capable of inspecting and guarding

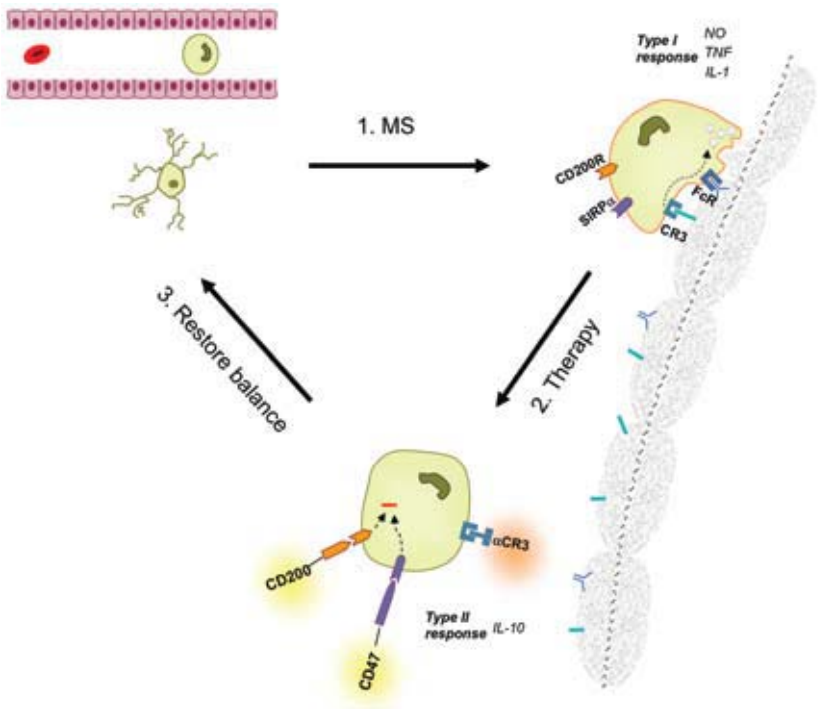

Fig. 1 The as yet unexplored therapeutic targets on myeloid cells in MS. Infiltrated macrophages and microglia are highly activated in MS (step 1). These classically activated cells secrete NO and type I cytokines such as IL-1β and TNF. Via high expression of the complement receptor CR3, these cells mediate complement-activated myelin phagocytosis. In addition, in MS lesions the ligands for the inhibitory receptors SIRPα and CD200R are not sufficiently present to suppress cellular activation. Thus, by blocking CR3 and triggering of SIRPα and CD200R, phagocytosis and pro-inflammatory activities of the cells are inhibited and the pathological phenotype is shifted towards alternatively activated macrophages (step 2). These interventions drive these cells to mediate type II immune responses (e.g. IL-10 production) that involve tissue repair and dampening of inflammation. Eventually, the imbalance in immune homeostasis in the brain will be restored by enhancing the brain's intrinsic immune suppressing potential (step 3).

their environment, as shown by Nimmerjahn *et al.*[11] Only when a certain threshold is exceeded, they become activated and typically show retraction of ramifications, rounding off of cells and up-regulation of a number of markers like CD45 and MHC class II. These cells will become motile, phagocytic and might even display local proliferation. A proper balance in immune activating and immune inhibitory signals thus regulates their activation. We and others have postulated that excessive microglia/macrophage activation in a pathological setting is due to imbalanced control, reflected by impaired immune activation, immune inhibition, or both, and could lead to disease progression as seen in MS.[111,112] This view subsequently implies that correcting the equilibrium by supplying extra inhibitory signals specific

for these cells may dampen the pathological inflammatory response and restore the immune suppressed environment of the CNS (Fig. 1). Eligible specific candidate therapeutic targets to diminish inflammation in MS via these routes are the immune inhibitory molecules CD47 and CD200, and their receptors signal regulatory protein (SIRP)α and CD200R.

CD47-SIRPα

CD47, also known as integrin-associated protein, is a membrane glycoprotein and belongs to the immunoglobulin superfamily (IgSF). It is broadly expressed in different cell types and abundantly present in the CNS, amongst others on neurons. Its receptor, SIRPα, is expressed on neurons and on myeloid cells. The effects of SIRPα-signaling in neurons is unknown, but CD47 ligation to myeloid SIRPα has been shown to mediate cellular inhibition in these cells, as phosphorylation of immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic tail leads to reduced MAPK activation.[113] CD47 can not only reduce macrophage activation, it can also prevent phagocytosis as erythrocytes derived from $CD47^{-/-}$ mice were instantly cleared by macrophages when transferred into wild type mice.[114,115] Furthermore, blocking CD47 also inhibits the migration of monocytes across brain endothelial cells.[116,117]

As excessive macrophage and microglia activation occurs in the CNS of MS patients, we recently hypothesized that in MS lesions, the immune suppressive signals such as from CD47 were reduced. Therefore we studied the expression patterns of CD47 in relation to several other (inflammatory) molecules in three different sub-areas from chronic active and inactive MS lesions.[111] Indeed, the expression of CD47, but not of SIRPα, was reduced in MS lesions. In the rim of chronic active lesions this coincided with increased complement levels, a profile that is known to promote phagocytosis. Interestingly, this profile was also found in the area surrounding chronic active lesions, where lesion expansion is likely to occur. In contrast, this expression profile was absent in the area surrounding inactive lesions, where lesion expansion has halted. These results indicate that in chronic active lesions, this pro-inflammatory and pro-phagocytic environment may facilitate and contribute to lesion development, and that SIRPα signaling can contribute to curtailing lesion expansion.

Collectively, these data suggest that treating MS patients with a SIRPα agonist could reduce monocyte migration into the CNS and decrease the activation and phagocytic capacity of macrophages (Fig. 1). A few obstacles however include that both CD47 and SIRPα can induce intracellular signaling pathways, which are not entirely understood. Hence a SIRPα agonist might turn out to also act as CD47 antagonist. As $CD47^{-/-}$ animals have no clear phenotype,[118] CD47 is a potential therapeutic target, however, in need of further experimental investigation.

CD200-CD200R

CD200 is also a membrane glycoprotein belonging to the IgSF. Its expression is extremely high in the CNS, where amongst others, neurons are positive.[111,119,120] Having a short cytoplasmic tail with no known signaling motifs, it is unlikely that CD200 itself exerts functions in the cell on which it is expressed. Its receptor, CD200R, is homologous to CD200, but its expression is confined mainly to myeloid cells like macrophages and microglia,[119] and to a subset of B and T cells.[121,122] CD200R has an extended cytoplasmic tail, which contains three tyrosine residues, one of which forms part of an NPXY motif. Upon binding to its ligand, the tyrosine residues become phosphorylated, and adaptor proteins downstream of tyrosine kinase (DOK)1 and 2 are recruited. This ultimately leads to inhibition of the MAPK p38, ERK and c-Jun N-terminal kinase (JNK), the common pathways involved in classical activation of macrophages and microglia. This in turn results in inhibition of release of several cytokines like TNF, IL-5 and IL-6.[123,124] Thus, through CD200-CD200R interaction, the activity of macrophages and microglia can be down-regulated. The strikingly high expression of CD200 in the CNS is therefore thought to be a mechanism of constitutive immune suppression in this sensitive organ. Indeed, blocking CD200-CD200R interaction in $CD200^{-/-}$ mice, leads to spontaneous activation and increased proliferation of microglia and macrophages, demonstrated by upregulation of CD45, which is a hallmark of microglial activation, and expression of inducible nitric oxide synthase (iNOS).[125] Induction of EAE and experimental autoimmune uveoretinitis (EAU) showed enhanced macrophage infiltration and a more rapid and severe disease course compared to wild type mice.[125-127] Also in rats, a blocking antibody against CD200R aggravated EAE which was reflected by enhanced infiltration of T cells and activated macrophages.[121,128] In the latter study, it was further shown that interruption of CD200-CD200R interaction significantly increased neuronal damage in macrophage-neuronal co-cultures. Taken together, these data implicate that CD200 is a potent immune suppressor and that reduced inhibitory input from CD200 causes a disturbed equilibrium, which subsequently results in cellular activation and tissue damage. Recent data from our lab confirmed this in MS. Besides a decreased expression of CD47, we also found decreased expression levels of CD200, but not CD200R.[111] Immune inhibition may thus be hampered in MS and could facilitate the activated state of macrophages and microglia with their demyelinating activity as a consequence.

A crucial finding in animal studies shows that increased CD200R signaling can be beneficial. In mice that have inherently elevated levels of CD200, EAE is ameliorated and is accompanied by enhanced neuroprotection.[129] As expected, in these animals fewer activated macrophages were present in the CNS and less evidence

was found for disrupted myelin sheets and axonal damage. Interestingly, a study recently conducted in our lab revealed that CD200R expression is increased on alternatively activated macrophages compared to classically activated macrophages (manuscript submitted). CD200R is very likely to mediate several actions of M2 cells such as the reduced secretion of inflammatory cytokines.[124] In addition, CD200R expression on T cells also appeared to be restricted to Th2 cells,[130] and finally, it may down-modulate the Th1 response as shown in a study where tumor cells overexpress CD200.[131] Conclusively, these results imply that using a CD200R agonistic agent in MS patients could suppress macrophage/microglia activation, restore the intrinsic immune suppressed environment of the CNS and could thereby restrain disease activity (Fig. 1). Furthermore, it may shift Th1 to Th2 responses, a paradigm many broad immune suppressants rely on. Although by suppressing macrophages caution should be taken with respect to the development of opportunistic infections, targeting CD200R would be more specific than most other immune suppressants because its expression pattern is restricted and its effects have been well studied, which would consequently decrease the probability of unexpected adverse events.

Conclusion

Inhibition of blood-brain barrier disruption, migration/infiltration or myelin phagocytosis by myeloid cells all seem plausible mechanisms to limit CNS inflammation in MS. Current therapies, although not directly tailored to do so, do have effects on macrophages and microglia. However, specifically targeting the activation of these cells would likely increase the effectiveness of the treatment, especially when using the CNS' intrinsic immune suppressive systems. Direct suppression of macrophages and microglia as MS therapeutic is an unexplored field. As of yet, CD200 seems the most suitable candidate to restore the immune suppressive status of the CNS in MS. Moreover, since it is not likely that CD200 and CD200R have other binding partners, the chances of multiple adverse effects as seen with the broad immune suppressants might be considerably lower. Interestingly, although beyond the scope of this review, these potential therapeutic targets may be equally well appropriate in preventing damage caused by activated microglia in other neurodegenerative disorders like Alzheimer's disease[132] and Parkinson's disease.

Scope of the thesis

As discussed above, activated macrophages and microglia are thought to be crucial in the development of MS lesions. How the activation of these cells is regulated in MS is not completely understood, while this knowledge could have a significant impact on treatment strategies. The inappropriate activation of macrophages and microglia in MS reflects an imbalanced immune regulation in the brain, which can be due to enhanced activating signals, decreased inhibition or a combination of both. Most of the studies addressing this issue in MS focused on activating signals. However, in the immune-privileged CNS, there are some powerful systems that continuously suppress local immune activities that have hardly been studied. Previous work showed that absence of only one of these inhibitory molecules, CD200, was sufficient to cause spontaneous activation of microglia in mice and to accelerate the development of EAE, the animal model for MS.[125] These results demonstrate the importance of constitutive immune inhibition in the brain. In the present thesis we hypothesize that immune suppressive signals in MS lesions are diminished, thereby contributing to imbalanced immunity. The consequence is facilitated activation of macrophages and microglia that in turn is associated with lesion formation and disease progression. The aim of the studies described in this thesis was therefore to study the expression patterns of immune suppressive systems in the brains of MS patients as compared to brains of healthy donors, as well as the regulation of these systems *in vitro*. These studies mainly focused on immune suppression via CD200-CD200R, but also CD47-SIRPα and, in the periphery, GITR-GITRL interaction, are regarded as potent immune regulatory mechanisms.

In the first study, described in **chapter 2**, we used laser dissection microscopy to isolate different areas of chronic active and inactive MS lesions to discriminate between lesion compartments that reflect different inflammatory stages in lesion development. Within different lesion areas, we analyzed the expression patterns of immune inhibitory molecules CD200-CD200R and CD47-SIRPα in relation to inflammatory mediators, and other molecules thought to be involved in lesion development. To further understand the interactions of CD200 and CD200R in the CNS, we investigated the anatomical and cellular localization of these molecules in the human CNS, as shown in **chapter 3**. Next, it was important to know if and how the expression of CD200 and CD200R can be influenced, which is important in view of the development of therapeutic approaches. In **chapter 4** we have studied the effects of different pro- and anti-inflammatory stimuli on CD200R expression on human monocyte-derived macrophages. However, not only macrophages, but also activated microglia play a predominant role in MS lesion formation. **Chapter**

5 describes a unique procedure to isolate and culture human post-mortem microglia. In a pilot study, we report on the expression and regulation of CD200R on these cells and compared them to other CNS-associated macrophages. In addition, we addressed the regulation of CD200 on a human neuroblastoma cell line. In **chapter 6** we focused on a peripheral mechanism of immune suppression by way of regulatory T cells. We show the effects of constitutive GITR-GITRL interaction and its functional consequences in EAE, the animal model for MS.

The main conclusions of the present thesis are that constitutive expression of immune suppressive molecules such as CD200 and CD200R is essential in maintaining an immune suppressed environment in the CNS. Diminished expression of these molecules in MS lesions can contribute to the imbalanced immunity in MS, reflected by hyperactive macrophages and microglia. Our data presented here suggest that targeting CD200-CD200R interaction would not only result in decreased immune activation, but may also cause the immune reaction to be shifted towards a more beneficial anti-inflammatory response, thereby affecting inflammation, demyelination and axonal damage, the hallmarks of MS.