CCR5 in multiple sclerosis : expression, regulation and modulation by statins

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Discussion

General Discussion

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General Discussion

8

CCR5 in MS

CCR5 expression on microglia/macrophages

In **chapter 3** we have demonstrated that the transcription factor CREB-1 is involved in the regulation of CCR5 expression. In earlier studies, we have observed that expression of CREB-1 is elevated in MS lesions, mainly in microglia and macrophages [1]. For example, CREB-1 expression is strongly enhanced in activated microglia in preactive lesions, compared to moderate expression of CREB-1 in microglia in NAWM. In addition, phagocytic macrophages inside active demyelinating lesions also express CREB-1. However, the level of CREB-1 expression in these macrophages is lower than that in activated microglia in preactive lesions. In **chapter 2** we have observed that CCR5 expression in microglia and macrophages greatly parallels this expression (Figure 1): NAWM microglia display moderate CCR5 expression, whereas both microglia in preactive lesions and phagocytic macrophages in active lesions express high levels of CCR5. The fact that macrophages in active lesions express high levels of CCR5, while CREB-1 expression in these cells is lower than that in activated microglia might implicate that CCR5 expression does not completely parallel CREB-1 expression. However, these observations might also indicate that CCR5 expression is switched off at a later stage than CREB-1 expression. Indeed, phagocytosing macrophages in active lesions still display high CCR5 expression, whereas CCR5 expression in macrophages present at a later lesion stage, at the expanding rims of chronic active lesions, is markedly decreased. This indicates that CCR5 expression in macrophages is lost during late lesion development.

The fact that the loss of CCR5 expression seems to be delayed compared to loss of CREB-1 expression, suggests that CCR5 expression on the cell membrane is fairly stable and has a low turnover rate. The observation in **chapter 6** that statin treatment only partly reduces CCR5 expression on cultured microglia supports this hypothesis: statin treatment only affects the trafficking of newly formed or recycled CCR5 molecules to the cell membrane and not the internalization or breakdown of CCR5 molecules present on the cell membrane. Therefore, if the rate of CCR5 turnover is low, simvastatin does not affect expression of CCR5 molecules that are already present on the cell membrane during statin treatment and are not internalized during treatment. The fact that these cells do not functionally respond to CCL5 or CCL3 anymore illustrates that although CCR5 protein is still present on the cell membrane, the signal transduction pathways and cellular mechanisms downstream of the CCR5 receptor are affected by simvastatin.

CCR5 expression on astrocytes

In **chapter 2** we have demonstrated that astrocytes in control and MS brain tissue express CCR5 molecules. In addition, we have observed CCR5 protein expression on cultured astrocytes. However, we have not been able to detect CCR5 mRNA in cultured primary astrocytes (**chapter 3**). There are two possible explanations for this apparent paradox. It could be that astrocytes do express CCR5 mRNA *in vivo*, but that CCR5 transcription is shut down by mechanisms such as DNA methylation and histone modifications when astrocytes are cultured. This would again suggest that the half-life of CCR5 proteins, produced by astrocytes *in vivo*, is long and therefore CCR5 protein expression persists in culture. Alternatively, astrocytes might not be able to synthesize CCR5 *de novo*, but obtain CCR5 molecules via another mechanism. CCR5 molecules could be transferred from CCR5 expressing cells, for example microglia/macrophages, to astrocytes. This hypothesis is supported by the observation that primary peripheral blood mononuclear cells (PBMC) are able to shed microparticles that specifically contain CCR5. Through the release of these microparticles, PBMC are able to transfer CCR5 molecules to several cell types, including monocytes and endothelial cells [2]. In addition, a similar transfer of MHC-II molecules by T cells has been reported [3]. It could be that astrocytes obtain their CCR5 expression from neighbouring microglia and macrophages by this mechanism. This would also account for the fact that CCR5 expression is enhanced on astrocytes at a later stage than that on microglia and macrophages (Figure 1). If we assume that astrocytes are the main source of chemokines in an early stage of lesion formation, activated microglia, which upregulate CCR5 expression and are attracted to the lesion site, logically come in close contact to these cells. In addition, migrating microglia encounter many astrocytes on their way. At a later stage, astrocytes could obtain CCR5 from macrophages, which

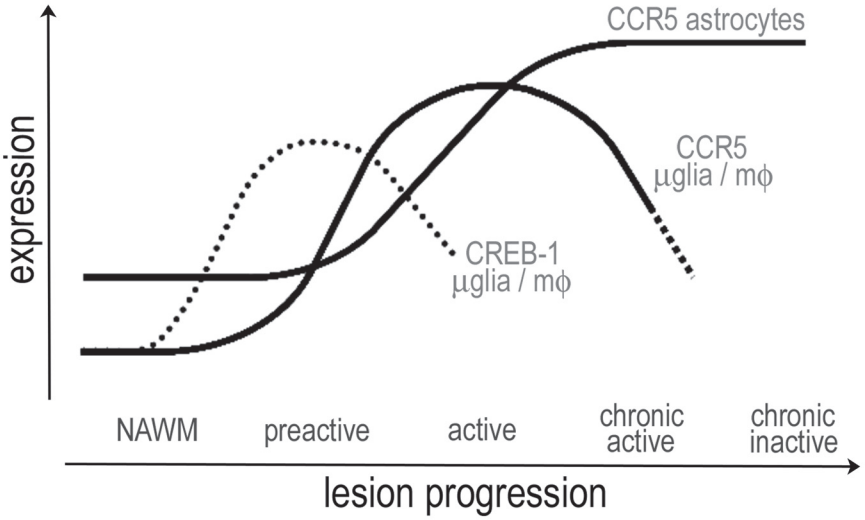


Figure 1. The kinetics of CCR5 and CREB-1 expression during MS lesion development. Expression of CREB-1 and CCR5 in microglia follow the same increasing-reducing pattern with peak expression in preactive and active lesions, respectively. CCR5 expression on astrocytes, which do not express CREB-1, is gradually enhanced and reaches maximal levels in the chronic stages of MS lesions.

are abundantly present in active lesions. Furthermore, at this stage, astrocytes might also obtain CCR5 from infiltrating inflammatory cells, for example CCR5 expressing T cells. As a result, initially, microglia and macrophages display increased expression of CCR5 and at a later stage astrocytes express enhanced levels of CCR5.

Another indication that astrocytes obtain CCR5 expression through another mechanism than enhanced or *de novo* transcription is that their transcription factor profile in MS lesions differs from that of microglia and macrophages. As discussed above, we have shown in **chapter 3** that the transcription factor CREB-1 is involved in the transcriptional regulation of CCR5. Whereas the expression profile of CCR5 correlates with that of CREB-1 in microglia/macrophages in MS lesions (Figure 1, **chapter 2** and [1]), hypertrophic astrocytes in and around MS lesions, which display enhanced expression of CCR5, do not express CREB-1. Provided that CREB-1 is necessary for CCR5 transcription, this again would argue for an alternative mechanism underlying CCR5 expression on astrocytes.

Of course, if the hypothesis that CCR5 molecules are transferred to astrocytes is true, the question still remains whether CCR5 expression on astrocytes is actually functional. In order to respond to, and migrate towards, a chemokine gradient, cells need intact intracellular signaling mechanisms in addition to chemokine receptor expression on their cell surface. These mechanisms consist of, amongst others, intracellular accessory molecules, such as G proteins that are linked to the intracellular part of the receptor, and second messenger systems leading to intracellular changes such as actin rearrangement [4,5]. The report of Mack *et al.* indicates that CCR5 molecules that are transferred by microparticles might indeed be functionally active. Their findings show that, depending on the recipient cell type, transferred CCR5 molecules can be actively

internalized, which indicates that they are fully integrated into the plasma membrane and connected to the intracellular pathways downstream of CCR5 [2]. In addition, Undale *et al.* have shown that transferred MHC-II molecules are functionally active [3]. However, it is unclear whether CCR5 functionality is determined by the presence of the intracellular machinery necessary for CCR5 function in the recipient cell or that these components are transported along with the CCR5 molecules.

In vivo and *in vitro* observations indicate that CCR5 expression by astrocytes might be functional. In response to injury, reactive astrocytes, along with microglia, proliferate and migrate towards the site of injury, forming a glial scar around the damaged area. This process is known as reactive gliosis [6]. In MS, high numbers of astrocytes can be found in and around late stage MS lesions, with a peak of astrocyte numbers in chronic inactive lesions, which are almost exclusively populated by astrocytes. Together, these observations suggest that at a certain point in the development of MS lesions astrocytes migrate towards the site of lesion. The expression of chemokine receptors most likely accounts for this recruitment. Since expression of the chemokines CCL3, CCL4 and CCL5 is enhanced in MS lesions [7-9], CCR5 is one of the candidates. In addition, astrocytes have been shown to functionally respond to CCL3 *in vitro* [10,11]. However, because the presence of both CCR5 mRNA and protein in astrocytes remains debatable, this response to CCL3 could also result from astrocytic expression of CCR1, which has been established [12]. Therefore the functionality of CCR5 expression in astrocytes has not been definitively confirmed.

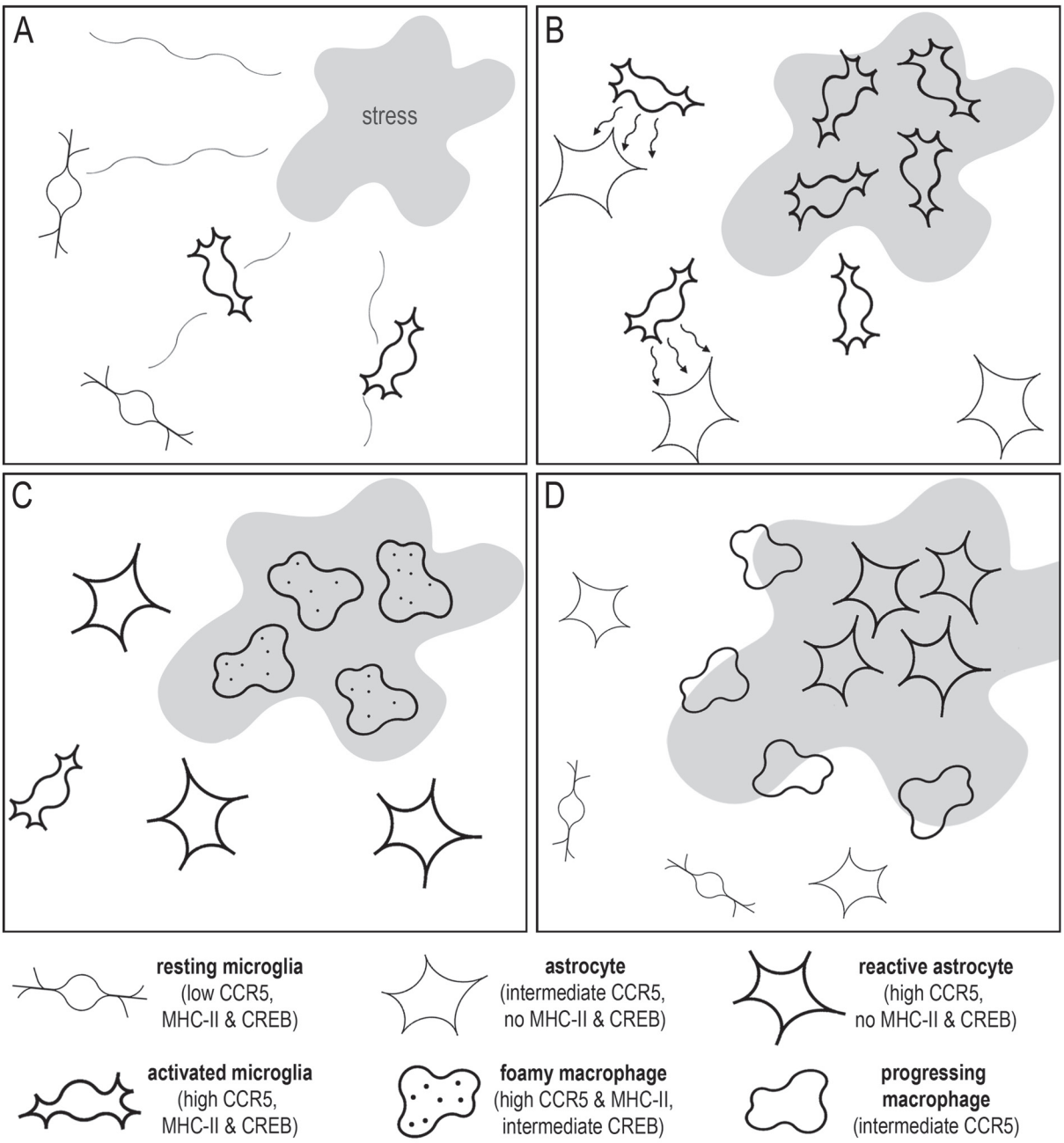
Role of CCR5 in MS lesion formation

Both microglia and astrocytes are thought to play an important role in the development of MS lesions [13,14]. In addition, enhanced motility of these cells is an important aspect of MS pathology: in early lesion stages microglia cluster at certain places in the NAWM and in active demyelinating areas accumulation of macrophages can be found. Furthermore, *in vitro* and *in vivo* indications that migration of astrocytes towards sites of injury also occurs in MS are supported by the fact that the final stages of lesion development are characterized by high numbers of astrocytes.

By evaluating the expression patterns of MHC-II and CCR5, molecules that can play a role in the initiation of an inflammatory reaction, and the transcription factors that

Figure 2. Schematic representation of MS lesion formation. A Upon neuronal damage or another event of injury, stress factors, including cytokines and chemokines are synthesized. Microglia are activated by, and attracted to, these factors. B Clusters of activated microglia are formed at the site of injury. On their way to the lesion microglia come in contact with astrocytes and induce CCR5 expression or transfer CCR5 molecules to astrocytes. C Activated microglia transform into foamy macrophages that phagocytose myelin and produce inflammatory mediators. Astrocytes expressing CCR5 are attracted to the lesion. D Macrophages that lose their activation state and subsequently CCR5 expression move outwards from the lesion center, leading to expansion of the lesion. CCR5 expressing astrocytes populate the center of the lesion.

contribute to this expression, such as CREB-1, a hypothetical scheme of the processes underlying MS lesion development can be proposed. This scheme is depicted in figure 2. The initial step in MS lesion formation is characterized by activation and clustering of microglia in otherwise unaffected white matter (Figure 2A and B). In theory, this microglial activation and migration is induced by the localized production of stress factors, including cytokines and chemokines, which is triggered by for example neuronal damage (Figure 2A). As discussed in **chapter 2**, these factors are probably produced by astrocytes and microglia present at the site of injury [8,15,16]. As we have discussed



above, we hypothesize that, when migrating to the lesion site, microglia transfer CCR5 molecules to astrocytes that they encounter (Figure 2B). Subsequently, in the active stage of MS lesions activated microglia transform into foamy macrophages that phagocytose myelin (Figure 2C) [14,17]. Activated microglia and foamy macrophages produce many cytotoxic and inflammatory mediators, including chemokines [8,16,18], which attract CCR5 expressing, and activated, astrocytes to the site of demyelination. When lesion development progresses, foamy macrophages move outwards from the center of the lesion, leading to progression of demyelination (Figure 2D). Gradually, their activated state is quieted, as observed by low expression of CREB-1 and decreased expression of CCR5, which allows them to move away from the source of chemokines inside the lesion. In contrast, because CCR5 expression on hypertrophic astrocytes is high, these astrocytes migrate towards and populate the lesion center (Figure 2D). Finally, the inflammatory reaction is stopped, either because macrophages return back to a resting state (as microglia), or because these cells are eliminated by apoptosis. Because of the supposed stability of CCR5 expression on astrocytes, these cells populating the center of the lesion still display high levels of CCR5 expression. Depending on the extent of demyelination, axonal damage and oligdendrocyte cell death, some degree of remyelination can occur [19], or MS lesions remain as demyelinated areas.

To understand where things go wrong in MS, a logical approach is to look into one of the first steps of lesion formation. One possibility is that microglia in MS patients are predisposed to be activated or that microglial activation is not switched off. A normally harmless trigger of microglial activation, such as for example neuronal cell death, will in this case initiate a cascade of aberrant microglial activation. This cascade is intensified by the factors that are secreted by these activated microglia, ultimately leading to damage to myelin and axons. Alternatively, it could be that the initial event that triggers microglial activation is deranged: a normally harmless event triggers excessive production of microglia activating factors, including chemokines, which excessively activate microglia, initiating the cascade of microglial activation leading to demyelination. Of course both these scenarios put an emphasis on the role of microglia in MS lesion formation. However, without proper understanding of the contribution of each glial (and neuronal) cell type to the pathogenesis of MS, these hypotheses remain speculative.

Potential of statins in MS treatment

Obviously, the main question of the second part of this thesis is whether statins can be used as a treatment for MS. The answer to this question depends on the etiology of MS, which unfortunately still has not been uncovered. In addition to the widely investigated effects of statins on the function of T cells (as discussed in **chapter 1** and shown in **chapter 5**), we have demonstrated that statins can also affect microglia

functions (**chapter 6**) and interfere with development of monocytes into professional APC (**chapter 7**). With regard to microglia and their function in the pathology of MS, it is still unclear whether these cells are friend or foe. The prevalent belief is that microglial activation is a key event in the development of MS lesions and that this activation results in demyelination and axonal damage [13,14,20-22], which would make this cell type the main player in MS pathology. If this notion is correct, statin treatment, targeted to the brain parenchyma could be advantageous in silencing microglia and blocking their migration to the site of lesion formation. In this respect, statins could prove to be a good treatment to prevent lesion formation and alleviate MS symptoms. However, microglial activation could also be a secondary effect and represent the activation of a survival mechanism. The CNS innate immune system would in this case attempt to minimize the extent of (bystander) damage by removing the debris of a primary destructive reaction targeted to the myelin sheet, mediated by the peripheral immune system. In addition, microglia might be involved in the tempering of this immune response. In this scenario microglia are important mediators of the CNS repair system and statin treatment could possibly hamper their beneficial functions in MS and prove to worsen the disease.

As I will discuss below, there is evidence supporting the potential of statins in the treatment several immune-mediated diseases, including MS. However, before extrapolating these findings safely to clinical treatments other than lowering cholesterol levels, we should gain more insight in the mechanisms underlying the beneficial effects of statins. None withstanding, because the safety of statin treatment has already been established for the therapeutic ranges used for cholesterol lowering, preliminary clinical trials with statin treatment of MS patients have already been performed on a small scale, with cautiously promising results.

Therapeutic potential of statins for treatment and management of immune-mediated diseases

Several recent *in vivo* studies, performed with different experimental animal models for immune-mediated diseases, provide evidence for a beneficial effect of statins in the treatment and management of immunological disorders (Table 1). A number of studies in mouse and rat experimental autoimmune encephalomyelitis (EAE), a Th1-mediated central nervous system (CNS) demyelinating disease with symptoms similar to MS, have shown that oral treatment of EAE susceptible animals with atorvastatin and lovastatin not only protects these mice from developing this disease, but also reverses already established disease. Statin treatment clearly results in a delayed disease onset, milder clinical signs, normalization of the symptoms and protection against loss of myelin and perivascular inflammatory infiltrates [23-26]. Adoptive transfer experiments revealed that this statin-induced protection is mediated through CD4+ cells and not CD8+ cells [23].

Table 1. List of selected in vivo and clinical experiments with statins

Disease model*	Species	Statin	Dosage	Administration, frequency**	Effect***	Ref.
CIA	mouse	simvastatin	40 mg/kg	ip, daily, prophylactic	suppression developing CIA (incidence 50%, severity 60%)	27
				ip, daily, therapeutic	reduction progression CIA (severity 70%)	
asthma	mouse	simvastatin	40 mg/kg	ip, three times	reduction cell infitration into lung (55%)	28
				oral, three times	reduction cell infitration into lung (34%)	
EAE	mouse	atorvastatin	1-10 mg/kg	oral, daily, therapeutic	reduction clinical symptoms (60-100%), normalization EAE	23
				oral, daily, prophylactic	reduction clinical symptoms (75-100%), normalization EAE	
EAE	mouse	atorvastatin	1-10 mg/kg	sc, daily, prophylactic	reduction clinical symptoms (30%)	24
				sc, daily, therapeutic	reduction relapse rate (57%), reduction symptoms (50%)	
			10 mg/kg	oral, daily, prophylactic	reduction clinical symptoms (30-70%)	
EAE	rat	lovastatin	2 mg/kg	ip, daily, prophylactic	reduction severity (40%), delay onset (2 days), normalization EAE	25
EAE	rat	lovastatin	4 mg/kg	ip, daily, prophylactic	reduction clinical symptoms EAE (69%)	26
MS	human	simvastatin	80 mg	oral, daily	reduction mean number (44%) and volume (41%) T1 lesions	88
MS	human	lovastatin	40 mg	oral, daily	reduction number of T1 lesions (mean: 43%)	92

* CIA – collagen-induced arthritis; EAE – experimental autoimmune encephalomyelitis; MS – multiple sclerosis
** ip – intraperitoneal; sc – subcutaneous; prophylactic – treatment from day 1 of induction of disease; therapeutic – treatment after development of first symptoms; three times – before each OVA challenge
*** T1 lesions – gadolinium-enhancing brain lesions on magnetic resonance imaging (MRI)

Another report demonstrates the therapeutic potential of statins in rheumatoid arthritis (RA). In a Th1-driven model of murine inflammatory arthritis, simvastatin is able to suppress developing arthritis and reduce progression of clinically evident collagen-induced arthritis [27]. It should be noted, however, that the effect of simvastatin on disease progression and severity in collagen-induced arthritis is less potent than the effect of atorvastatin treatment in EAE (60-70% reduction of clinical signs at a dose of 40 mg/kg vs. 60-100% reduction at a dose of 10 mg/kg, and only reduced progression of arthritis was observed, no reversal of disease) [27].

Lastly, in a murine model of allergic asthma, a Th2 driven airway inflammation, simvastatin treatment reduces inflammatory infiltration and the production of cytokines in the lungs [28]. In the studies discussed above, orally given simvastatin seems to be less potent compared with atorvastatin. This difference could be due to first-pass hepatic metabolism of simvastatin after absorption from the gastro-intestinal tract, giving rise to several metabolites and reducing the effective dose of available simvastatin [29]. However, differences in observed *in vivo* effects of statins and potency of these effects could also reflect differences in treatment regime (high-dose vs. low-dose, one time vs. daily administration).

Molecular mechanism of immune regulation by statins

Regulation of MHC2TA transcription

It has been reported that statins affect cell surface expression of MHC-II molecules by inhibiting the transcription of the *MHC2TA* gene [23,30-32] encoding the class II transactivator (CIITA), the master switch for MHC-II transcription [33-35]. The transcriptional regulation of CIITA is controlled by the differential usage of at least three independent CIITA promoter units, each encoding a unique first exon: CIITA-PI, -PIII and –PIV [36]. CIITA-PI and –PIII are used for the constitutive expression in DC and B cells, respectively. CIITA-PIV is the promoter that is predominantly involved in IFN-γ-induced expression of CIITA in a variety of different cell types [36,37]. However, CIITA-PIII can also be induced by IFN-γ, but this induction requires an additional 4 kb IFN-γ regulatory region, which is located 2 kb upstream of the CIITA-PIII core promoter [38,39].

The fact that statins would reduce cell surface expression of MHC-II molecules by inhibiting *MHC2TA* transcription is of interest because this would infer that the mode of action of statins on MHC-II expression involves interference in the transcriptional control of *MHC2TA*. In particular, it was suggested that statins specifically mediate their effect on IFN-γ-induced MHC-II molecule expression in non-bone marrow-derived cells through downregulation of transcription of the IFN-γ-induced CIITA-PIV-isoform [30,31]. Besides impairing IFN-γ-induced MHC-II molecule expression, statins also downregulate membrane expression of MHC-II molecules in B cells and activated T cells ([40] and **chapter 5**). Expression of MHC-II in activated T-cells, as well as

constitutive expression of MHC-II in B cells, is mediated by the PIII-isoform of CIITA [36,39,41-43]. This would imply that statins also affect the PIII-isoform of CIITA. Additionally, in microglial cells, statins have been shown to affect the IFN-γ-mediated induction of both the CIITA-PI and the PIV-isoform [23]. As we discuss in **chapter 4**, together, these observations infer that statins interfere in transcription of all CIITA isoforms, which would explain the observed reduction in MHC-II molecule expression at the cell surface of a variety of hematopoietic and non-bone marrow-derived cells. However, we argue that this clearly presents an enigma, because the different CIITA promoters are mostly activated through non-overlapping regulatory pathways.

As we have shown in **chapters 4** and **5**, which describe our analysis of the effect of statins on cell surface expression of MHC-II molecules, we did not find such a downregulatory effect of statins on CIITA mRNA levels or on the activity of the CIITA-PIII or CIITA-PIV promoter in various cell types. In addition to our findings, it has been shown that treatment of mice with simvastatin does not alter draining lymph node CIITA mRNA-levels, whereas under the same conditions an effect on cytokine production can be observed [27,28]. Therefore, it seems that statins do not inhibit cell surface MHC-II expression by interference in *MHC2TA* gene transcription.

JAK/STAT signaling

Activation of the JAK/STAT pathway by IFN-γ is required for the induction of various immuno-regulatory molecules [44]. Signaling by IFN-γ through its receptor and associated Janus kinases (JAK) 1 and 2 leads to activation of signal transducer and activator of transcription 1 (STAT-1), followed by dimerization of this protein, translocation to the nucleus and binding to IFN-gamma activated site (GAS) elements in the promoters of IFN-γ responsive genes [44]. In the case of *MHCT2A*, the STAT-1 homodimer binds to the GAS element in promoter PIV [37]. This would imply that if statins selective inhibit IFN-γ-induced CIITA-PIV activity [30,31], they would most likely interfere with JAK/STAT signaling. Indeed, statins have been shown to have an effect on some members of the STAT-family. For example, *in vivo* treatment with atorvastatin results in decreased phosphorylation of STAT-4 and induction of phosphorylation of STAT-6, which are required for Th1 and Th2 lineage commitment, respectively [23]. However, the phosphorylation, nuclear translocation and DNA binding of STAT-1, which is required for CIITA-PIV activation upon IFN-γ triggering, is not affected by simvastatin [32]. It has also been suggested that the suppression of IFN-γ-induced CD40 expression in microglia by lovastatin is mediated through inhibition of IFN-γ-induced phosphorylation of STAT-1 following JAK-1 and JAK-2 activation [45]. Contradictory, the inhibition of IFN-γ-induced expression of ICAM-1 on endothelial cells by lovastatin shows that lovastatin does not affect phosphorylation of STAT-1 by JAK-1 and JAK-2 [46]. Together, these studies do not favor a direct involvement of statins in regulatory pathways affecting the transcription of genes encoding molecules that are important for antigen presentation and immune regulation.

Lipid raft disruption

Because cholesterol plays an important role in the recruitment and concentration of proteins to the cell membrane, statins could affect the expression of membrane bound proteins in a way distinct from suppressing gene transcription. It has become clear that the plasma membrane is not a homogeneous environment of lipids, but rather a discontinuous field containing numerous microdomains that are essential for cellular function. Lipid rafts, also known as detergent-insoluble glycolipid-enriched complexes (DIGs) or glycosphingolipid-enriched membrane microdomains (GEMs), which are highly enriched in cholesterol and glycosphingolipids, are the best studied of these microdomains [47-49]. Lipid rafts range in size from 50 to 70 nm and the proteins that are present within these microdomains are severely limited in their capacity to diffuse freely over the plasma membrane [49]. The most important role of lipid rafts therefore seems to be the recruitment and concentration of cell signaling molecules to the plasma membrane [48]. Glycosylphosphatidylinositol (GPI)-linked proteins are in general characteristic components of lipid rafts and rely for transport, recycling and function at the cell surface on the integrity of these cholesterol-containing vesicles [47]. Lipid rafts have already been shown to be very important in both T and B cell signaling [50-52]. Moreover, recent studies have shown that surface MHC-II molecules are also present in lipid rafts and that this association serves to increase the local concentration of MHC-II molecules in distinct plasma membrane microdomains and so facilitate antigen presentation [53,54]. Because cholesterol is one of the major components of lipid rafts, statin-mediated inhibition of cholesterol biosynthesis results in the disruption of these rafts [55]. In **chapter 5** we have shown that statins might affect membrane expression of MHC-II and other molecules associated with lipid rafts by disrupting these structures. Indeed, we have shown in **chapter 5** that statin treatment of cells affects membrane expression of a variety of molecules, including MHC-II, of which the function depends on the integrity of lipid rafts [51,53,56-61]. That statins affect the integrity of lipid rafts is underscored by the observation that statin treatment of lymphoid cells does not affect membrane expression of CD45 or the transferrin receptor (CD71) (**chapter 5**), molecules known not to be associated with lipid rafts [57,62,63]. In addition, it has been shown that *in vitro* statin treatment results in disruption of membrane rafts and so impairs Fcγ-receptor signaling at the level of tyrosine kinase activation [64]. Moreover, oral simvastatin treatment at standard therapeutic dosages leads to a reduction in lymphocyte lipid raft levels in healthy volunteers [65]. Obviously, impairment of immune receptor signaling through disruption of lipid rafts leads to reduced activation of downstream signal transduction cascades important for the induction of inflammatory mediators, so affecting immune activation at another level. Indeed, it has been shown that fluvastatin blocks Fcγ-receptor mediated activation of ERK and p38 MAP kinases in monocytes, resulting in lowered cytokine release [64].

Isoprenylation

In addition to the effect of statins on lipid raft integrity, cholesterol depletion by statin treatment may affect not only the composition of cellular membranes and the expression of membrane-bound molecules, but also various other cellular processes in which cholesterol plays an important role, such as the production of steroid hormones and vitamin D, and the regulation of receptor function and signaling molecules [66-68]. Moreover, since the isoprenoid intermediates of the cholesterol biosynthesis pathway also play important roles in various cellular processes, the inhibition of mevalonate synthesis most likely also results in interference in these processes. However, it should be noted that the enzymes involved in the side pathways of cholesterol synthesis, leading to non-sterol products, are less sensitive to inhibition of mevalonate synthesis than the enzymes leading to cholesterol synthesis [69]. Statins could, in addition to inhibiting cholesterol synthesis, affect for example isoprenylation processes, such as farnesylation and geranylgeranylation. These mechanisms consist of post-translational lipid modifications leading to targeting of proteins to the cell membrane or other subcellular compartments and their consequential activation. As a result, these processes also play a role in protein-protein interaction and the assembly of multi-subunit complexes. Targets of isoprenylation include nuclear lamins, Heme-A, the γ subunit of G-proteins, and small GTP binding proteins such as Ras and the Ras-like proteins Rac, Rab and Rho [70-72]. These Rho GTPases play a central role in various cellular events such as cytoskeletal organization, membrane trafficking, transcriptional activation, cell growth control and development [73,74]. Statins could interfere with these processes by inhibiting their activation by isoprenylation. In fact, statins have been shown *in vitro* and *in vivo* to inhibit RhoA isoprenylation and activation, and Rho/Rho-kinase signaling, leading to disruption of the actin cytoskeleton and disturbance of cell adhesion [75-80]. For example, statins inhibit ICAM-1 expression on endothelial cells by preventing RhoA activation and subsequent ERK-1/2 phosphorylation [46,81], and affect T cell proliferation by reducing functional activity of Ras-dependent ERK pathways and Rho-dependent p38 activation [82]. In addition, atorvastatin reduces lipoprotein-induced actin polymerization, and RhoA and FAK activation in monocytic cells, which is essential for their adhesive and migratory capacities [75,76].

NF-κB activation

Some reports propose the inhibition of NF-κB activation as the mechanism by which statins reduce the expression of immune modulatory molecules. The transcription factor NF-κB induces transcription of several immune genes, including MHC class I genes and chemokines such as interferon-inducible protein 10 (IP-10) and MCP-1. Indeed, atorvastatin has been show to reduce the expression of MCP-1 and IP-10 presumably by inhibition of NF-κB activation [83]. Because TNF-α is a potent inducer of NF-κB, statins might interfere in the induction of immunomodulatory molecule

expression by TNF-α. This is illustrated by the observation that simvastatin inhibits TNF-α-induced activation of NF-κB and so decreases VCAM-1 and ICAM-1 expression in human endothelial cells [84].

LFA-1 interaction

In addition to the inhibition of mevalonate synthesis, an anti-inflammatory effect of statins unrelated to inhibition of HMG-CoA reductase has been uncovered recently. It has been shown that statins (mainly lovastatin) can inhibit leukocyte adhesion by blocking interaction with leukocyte function antigen 1 (LFA-1, CD11a) and ICAM-1. By binding to a novel allosteric site within LFA-1, the L-site, statins can block the adhesion of lymphocytes to ICAM-1 and so impair T-cell costimulation [85].

Treatment potential of statins for MS

As discussed above, statins affect the expression and functionality of a myriad of membrane-bound molecules and the release of various immune mediators. In this way, statins interfere in processes that are important for both adaptive and innate immune functions. These anti-inflammatory properties of statins would potentially be beneficial for the treatment of patients with immune-mediated disorders. Therefore, statins are currently considered as possible treatment agents for MS and other neurodegenerative diseases [86-92]. Because activation of microglia is thought to be a key element in the development of MS [13,20,21], it could be argued that statin interference in the processes that result in microglia activation would benefit these patients. In **chapter 6** we have shown that simvastatin is able to inhibit the activation-induced cell surface expression of various immunomodulatory molecules on microglia *in vitro*. For example, simvastatin treatment reduces the expression of MHC-II, thereby potentially interfering in APC capacity of microglia. In addition, we have shown in **chapter 6** that simvastatin reduces expression of the chemokine receptors CCR5 and CXCR3 on microglia and abolishes chemokine-induced cell motility of microglia. Moreover, early results from clinical trials show that statin treatment might have favorable clinical effects in MS patients. Vollmer *et al.* have shown that in MS patients receiving simvastatin treatment the number and volume of gadolinium-enhancing (T1) lesions in the brain decrease significantly (Table 1) [88]. However, yearly relapse rates and disability status scores did not change during the treatment and no changes in the immunological status of patients were observed. Moreover, because this study was short, small scale and non placebo-controlled, and patients were enrolled on the basis of disease activity (presence of lesions), conclusions from this study should be drawn with caution [88,89]. Likewise, Sena *et al.* have shown in an earlier study that lovastatin treatment of MS patients reduces the number of T1 lesions in a number of patients, whereas disability scores remain stable and the majority of patients develop new T2-weighted lesions [92].

Although follow-up in this study lasted for 2 years [90], patient number was very low (n=7). In conclusion, more detailed knowledge on the actions of statins in the brain and larger, long-term, placebo-controlled clinical studies are needed before statins can truly be considered as save and effective treatment options for MS.

Conclusions and recommendations for future research

In this thesis I have shown that CCR5 expression in MS brain tissue greatly overlaps expression of MHC molecules and the expression of transcription factors involved in MHC regulation. In addition, I have determined that at least one of these transcription factors, namely CREB-1, is involved in the transcriptional regulation of CCR5. Many genes contain consensus sites for CREB binding and as such the transcription factor CREB-1 has been implicated in a wide variety of cellular processes [93]. In the CNS, CREB-1 plays an important role in various neuronal processes, such as neuronal development, neuroprotection and disease. In addition, various signaling pathways, including neurotrophin-mediated signaling, result in enhanced CREB-1 expression [94]. Furthermore, CREB-1 has also been implicated in axonal regeneration in the injured CNS [95]. Together this indicates that there indeed might be a general state of activation of glial cells in MS brain tissue, resulting in an excessive reaction to a small injury in the brain parenchyma. However, to strengthen this hypothesis, we have to obtain a complete overview of this activation status and confirm that this common activation causes the aberrant expression of various immune molecules in MS. Ultimately, understanding of the factors that are activated in MS and the pathways by which these factors could be activated might provide clues to the source that triggers the activation process and the subsequent cascade of excessive reactions.

In addition to determining the regulatory pathways that might be affected in MS, the function of CCR5 in normal and diseased brain should be further investigated, in order to obtain more insight in the role of CCR5 in MS pathogenesis. Particularly the function of CCR5 expression on astrocytes is a very interesting topic to explore, because it might shed some light on the role of astrocytes in MS pathogenesis.

Finally, there is ample experimental support that statins are able to function as immunomodulatory mediators. Because statins interfere in the mevalonate pathway, which controls isoprenylation and cholesterol biosynthesis, it can be argued that, besides immunoregulatory properties, statins have an impact on a variety of other cellular functions. These include lipid raft-mediated processes, which involve cell signaling events needed for activation and intracellular communication, and processes in which isoprenylated proteins play critical roles, such as those involved in actin rearrangement and cell motility. Whether the therapeutic doses used for treatment of hypercholesterolemic patients are sufficient to (safely) interfere in the inflammatory processes involved in various immune-mediated disorders and ameliorate disease

remains to be investigated. In addition, to pursue the potential of statins in the treatment of MS, we first have to determine the role of each (CNS) cell type in the pathogenesis of MS and subsequently the effect of statins on the abnormal cell functions found in MS patients. Given the general nature of the working mechanism of statins it is very important not to overlook potential adverse effects of statin treatment and the dosage at which these might occur. Therefore, great efforts should also be made in the targeting of statins to their intended place of action (and possibly their target cell), resulting in maximum efficacy of treatment with minimal incidence of adverse effects. Alternatively, agents that specifically interfere with downstream branches of the mevalonate pathway might be developed and examined for effectiveness in inhibiting specific (immune) functions of glial cells.

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