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

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Introduction

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Central nervous system immunity & immunomodulation by statins

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Central nervous system immunity & immunomodulation by statins

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Immunity in the central nervous system

Introduction

Traditionally, the central nervous system (CNS) was considered an immunologically privileged organ. This viewpoint was based on several observations: the CNS lacks conventional lymphatic drainage and dendritic cells, the brain is carefully shielded by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier, rejection of transplants is impaired in the CNS and, under normal conditions, the expression level of major histocompatibility complex (MHC) and adhesion molecules in the CNS is very low. However, over the years it has become clear that immune privilege of the CNS is not absolute, allowing restricted entry of peripheral immune cells, having a lymph-like system enabling drainage of soluble antigens and being capable of participating in CNS immune reactivity by activation of its resident cells [1,2]. Macrophages and dendritic cells have been found in the dura mater, leptomeninges and choroid plexus, strategically located to protect the ventricular/subarachnoid compartment [3], and sentinel function at the BBB is ensured by perivascular macrophages, in addition to pericytes [4]. So, it seems that innate immune cells survey all entry sites for possible blood-derived pathogens and immune cells into the CNS. However, in addition to this, certain resident cells within the brain parenchyma are able to process and present antigens to T cells and activate these immune mediators. For example, both microglia and astrocytes, present within and distributed throughout the CNS parenchyma, are able to -when activated- phagocytose pathogens and cell debris, change their microenvironment to a more favourable environment for infiltrating T cells by secreting cytokines, and activate T cells [5-7]. After a brief introduction of the cell types inherent of the CNS, I will discuss the immune function of CNS resident cells and expression regulation of the mediators of these immune functions.

Cells of the CNS

The CNS consists of neurons, which constitute about 50% of the volume of the CNS, and glial cells, which make up the rest. Glial cells are considered to be the supporting cells of the central nervous system. Their main functions consist of surrounding and supporting neurons, supplying nutrients and oxygen to neurons, producing myelin for the insulation of neurons, and the removal of critically damaged neurons. Glial cells are divided into three types: astrocytes, oligodendrocytes and microglia.

Astrocytes

Astrocytes constitute the major component of glial cells in the CNS and are critical for the development and support of neurons, the repair of injured neurons, the formation and maintenance of the BBB, and the uptake of neurotransmitters such as glutamate. For example, astrocytes increase the number of functional synapses of CNS neurons and are required for synaptic maintenance *in vitro*. Therefore, astrocytes may have an active role in synaptic plasticity [7,8]. In addition, astrocyte processes are in close contact with endothelial cells and cover the entire abluminal surface of the BBB. Soluble factors secreted by astrocytes also seem to be important for the maturation of endothelial cells into and the maintenance of the BBB. Therefore, astrocytes contribute to both the structural and the functional integrity of this barrier [9].

Oligodendrocytes

The principle function of oligodendrocytes is to provide support to axons and to produce the myelin sheath, which insulates axons. Myelin consists of 80% lipid and 20% protein and allows for the efficient conduction of action potentials down the axon. During development, oligodendrocyte progenitor cells differentiate into myelin-producing oligodendrocytes that radially extend multiple processes. These processes wrap themselves around portions of the surrounding axons, forming layers of myelin. Each process thus becomes a segment of the axon's myelin sheath. In this manner, oligodendrocytes provide multiple neurons with myelin sheaths at once [10].

Microglia

Microglia are considered the resident macrophages of the CNS and in the normal brain are involved in the removal of cells undergoing apoptosis, a normal feature of brain development. In addition, because of their phagocytic, cytotoxic, cytokine/chemokine secreting and antigen presenting capacities, microglia also play a key role in immune reactions within the CNS [11]. They constitute approximately 10-20% of the total glial cell population and are distributed ubiquitously in non-overlapping regions in the central nervous system [11]. Microglia are of the myelo-monocytic lineage and share phenotypic and functional characteristics with blood-borne macrophages. In an

early stage of embryonic development, cells of the mononuclear phagocyte lineage enter the developing CNS and differentiate, via an intermediate amoeboid microglia stage, into the typical ramified microglia [12-14]. However, recently it has become clear that, in addition, the microglia population in the CNS is replenished with circulating peripheral monocytes that infiltrate into the brain parenchyma and transform into microglia [14]. Microglia are able to respond quickly to stress or immune signals and pathogen infiltration. This response leads to tissue repair and the induction of protective immune responses. For this, microglia need activation and maturation during which they obtain macrophage differentiation markers and effector properties [6].

Immune function of glial cells

Microglia and astrocytes are considered the main immune effector cells of the CNS. By evaluating the immune function of these cell types the importance of their role in (regulating) immune responses in the CNS parenchyma becomes clear. Therefore, following a short introduction on general mechanisms of immune activation and immune mediators, I will discuss the immunological properties of these two CNS cell types in more detail.

Mechanisms of antigen presentation and immune activation

Antigen presentation by major histocompatibility complex class II (MHC-II) and class I (MHC-I) molecules, expressed on professional antigen presenting cells (APC), is a crucial phase in the initiation and maintenance of an antigen-specific immune response. While MHC-I molecules are constitutively expressed in almost all nucleated cell types, constitutive expression of MHC-II molecules is restricted to professional APC, such as dendritic cells (DC) or B cells. However, upon inflammatory cytokine stimulation, several cell types start expressing MHC-II molecules and may function as APC. Recognition of pathogen-derived peptide bound to MHC-II or MHC-I by $CD4^+$ or $CD8^+$ T cells, respectively, requires accumulation of antigen presenting molecules in membrane microdomains at the surface of APC, which act as signaling platforms for MHC-peptide-T cell receptor (TCR) interactions in the immunological synapse. This process is important for efficient T cell activation and requires the assistance of MHC - CD4/CD8 coreceptor interactions and interactions between costimulatory molecules, such as CD80/CD86 - CD28/CTLA4 and CD40 - CD154, and adhesion molecules, including ICAM-1 - LFA-1 [15-17].

In addition to engagement of APC with T cells through adhesion molecules, fine-tuning of antigen-specific T cell activation not only requires MHC-TCR and costimulatory molecule interactions, but also relies on cytokine signaling by APC to meet with local requirements for adequate immune reactivity. This cytokine signaling is an important factor in T cell activation and determining T cell fate [18]. In addition, cytokines play an important role in the regulation of cell survival and differentiation.

Chemokines and chemokine receptors

Chemoattractant cytokines or chemokines are a group of small secreted proteins that are able to attract various cell types towards sites of inflammation. Chemokines initiate chemotaxis by binding to G protein-coupled receptors, which leads to activation and migration of the responding cell. Interactions between chemokines and chemokine receptors are promiscuous, i.e. most chemokines activate more than one receptor and most chemokine receptors can bind several chemokines. Based on their structure, chemokines and their receptors have been classified into four subfamilies: C, CC, CXC and CX_3C [19,20]. In addition to their role in inflammation and immune activation, chemokines mediate various other processes, such as cell development, maturation and differentiation, angiogenesis, tumor growth, metastasis, neurodevelopment and neurophysiological signaling [19,21,22]. In the normal brain, chemokines and chemokine receptors are present at low levels. However, chemokine production and chemokine receptor expression is upregulated during CNS injury or inflammation and in various neurodegenerative diseases [21,22]

The CC chemokine receptor 5 (CCR5) is one of the best described CC chemokine receptors and mainly regulates trafficking and effector functions of memory/effector T-lymphocytes, macrophages, and immature DC. CCR5 binds the chemokines CCL5 (RANTES), CCL3 (MIP-1α) and CCL4 (MIP-1β) [19,20,23,24]. Because of its important immune regulatory role, CCR5 is implicated in the pathogenesis of various inflammatory diseases such as atherosclerosis, transplant rejection, autoimmunity, and neurodegenerative diseases [21,22,25-27]. In addition, CCR5 also serves as a coreceptor for viral entry of HIV-1, enabling the virus to enter its target cells [24,28].

Immune function of microglia

The immunological functions of microglia have been reviewed extensively by Aloisi [6]. Microglia are the first cell type to respond to a variety of CNS injuries and can even display an activated phenotype without the occurrence of obvious neuropathological changes. Therefore, microglia seem to respond very rapidly to changes in the microenvironment within the CNS. In the normal (adult) CNS ramified microglia, in contrast to peripheral macrophages, display a downregulated/quiescent phenotype, characterised by a lack of phagocytic activity, low CD45 expression and low expression of membrane bound molecules essential for the induction of macrophage functions [6,11].

Upon activation, microglia display a range of innate immune functions, such as proliferation, increased or de novo expression of immunomodulatory molecules, recruitment to the site of damage and the release of cytokines. In addition, microglia are highly capable of presenting antigens [6,11]. This response of microglia, however, depends on the expression of receptors such as pattern recognition receptors, opsonic receptors, cytokine and chemokine receptors, and receptors needed for macrophage

effector functions. Pattern recognition receptors expressed by microglia, either constitutively or induced through activation, include the integrin CD11b, the mannose receptor and the LPS-binding receptor CD14, and are important for the recognition of foreign entities, leading to phagocytosis and elimination of pathogens [6,29]. Receptors that bind the constant region of immunoglobulins, the Fcγ receptors I, II and III, and the complement receptors CR-1, -3 and -4 and C1qRp are all expressed on resting microglia at low levels, which are increased upon activation [30-34]. These opsonic receptors mediate or enhance microglial phagocytosis through recognition of serum components deposited on invading micro-organisms. In some multiple sclerosis (MS) lesions, antibody and complement mediated phagocytosis of myelin by microglia can be observed and could represent a mechanism of demyelination, which is the hallmark of this disease [35].

IFN-γ is considered to be the best known inducer and amplifier of microglial antimicrobial, pro-inflammatory and antigen presenting functions. Therefore it is not surprising that the IFN-γ receptor (IFN-γR) is expressed constitutively on resting microglia. Binding of IFN-γ to the IFN-γR triggers the activation of the classical IFN-γ signaling cascade, which ultimately leads to transcription of IFN-γ responsive genes [36]. TNF-α, another potent pro-inflammatory cytokine, is produced during CNS inflammation by Th1 cells, macrophages and microglia and induces microglial phagocytosis and production of pro- and anti-inflammatory cytokines and nitric oxide, representing a possible autocrine activator [6,37]. The TNF receptors type I and type II are expressed by microglia *in vitro* and in HIV-infected individuals [38,39]. Triggering of these receptors induces activation of a number of transcription factors, of which NF-κB is the best known. NF-κB can be found in resting microglia in human normal white matter and is highly expressed in activated microglia in MS lesions and experimental autoimmune encephalomyelitis (EAE), an animal model of demyelinating disease [40-42]. Microglia also express the receptor for the pro-inflammatory cytokine interleukin (IL) -1, IL-1RI, since microglia respond *in vitro* to IL-1, resulting in enhanced production of cytokines, amongst which TNF-α [43]. In addition, microglia respond to anti-inflammatory cytokines: most of the immune functions exhibited by microglia upon activation by LPS or IFN-γ are blocked by anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and TGF-β [44]. Therefore, microglia probably express receptors for all of these cytokines. In addition to receptors for cytokines, microglia also express a variety of chemokine receptors. *In vitro* and in various CNS diseases microglia express the chemokine receptors CCR2, -3 and -5, CXCR4 and CX_3CR1 [21,45-48]. Since most chemokine receptors can be bound by multiple chemokines, microglia can functionally respond to a variety of chemokines.

In addition to responding to cytokines and chemokines, microglia are also a source of immune mediators. In CNS diseases as well as in culture, microglia are the main source of pro-inflammatory and immune regulatory cytokines. These include IL-1, IFN-γ, TNF-α and IL-6 [11,49]. The production of TNF-α is enhanced by IFN-γ stimulation.

IL-1 produced by microglia has been shown to induce proliferation of astrocytes. The production of IFN-γ would induce an activation of both microglia and astrocytes, and possibly other cell types present at the site of action, so amplifying the immune response. TNF-α on the other hand, in addition to inducing and enhancing microglial and astrocyte immune functions, seems to have a cytotoxic effect on oligodendrocytes and as such contributes to demyelination [11]. *In vitro*, microglia are also a source of IL-12 and IL-18, cytokines that, like IL-6, enhance the immune response and are produced within the CNS during inflammation and autoimmune diseases [6]. In addition, microglia produce various chemokines, such as CCL2, -3, -4 and -5, CXCL8 and -10 [6,45,46,50]. In contrast, microglia also produce anti-inflammatory cytokines such as TGF-β, IL-10 and the IL-1 receptor antagonist [6,11,49]. These factors inhibit microglial activation by downregulating MHC-II and costimulatory molecules, and the production of cytokines and nitric and oxygen radicals.

Most of the literature suggests that microglia are the most efficient APC within the brain parenchyma. It has long been known that microglia, when activated, express high levels of MHC-II [51-54]. However, it seems that microglia already express low levels of MHC-II in their resting state and readily upregulate MHC-II expression upon activation [52]. Gobin et al. have found that MHC-II molecules are expressed at moderate to strong levels in nonactivated microglial cells of normal white matter of the CNS, and at intense levels in activated microglial cells in MS lesions [40]. In addition, several studies have demonstrated that activated microglia are able to prime naïve $CD4^+$ T cells and activate memory $CD4^+$ T cells in an antigen-specific, MHC-II restricted manner [53-55]. Aloisi et al. compared the ability of microglia and astrocytes to process and present antigens to Th1 and Th2 cells and found that activated microglia were more efficient in antigen processing and restimulation of both Th1 and Th2 cells compared to astrocytes [54,55]. For optimal antigen presentation and T cell activation, however, additional interactions between adhesion molecules and costimulatory molecules are needed. In resting microglia, expression of these molecules is low or not detectable. However, during CNS inflammation, upon stimulation with IFN-γ and in virtually all neurodegenerative diseases activated microglia express adhesion molecules, such as CD11a, CD58 and CD54, and the costimulatory molecules CD40, CD80 and CD86 [54,56-60]. Indeed, Aloisi et al. have shown that microglial activation of Th1 cells was dependent on the expression of MHC-II molecules and CD40 – CD40L (CD154) interactions [54]. In addition, CD40 expressing activated microglia and CD40L expressing T cells have been identified in both MS and EAE brain lesions [58]. However, the capacity of microglia to act as APC seems to vary amongst species. Rat microglia are found to incompletely present antigen, leading to T cell cytokine production, but not clonal expansion [5,61,62]. This incompetence could be due to the lack of costimulatory molecules that, in contrast, are expressed on human and murine microglia.

Immune function of astrocytes

Reports on the ability of astrocytes to express MHC-II and costimulatory molecules, and to act as APC, are quite conflicting. Astrocytes were the first CNS cell type shown to be inducible for MHC-II expression [63]: *in vitro*, astrocytes express MHC-II upon INF-γ stimulation, which is enhanced by TNF-α [7,64-66]. *In vivo*, astrocytes respond to intrathecal IFN-γ injection by expressing MHC-II molecules, albeit that this expression is less intense and its onset is at a later time point compared with microglia [52]. It should be noted however that the presence of MHC-II expressing astrocytes in the diseased brain remains controversial [40,67-72].

The inducibility of costimulatory molecule expression on astrocytes seems to vary between species. Most studies suggest that human astrocytes do not express CD80 or CD86, either constitutively or following IFN-γ exposure [60,73]. However, in some MS lesions, CD80/CD86 expressing astrocytes can be found [74]. In rodent astrocytes, constitutive and IFN-γ inducible expression of either CD80 or CD86, or both can be found. Murine astrocytes have been shown to express CD86 constitutively [75]. Several studies report IFN-γ-induced expression of CD80 [76], CD86 [75] or both [75,77]. The discrepancies in these findings could be due to temporal differences in CD80/CD86 expression by astrocytes. Soos et al. have shown that IFN-γ inducibility of CD80 expression in astrocytes depends on the time of *in vitro* culture, which may reflect phenotypical changes during different stages of astrocyte differentiation [75]. Despite the debate on costimulatory molecule expression on astrocytes, however, these studies do demonstrate that murine astrocytes are capable of processing and presenting encephalitogenic antigens to Th1 cells and induce T cell proliferation and activation, dependent on either CD80 or CD86 expression [55,75-78].

Taken together, it seems that astrocytes are able to act as APC, however much less efficient than microglia [54,55,79]. In general, efficient T cell activation *in vivo* relies on expression of the complete repertoire of costimulatory and adhesion molecules. Insufficient expression of these molecules could result in anergy or even apoptosis of T cells. However, it has been shown that upon stimulation with proinflammatory cytokines or in neurodegenerative diseases, astrocytes are able to express CD40 and the adhesion molecules CD54 and CD106 [76,80-82]. In contrast, there are also reports that MHC-II positive astrocytes tolerise CD4+ T cells and can even induce apoptosis in these cells, possibly due to insufficient expression of costimulatory molecules [53,83]. This raises the question whether under normal physiologically conditions and in disease state astrocytes do act as APC and activate T cells, or whether they lack the capacity to fully induce T cell activation and possibly inhibit T cell activation or induce T cell anergy or apoptosis.

Although astrocytes may not be able to display professional antigen presenting capacities, these glial cells are capable of influencing immune reactions in the CNS. In many neurodegenerative diseases astrocytes produce a wide variety of cytokines

and chemokines. Astrocytes have been shown to produce either *in vitro* or *in vivo* the proinflammatory cytokines IL-1, IL-6 and TNF-α, and the chemokines CCL2, CCL5, CXCL8, CXCL10 and CX_3CL1 [7,11,48]. In addition, astrocytes are an important source of colony stimulating factors, such as M-CSF, GM-CSF and G-CSF, which are strong stimulators of microglial proliferation [11]. Astrocytes could also play an important role in moderating the immune response by the production of anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β [7,84]. Indeed, in MS lesions, astrocytes seem to be the main source of both IL-4 and IL-10 [84]. In addition, astrocytes can respond to and migrate towards several chemokines through their expression of the chemokine receptors CCR1, CCR5, CXCR4 and CX_3CR1 [48,85].

Regulation of glial immune functions

Transcriptional regulation of MHC-II

The regulation of MHC-II expression occurs primarily at the transcriptional level. A set of conserved cis-acting regulatory promoter elements, located in a region within 150 bp from the transcription start site, mediates transcription of all MHC-II genes. This set of conserved promoter elements , the SXY-module, is bound by several regulatory proteins, i.e. RFX, CREB/ATF and NFY. However, binding of these factors is not sufficient for MHC-II transcription. Essential for activation of MHC-II promoters is the class II transactivator (CIITA), which functions through protein/protein interactions with the transcription factors bound to the SXY module. CIITA has been shown to be the master switch for MHC-II transcription, required for both the constitutive and IFN-γ-inducible expression of MHC-II genes [86-88]. Concurrent with MHC-II expression, the constitutive expression of CIITA is confined to APC only. However, CIITA expression can be induced in various other cell types by several inflammatory cytokines, of which IFN-γ is the most potent.

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 [89]. 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 [89,90]. 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 [91,92].

The induction of various immuno-regulatory molecules, including MHC-II and CIITA, by IFN-γ involves activation of the JAK/STAT pathway. Signaling by IFN-γ through its receptor and associated Janus kinases (JAK) 1 and 2 leads to tyrosine phosphorylation and activation of signal transducer and activator of transcription 1α (STAT-1α), followed by dimerisation of this protein, translocation to the nucleus and

binding to gamma interferon activation site (GAS) elements in the promoters of IFN-γ responsive genes [36]. In the case of CIITA, the STAT-1α homodimer binds to the GAS element in promoter PIV. This element is present in the proximal region of the CIITA-PIV promoter, spanning 154 bp and containing three *cis*-acting elements essential for its IFN-γ inducibility: a GAS element and adjacent E-box, and a proximal interferon-stimulated response element (ISRE). The binding of STAT-1α to the GAS is stabilized by and dependent on cooperative interaction with upstream stimulatory factor 1 (USF-1), a ubiquitously expressed transcription factor that binds to the E-box element adjacent to the GAS box. In addition, binding of the IFN-γ-induced transcription factor interferon response factor 1 (IRF-1) to the proximal ISRE is essential for activation of CIITA-PIV [90].

Regulation of MHC-II on microglia and astrocytes

It has been shown that in the murine microglial cell line EOC20 and in the murine macrophage cell line RAW 264.7, IFN-γ induction of CIITA-PIV is dependent on the three elements discussed above. Figure 1 shows the transcriptional regulation of IFN-γ-induced CIITA-PIV and MHC-II expression in microglia (A) and astrocytes (B). In both cell types binding of STAT-1α to the GAS element is induced by IFN-γ stimulation and depends on the constitutive binding of USF-1 to the E-box [93,94]. In EOC20 and RAW264.7 cells, IRF-1 binding to the ISRE is constitutively present at low levels and

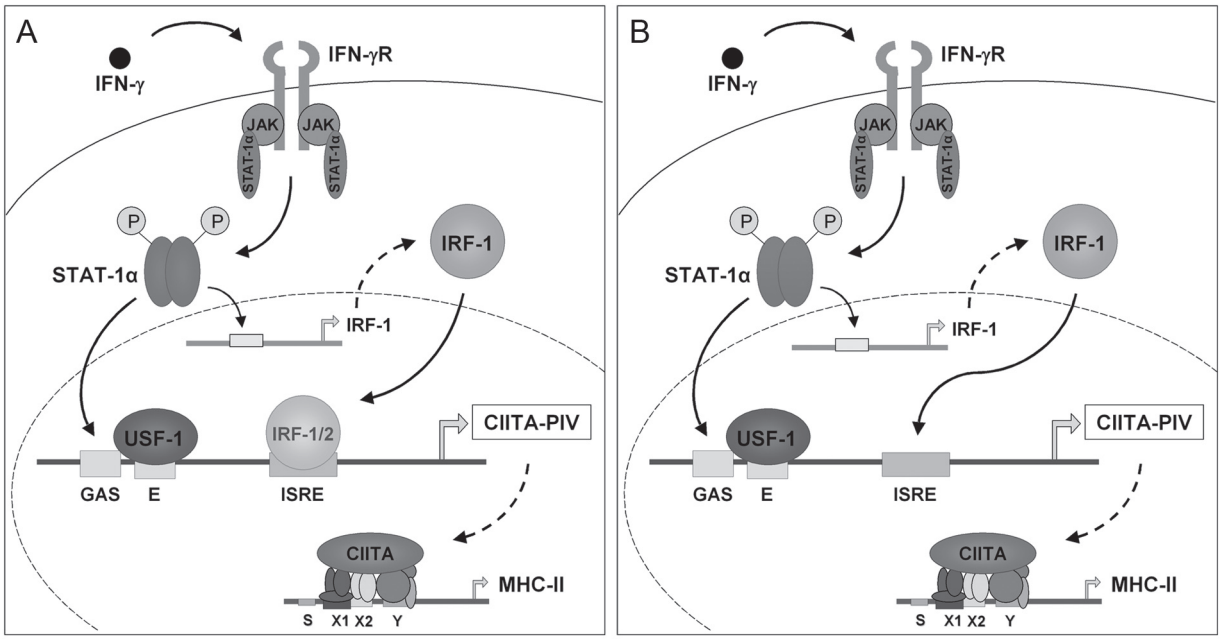

Figure 1. Transcriptional regulation of IFN-γ-induced CIITA-PIV expression in (A) microglia and (B) astrocytes. **A** In microglia, constitutive binding of both IRF-1 and IRF-2 to the ISRE of CIITA-PIV has been observed. Upon IFN-γ treatment, IRF-1 expression and its binding to the CIITA-PIV ISRE is enhanced, leading to enhanced expression of CIITA-PIV. **B** In astrocytes, no constitutive binding of IRF factors to the CIITA-PIV ISRE has been reported. However, binding of IRF-1, and subsequent CIITA-PIV expression, is induced in these cells by IFN-γ.

is markedly enhanced upon IFN-γ stimulation. Also low levels of constitutive IRF-2 binding are observed in EOC20 and RAW264.7 cells. However, this binding is not increased after IFN-γ treatment [94].

In primary rat astrocytes, the ISRE of CIITA-PIV is not constitutively occupied, whereas upon IFN-γ activation binding of IRF-1 is induced. This occupation has been shown to be essential for the IFN-γ induction of CIITA-PIV in astrocytes, whereas the GAS and E-box elements contribute to the maximal response of CIITA-PIV to IFN-γ stimulation [94]. This is in contrast to another report stating that occupation of the GAS is not required for CIITA-PIV activity in primary rat astrocytes whereas in murine macrophage cells CIITA-PIV activity is partially dependent on GAS occupation [95]. Taken together, it seems that whereas in microglia the activation of CIITA-PIV relies on both the GAS and ISRE elements, in astrocytes the ISRE has a more prominent role. This difference in importance of the ISRE could be due to the constitutive binding of IRF-2 to the ISRE that is present in microglia and not in astrocytes.

Whether the CIITA-PIII promoter (by activation through the upstream IFN-γ regulatory region) is also utilized in microglia and astrocytes after exposure to IFN-γ is still not completely clear. Nikcevich et al. have shown that in primary rat astrocytes CIITA-PIV is activated by IFN-γ and CIITA-PIII not, whereas in murine macrophage cell line RAW 264.7 both IFN-γ-inducible CIITA promoters are utilized, albeit CIITA-PIII to a lesser extent than CIITA-PIV [95]. The low level of CIITA-PIII induction could be related to structural differences in the CIITA multi-promoter region, including the upstream IFN-γ regulatory region, between the different rodent species and man.

Negative regulators of glial immune function

Neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) inhibit IFN-γ-induced microglial expression of MHC-II, CD86 and CD40 and might so prevent effective antigen presentation by microglia [6,96,97]. In addition, norepinephrine and neuropeptides, such as α-melanocyte stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), inhibit the production of pro-inflammatory cytokines, chemokines and nitric oxide and the expression of MHC-II and CD40 by activated microglia and MHC-II on IFN-γ stimulated astrocytes [31,98-103]. VIP and PACAP inhibit IFN-γ signal transduction cascades, specifically STAT-1α phosphorylation and subsequent binding to GAS elements [101] and the activation of NF-κB by inhibiting IκB-kinase, upstream of this activation [99,102]. Recently, the membrane-bound glycoprotein CD200 (OX-2) has received much attention as a possible inhibitor of microglial activation. This molecule, which regulates macrophage function in various tissues, is expressed at high levels at the cell surface in neurons. Its receptor, CD200R, is restricted to cells of the myeloid lineage, including DC, macrophages and microglia. CD200-CD200R interactions lead to inhibition of the activated state of microglia, providing a mechanism for neuronal control of microglia function [104,105].

Astrocytes have also been shown to inhibit microglia functions. For example, astrocytes can downregulate microglial secretion of cytokines [106,107]. This could possibly be due to astrocytic secretion of TGF-β or other anti-inflammatory cytokines such as IL-4 and IL-10, or by direct interaction between astrocytes and microglia via for instance CD200-CD200R, or other (unknown) membrane-bound signal transducing molecules.

Anti-inflammatory cytokines are potent inhibitors of microglia and astrocyte immune functions. IL-4 and TGF-β suppress IFN-γ-induced MHC-II expression on microglia and astrocytes, while IL-10 has been shown to inhibit this expression on microglia only [31,44,108-111]. O'Keefe et al. have investigated the effect of IL-10, IL-4 and IL-13 and the cytokine TGF-β on IFN-γ-induced MHC-II expression in microglia. They show that IL-10, IL-4 and TGF-β inhibit IFN-γ-induced MHC-II expression in EOC20 cells by inhibiting CIITA expression. IL-13 does not affect either CIITA or MHC-II expression in these cells. Interestingly, whereas IL-10 and TGF-β are potent inhibitors of CIITA and MHC-II expression in both murine microglial cell lines and primary murine microglia, in the latter, IL-4 has an enhancing effect on expression of CIITA and MHC-II [44]. However, this is in contrast to other reports in which IL-4 has been found to inhibit MHC-II in primary microglia and astrocytes [108,109]. In addition, IL-10, IL-4 and TGF-β inhibit either GM-CSF or IFN-γ-induced CD40 and CD86 expression in microglia [97,112,113]. This inhibition of IFN-γ-induced CD40 expression mediated by IL-4 or TGF-β is due to impaired gene transcription and destabilization of CD40 messenger RNA, respectively [112,113].

Immunological signaling network in the CNS

It is clear that, in addition to infiltrating immune cells, there is a large role for CNS resident cells in immunity of the brain. Two CNS cell types, microglia and astrocytes, are able to respond to (immune) signals within the brain and interact with cells of the immune system and the CNS. Both microglia and astrocytes are able to act as professional APC, though microglia seem to be more efficient in activating infiltrating T cells in comparison to astrocytes. In addition, both cell types produce an array of cytokines, chemokines and other immune mediators. These substances, in turn, affect the expression of immune receptors and other immune modulatory molecules on the cell surface and the secretion of cytokines by these cells. A complex of positive and negative feedback mechanisms determine the reactivity and response of glial cells to immune mediators. Figure 2 provides a simplified representation of this complex interplay.

The main focus of this introduction has been the effect of immune mediators on microglia and astrocyte immune function. However, the intercellular environment of the brain is filled with a plethora of signaling molecules. In addition to cytokines, chemokines and other immune mediators, constitutive and inducible factors such as neurotransmitters, growth factors, ion concentrations, adenosine, ATP, etc. influence not only homeostatic and reparative functions, but certainly also immunological

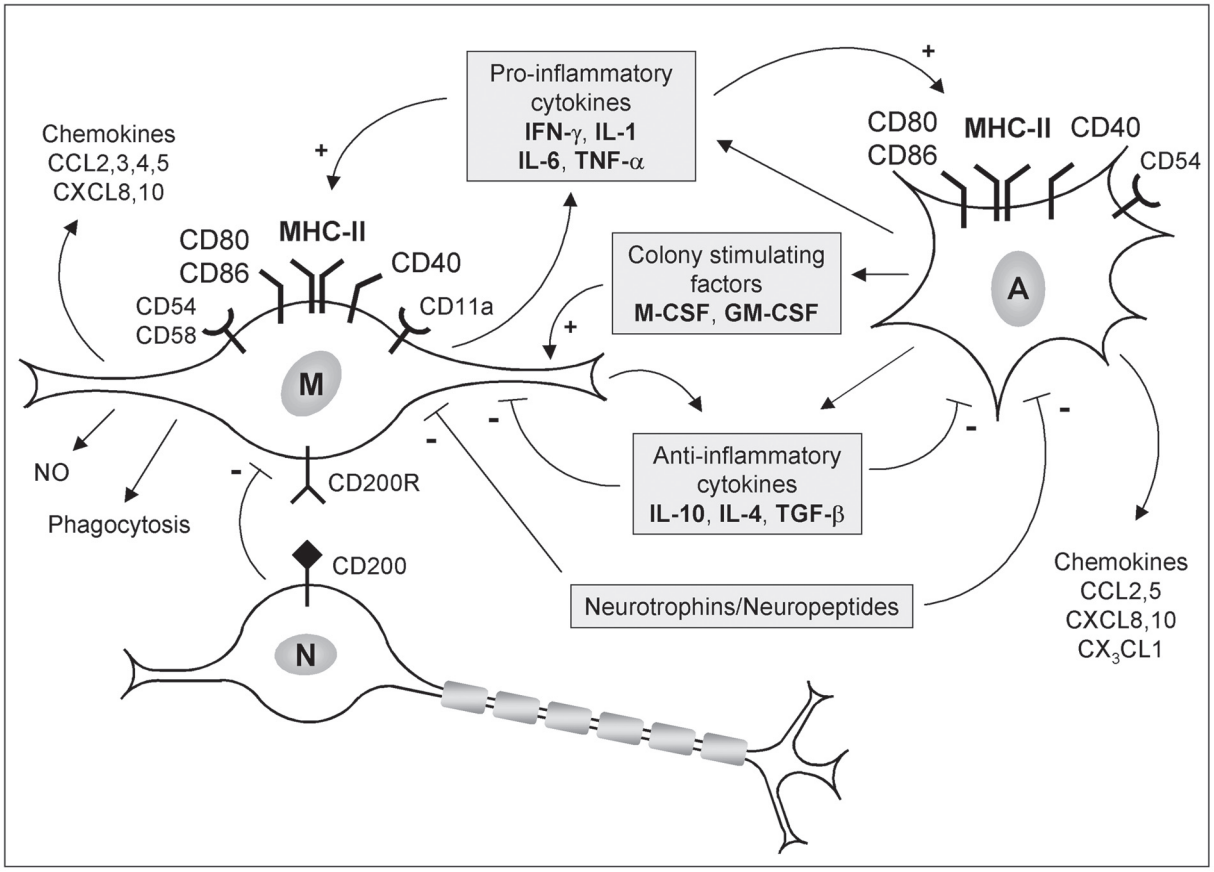

Figure 2. Schematic representation of the immunological interactions between microglia (M), astrocytes (A) and neurons (N). Microglia and astrocytes can affect each others and their own immunological functions by secretion of various factors, such as cytokines and colony stimulating factors. In addition, neurotrophins may inhibit microglia and astrocyte immune functions. Microglial (immune) functions can also be inhibited by direct contact with neurons, through interaction of CD200 with its receptor.

functions of microglia, astrocytes and other CNS resident cells. Many of these interactions have not been unraveled yet, let alone the interaction of brain parenchymal cells with infiltrating peripheral cells. In addition, it should be noted that the reported contradictions in findings and apparent complexity of the issues detailing the immune functions of CNS resident cells and the regulation of immune modulatory molecules in these cells may reflect the variability of activation pathway components amongst the species used in the various studies. Furthermore, it may also reflect differences in isolation methods and culture conditions, which may influence the activation state of CNS-derived cells, and the number and type of molecules expressed. Moreover, former and current research methods are limited in the capacity of model systems to mimic the precise situation in the healthy and diseased human CNS. Therefore the question remains whether and to what extent brain parenchymal cells *in vivo* do indeed play a role in the local immune response and the development of neurological disorders.

Multiple Sclerosis

Multiple sclerosis (MS) is the major cause of neurological disability among young adults in Western countries. This neurodegenerative condition is a chronic demyelinating disease of the central nervous system (CNS), hallmarked by inflammatory lesions throughout the brain and spinal cord. MS is a complex disease due to its heterogeneity in clinical course, neuropathological and radiological appearance, and response to therapy [111,114]. In the majority of MS cases, the disease starts around the age of 30 with a relapsing-remitting (RR-MS) course, characterized by episodes of neurological symptoms followed by full or partial recovery. Over a variable period of time, this disease progresses into a secondary progressive form, with neurological deterioration progressing at a consistent rate. In addition, approximately twenty percent of patients have a primary progressive form of MS, characterized by constant progression of clinical symptoms from the onset of disease [114].

The pathology of MS is characterized by infiltration of inflammatory cells into the CNS, localized destruction of myelin (demyelination), oligodendrocyte death and axonal degeneration [115]. Depending on the location of the lesion in the CNS, loss of myelin and axonal transection result in various neurological impairments. Microglia and macrophages are thought to play an important role in demyelination by producing toxic agents and phagocytosing myelin proteins [115,116]. In addition, astrocytes have also been implicated in the pathogenesis of MS [115]. Furthermore, recent studies have revealed that oligodendrocyte cell death is an important feature of MS lesion formation, possibly triggering the destruction cascade leading to demyelination and axonal damage [117,118]. MS lesions are categorized in four stages [117]: preactive lesions, active (demyelinating) lesions, chronic active demyelinating lesions and chronic inactive lesions. Preactive lesions are distinguished by clusters of activated microglial cells within normal appearing white matter. These microglia display an activated phenotype, characterized by enhanced expression of MHC-II, CD45 and CD68 and an enlarged morphology, to a variable degree resembling macrophages. In this type of lesion no signs of demyelination and only occasionally perivascular infiltrates are present. Active MS lesions, on the other hand, are characterized by demyelinated areas abundant in MHC-II expressing macrophages containing phagocytosed myelin fragments. Reactive astrocytes are distributed evenly throughout these demyelinated regions. Chronic active lesions are composed of a demyelinated hypocellular center containing only a small number of macrophages with some residual myelin components, surrounded by a hypercellular border of MHC-II expressing macrophages of which a variable number contain myelin degradation products. In these lesions reactive astrocytes are predominantly located at the edge of the lesion center and within the hypercellular rim. Lastly, chronic inactive lesions are characterized by hypocellular demyelinated regions with low numbers of inflammatory cells, widespread astrogliosis and enlarged extracellular spaces.

Although over the last decades the disease course of MS has become well-known, enabling early clinical diagnosis, and the pathogenesis of MS is being unraveled more and more, no curative treatment for MS has been developed yet. Considering the inflammatory component of, and the involvement of the peripheral immune system in the disease, the main therapies of MS are based on peripheral immunosuppression and immunomodulation [114,119]. However, these treatments, of which corticosteroids and interferon beta are commonly used, only have a partial effect in reducing relapse rate and accelerating recovery. Moreover, these therapies do not prevent or delay the progression of disability in the progressive phase of the disease. Therefore, extensive research is aimed at trying to find leads for the development of new MS therapies.

Immunomodulation by statins

Statins

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are potent inhibitors of the endogenous mevalonate pathway [120]. Statins, therefore, interfere in the biosynthesis of isoprenoids such as geranylgeranyl-pyrophosphate (GGPP) and farnesylpyrophosphate (FPP), as well as cholesterol. GGPP and FPP are important lipid attachments for the post-translational modification of several proteins, including the small GTP-binding proteins Ras, Rac, and Rho (Figure 3). Attachment of these lipids, so-called isoprenylation, is essential for activation and intracellular transport of proteins crucial for various cellular functions, such as maintenance of cell shape, motility, factor secretion, differentiation, and proliferation [121,122].

Due to their ability to inhibit the synthesis of cholesterol, statins are widely used in medical practice and are the principal therapy for hypercholesterolemia. Statins have been shown to induce a regression in vascular atherosclerosis and a reduction in cardiovascular-related morbidity and mortality in patients with and without coronary artery disease [123-126]. In addition to the effect of statins on atherosclerosis, various findings suggest that statins also exert anti-inflammatory properties and may so play a role in regulating the immune system. In the final part of this introduction, I will discuss the immunomodulatory properties of statins and possible underlying mechanisms of these activities. In this discussion I will also evaluate the effects of statins on immunocompetent cells of the central nervous system, microglia and astrocytes, to emphasize the potential of these agents in the treatment of neuroinflammatory disorders.

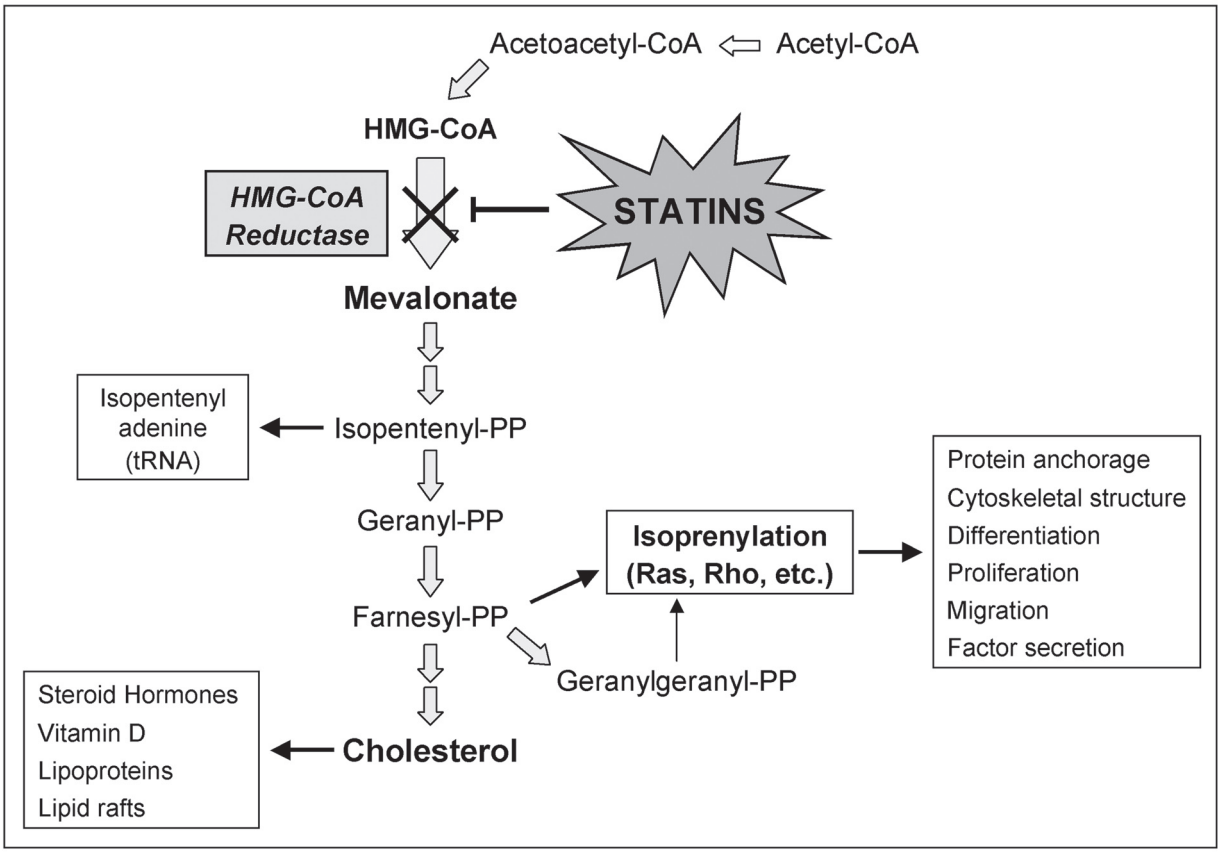

Figure 3. The endogenous mevalonate pathway leading to cholesterol biosynthesis. Statins block the conversion of HMG-CoA to mevalonate by inhibiting the enzyme HMG-CoA reductase. This inhibition leads to decreased production of cholesterol and isoprenoid intermediates, such as farnesyl-PP and geranylgeranyl-PP. The inhibition of endogenous cholesterol synthesis by statins leads to lowered production of sterol products and impaired formation or disruption of lipid rafts. By inhibiting the production of isoprenoids, which serve as lipid attachments for intracellular signaling molecules such as the GTP-binding proteins Ras and Rho, statins interfere with a number of cellular processes, including cell proliferation, differentiation and migration, and factor secretion.
Abbreviations: HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; PP: pyrophosphate.

Statins and immune activation

As discussed before, even though constitutive expression of MHC-II molecules is restricted to professional APC, several cell types start expressing MHC-II molecules upon cytokine stimulation. In the last few years, a number of studies have shown that statins are able to inhibit MHC-II expression on a variety of cell types. It was first shown that statins inhibit IFN-γ-induced expression of MHC-II on endothelial cells, macrophages and microglia [127-131]. Next, statins were shown to not only affect IFN-γ-mediated induction of MHC-II expression, but also to inhibit constitutive MHC-II expression on B lymphocytes and MHC-II expression by activated T lymphocytes [131,132]. Recently, it has become clear that statin treatment also inhibits the enhancement of MHC-II expression during DC maturation [133]. Regarding the effect of statins on MHC-I expression, Kwak et al. have shown that atorvastatin does not affect MHC-I

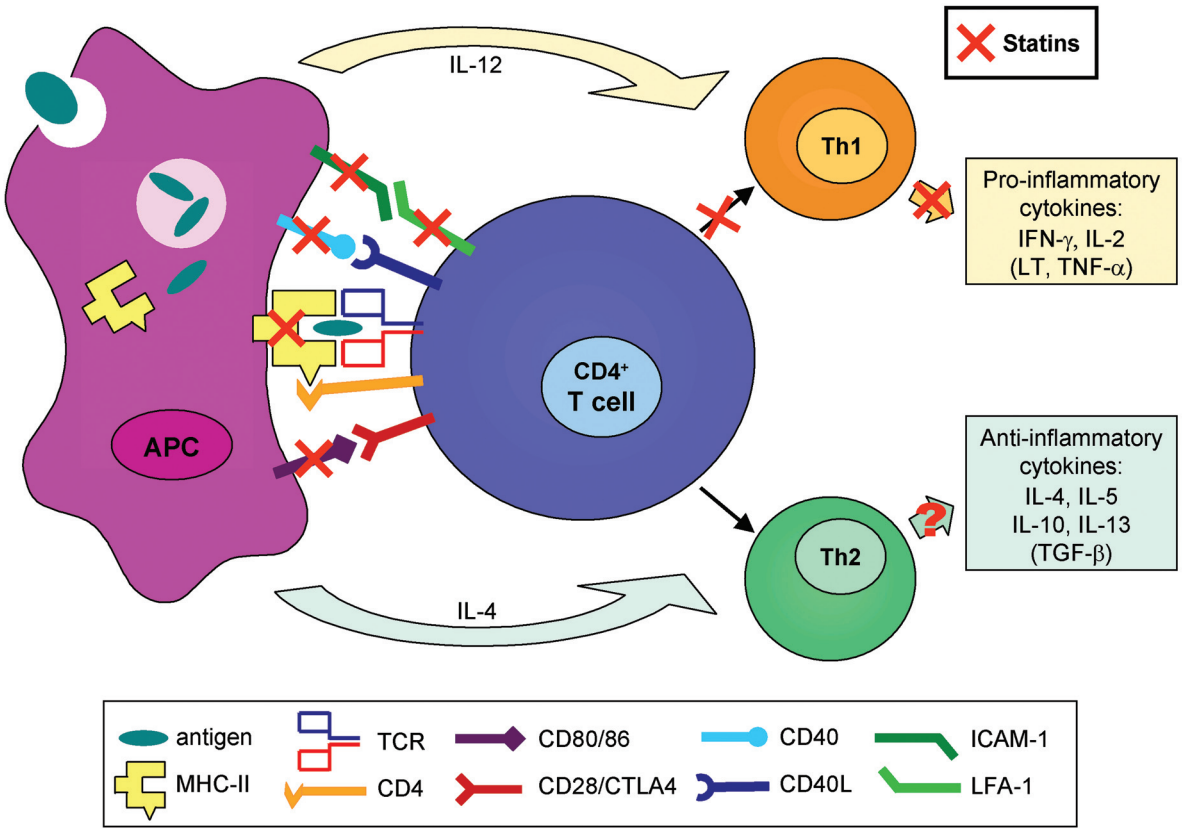

Figure 4. Statins interfere with immune activation. Statins interfere with T cell activation at various levels. First, statins inhibit cell surface expression of MHC-II on APC and so hamper antigen presentation to T cells. Second, statins block cell surface expression of costimulatory molecules on APC and cell surface expression of adhesion molecules on both APC and T cells, thereby disabling efficient T cell activation. Finally, statins inhibit the production of pro- (and anti-) inflammatory cytokines by APC and T cells, which are needed for the initiation of an adequate immune response. Adapted from Stüve et al. [144].

expression on endothelial cells [127]. However, we have observed that, in addition to inhibiting MHC-II expression, simvastatin reduces not only IFN-γ-induced, but also constitutive cell surface expression of MHC-I on various cell types [132].

In addition to reducing expression of both classes of MHC molecules, statins are able to suppress constitutive and IFN-γ-induced expression of the costimulatory molecules CD40, CD80 and CD86 on lymphocytes, macrophages, microglia and endothelial cells, and inhibit the upregulation of these molecules during maturation of DC [130,132-136]. As a result, it has also been shown that statin treatment of DC suppresses their ability to stimulate T cell proliferation [133].

The effect of statins on the production and secretion of cytokines has been studied extensively *in vitro* and *in vivo*. Statins inhibit the production of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1β and IL-6 by mononuclear cells, microglia and astrocytes [135,137-141]. In addition, serum levels of TNF-α, IL-6, IL-1 and IL-8 are lowered in hypercholesterolemic patients treated with statins [142,143]. Moreover, statins also reduce the secretion of other inflammatory mediators, including matrix metalloproteinases (MMPs) and nitric oxide (NO), by monocytes, macrophages, activated T cells, microglia

and astrocytes [131,138,140,141,144] and serum MMP-9 levels in mildly hypercholesteremic men [145]. Figure 4 provides a scheme indicating at which levels statins could intefere in immune activation.

Statins and T/NK cell function

Statins are able to suppress T cell proliferation *in vitro* [130,131,139,147-149], which is illustrated by the fact that treatment of antigen-specific T cells inhibits their production of IFN-γ, TNF-α, IL-2, IL-12 and IL-6 [130,139,147,150]. In a murine model of allergic asthma, a similar statin-induced reduction in *in vitro* production of IL-6 and IFN-γ by antigen-specific T cells is observed. However, this reduction is reported not to be associated with a decrease

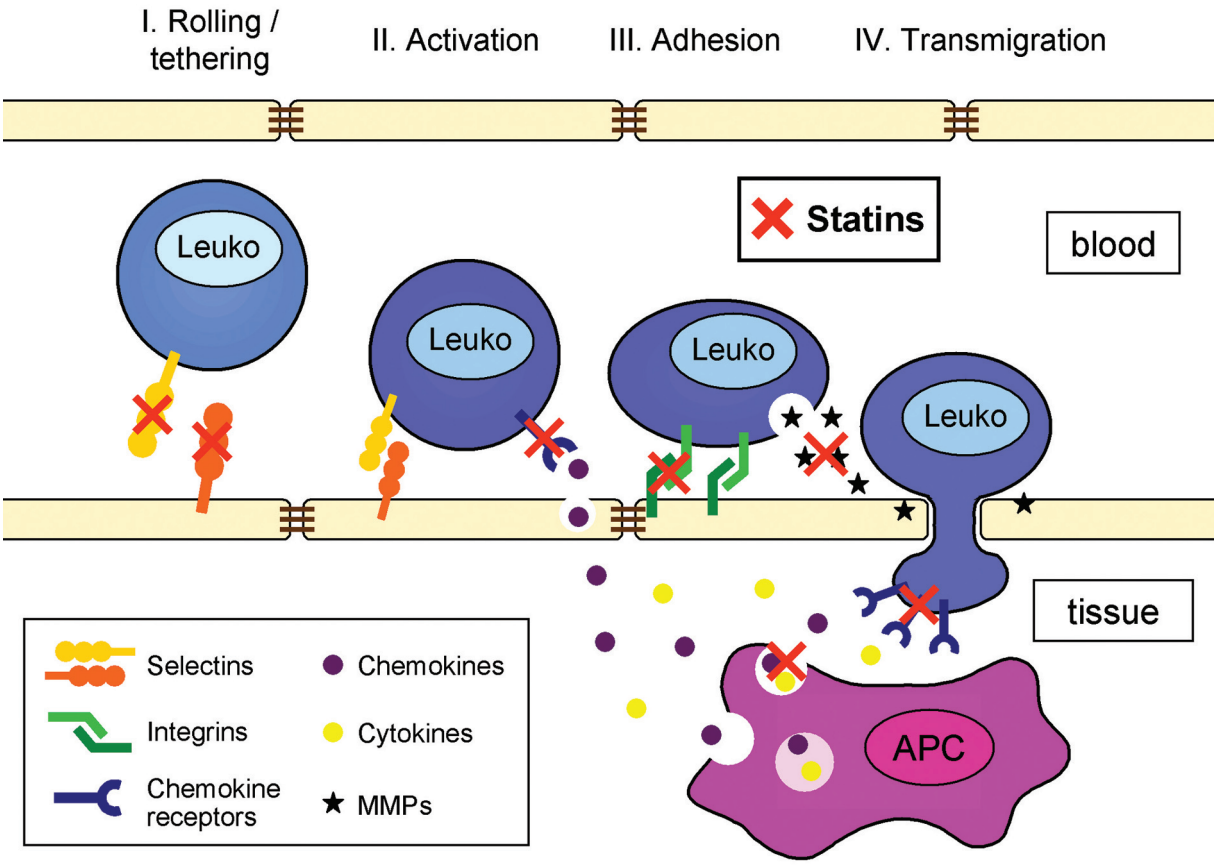

Figure 5. Statins interfere with cell recruitment to inflammatory sites. In the recruitment of blood-borne leukocytes to inflammatory site 4 stages are distinguished: I. Rolling/tethering, slowing down of leukocytes through selectin interactions; II. Leukocyte activation initiated by chemokines and cytokines secreted by APC at the site of inflammation; III. Firm adhesion and spreading of leukocytes to the endothelium through integrin interactions; IV. Transmigration through the endothelium, which is propagated by the chemokine gradient that is present at the site of inflammation, and facilitated by matrix metalloproteinases (MMPs). MMPs, secreted by leukocytes, degrade the endothelial extracellular matrix and open tight junctions between endothelial cells. Statins interfere with these processes at various stages: statins inhibit cell surface expression of selectins and integrins on both leukocytes and endothelial cells, block the secretion of chemokines and cytokines by APC, down regulate cell surface expression of chemokine receptors on leukocytes, and reduce MMP secretion by leukocytes. Adapted from Stüve et al. [144].

in antigen-specific T cell proliferation [151]. In addition to *in vitro* activity, simvastatin reduces *in vivo* serum levels of IL-6 in mice [139]. In humans, simvastatin reduces *in vitro* the proliferation of, and IFN-γ production by PBMC of healthy donors and Rheumatoid Arthritis (RA) patients as well as synovial fluid-derived mononuclear cells from RA patients. In addition, simvastatin decreases TNF-α production by healthy donor and RA patient-derived monocytes/macrophages [139]. With regard to natural killer (NK) cell function, simvastatin treatment of healthy volunteers reduces both *ex vivo* and *in vitro* NK cell cytotoxicity [149]. Moreover, in patients with cardiovascular disease, both T-cell proliferation and NK cell cytotoxicity are reduced by statin treatment [149,152].

Statins are also implicated in inducing a switch from a Th1 response to a Th2 response. This is illustrated by the observation that *in vivo* administration of statins induces the secretion of the Th2 cytokines IL-4, IL-5, IL-10 and TGF-β by antigen-specific T cells [130,147,150]. In addition, *in vitro*, statins are able to promote Th2 differentiation of naïve Th0 cells [130,153]. Also, enhanced serum levels of TGF-β and production of this cytokine by monocytes have been found in hypercholesterolemic patients treated with pravastatin [154]. However, the question remains whether the induced concentrations of these cytokines really reflect a switch from Th1 to Th2, given that the concentration of Th1 cytokines after statin treatment are in the same range or even higher than those of Th2 cytokines [130]. Several findings support the notion that statins might not completely induce a Th1 to Th2 switch. For example, in a murine inflammatory arthritis model, IL-10 production by antigen-specific T cells was unaltered or suppressed by simvastatin treatment, production of IL-4 and IL-5 was not detected and both serum collagen specific IgG1 and IgG2a levels were reduced, suggesting that simvastatin suppresses the Th1 response without enhancing a Th2 response [139]. Moreover, in a murine model of allergic asthma, simvastatin treatment even results in *in vivo* reduction of IL-4 and IL-5 production in the lung [151].

Statins and cell recruitment

Leukocyte–endothelium interaction and accumulation of inflammatory cells is a key element in the induction of inflammation. These processes are mediated by various cell surface receptors. Statins can interfere with adhesion and migration of inflammatory cells by modulating the expression and function of these receptors on both inflammatory and endothelial cells. Several statins have been shown to reduce cell surface expression of adhesion molecules such as ICAM-1, LFA-1, CD11b, CD18 and CD49 *in vitro* and *in vivo* on monocytes and activated T cells [131,155-157]. In addition, statins inhibit cell surface expression of ICAM-1, VCAM-1, LFA-1 and CD18 on activated endothelial cells [156,158-160], although one report shows enhanced expression of these adhesion molecules on endothelial cells following statin treatment [161]. It has also been shown that adhesion of monocytes to endothelial cells is reduced when the

latter cells are treated with simvastatin [156,160]. Furthermore, *in vitro* or *in vivo* treatment with statins reduces human monocyte adhesion to VCAM-1 and endothelial cells, and CD11b-dependent adhesion of unstimulated monocytes or monocytes stimulated with monocyte chemo-attractant protein-1 (MCP-1) to endothelium [155,157,162,163].

Following leukocyte-vascular endothelium interaction, another key element in inflammation is leukocyte trafficking. This process is mediated by chemo-attractant cytokines, also known as chemokines. Several studies have shown that statins inhibit the production of MCP-1 (CCL2) in a variety of cell types and decrease serum levels of this chemokine and IL-8 (CXCL8) in hypercholesteremic patients [138,142,164]. In addition, spontaneous release of MIP-1α (CCL3) and IL-8 by PBMC is decreased in patients with coronary artery disease treated with atorvastatin [165]. Interestingly, while mRNA expression of the chemokine receptors CCR1 and CCR2 (the receptor for MCP-1) was decreased in the above mentioned group of patients, the mRNA expression levels of the chemokine RANTES (regulated upon activation, normal T cell expressed and secreted) and the chemokine receptors CCR4, CCR5, CXCCR1, CXCR2 and CXCR4 was not affected [165]. However, statins do inhibit cell surface expression of CCR5, CXCR3 and to a lesser extent CXCR4 and CXCR5 on human activated T and B cells, CCR2 on monocytic cells, and CCR5 and CXCR3 on microglia [131,136,163], indicating that statins most likely do not interfere with chemokine receptor expression on the level of gene transcription, but rather inhibit cell surface expression of these molecules. Figure 5 shows a model of statin-mediated inhibition of leukocyte adhesion to and migration through blood vessel endothelium. In addition to chemokine/chemokine receptor expression and interactions, functional chemotaxis of leukocytes to inflammatory sites also depends on actin cytoskeleton reorganization and cell polarization. Statins have been shown to inhibit actin polymerization [155,166]. As a result of this, statins interfere with cell migration by inhibiting cytoskeletal rearrangement, in addition to down regulating chemokine receptor expression.

Thesis outline

As discussed above, microglia and astrocytes are thought to play an important role in the development of MS lesions [115,116]. Within normal appearing white matter of MS patients clusters of activated microglia - preactive lesions - might represent a very early stage of MS lesions [117]. In addition, active demyelinating lesions are occupied by large numbers of macrophages, and at later stages astrocytes. Therefore it seems that enhanced motility is a key factor in the formation and development of MS lesions.

It has been shown that production of certain chemokines is enhanced in MS lesions. For example, enhanced expression of CCL3, CCL4 and CCL5 has been found in MS lesions [47,167,168]. Furthermore, levels of CCL5 and CCL3 are elevated in the cerebrospinal fluid of MS patients [169,170]. These molecules are efficient mediators of cell migration, and

therefore enhanced expression of chemokine receptors might underlie the trafficking of immunologically active glial cells to lesion sites.

In earlier studies our group has shown that expression of MHC-II and MHC-I molecules is enhanced in various MS lesion stages [40]. This enhanced expression is the result of enhanced expression of general and MHC-specific transcription factors. Some of these transcription factors are the downstream effector molecules of stress-induced signaling pathways. Therefore, we have raised the hypothesis that MS lesions, including very early stages, are characterized by a general state of glial cell activation. Enhanced expression of MHC molecules could be just one of the phenotypic features of this altered state and other immunologically relevant molecules, such as chemokine receptors, might also be induced during this activation. This would consequently account for the enhanced motility observed in MS brain tissue. Therefore, we investigated chemokine receptor expression and regulation in MS lesions.

Furthermore, given the importance of cell motility in the development of MS, modulation of aberrant chemokine receptor expression could prove to be a possible treatment option for MS. Because statins exert anti-inflammatory and immunomodulatory actions, we further investigated the immunomodulatory capacities of statins and the effect of statins on the alleged villain in MS lesion development: microglia.

First, we have first examined the expression of CCR5 in MS brain tissue. In **chapter 2** we have determined the cell types expressing CCR5 and the level of CCR5 expression in various stages of MS lesions and compared this with expression in normal controls. Subsequently, we have investigated the transcriptional regulation of CCR5. To find possible similarities between the regulation of MHC molecules and CCR5, we have investigated whether stress-induced signaling pathways and the subsequent transcription factors are involved in CCR5 transcriptional regulation in **chapter 3**.

Next, we have explored the immunomodulatory capacity of statins. In **chapters 4** and **5** we describe the inhibitory effect of statins on cell surface expression of MHC-II and other immunologically relevant molecules on several cell types, and in **chapter 5** we have elucidated the mechanism involved in this downregulation of cell surface expression. Finally, we have explored the effects of statins on cell functions of immune cells of the CNS: in **chapter 6** we have examined the effect of the statin simvastatin on the expression of immunologically relevant molecules, including MHC-II and CCR5, on microglia. Furthermore, in this chapter we have investigated the effect of simvastatin on the ability of microglia to migrate towards chemokines and we have searched for the underlying mechanism of this effect. Finally, we have investigated the effect of simvastatin on the differentiation of blood-derived monocytes in to dendritic cells and the maturation of these cells in **chapter 7**.

To conclude, I will summarize and discuss our results in **chapter 8**. I will clarify and further elaborate on conclusions stated in the previous chapters and discuss the implications of our findings for the understanding and possible treatment of MS.

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