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Karolinska Institutet, Stockholm, Sweden**

CHEMOKINE RECEPTOR EXPRESSION AND FUNCTION IN EXPERIMENTAL AUTOIMMUNE NEUROINFLAMMATION

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To Anwaar Kordofani & Mustafa Dafalla,
To the soul of my friend Munira Kamblawi,
To my wonderful parents

In the name of Allah, the Most Gracious, Most Merciful

God says:

- It is only those who have knowledge among his slaves that Allah fear-(Fatir, 28)

- The life of this world is only the enjoyment of deception-(Al-Hadîd, 20)

- And Allah likes not any prideful boaster-(Al-Hadid, 23)

ABSTRACT

Neuroinflammatory lesions in the central nervous system (CNS) are characterized by the presence of leukocytes, mainly monocytes/macrophages, derived from the systemic compartment. We believe CC inflammatory chemokine receptors (CkRs), CCR1, CCR2 and CCR5 are prerequisite for controlling the migration of monocytes/macrophages into inflammatory foci. The role of fractalkine (CX3CL1) and its receptor CX3CR1 in the CNS is more unclear, but their expression pattern suggests a role in neuron-microglia interaction.

Chronic rat model for Multiple Sclerosis (MS), myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (MOG-EAE), was employed here to determine the role of these CkRs during neuroinflammation. *In situ* hybridization histochemistry combined with immunohistochemistry (ISH/IHC) demonstrated high-level of CCR1/CCR5, and moderate-level of CCR2 mRNA expression on mononuclear phagocytes ($ED1^+$ $GSA/B4^+$) correlated with active demyelination. Expression of CCR1, CCR2 and CCR5 was substantially reduced during clinical remission. CX3CR1 displayed a low constitutive expression on microglia on the basis of their cellular morphology and positive lectin staining. There was a notably increased density of CX3CR1 mRNA expressing cells within the inflammatory areas, especially during the acute and relapse phase of the perivascular and submeningeal lesions. CCR1 and CX3CR1 were also found to be differentially expressed at the protein level on monocyte/ microglia populations by flow cytometric analysis of leukocytes extracted from the inflamed CNS tissue, confirming non-overlapping expression. CkR´s ligands, MIP-1α (CCL3) and RANTES (CCL5), were found abundantly expressed in neuroinflammatory lesions, but not in the healthy CNS. There was a neuronal expression of CX3CL1 throughout the CNS at all time points examined (constitutive expression), with induced expression on astrocytes within inflammatory lesions.

A low-molecular weight CCR1 selective antagonist was able to potently abrogate both clinical and histopathological signs of the disease during the effector stage of EAE without any signs of peripheral immune compromise. The antagonist also reduced the severity of ongoing disease when given after clinical onset of disease.

In conclusion, the thesis shed light on some of CkRs and provide new information about mechanisms controlling their mRNA expression during neuroinflammation. Because this research carries potential medical implications, detailed knowledge of the roles of specific CkRs and ligands in the CNS in health and in disease and identifying their cellular phenotypes could be of use in the identification of disease mechanisms, and enabling more specific future therapeutic interventions.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Dan Sunnemark, **Sana Eltayeb**, Erik Wallström, Lena Appelsved, Åsa Malmberg, Hans Lassmann, Anders Ericsson-Dahlstrand, Fredrik Piehl, Tomas Olsson. Differential Expression of the Chemokine Receptors CX3CR1 and CCR1 by Microglia and Macrophages in Myelin Oligodendrocyte Glycoprotein Induced Experimental Autoimmune Encephalomyelitis. **Brain Pathol. 2003, 13:617-29.**

II. Dan Sunnemark*, **Sana Eltayeb***, Maria Nilsson, Erik Wallström, Hans Lassmann, Tomas Olsson, Anna-Lena Berg and Anders Ericsson-Dahlstrand. CX3CL1 (fractalkine) and CX3CR1 expression in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis: kinetics and cellular origin**. J Neuroinflammation. 2005, 2:17.**

III. **Sana Eltayeb**, Anna-Lena Berg, Hans Lassmann, Maria Nilsson, Erik Wallström, Tomas Olsson, Anders Ericsson-Dahlstrand and Dan Sunnemark. Temporal expression and cellular origin of CC chemokine receptors CCR1, CCR2 and CCR5 in the central nervous system: insight into mechanisms of MOG-induced EAE**. J Neuroinflammation. 2007, 4:14.**

IV. **Sana Eltayeb***, Dan Sunnemark*, Anna-Lena Berg, Gunnar Nordvall, Åsa Malmberg, Hans Lassmann, Erik Wallström, Tomas Olsson, Anders Ericsson-Dahlstrand. Effector stage CC chemokine receptor-1 selective antagonism reduces multiple sclerosis-like rat disease. **J Neuroimmunol. 2003, 142:75-85.**

*Contributed equally to the work.

OTHER RELATED PAPERS NOT INCLUDED IN THE THESIS

I. Anna Lobell, Robert Weissert, **Sana Eltayeb**, Cecilia Svanholm, Tomas Olsson, Hans Wigzell. Presence of CpG DNA and the local cytokine milieu determine the efficacy of suppressive DNA vaccination in experimental autoimmune encephalomyelitis**. J Immunol. 1999, 163:4754-62.**

II. Robert Weissert, Anna Lobell, Katrien de Graaf, **Sana Eltayeb**, Ronald Andersson, Tomas Olsson, Hans Wigzell. Protective DNA vaccination against organ-specific autoimmunity is highly specific and discriminates between single aminoacid substitutions in the peptide autoantigen. **Proc Natl Acad Sci U S A. 2000, 97:1689-94.**

III. Anna Lobell, Robert Weissert, **Sana Eltayeb**, Katrien de Graaf, Judit Wefer, Maria Storch, Hans Lassmann, Hans Wigzell, Tomas Olsson. Suppressive DNA Vaccination in MOG-peptide-induced Experimental Autoimmune Encephalomyelitis Involves a T1-biased Immune Response. **J Immunol. 2003, 170:1806-13.**

IV. Henrik Hammarberg, Olle Lidman, Cecilia Lundberg, **Sana Eltayeb**, Sander Gielen, Saad Muhallab, Anders Svenningsson, van Der Meide, Tomas Olsson, Fredrik Piehl. Neuroprotection by encephalomyelitis: Rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells**. J Neurosci. 2000, 20:5283-91.**

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
Ag	Antigen
BBB	Blood-brain barrier
Bp	Base Pair
C	Cysteine
CC	β-chemokine
CCR1	Chemokine receptor 1
CCR2	Chemokine receptor 2
CCR5	Chemokine receptor 5
cDNA	Complementary DNA
Ck	Chemokine
CkR	Chemokine receptor
CNS	Central nervous system
cRNA	Complementary RNA
CX3C	δ-chemokines
CX3CL1, FKN	Fractalkine
CX3CR1	Fractalkine receptor
DA	Dark Agouti
EA	Early active (lesion)
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
GPCR	G protein-coupled receptor
IA	Inactive (lesion)
DM	Inactive completely demyelinated lesions
IFA	Incomplete Freund's adjuvant
IHC	Immunohistochemistry
ISH	In situ hybridization
LA	Late active (lesion)
MCP-1	Monocyte chemoattractant protein-1 (CCL2)
MIP-1α	Macrophage inflammatory protein-1 alpha (CCL3)
MIP-1β	Macrophage inflammatory protein-1 beta (CCL4)
mRNA	Messenger ribonucleic acid
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NAWM	Normal appearing white matter
p.i.	Post immunization
PPWM	Periplaque white matter
RANTES	Regulated on Activation, Normal T cells Expressed and Secreted (CCL5)
RM	Remyelinated shadow plaque
7-TM	Seven transmembrane
X	Any amino acid

PREFACE

The challenge of working backwards to identify and predict common factors that may involve in complex humans diseases is immense. This thesis examines the expression pattern of CkRs, to get better insight into their mechanism in the CNS and in lesion evolution. These studies are motivated by similarities of immunological and histopathological features among EAE and MS, where peripheral leukocyte recruitment and activation are critical for disease induction. Therefore, I focused my studies on chronic rat model of the human disease MS; MOG-induced EAE.

Identifying chemokines and receptors associated with this rat model might provide useful additions to the therapeutic armamentarium for MS, and it gives the pharmaceutical industry the clue about why, when, where, which chemokine/receptor to inhibit, since they are potential targets for therapeutic intervention.

Enjoy your reading!

1 INTRODUCTION

Neuroimmunology research encompasses investigations on functional interactions between the nervous system and immune system. Although the CNS has been characterized as an immunologically privileged site in the past, it should more accurately be viewed as immunologically specialized, since both local innate and systemic immunity do occur in the brain.. Neuroinflammation and demyelination often has severe consequences on the afflicted individual, resulting in impaired nerve conduction and paralysis. During the past decade, crucial components that are involved in the process of leukocyte migration have been identified and progress has been made in understanding the mechanisms of neuroinflammatory reactions. Considerable research effort appears to confirm that Cks have largely to do with inflammation and host defence, and that leukocyte recruitment and traffic is their main task Understanding the molecular networks that regulate these processes is of key importance in the development of appropriate therapy in neuroinflammatory disorders including MS.

1.1 The interplay between the CNS and the immune system

Leukocyte entry into the CNS is restricted, in part, because of the blood–brain barrier (BBB), as such, the CNS has been regarded as an immune privileged organ with the barrier that restricts and modifies inflammatory cell recruitment [1]. Recently, much progress has been made with respect to the control of leukocyte extravasation [2-6], and components that are involved in the process of leukocyte migration in neuroinflammatory reactions have been identified [5]. In recent years, some experiments have indicated that the CNS itself may have an innate immune system comprised of astrocytes and microglia capable of regulating the initiation and progression of immune responses. Thus, immunological privilege should be considered as an intrinsic property of the CNS that could involve direct CNS immune cell interactions [7]. Moreover, Lassmann et al., have suggested that microglial cells may play an important role in antigen (Ag) recognition at the BBB [8].
Recently, three distinct routes were found for leukocytes to enter the CNS; i.e., from the blood to cerebrospinal fluid (CSF) across the choroid plexus; from the blood to the subarachnoid space through meningeal vessels; and from the blood to parenchymal perivascular spaces [5]. Attachment of leukocytes to endothelium that enables leukocytes to roll along the blood vessel wall is the early step of leukocyte extravasation (see Fig.1). However, the maintenance of leukocyte–endothelial interactions is further needed to complete transendothelial migration and is believed to be triggered mainly by Cks [9], which govern leukocyte–endothelial migration in a gradient-dependent fashion by regulating integrin adhesiveness. Thus, such chemoattractant gradients specify the leukocyte migration into the CNS [10, 11].

1.2 Trafficking determinants for leukocytes

Leukocyte extravasation is associated with selectins and their ligands, integrins and cell-adhesion molecules (CAMs), Cks and CkRs (Fig.1). The initial contact between

leukocyte and endothelial cell is referred to as **tethering**, subsequent interactions are referred to as **rolling**. Both steps are associated with selectins and their carbohydrate ligands. These molecules distributed both on leukocyte microvilli; where they mediate both tethering and rolling, and on the cell soma; where only rolling occurs [12-14].
Later stages of extravasation, require the engagement of a G-protein-coupled receptor (GPCR) expressed by the leukocyte with an appropriate ligand [4]. This thesis focuses on the Cks and their receptors, which mediate the activation of integrins [15]. The final stage of extravasation is **diapedesis,** that depends on a second set of signals through Cks and their receptors [16-18]**.**

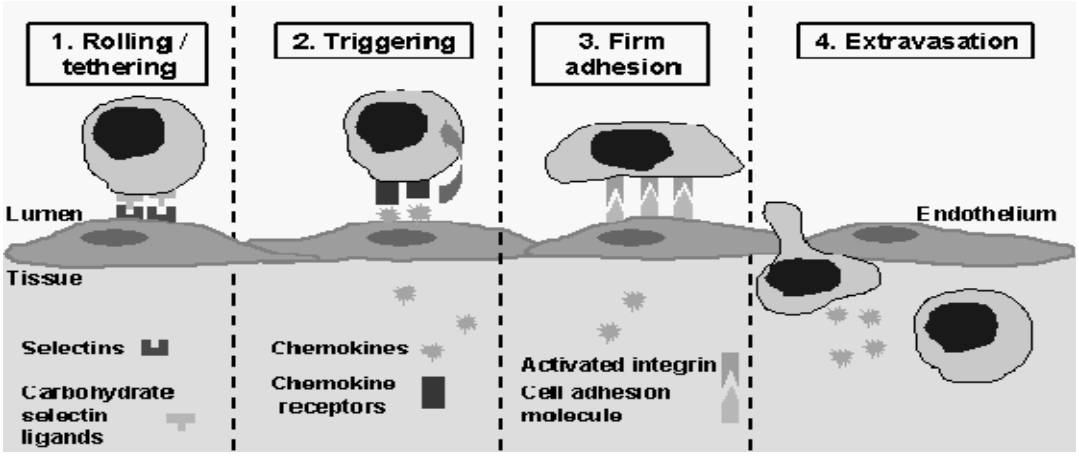

Figure 1: The four events of leukocytes transendothelial migration from blood stream to site of inflammation

1.3 Chemokines (Cks)

Cks are a family of structurally-related and secreted glycoproteins with potent leukocyte activation and/or chemotactic activity [19, 20]. They are 70 to 90 amino acids (aa) in length and approximately 8 to 10 kDa in molecular weight. Most of them fit into two subfamilies with four cysteine residues. These subfamilies are base on whether the two amino terminal cysteine residues are immediately adjacent or separated by one aa.
The **CXC** subfamily, also known as (**α**-Cks) contain a single aa between the first and second cysteine residues (see Fig. 2). **CC** subfamily (or **β**-Cks) have adjacent cysteine residues. Most **CXC** Cks are chemoattractants for neutrophils, whereas CC Cks generally attract monocytes, lymphocytes, basophils, and eosinophils.
There are also two other small sub-groups [21, 22]. **C** subfamily (or **γ**-Cks) has one member lymphotactin. It lacks one of the cysteines in the four-cysteine motif. The **C** subfamily seems to be lymphocyte specific, and attract T cell precursors to the thymus.

The fourth subfamily is the **C-X3-C** (or **δ**-Cks), has three aa residues between first two cysteine (Fig. 2). It is both secreted and tethered to the surface of the cell that expresses it, thereby serving as both chemoattractant and as an adhesion molecule. The only CX3C Ck discovered to date is called CX3CL1 (neurotactin or FKN) [23]. FKN

receptor is known as CX3CR1 [24, 25]. FKN directs migration of monocytes, T cells and NK cells *in vitro* [26]. The known Ck system in humans comprises approximately 50 ligands and 19 couple receptors [27, 28]. They signal through seven transmembrane (7-TM) domain GPCRs [29].

1.3.1 Chemokine function

The major role of Cks is to guide the migration of cells. Cells that are attracted by them follow a signal of increasing Ck concentration towards the source of the Ck. Some Cks control cells of the immune system during processes of immune surveillance, such as directing lymphocytes to the lymph nodes, known as **homeostatic** Cks that are produced and secreted without any need to stimulate their source cell(s). Some Cks have roles in the development and promote **angiogenesis** or guide cells to tissues that provide specific signals that are critical for cellular maturation. Other Cks are **inflammatory,** that are released from a wide variety of cells in response to infection, viruses and agents that cause physical damage. **Inflammatory** Cks function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or tissue damage. Certain inflammatory Cks can also activate cells to initiate an immune response or can promote wound healing. They can be released by many different cell types and serve to guide cells of both innate immune system and adaptive immune system [10, 30-32].

Characteristic	**Definition**
Pleiotrophy	A single Ck acts on several different types of target cells and stimulates different responses
Redundancy	Two or more different Cks induce a similar response
Synergism	The combined effect of two Cks is greater than might be expected for only one Ck
Antagonism	A drug or other molecule that blocks receptors; antagonists inhibit the effects of agonists.

Table 1. Functional characteristic of chemokines

1.3.2 Chemokine receptors and their interactions with chemokines

Cks can not exert their functions unless they interact directly with cell surface CkRs that belong to GPCRs [29]. CkRs are members of a superfamily of 7-TM loops with an extracellular amino terminal (NH2) segment (3 extracellular loops) and cytoplasmic carboxylic terminus (3 intracellular loops) [33]. The N-terminal portion of CkRs is key to determining ligand binding specificity (see Fig. 2). They transduce their signals through heterotrimeric G proteins (Gαβγ) leading to multiple biologic functions. They produce their effect through a cascade of intracellular events that begin with binding of Cks to their respective receptors, leading ultimately to activation and/or directed migration of the cells. CkRs are structurally related and can be categorized into specific, shared, promiscuous, and are expressed in immune cells which are their major

target [34, 35]. Because of this feature, they may be potent mediators of inflammatory processes. Many other cell types express CkRs, these include endothelia, smooth muscle cells, stromal cells, neurons and epithelial cells [36]. Targeting CkRs offer a novel approach with the rationale of preventing the recruitment of undesirable leukocytes to site of inflammation [37]. There are 19 known CkRs, whose functional ligands are identified CXCR1-CXCR5, CCR1-CCR11, XCR1 and CX3CR1 [35, 38, 39].

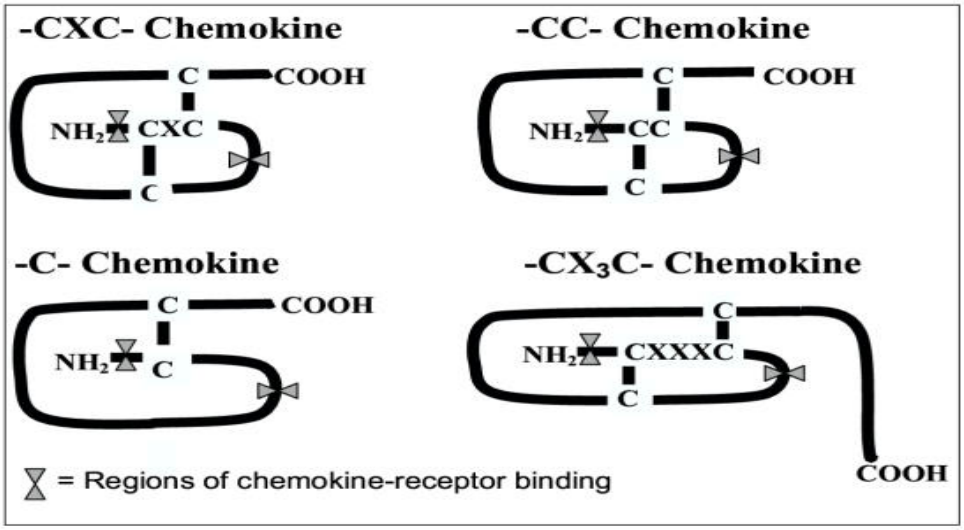

Figure 2: The four subfamilies of chemokines, CXC, CC, C, and CX3C, with their region binding to chemokine receptor (adopted from © 2003 Townson and Liptak).

1.3.3 G-proteins signalling

When GPCRs are activated by agonists, heterotrimeric **G** proteins (Gαβγ) undergo their own activation steps. Usually, in its resting state, **Gα** is the GDP-bound form and associates tightly with **Gβγ**. Upon activation of the receptor, **Gα** becomes GTP bound via intrinsic GDP/GTP exchange activity and dissociates from **Gβγ**. Activated **Gα** or free **Gβγ** subsequently activates downstream targets [40]. Major effectors for **Gα** are adenylyl cyclase and phospholipase β depending on Gα coupling to receptors, whereas Gβγ is believed to play a major role in activating an array of downstream targets [41].

1.3.4 Receptor modulation post-chemokine binding

When CkR-expressing cells are repeatedly stimulated with a Ck, signaling becomes attenuated by a process known as receptor desensitizations or **sequestrations** [42]. Mechanisms for such receptor downregulation after ligands binding to GPCRs have been established [43]. Upon ligand binding, the C-terminal tails of GPCRs are rapidly phosphorylated at serine or threonine residues. Phosphorylation directly alters receptor conformation in such a way that the interaction with heterotrimeric G protein is impaired. Turnication or mutation of these serine or threonine residues of the C-terminal tails of CkRs results in impairment of receptor internalization [44].

1.4 Experimental models of neuroinflammation

Experimental studies to find disease mechanisms and etiological factors in human diseases such as MS, would be virtually impossible if no animal models were used. Furthermore, the CNS is relatively inaccessible for sampling in humans. EAE models offer clues in elucidating the etiopathogenesis of MS. Myelin antigens such as myelin basic protein (MBP) [45], proteolipid protein (PLP) [46] and myelin oligodendrocyte glycoprotein (MOG) [47] are most commonly used Ags for induction of EAE in rodents. EAE is a disease with proven autoimmune pathogenesis [48, 49]. The origin of EAE was discovered when humans received a vaccine against rabies virus that contained CNS homogenate. Genetic underlies the second line of evidence. The major histocompatibilty complex (MHC), in particular MHC class II genes [50, 51] is so far, the only gene region associated with disease susceptibility. The use and effect of immunomodulatory therapies strengthens the concept of autoimmunity.

1.4.1 MOG induced EAE

MOG comprises only 0.05 % of the myelin sheath and is exposed on the outer surface of myelin sheath [52-54] making it accessible to autoantibodies [55]. Dark agouti (DA) is a susceptible rat strain, displaying a relapsing-remitting clinical course as well as histopathological MS-like features when immunized with MOG in Incomplete Freund´s adjuvant (IFA) [56]. The disease is characterized by early (day 12) acute paralysis, followed by a sustained chronic clinical course, extensive inflammation and demyelination coincided with clinical signs of the disease [57, 58]. Some studies are suggesting that B cells and antibodies (Abs) are necessary for the development of EAE [59-62], as well as studies implying the opposite [63, 64].

1.4.2 Leukocyte trafficking in EAE

Leukocyte migration into and through tissues is fundamental to normal physiology, immunopathology and host defence. Accumulation of leukocytes in tissues contributes to a wide variety of diseases [65]. Some immune reactions are suspected of being deleterious, such as the inflammatory demyelinating disease MS [66]. Conversely, in response to tumors of astroglia, the immune reaction in the brain seems inadequate to protect the host [67]. In EAE, circulating leukocytes penetrate the BBB and damage myelin, resulting in impaired nerve conduction and paralysis [68].

After following leukocyte traffic into the CNS of rats with EAE [69] a limited repertoire of T cells named the primary influx found to interact with their target Ag leading to the activation of the BBB to express various adhesion molecules and to increase its permeability to circulating leukocytes [69, 70]. Enhanced permeability of the barrier further allowed a nonselective influx of leukocytes that named as the secondary influx. The later influx was found to correlate with disease onset [69, 71] defining the process as a sequential multistep event. Subsequently, Ag-specific autoimmune T cells either become anergic or undergo programmed cell death (apoptosis), leading to a remission in disease severity [72]. Inhibition of the secondary influx, by either soluble peptide therapy or anti-adhesion molecule blockade, effectively prevented, or even reversed, an ongoing disease, even though, the primary influx remained apparent at the site of

inflammation [69-71, 73]. The aforementioned study emphasized the important role of the nonselective leukocyte influx to site of inflammation. However, immune cell migration is also critically important for the delivery of protective immune responses to tissues. Thus, the challenge for the future will be to identify the trafficking molecules that will most specifically inhibit the key subsets of cells that drive disease processes without affecting the migration and function of leukocytes required for protective immunity [65, 74].

1.5 Resident CNS cells with immunological functions

1.5.1 Nerve cells

Nerve cells or **neurons,** are specialized to carry "*messages*" through electrochemical process and constitute the signaling units of the CNS. They come in many different shapes and sizes. The human brain has approximately 100 billion neurons [75]. Morphology of the nerve cell include its **cell body**, specialized extensions called **dendrites** and **axons**. Dendrites bring information to the cell body and axons take information away from the cell body. Neurons also contain some specialized structures known as (synapses) and chemicals (neurotransmitters). They communicate with each other through electrochemical process, and communicating information is done by two methods. *Electric signals* process and conduct information within the cell, *chemical signals* transmit information between cells. Neurons are fragile and fastidious cells unable to withstand long-lasting exposure to toxic molecules. Under normal and under pathological conditions, neuronal well being and functioning are highly dependent on glial cells that sustain the abundance of neuron-supporting function [76].

1.5.2 Glia

Comparing to neurons, there are about 10 to 50 times more glial cells in the brain. Glia cells do not carry nerve impulses (action potentials), but in fact, without glia, the neurons would not work properly. Glial cells are classified into **macroglia** and **microglia**. Macroglia includes astrocytes, oligodendrocytes, and ependymal cells.

1.5.3 Astrocytes

Star-shaped glial cells of neuroectodermal lineage that provide physical and nutritional support for neurons, they: 1) clean up brain "debris"; 2) transport nutrients to neurons; 3) hold neurons in place; 4) digest parts of dead neurons; 5) regulate content of extracellular space, and perhaps performing immune function in the adult CNS [77]. 40-50% of glial cells are astrocytes. Under pathological conditions, astrocytes respond to changes in the CNS tissue and play a major part in reactive gliosis and the formation of scar tissue [78]. The phenotype exhibited by reactive astrocytes is characterized by hypertrophy and proliferation, as well as elevated expression of intermediate filaments glial fibrillary acidic protein (GFAP) [79]. GFAP was originally purified from MS plaques and has become one of the indicators of reactive astrogliosis [80].

1.5.4 Microglia

Microglia are a hybrid between white cells and glial cells and account for approximately 10% of all glial cells in the adult CNS [81]. The fascinating point about them is that they are supportive and immunocompetent defense cells, and digest parts of dead neurons [82]. Microglia are most likely to function as antigen- presenting cells (APCs) [83-85] but the immune competence of microglia within the CNS is limited compared with APCs within other organs [86, 87]. When the BBB is disrupted during autoimmune disease, cells from the blood gain access to the brain parenchyma and to brain Ags, but microglia reside in the CNS parenchyma does not usually encounter T cells. This is perhaps not surprising, since the CNS also harbors other macrophages that are not part of parenchyma, populate the critical interface between the CNS parenchyma and the blood known as perivascular cells [88].

1.5.5 Perivascular macrophages

The CNS contains various macrophage subsets, including microglia, perivascular macrophages, meningeal macrophages, choroid plexus macrophages and epiplexus cell [5, 89, 90]. The so-called "other CNS macrophages" or perivascular macrophages [91] are derived from the bone marrow and situated in the vicinity of the blood vessel of the CNS, separated from the CNS parenchyma by the perivascular basement membrane. They represent a subset that is phenotypically distinct (CD11b/c+ and $CD45^{hi}$) from parenchymal microglia which are (CD11b/c+ and $CD45^{low}$), and they have immunological properties of peripheral accessory cells. Because of their location, these cells are likely to come into contact with CNS Ag and present it to T lymphocytes. Perivascular macrophages express constitutively MHC class II and have been proposed as to be among the first cells to be involved in immune reactions in the CNS. They also express the marker ED2, in contrast to the ED2-negative parenchymal microglia. The functions of perivascular cells have been reviewed by [92-95]. Meningeal macrophages are thought to be rapidly replaced by cells of bone-marrow origin, whereas the replacement of perivascular and choroid-plexus macrophages is slower, indicating that replacement of the individual populations might occur through different mechanisms. Microglial cells can proliferate *in situ*, and this might be one of the main sources of microglia in adults. Bone marrow-derived cells can enter the CNS across the BBB and populate the microglial-cell compartment. Monocytes are assumed to be capable of entering the CNS and differentiating into microglia [90].

1.6 How to antagonize the chemokine system?

There are several levels to prevent inflammation by interfering with the Ck system. The expression of either ligand or receptor could be prevented by inhibiting their transcription. Another possibility to block the action of ligand or receptor is by using neutralizing mAbs, or to prevent Ck binding to receptor and subsequent activation is by using CkR antagonists that may take the form of small molecule antagonists or modified Cks. Lastly, it could be by inhibiting the signal transduction pathways that are triggered by activation of receptor by its ligand [96], [37].

1.6.1 Chemokine receptor antagonist

7-TM receptors are favorite target for the pharmaceutical industry since numerous drugs currently in use as therapeutics act on this class of receptors.
The approach to identify an antagonist is to screen the compound library with a high throughput ^{125}I-MIP-1α binding assay [97].
Molecular basis of Ck/receptor interaction as a scope for a design of antagonist, suggested two-step mechanisms [98]. The main body of the Ck interacts with the outside of the receptor (**called site 1**), this interaction directs **receptor selectivity**; subsequently, the flexible amino-terminus of the Ck interacts with the receptor core (**called site 2**) to initiate **signaling response** [98].

Cks have an *in vivo* requirement to bind to glycosaminoglycans (GAGs) in order to mediate directional cell migration. Preventing of GAG interaction has been shown to be a viable therapeutic strategy. Targeting Ck intracellular signaling pathways offers an alternative small-molecule approach. One of the key signaling targets downstream of a variety of CkRs is phosphoinositide 3-kinase gamma (PI3Kgamma), a member of the class I PI3K family. Thus the Ck system offers many potential entry points for innovative anti-inflammatory therapies for autoimmune diseases such as MS [99]. Recently, a number of pharmaceutical companies have disclosed significant efforts in discovering CkR antagonists to combat inflammatory diseases models [89, 100-104]. Recent data from Berlex group has appeared in the literature about CCR1 antagonists [97]. The compound per se, was found to be effective in reducing the severity of attack and delaying onset of disease in Lewis rat, the monophasic model for MS [105] Moreover, much work has focused on the development of CCR5 inhibitors as anti-HIV agents from Takeda laboratory [106, 107].
The Ck system appears problematic as a therapeutic target, in view of its apparent redundancy and overlapping receptor-ligand profiles. But despite the large number of CkRs, it is possible to find molecules that are selective for one CkR compared with another, whether such selectivity is needed *in vivo* is still not clear [37].

1.7 Multiple Sclerosis (MS)

MS is a chronic inflammatory demyelinating disease of the human CNS, and is the most common cause of neurological disability in young adults in the western world [108, 109]. The etiology of MS is still unknown, but it is generally accepted that it involves CNS-directed autoimmune responses. Whether these responses are primarily raised against "self" CNS components or whether they are induced by cross-reactivity between CNS components and infectious agents (molecular mimicry) is unclear [110-113]. Genetic susceptibility also show a major genetic component in susceptibility to MS [114, 115].
The disease is mediated by extensive infiltration of monocytes and T lymphocytes into the CNS followed by resident macrophage and microglia activation (Fig. 3). This results in extensive inflammation and subsequent demyelination of white matter. The disease usually manifesting its self as discrete with recurrent attacks. These attacks are followed by remission (relapsing-remitting RRMS). Later, however, the relapsing-remitting course often blends into a slow, but permanent progression (secondary progressive SPMS) [116, 117]. Lucchinetti et al., 1998 have speculated that MS might

be a neurological syndrome with different immunopathological mechanisms triggering a common pathway, rather than a single disease with a uniform mechanism of myelin destruction.
Many immune abnormalities have been described in MS, which indicate that the immune system plays a central role in its pathogenesis [118-120]. The current consensus is that MS pathogenesis comprises an initial inflammatory phase, which fulfils the criteria for an autoimmune disease [121], followed by a phase of selective demyelination and at last a neurodegenerative phase [118, 122]. The crucial role of the immune system in disease pathogenesis has important therapeutic implications [123-125]. Cks participate in the development of MS by guiding immune cells into the brain, and several studies have shown that Ck and their receptors are involved in the pathogenesis of MS [126-130].

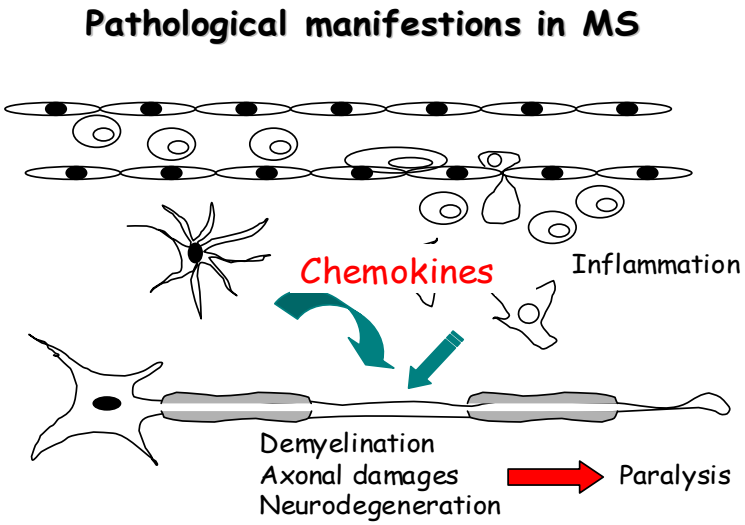

Figure 3: This figure illustrates hypthetical points about the pathogenesis of MS and EAE, where circulating leukocytes (monocyte/macrophages) penetrate the BBB and damage myelin, resulting in impaired nerve conduction and paralysis

1.7.1 Macrophage and microglia in MS

A key issue in understanding the pathogenesis of MS is the reliable identification of phagocytes capable of degrading myelin and presenting autoantigen to T cells at the onset of demyelination. Most MS research has been focused on T cells, although evidence is accumulating for macrophages as important effector cells involved in the demyelination [131-133]. Highest numbers of macrophages and intense infiltration was seen in early and late active lesions in acute MS cases, while lower numbers were encountered in inactive, demyelinated, or remyelinated lesions. These findings indicate a differentiated pattern of macrophage activation in MS depending on the stage of the demyelinating activity [131]. Evidence for activation of microglia in the immediate vicinity of the lesion has also been obtained [134-136]. Some results indicate that microglia are the main population of phagocytes in the early stages of demyelination

and may play an important role in the pathogenesis of MS [137]. Loss of myelin appears to involve the active stripping of myelin lamellae by macrophages and/or microglia [138-140]. In accordance with this, elimination of macrophages or microglia have been shown to suppress clinical and histopathological manifestations in rodent models for MS [141, 142]. Thus, the central role of macrophages/microglia as active mediators of demyelination suggests that pharmacological intervention (infiltration and activation) of these cells may reduce or halt the clinical progression in MS.

1.7.2 Hallmarks of histopathology in MS

MS selectively affects the myelin sheaths that help speed the conduction of nerve signals from point to point, and damage to the myelin is eventually replaced by scar-like tissue, which further interferes with nerve signaling. The BBB loses barrier function during large-scale of leukocyte transmigration and permits exposure of parenchymal CNS cells to serum components. Thus, an MS lesion consists of Ag-specific autoreactive T-cells, secondary recruited inflammatory monocyte/ macrophages, activated microglia and activated cerebrovascular endothelium [109, 120, 143]. Beside the myelin sheaths being primary target of tissue destruction, axons, nerve cells (leaves the nerves cells and axons-at least in part-intact) and astrocytes were also affected. Active disease can usually be shown by gadolinium-enhanced magnatic resonance imaging (MRI) [144]. The pathology underlying MS is the formation of multiple demyelinated plagues. Lesions are widely disseminated in the CNS, with the predilection of myelinated areas; the optic nerve, periventricular white matter, corpus callosum, cerebellum and cervical cord [145]. The demyelination is associated with persistent inflammation of the white matter. Therefore, our knowledge concerning the histopathology of MS is derived mainly from studying white matter plaques. During the disease course new lesions are formed and old lesions persist, therefore, the inflammatory and demyelination activity often varies between the plaques [140]. New lesions typically evolve around small and medium sized vessels.

Four different patterns of lesions have been described [133]. Pattern (I and II) are the most prominent lesions in MS, with close similarities to EAE lesions. **Pattern I;** and **pattern II;** display a T cell mediated inflammation and macrophage-mediated demyelination. The other patterns (III and IV) were highly suggestive of a primary oligodendrocyte dystrophy, reminiscent of virus or toxin-induced demyelination rather than autoimmunity. The patterns of demyelination were heterogeneous between patients, but were homogenous within multiple active lesions from the same patient. This heterogeneity of plaques from different MS patients may have fundamental implications for diagnosis and therapy of this disease [123, 132].

Actively demyelinating lesions are heavily infiltrated by macrophages and activated microglial cells (involved in disintegration, uptake and removal of myelin sheaths), and invading T cells [146]. Macrophages contain myelin degradation products due to recent myelin phagocytosis and show a foamy phenotype [131]. Ramified microglial cells secrete high amounts of cytotoxic mediators, which are directly responsible for demyelination and oligodendroglial and neuroaxonal injury [147]. Astrocytes are large, often multinucleated and show strong immunreactivity for GFAP, and also engage demyelinated axons, thus substituting in part for the lost myelin sheath [148].

The extent of acute axonal damage correlates with the existence of macrophage/microglia [149]. Axonal damage and loss is an irreversible process that leads to severe and permanent neurological deficits in MS. Recently, an evidence postulated that axonal loss rather than demyelination underlies the progression of MS, and thus, constitutes a therapeutic target [150]. Oligodendrocytes perform some degree of remyelination in humans [151] and in animal lesions also [152].

1.7.3 MS Treatment-what more can we do?

The ultimate goal of research in MS is the development of intervention that could improve the life of those living with MS and possibly cure them. However, the understanding of MS disease process is not yet sufficient to predict which therapeutic strategies will be most effective. Even though, several therapeutic drugs are available, none have emerged as an ideal treatment that delays myelin destruction and halts disease progression. The crucial role of the immune system in the disease pathogenesis has important therapeutic implications.

Two classes of **immunomodulatory** agents, **interferon-β** and **glatiramer acetate** (Copaxone), since the 1990:ies have been approved for the treatment of MS [153, 154]. They reduce disease activity, and also reduce the progression of disability in relapsing-remitting MS. These disease-modifying drugs are neither a cure, nor are effective for all patients. **Natalizumab** (Tysabri) is the most recently approved monotherapy for the relapsing forms of MS. Natalizumab exerts its immunologic effects by targeting the **α-4 integrin** receptor, the molecule responsible for the migration of leukocytes from the blood into inflamed tissues.

Immunomodulators which shift immune responses from Th1 towards more beneficial anti-inflammatory Th2 that are secreted by regulatory T cells have been partially effective in clinical trials [155, 156]. In the search for more efficacious agents, many new drugs are under investigation in preclinical and clinical trials, but several promising approaches have failed [157-160].

One of the characteristics of inflammation is the recruitment of leukocytes to the site of inflammation. The Ck system has been validated as providing good therapeutic targets by several approaches [101, 105-107]. The important role-played by Cks in MS pathogenesis is the main rationale behind using them for treatment [124, 161]. CkRs have proven to be excellent targets by the pharmaceutical industry for many inflammatory diseases models. They have shown to be effective in rat heart transplant rejection [102], rabbit allograft rejection [162] monophasic rat model for MS [163] as well as inhibiting HIV-1 infection [106].

Recently CkR antagonist, as a new class of drugs, has been introduced in clinical practice in MS [164]. A novel CCR1 antagonists, **BX471** (also known as ZK811752), is a potent selective orally available agent that was safe in Phase 1 clinical trials in MS [165], but unsuccessful in larger Phase II trials. In addition, a small molecule CCR1 antagonist BX 471, is currently in Phase II clinical trials [166, 167].

2 AIMS OF THE THESIS

The aim of this thesis was to determine the role of the Ck system specifically the CCR1, CCR2, CCR5 and CX3CR1 receptors in the development of autoimmune neuroinflammation. A key issue in understanding the pathogenesis of MS is the reliable identification of phagocytes capable of degrading myelin

Specifically we aimed to:

I. Characterize the pre-symptomatic, acute and remission stages of MOG-EAE, as well as rats at various stages of relapse, regarding CCR1, CCR2, CCR5 and CX3CR1 expression.

II. Quantify CCR1, CCR2, CCR5 and CX3CR1 mRNA-expressing cells in relation to the stage of demyelinating activity: EA lesions, LA lesions, DM lesions and PPWM.

III. Map if there is a differential CCR1, CCR2, CCR5 and CX3CR1 expression in resident microglia cell and blood-derived monocytes/macrophages.

IV. Explore the effects of a low-molecular weight CCR1 selective antagonist on the course of rat MOG-EAE.

V. Determine the expression of the receptor ligands CCL3, CCL5 and CX3CL1 in relation to the findings above.

3 METHODLOGICAL CONSIDERATIONS

The main materials and methods used in papers included in this thesis are listed below. For specific details, see each separate paper.

3.1 EAE induction and profile

Chronic relapsing EAE was induced in female DA rats at 10-14 weeks of age by intradermal immunization with the N-terminal sequence of recombinant rat MOG (aa 1-125) [168] in Incomplete Freund´s Adjuvant (Difco, Detroit, MI), in rats anaesthetized with methoxyflurane (paper **I-IV**). Chronic of long duration, a term often used to describe a disease that becomes progressively worse. Seven days after immunization, body weight and clinical score were daily recorded. Ataxia is unsteadiness and lack of co-ordination that result from the brain's failure to regulate the body posture and the strength and direction of limb movements. Ataxia is most often caused by disease activity in the cerebellum. A disease remission was defined as a lessening in the severity of symptoms or their temporary disappearance during the course of the illness. A relapse is return of the manifestations of a disease after an interval of improvement.

3.2 Treatment strategy

Disease-modifying therapy is a treatment intended to influence the course of the disease and alter its natural history. Treatment with CCR1 selective antagonist (paper **IV**), was run on two different experimental occasions: Group **(A)** before the onset of clinical disease; rats were injected subcutaneously twice a day with 13.3 mg/kg of CCR1 antagonist for 5 consecutive days; and Group **(B)** after clinical onset of disease; rats were subjected to same treatment protocol. There were 12 rats per treated group. Vehicle-treated MOG-EAE rats were served as controls.

3.3 Histopathology

At different time points, brain, spinal cord and peripheral organs (heart, lung, liver, spleen, and kidney) were removed from rats, transcardially perfused with PBS followed by 4% paraformaldehyde and processed for light and electron microscopy. Paraffin-embedded tissue sections (4μm) were stained with haematoxylin and eosin (H&E), Luxol fast blue (LFB)/periodic acid Schiff (PAS), and Bielschowsky silver impregnation, to detect inflammatory infiltrates, demyelination, and axonal loss, respectively [58], (paper **I-IV**).

3.4 Tissue in situ hybridization histochemistry (ISH)

Nucleic acid hybridization is a fundamental tool in molecular genetics which takes advantage of the ability of individual single-stranded nucleic acid molecules to form double-stranded molecules (that is to hybridize to each other). The interacting single-stranded molecules must have a sufficiently high degree of base complementary. Standard nucleic acid hybridization assays involve using a labeled nucleic acid probe to

identify related DNA or RNA molecules (that with a significantly high degree of sequence similarity) within complex mixture of unlabeled nucleic acid molecules (the target nucleic acid). There are many different ways to do ISH including probing with cDNAs, cRNAs, or synthetic oligonucleotides. There are also multiple ways of labeling these probes using radioactive (^{3}H, ^{32}P, ^{33}P, ^{35}S) or non-radioactive (biotin, alkaline phosphatase, digoxigenin, fluorescent) nucleotides. ^{35}S riboprobes represent the most sensitive method for the detection of mRNA in tissue sections [169].

Table 2. Comparison of different labeling strategies for *in situ* hybridization ranked according to their relative sensitivity (according to Wilcox 1993).
Riboprobes > cDNAs > Synthetic oligomers ^{35}S > ^{32}P > ^{3}H >> biotin/digoxigenin Frozen > Paraffin

In this thesis work (paper **I-IV)**, we mainly used radioactive single-stranded RNA probes (^{35}S), also called complementary RNA (cRNA) or riboprobes, to study the mRNA expression of a single protein. cRNA or riboprobes (Riboprobe® is actually a registered trademark of Promega Corporation) are often used for in situ hybridization because:

1. They are extremely sensitive (due to the fact that they can be labeled to high specific activity during probe synthesis)

2. RNA-RNA hybrids are more stable than DNA-RNA hybrids and

3. Non-specific tissue signals can be removed after hybridization with RNase A since RNA duplexes (representing specific binding) are resistant to degradation by RNase A.

In addition, it allows quantification of the hybridization signals, either on film autoradiographs or at the cellular level.

One major disadvantage of using RNA probes is the amount of effort required to actually prepare these probes.

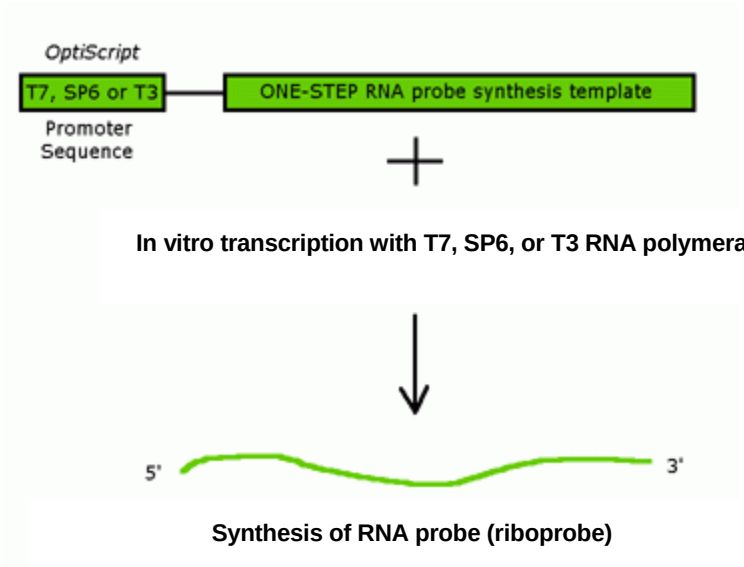

Figure 4: Preparation of RNA probe for in situ hybridization by *in vitro* transcription.

3.4.1 Preparation and labeling of cRNA riboprobes

RNA probes are usually prepared by *in vitro* transcription (Fig. 4). The RNA probe is transcribed from a linear DNA template using highly specific bacteriophage DNA-dependant RNA polymerases from the Salmonella bacteriophage **SP6**, and the E. coli. bacteriophages **T3** and **T7** (RNA polymerase T7, T3 or SP6). Therefore, sufficient quantities of a plasmid carrying the gene sequence of interest that can be used as the template for RNA probe synthesis must be obtained. Furthermore, before a riboprobe can be transcribed the correct RNA polymerase promoter sequences must be available in the plasmid in the correct orientation with respect to the template sequence. If the cloned gene exists in a plasmid lacking these promoter regions the investigator is forced to subclone the gene into a more suitable vector. For example the transcription vectors pGEM (SP6 and T7 promoters, Promega) and pBluescript (T3 and T7 promoters, Stratagene) are commonly used. For *in vitro* transcription reactions the plasmid must also be in a linear form. Restriction enzymes should be used to linearize the plasmid. Even after the RNA probe has been transcribed successfully from the template, if the RNA probe is greater than 300-400bps in length it should be hydrolysed into shorter fragments since the optimal upper length for riboprobes for ISH is 150-200bps. Longer probes have poor tissue penetration. Lastly, and perhaps most limiting is the possibility that the investigator may not be able to easily source the cloned gene sequence required for RNA probe generation. Reviewed in [170].

Riboprobes that are complementary to the mRNA of a gene are known as antisense and are obtained by cloning a gene in the reverse orientation. In such cases, the phage polymerase will synthesize labeled transcripts from the opposite DNA strands to that which is normally transcribed *in vivo*. Useful controls for such reactions include sense riboprobes, which should not hybridize to mRNA

A labeled probe is hybridized against RNA in tissue sections that either is paraffin-embedded or frozen tissue using a cryostat, then mounted on to glass slides. A hybridization mix including the probe is applied to the section on the slide and covered with a glass cover slip. Typically, the hybridization mix has formamide at a concentration of 50% in order to reduce the hybridization temperature and minimize evaporation problems.

It is important that the ^{35}S -labeled UTP is used immediately after thawing and any remaining label should be discarded rather than re frozen for use at a later time. Greatest contribution to high backgrounds using ^{35}S-labeled probes comes from repeated freezing and thawing of the isotope prior to use. According to Amersham there is about a 5% degradation of the nucleotide with freezing and thawing that might contribute to background problems Reviewed in [169, 170].

Hybridized probe is visualized using autoradiographic procedures. The localization of the silver grain is often visualized using only dark-field microscopy.

3.5 Immunohistochemistry (IHC)

IHC refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies (Abs) binding specifically to antigens (Ags) in biological tissues. It takes its name from the roots "*immuno*," in reference to Abs used in the procedure, and "*histo*," meaning tissue. Visualising an **Ab-Ag** interaction can be accomplished in a number of ways. In the most common instance, an Ab is conjugated to an enzyme, such as peroxidase that can catalyse a colour-producing reaction.
Abs can also be classified as primary or secondary reagents. Primary Abs is raised against an Ag of interest and are typically unconjugated (unlabelled), while secondary Abs are raised against primary Abs. Hence, secondary Abs recognizes immunoglobulin of a particular species and is conjugated to either biotin or a reporter enzyme such as alkaline phosphatase or horseradish peroxidase.
Paraffin-embedded tissue were easier to section serially (comparing with acetone-fixed frozen sections), show superior morphology, and stained cells were readily identified [171]. Ag preservation can be improved by the use of low-temperature paraffin and reducing the embedding time by infiltration under vacuum [172]. The most common techniques for immunohistochemical detection of Ags are indirect immunofluorescence and the avidin-biotin-complex (ABC) procedures [173]. Indirect labelling by Abs conjugated to biotin can be observed by avidin detection of biotin. Biotin is a vitamin with very high affinity to the glycoprotein avidin. Different fixatives were used and staining was performed according to the methods described in detail in paper **(I-IV)**. With proper fixation, immune cell surface markers can be easily identified in paraffin-embedded tissue [174].

3.6 Combined ISH/IHC

In this thesis work (paper **I-IV)**, we mainly used combined ISH/IHC to identify the phenotype of cell expressing certain mRNA. Combination of ISH and IHC allows the simultaneous detection of specific mRNAs and Ags in same section or in adjacent sections. However, when ISH is coupled to IHC on a same section, a loss of signal in ISH is often observed [175]. Since ISH is a histological technique cellular relationships are maintained and it is possible to precisely identify cell types expressing the gene of interest and interactions between cells that express different proteins may be uncovered. IHC staining was directly applied following the ISH steps, although, post-fixation and treatment with acetic anhydride and proteinase K were replaced with an antigen retrieval technique according to [176].

3.7 Demyelinated plaque and definition of lesional staging

Demyelination is defined as loss of myelin in white matter of the CNS (brain and spinal cord). The plaque (lesion); is an area of inflamed or demyelinated tissue in the CNS. Categories for demyelinated plaques in EAE were selected in H&E and Klüver-PAS stained slides and defined in this thesis work **(**paper **I-III)**, as normal appearing white matter (NAWM), periplaque white matter (PPWM), early active lesions (EA), late active lesions (LA), inactive complete demyelinated lesions (DM), and remyelinated shadow plaques (RM), according to [117, 177].

3.7.1 Early active lesions (EA)

These lesions (pink labeled in Figure 5), were heavily infiltrated by macrophages, lymphocytes and microglia. Myelin sheaths were being disrupted and macrophages contained degradation products, which were stained by Luxol fast blue (LFB).

3.7.2 Late active lesions (LA)

Myelin was already destroyed and removed from axons in these lesions (green labeled in Figure 5). Numerous macrophages contained PAS positive degradation products.

3.7.3 Inactive complete demyelinated lesions (DM)

These lesions showed no evidence for ongoing myelin destruction at their borders, but contained some infiltrating inflammatory cells; their numbers were much lower compared with active lesions. Macrophages without LFB or PAS staining and revealed empty vacuoles after complete destruction of internalized myelin.

3.7.4 Remyelinated shadow plaques (RM)

These lesions were characterized by myelin pallor due to abnormally thin remyelinated sheaths and the presence of low numbers of inflammatory cells. Microglia activation was present.

3.8 Flow Cytometry

Flow cytometry is the process in which measurements are made while cells in a liquid suspension are forced to flow one at a time through a measuring device. Each of the types of immune cells has a unique combination of size, shape, DNA content, and proteins. A flow cytometer equipped to separate the identified cells is called a fluorescence-activated cell sorter (**FACS**). These instruments are used to study the properties of cell subsets identified using monoclonal Abs to cell-surface proteins. Individual cells within a mixed population are first tagged by treatment with specific monoclonal Abs labeled with fluorescent dyes, or by specific Abs followed by labeled anti-immunoglobulin Abs. The mixture of labeled cells is then forced with a much arger volume of saline through a nozzle, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell are excited and will fluoresce. This method was used in paper (**I**).

3.9 Statistical analysis

Comparisons between pair of normally distributed groups were tested with Student's t-test. The non-parametric Mann-Whitney U test was used to evaluate significant statistical differences between abnormally distributed groups. A p value < 0.05 was considered to be statistically significant (paper **I-IV**).

4 RESULTS AND DISCUSSION

To choose an appropriate experimental model for studying and functionally validating pathogenic mechanisms of a human disease such as MS is difficult. We advocate that in order to identify mechanisms of relevance for human disease, the selected model should display as many features in common as possible. MOG-induced-EAE in rats is closely resembles the clinical and pathological features of MS and provides an excellent platform for investigating mechanisms of disease and evaluating efficacy of therapies, [50, 58]. Several hypotheses have been proposed for the etiopathogenesis of MS, which still remains elusive. Several studies have shown that Ck and their receptors are involved in the pathogenesis of MS, and. Cks, we believed, participate in the development of MS by guiding immune cells into the brain.

It is almost certain that in inflammatory diseases and at different stages of inflammatory process, the relative contribution of CkRs to the selective accumulation of leukocyte subsets to an organ site might vary. However, an emerging picture that overlays differential expression regulation with distinct cellular sources for individual receptors with functional bias for the recruitment of regulatory and/or effectors leukocyte subsets provides a framework to understand the localization/migration of these cells during the inflammatory reaction. The specific sites where these cell populations localize are critical to their ability to exert their regulatory/effector functions. This in turn is responsible for the induction of the characteristic immunological and pathophysiological endpoints that result in disease. This general view is illustrated by the waves of CkRs that might be responsible for the recruitment of monocytes-derived macrophages and/or microglia to the brain during the development of EAE.

4.1 Paper I: Expression of CX3CR1 by microglia and CCR1 by macrophages in MOG-EAE

One of the characteristics of ongoing demyelination in MS is the accumulation of macrophages in active lesions. Little is known about the source of these macrophages in early stages of plaque evolution, as microglial-derived and haematogenous macrophages share morphological characteristics and cell surface Ags. Understanding the pathogenesis of MS is the reliable on identification of phagocytes that capable of degrading myelin and cause onset of demyelination. However, more detailed data on the identity of CCR1 and CX3CR1 expressing cells during the course of autoimmune neuroinflammation has been lacking. For this reason, we have herein studied their mRNA expression in a model that shares several important features with MS to identify their cellular phenotypes that could be of use in future identification of disease mechanisms.
By utilizing combined ISH/IHC during different phases of MOG-induced EAE, we found CCR1 mRNA was preferentially expressed by macrophages (ED1+ *Griffornia simplifolica* isolectin B4+ (GSA/B4+). In contrast, CX3CR1 mRNA expressing cells were identified as microglia on the basis of their cellular morphology and positive (GSA/B4 lectin staining) in spinal cord lesions.

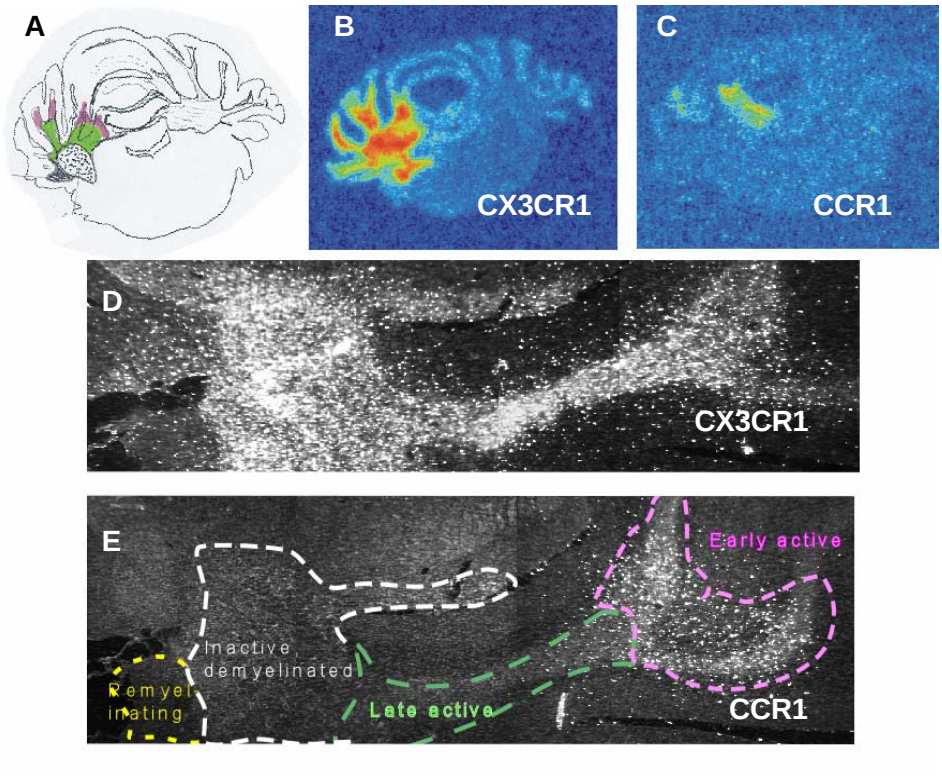

Figure 5. Lesional map made from Klöver-PAS stain and darkfield expression pattern of CX3CR1, CCR1. (A) Camera lucida drawing of huge plaque in cerebellum of DA rat at day 40 p.i. with MOG; EA :(lesion): pink label, LA: green label. DM: grey dotted label, RM: filled grey label. (B, C): Phosphorimager depicting expression of CX3CR1 (generalized expression) and CCR1 (focal expression) mRNA (D, E): Lesinal mapping of demyelination grade mirrored by CX3CR1 and CCR1 mRNA expression. NOTE CCR1 is only expressed in EA areas while CX3CR1 is expressed in all areas except remyelinated area (yellow) (paper I).

Next, actively demyelinating plaques were mapped into subareas representing NAWM, PPWM, DM, and RM in H&E and Klüver-PAS stained slides, using camera Lucida drawings for the correlation of CkR distribution with histopathology (Fig.5). We found CCR1 mRNA was been heavily upregulated in areas of EA demyelination, whereas mRNA for CX3CR1 demonstrated highest expression level in areas of DM.

In this study, we have also analyzed the expression of CCR1 and CX3CR1 at the protein level to determine which cell population expressed these receptors. By using flow cytometric analysis of cells infiltrating the spinal cord (gating was used for the surface markers CD45, ED-2 and CD11b), we found distinctly non-overlapping expression of CCR1 and CX3CR1 in macrophages and microglia respectively, suggesting a differential receptor expression between microglia and monocyte-derived macrophages, and provided insight into pathogenic mechanisms during early demyelinating events in EAE. Furthermore, the intimate association between CCR1 expressing phagocytic cells and the actively demyelinating edges of lesions strongly argues for a pathogenic role for blood-derived macrophages, and identified targets for future therapy by targeting monocyte recruitment.

In line of our finding here, expression of CCR1 in early stage of macrophage-mediated demyelinating activity and in active demyelinating border zone are consistent with previous studies in MS [127, 128, 177-179] and in EAE [180-184]. Moreover, recent reports demonstrate monocyte-derived macrophages in MS responsible for active demyelination [140]. Targeting monocyte recruitment in CNS develop a rationale for targeting the chemokine axis in order to treat CNS inflammatory disease [185].

In regard to the increased expression of CX3CR1 displayed by microglia in demyelinating plaque, hybrid studies have shown that CX3CR1 was constitutively expressed by microglia [186] and induction of EAE in the rat was accompanied by increased levels of CX3CR1 mRNA in spinal cords of animals displaying disease [187]. Taken together, this increased expression during the peak of disease might fulfill a proinflammatory role for CX3CR1 receptor in EAE.
In conclusion, the paper have showed macrophages and microglia present within demyelinating lesions in chronic EAE are expressing CCR1, CX3CR1 respectively, and imply them to the pathogenesis of EAE. These receptors may thus represent promising targets for therapeutic modulation.

4.2 Paper II: Fractalkine is expressed by neuronal-like cells and astrocytes, and CX3CR1 is expressed by microglia in the CNS

Earlier studies have suggested a continuous dialogue between neurons and microglia under basal conditions via FKN-receptor pair [129, 188, 189]. However, their function has remained enigmatic. Paper II temporally compared the expression of FKN and its receptor CX3CR1 in brain tissue derived from healthy controls versus rats with MOG-EAE. cRNA probes directed against rat FKN and CX3CR1, have revealed that FKN is constitutively expressed in the brain within neuronal-like cells (pan-neuronal marker NeuN), and located throughout the neuraxis of the healthy rat. Its expression remained unaltered in the CNS of rats with MOG-EAE, with exception of an induced expression on astrocytes within inflammatory lesions (double-labelling experiments showed those cells to stain positive for the astroglial marker GFAP). The receptor CX3CR1 was expressed by microglial cells in all regions of the healthy brain, and the morphology of these cells, as well as their positive labeling with *Griffornia simplifolica* isolectin B4 (GSI-B4); stains microglia, macrophages and endothelial cells, identified them as being inactive microglia. Induction of MOG-induced EAE was associated with a distinct accumulation of CX3CR1 mRNA expressing cells within the inflammatory brain lesions at all stages of disease. At relapse, the aggregation of cells expressing high levels of CX3CR1 mRNA closely followed the areas of expanding lesions, which the majority stained positive for markers of the microglia-macrophage lineage. Thus, this Ck-receptor pair may fulfill important roles in normal CNS physiology as well as in CNS pathology. In line of our findings here, a recent report demonstrates that human neurons and astrocytes were also found to express FKN mRNA, and human microglia expressed CX3CR1 mRNA [190].
Next, mRNA expression of CX3CR1 in time-staged brain lesions, was analyzed in H&E and Klüver-PAS stained slides. Elevated levels of CX3CR1 mRNA in microglia in the periplaque zone, as well as EA, LA and DM lesions were found, confirming a results by Sunnemark et al.[136]. The actual number of CX3CR1 mRNA expressing

cells was three-fold higher in lesional areas compared to PPWM and NWM. The fact that CX3CR1 mRNA was only detected within areas of plaque in EAE lesion but not in remyelinated zone [136] argues for a role of FKN signaling in the pathogenesis of inflammation. Alternatively, degeneration of neurons could be the cause of increased CX3CR1 expression due to priming of microglia to FKN shedded from injured neurons by a yet undefined neuronally-derived signal. Interestingly, an anti-inflammatory and neuroprotective effect of FKN was recently proposed [191, 192].
Thus, communication between microglia and other resident CNS cell as well as invading cells, involves Cks. Cellular communication between neurons and microglia, involving FKN and its receptor, occurs in both normal and pathological states of the CNS.

4.3 Paper III: β-Chemokine receptors CCR1, CCR2 and CCR5 are expressed by macrophage/microglia in the CNS

MS is heterogeneous in its clinical course, immunopathological mechanisms and response to treatment [123]. Identification of immunological markers associated with clinical subtypes, phase of disease activity would be an important progress in MS research. Although Cks involved in the regulation and immigration of immune cells by forming a concentration gradient at site of inflammation, still little is known regarding the in vivo expression and phenotype of receptor expressing cells in the CNS. Most of the cells infiltrating the CNS of animals with EAE are macrophages [141], and activated macrophages within lesions have been found to differ at different stages of lesion development towards repair and remyelination or towards chronic demyelination [140, 177]. In fact, macrophage-mediated injury may simply induce higher expression of CkRs or even new CkRs, creating a feed-forward scenario that results in more extensive tissue damage. This study was designed to give information on the role of β-CkRs CCR1, 2 and 5 in acute and chronic phase of MOG-EAE as well as in demyelinating activity and to compare them to CCR1-expressing macrophages.
By combined ISH/IHC, we have showed that acute and chronic-relapsing disease was associated with a distinct expression of CCR1, 2, and 5 within the spinal cord lesions by cells of the macrophage/microglia lineage. Next, actively demyelinating plaques were mapped into subareas representing NAWM, PPWM, active demyelinated plaques, inactive demyelinated plaques, and remyelinated shadow plaques in H&E and Klüver-PAS stained slides, and revealed that CCR1 was detected within lesions representing EA or LA demyelinating activity. The morphology, and the co-labelling for CCR1 with isolectin and/or the marker for phagoctosis ED-1, identified those cells as infiltrating macrophages and/or reactive microglia. These data confirm recent findings from our laboratory (study I), where CCR1 mRNA was found to be preferentially expressed by macrophages in areas of active demyelination.
CCR2 mRNA was detected within EA and LA demyelinating activity. Co-labelling for the isolectin and the marker for phagocytosis ED-1, as well as their amoeboid morphology identified those cells as infiltrating macrophages. Thus, CCR2 expression is distinctively associated with phagocytic properties of monocytes-derived macrophages in EAE lesions. Our findings confirm previous studies describing expression of CCR2 and its ligand CCL2 within inflamed brain lesions of rodents with

EAE [193]. In addition, expression of CCR2 on circulating monocytes have been demonstrated during MS relapses [194, 195].
Expression of CCR5 mRNA was found strikingly variable and associated with phagocytic macrophages and microglial cells in the spinal cord.. CCR5 mRNA was detected within lesions primarily representing EA, LA, DM lesions, and in PPWM areas of demyelinating activity. Co-labelling for the isolectin and their morphological characteristics identified those cells as infiltrating macrophages and reactive microglia. These data complement recent findings in active MS lesions where monocytes and microglial cells were found to express CCR5 [178]. Moreover, in pattern II lesions in MS, number of cells expressing CCR1 significantly decreased while CCR5 increased in LA compared with EA demyelinating regions [196]. Thus, expression of CkRs CCR1 and 5 reflects differential activation of mononuclear phagocytes in pattern II MS lesions.
Our further findings have indicated that CCR5 receptor demonstrated a strong expression in demyelinating activity compared to CCR1 and 2. Its expression tended to be more widespread to include all lesional areas as well as to cell located in the periplaque zone. Thus individual CkR could attract different cell types. Low expression of CCR2 was particularly evident in the acute phase, whereas during the active relapse the expression levels per cells reached more prominent levels by cells belonging to the macrophage/microglia cell population. However, these data are in agreement with data by others, where immunoneutralization of the CCR2-selective Ck ligand CCL2 [181, 197] reduced paralysis and brain inflammation at relapse phase in mice with transfer EAE.
In line with these, recent reports show that mice deficient in CCR1, CCR2 or their ligands[184, 198, 199] but not the CCR5 [200] genes, give a clear definition of the role of Cks and their receptors in EAE and suggested rationale for therapeutic intervention. Thus, we could speculate that macrophage recruitment to the CNS was found to be a necessary step in the development of pathologic inflammatory lesions in EAE. This is the context in which Ck inhibition or antagonism makes therapeutic sense. In conclusion, understanding the complex role of these CC CkRs receptors in the initiation, perpetuation and possibly also resolution of the lesion may enable the design of more efficacious treatment of MS.

4.4 Paper IV: Effector stage CCR1 selective antagonist reduces MS-like rat disease

This thesis suggests a rationale for interference with the Ck system at various points as therapeutic intervention using small-molecule inhibitors. The mechanism responsible for causing the immunological damage in the CNS is still unknown. In this way, the protective effects of leukocytes can be subverted in a manner that causes disease. Regulation of the recruitment of activated immune cells and extravasation of these cells into the CNS by CkR antagonist might be strong candidate for therapeutic intervention in MS. According to this, the hypothesis was that antagonism of CCR1 might lead to reduction of inflammation *in vivo*.
Antagonism of CCR1-mediated infiltration of macrophages (Fig.6), gave a powerful inhibition of the onset of paralytic disease and the associated inflammation *in vivo.* In addition, the antagonist potently reduced the severity of ongoing disease when given

after the clinical onset, provided solid evidence of the pathological role of CCR1 in rat MOG-EAE. In vehicle treated-rats, CCR1 mRNA and its ligand CCL3 were expressed within the inflammatory lesions during the acute phase of the disease compared to drug-treated rats. Our data are in line with previous data where blockade of CCL3 (ligand for CCR1), prevented the development of both acute and relapsing paralytic symptoms and infiltration of mononuclear cells into the CNS [180, 181]. Moreover, DNA vaccination encoding for CCL3 effectively inhibited the disease induction in EAE rats [201], and genomic deletions of CCR1 resulted in a partial disease protection in mouse models for MS. These data, collectively combined with our data presented here, provided strong evidence that CCL3 mediated activation of CCR1-expressing macrophages is central to the neuroinflammatory processes in EAE. One may speculate that at the initial phase of the disease more CCR1-expressing cells piled up in the lymphoid organs. As long as we treated the rats, the CCR1 expressing cells are retained in the peripheral organs. When we relieved the pressure on the migration, pathogenic cells are released to enter the brain, thus, we see the overshooting disease after cessation of treatment in group A-treated rats. Whether the antagonist might inhibit the disease at later stages, is still to be proven.

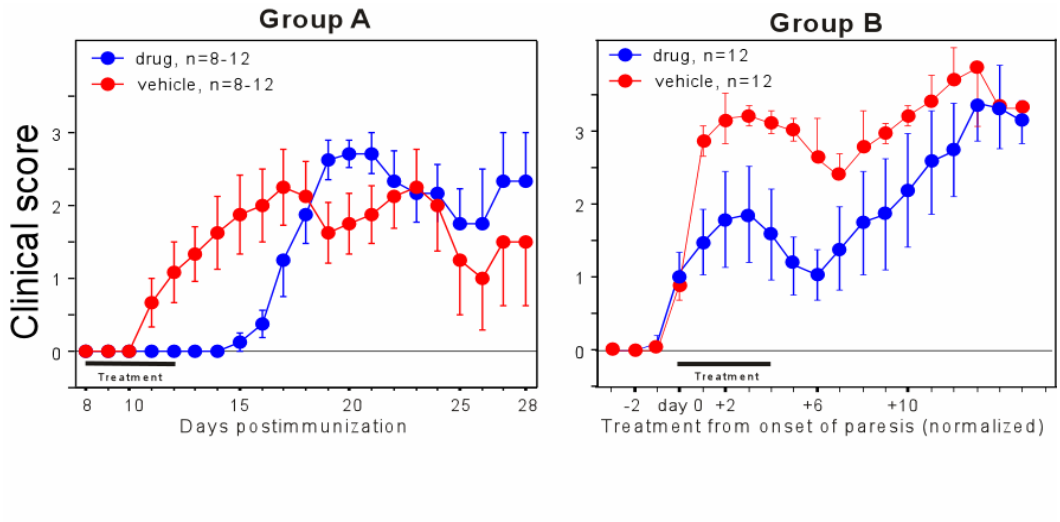

Figure 6:.Clinical effects of CCR1 antagonist. Rats were injected with MOG and treated from day 8 post immunization (group A, *n*=20), or from individual onset of disease (group B, *n*=16) with either drug or with vehicle (cyclodextrin/H_2O). Rats were treated twice daily for five consecutive days and scored on a daily basis. The data are combined from two independent experiments and expressed as mean ±2 S.E.M. Black bar below each graph indicates period for treatment.

To be able to compare various indices of immune system functions in vehicle- and drug-treated rats, histopathological evaluation of kidney, lung, liver, heart, spleen and adrenal glands in drug-and vehicle-treated rats were examined. There were no pathological alterations or lesions in any of these organs. Moreover, no signs of atrophy or lymphoid depletion were detected in the spleen in the drug-treated group. ISH with cRNA antisense probes was done to compare the numbers of CCL3, CCL5, CCR1 and CCR5 mRNA-expressing cells within the kidney, lung, liver, heart, spleen and adrenal gland of vehicle- and drug-treated rats. The number of Ck and receptor-expressing cells did not visibly differ between the drug- and vehicle-treated rats, suggesting that, treatment with the CCR1 antagonist does not cause systemic immune abnormalities

rather than a selective inhibition of CCR1-dependant infiltration of leukocytes into the CNS parenchyma. This confirms a study by [180] where anti-CCL3 did not affect the activation of encephalitogenic T cells, suggesting specificity of CCL3 for chemoattraction of mononuclear inflammatory cells into the CNS of EAE mice. Finally, to detect if the effect of CCR1 antagonist treatment was associated with a suppression of specific Ab responses. By ELISA, sera obtained from drug- and vehicle-treated rats (collected from group A and B animals 12 h after the last injection), have showed IgG, IgG1, IgG2a, IgG2b and IgG2c of anti-MOG-specific Abs similar in group A- vehicle and-treated rats, whereas in group B, levels of total IgG as well as IgG2b and c isotypes were found significantly lower in the vehicle-treated rats. In the rat, we believe Th1 cells induce switching to IgG2b and IgG2c. The higher levels of anti-MOG IgG2b and IgG2c therefore support the hypothesis that Th1 was present in both vehicle and treated rats argues against a Th2 deviation.

These findings suggest that treatment with CCR1 antagonist is not associated with a general suppression of B cell functions and or a shift to a Th2 driven immune response.

In conclusion, the therapeutic ability of a newly discovered CCR1 selective antagonist was explored in this thesis, and it seems that non-peptide CkR antagonist may have a disease-reducing effect in an animal model of autoimmune neuroinflammation.

5 GENERAL DISCUSSION

5.1 CCR1 and CCR1 antagonist in EAE and MS

One of the aims of the present thesis is to better understand the role of 7-TM GPCR at the target site tissue the CNS, and to explore the role of low molecular weight CkR selective antagonist in MOG-EAE. Hope such an approach would offer more insights into disease pathogenesis and potentially identify new therapeutic targets for MS. In this thesis work, CCR1 mRNA expression was found intensely upregulated in EA plaque (paper I) (Fig.5), and also was abundantly been expressed within the inflammatory lesions in EAE in the acute phase (day 13 p.i.), early relapse (day 21 p.i.), mid-relapse (day 24 p.i), late relapse (day 29 p.i n=3), as well as in the demyelinating activity by cells of the macrophage/microglia lineage (paper III). Thus, CCR1 is likely to play a role in the recruitment of secondary influx of leukocytes to the CNS. Ransohoff and co-workers recently reported that CCR1 is abundantly been expressed by infiltrating monocytes in perivascular cell cuffs and at demyelinating edges of evolving lesions in MS, arguing for a direct role of CCR1 mediated signaling in attracting monocytes/macrophages into the brain compartment [178].
This motivated us to use a newly developed CkR antagonist [97] to explore its therapeutic potential in MOG-EAE (paper IV) (Fig.6). We demonstrated that the antagonist alone was able to control the disease progression at the effector stage in chronic rat model for MS, suggesting that this CkR is non-redundant at this stage of the disease and could affect the early active zone of demyelinating activity for the entire treatment period. The antagonist has previously been shown to bind to the human CCR1 with high affinity (K_i = 40 nM) and selectivity, and it displays potent inhibition of CCL3 and CCL5 triggered functional responses [97]. Although the affinity of the antagonist to rat CCR1 is 10-20 fold lower, our results convincingly demonstrated that it was highly effective in preventing all signs of paresis as well as CNS inflammation when given just prior to onset of the disease. Even though, when given at individual onset of the disease, it reduced the clinical signs. Of particular note is the finding that the drug also prevents infiltration of T cells, perivascular expression of CCL3 and CCL5 and visible signs of activation of perivascular macrophages. This suggests that CCR1-dependent processes are central also in the, presumably, most early events of neuroinflammation, i.e. infiltration and activation of pathogenic T cells and/or activation of perivascular macrophages, and based on this, we provided evidence of the pathological role of CCR1 receptor in early proinflammatory events in rat MOG-EAE.
More recently, data published from small clinical studies has shown efficacy of an oral CCR1 antagonist in treating RA [202] but failed to meet therapeutic endpoints in larger clinical trials. Moreover, a novel CCR1 antagonists, BX471 (also known as ZK811752), is a potent, selective, orally available agent that was safe in Phase 1 clinical trials in MS [203], but unsuccessful in larger, Phase II trials. One draw back of the lack of CCR1 was only reported in mice lacking this receptor showed increased susceptibility to Toxoplasma gondii infection [204].

5.2 Fractalkine and its receptor CX3CR1 in EAE and MS

Neurons and glial cells are the two major types of cells present in the CNS. For many years, microglia and astrocytes residing within the CNS compartment were thought to

only support the activities of neurons. Astrocytes fill in the space between neurons, take up neurotransmitters that are released by neurons, and help maintain the correct chemical concentrations around neurons. Microglia provides physical and nutritional support for neurons and digests parts of dead neurons. Thus, neurons would not work properly without them. They normally reside in the CNS in resting state, but are rapidly activated by virtually all types of disturbances to the CNS homeostasis [205]. Reviewed in [206, 207]. Disturbances in the normal homeostasis of the CNS induce a localized activation of microglia. This activation serves to isolate pathological processes from surrounding, intact nervous tissue. Concomitantly, healthy or minimally damaged neurons nearby may be negatively influenced by potent molecules released by activated microglia. This situation appears to exist e.g. in MS [208]. In their final stage of activation, microglia becomes phagocytic cells with very high phenotypical resemblance to peripherally-derived macrophages which is sharing a common mesodermal origin with. One exception is the level of CD45 expression, which can be used to distinguish between these two cell populations [209]. It has been hypothesized that monocyte-derived macrophages are more actively involved in damaging consequences of MS than microglia-derived phagocytes[131, 185, 210] as indicated by the preferential appearance of plaques near and around blood vessels. However, phagocytic microglia are also abundant in inflammatory lesions and may exert important effector functions, especially in early stages of MS disease [137, 146]. Therefore, pharmacologic regulation of microglial activity is therefore a rational approach to treatment of many CNS disorders [208]. FKN is a membrane-tethered Ck that functions as a chemoattractant and adhesion protein by interacting with the receptor CX3CR1[211].

In this thesis work, expression of FKN and CX3CR1 in naive rats and in rats with MOG-EAE was determined (paper II). We speculate that the constitutive expression of FKN and its receptor on neurons and microglia are not normally promoting inflammation and indicate a potential mechanism whereby neurons control microglia functions in the intact brain as well, and that FKN appears to restrain the neurotoxic capabilities of microglia under normal circumstances. With the finding that the expression of neuronal FKN is reduced in intensively inflamed areas of the brain, combined with a distinctly induced expression of this ligand within GFAP-positive astroglia (paper II), we speculate that mechanism by which neurons and reactive astrocytes may control migration and function of the surrounding microglia can be proposed. In MS, FKN levels were found significantly increased in patient's serum versus controls [212]. One study showed different patterns of activation cascades depending on FKN concentration on microglia and astrocytes when FKN been analyzed *in vitro*; they found it exist as a cell regulator at low concentration, while increased (de novo) release of FKN suggested to fulfill a proinflammtory role [213]. Again, we propose that FKN may play a role in inflammation processes in the brain (increased expression) and might be involved in the generation and progression of MS. On the other hand, FKN significantly suppressed neuronal cell death induced by microglia activated with LPS and IFN-γ in a dose-dependent manner. This suggest the possible functions of FKN as an intrinsic inhibitor against neurotoxicity by activated microglia [214]. CX3CR1 mRNA in (paper I and paper II) was revealed elevated levels of expression by microglia in demyelinating activity (Fig.5). In addition, the accumulation of CX3CR1 expressing cells other than microglia within the

inflammatory brain lesions indicate a possible role for FKN in controlling invasion of peripheral leukocytes to the brain (paper II). These cells may be speculated to correspond to infiltrating leukocytes, such as NK, γδ T cells and/or terminally differentiated CD4+ and CD8+ T cells, which previously have been described to express FKN receptors and/or to respond functionally upon FKN stimulation [215-220]. However, the majority of the lymphocyte-like cells within the inflammatory lesions of these rats did not express CX3CR1 mRNA.
We could speculate in line of our finding here, that healthy neurons may inform microglia via constitutive calming inputs (FKN) about their normal activity. Upon activation by dangerous signals, microglia chemoattract leukocytes via Cks and produce cytokine and other factors with potential toxicity for neurons. Signals for microglial activation may also derive from severed damaged neurons by disruption of calming inputs (disturbed FKN signaling). Lymphocytes may send feedback to microglia to adjust the profile of invasion-supporting signals. Astrocytes will have input on microglia and on neurons by releasing the harm FKN. In studies by others, astrocytes have been found to have input on microglia and on neurons by releasing the harmful glutamate [221].

With the finding that FKN-/- mice [222, 223], and CX3CR1 -/- mice did not demonstrate any differences to disease severity compared to wild type mice [224], moreover, and in a recent study, CX3CR1-/- mice showed more extensive neuronal cell loss than CX3CR1+ littermate controls, augmenting CX3CR1 signalling may protect against microglial neurotoxicity, whereas CNS penetration by pharmaceutical CX3CR1 antagonists could increase neuronal vulnerability [225], with these controversial findings, we believed that the role of FKN and its receptor in neuroinflammation remains elusive and enigmatic.
A definite assignment of the role of FKN and its receptor in normal and inflamed or injured CNS conditions will await conceptual testing in animal disease models using more informative tools such as selective immunoneutralizing antisera, peptide antagonists or non-peptide CX3CR1 antagonists delivered at various stages of the disease. Development of CX3CR1 antagonists may allow reductions in the doses of immunosuppressive drugs such as that used in transplantation models as suggested by Haskell et al.

5.3 CCR2 in EAE and MS

We presented in (paper III) data to show that CCR2 mRNA (ligand for CCL2) is expressed in spinal cord lesions and in demyelinating activity and associated with phagocytic properties of monocytes-derived macrophages. Early studies have shown CCR2 plays an important role in the recruitment of inflammatory infiltrate into the site of inflammation and/ or into the CNS [226-229]. Mice deficient in CCR2, and CCL2 are resistant to EAE, indicating that these chemokines are required for the induction of EAE, consistent with a nonredundant role of CCL2 and CCR2 in EAE [230, 231]. Thus, the process of CNS infiltration appears to be separated into discrete phases, each controlled by various chemokines. Moreover, CCL2 is considered an excellent candidate for a mediator of leukocyte recruitment in a variety of models of neural trauma and immune-inflammation [31, 232].

In MS patients, no significant difference between the majority of monocytes expressing-CCR2, and the minority of T cells-expressing CCR2 were seen among MS patient compared to control, and also in other non-inflammatory CNS disorders [195, 233]. While in other studies, expression of CCR2 on circulating monocytes and on T cells were found to be significantly elevated during MS relapse compared to control [130, 194]. Moreover, *in vivo* treatment with IFN-β has shown increased expression of CCR2 in MS patients compared to control [234]. However, the significance of CCL2 and CCR2 in MS is enigmatic, because CCL2 levels are consistently decreased in the CSF of patients with this disease and other chronic neuroinflammatory conditions, despite abundant expression within lesional MS tissues [235]. These interpretations are limited, however, by insufficient knowledge and paucity of studies concerning distribution of CCR2 in MS lesions, despite the nonredundant role of CCR2 that demonstrated by using animal models. This might partly be due to technical reasons, such as restricted availability of commercial antibodies. For this reason, the spatial relationship between expression of CCR2 and CCL2 in MS tissue and co localization of CCR2-expressing cell in MS tissue is yet to be fully defined.

The present data do not support a role for CCR2 receptor as a Th2, since several studies have suggested that CCR2 plays a down-regulatory role in EAE [226, 236], and contradict recent reports of the importance of CCL2 in mounting T helper 2 (Th2) responses [199]. CCR2 was not found on T cells, making it unlikely that CNS entry by polarized type 2 lymphocytes or $CD4^{+}$/CD25+ T cells is mediated by CCR2. Based on our findings in this thesis work in regard to the high expression of CCR2 during the active relapse in EAE, we could conclude that, CCR2-mediated effects are likely to play an important role in the disease pathology especially at relapsing EAE. Thus, our data suggested an important and unexpected role for CCR2 activation in modulating the immune response, as well as recruiting monocytes/macrophages to sites of inflammation.
Taken in the context of reduced EAE severity in CCR2-/- mice, and the role of CCR2 antagonist (INCB3344) [237], in immune-based inflammatory reactions and in inflammatory disease models including EAE, these findings suggest that CCR2 considered a potential target for therapeutic intervention in CNS diseases, in particular MS, in which phagocytic macrophages are deemed pathogenic.

5.4 CCR5 in EAE and MS

In paper III, we showed that CCR5 mRNA is expressed by phagocytic macrophages and microglial cells. In line of our findings, monocytes-derived macrophages characterize brain lesions in MS and are believed to be effector cells causing demyelination and axonal injuries in MS [177, 238]. The abundant expression of a variety of CkRs by cells of monocyte/macrophage lineage is suggestive of a redundancy in the Ck-mediated control of macrophage function [239]. Most leukocytes found in MS lesions are macrophages, derived either from monocytes or microglia [240]. Despite different activation mechanisms and origins (i.e., resident microglia *versus* hematogenous monocytes), most phagocytic macrophages in MS, expressed CCR5 within demyelinated lesions [178, 241], and its expression on mononuclear phagocytes increased during MS lesion evolution, a finding that was interpreted as indicating up-regulation of CCR5 on resident microglial cells and haematogenous

monocytes upon activation [178]. CCR5 is another CkR associated with Th1 inflammatory reactions [242]. Unexpectedly, we did not observe CCR5 positive CD3 T cells in spinal cord sections of EAE. Our results contradict recent findings by [242, 243] where they found preferential expression of CCR5 on Th1 cells in MS. The profile of CkR on T helper cells may, in part, explain selective recruitment of T cell subpopulations to sites of inflammation, including the predominance of Th1 cells expressing CCR5 in MS lesions [244, 245].

Interestingly, microglia appears to express preferentially other members of CC Ck family, including CCL3 and CCL4 [239]. Various types of injury to the CNS, such as infection, trauma, autoimmune inflammation, and neurodegeneration are known to elicit microglial activation [240]. It has become clear that microglia may display different activity states and have different functional properties under different pathological conditions [246]. These results indicated varied pathways to destruction of neural tissue, and myelin in particular. Regardless of the nature of damage inflicted on the CNS, microglial activation is generally associated with a change in morphology into an amoeboid appearance with shortened cytoplasmic processes and a rounded cell body accompanied by increased expression of genes involved in immune reactions.

CCR5 delta32 (Δ32) is a 32-basepair deletion of CCR5 gene. A CCR5 Delta32 deletion mutation abolishes functional CCR5 on the cell surface and may reduce cell entry into the lesion sites [247]. Individuals homozygous for a non functional Δ32 CCR5 develop MS [248] and individuals heterozygous for the Δ32 non-functional CCR5 allele experienced prolonged disease free intervals, compared to ones with a fully functional CCR5 receptor [249, 250]. More recently, Pulknin [247] from Finland suggested that the lack of CCR5 does not protect from MS, but rather it may predispose to the chronic course of the disease. This would further imply that in view of the redundancy in the Ck system, CCR5 ligands must be assumed to function through other closely related CkRs. Moreover, among many other same studies, Ristic from Croatia in 2006 [251] concluded that the CCR5Δ32 mutation is neither protective of, nor a risk factor for MS, where he showed no significant differences in the distribution of this mutations between MS and control, indicating that this mutation does not influence susceptibility to MS. The presence of these two CCR5 Δ32 homozygous in the MS patients indicated the absence of CCR5 is not protective against MS, and suggested that CCR5 is not an essential component in MS expression. Although this may be due to redundancy in the Ck system where different CkRs may substitute for CCR5 when it is absent [248]. In this regard, CCR5 expression may be a useful marker to identify effector cells in MS and could be used as a tool for monitoring disease activity [252], and response to treatment [253]. Moreover, the lack of effect of MIP-1α deficiency [200] illustrates redundancy in the chemokine system. Further research into the specific functions of chemokines will elucidate the role of these molecules in CNS disease. This data collectively suggests a role in the recruitment of inflammatory cells from the circulation and promoting tissue injury in MS and EAE lesions.

In conclusion our findings imply that C-C CkR could all potentially activate and recruit both resident glia and infiltrating haematogenous cells to sites of CNS inflammation., and provide several potential CkRs targets for therapeutics that affect the disease at different times in the process, allowing the lessons learned from this model to be applied to human MS.

6 CONCLUDING REMARKS

Several conclusions can be drawn so far:

I. The abundant expression of CkRs on microglia and monocyte-derived macrophages in EAE plaque support the hypothesis that Cks may control the formation of inflammatory brain lesions by facilitating local accumulation of non-specific inflammatory cells. Thus, they may serve as potential targets for therapeutic intervention in EAE, and in human MS (paper I).

II. The expression of FKN by neurons and astrocytes and and its cognate receptor by microglia shed light on mechanism by which neurons and reactive astrocytes control migration and function of surrounding microglia in pathological states of the CNS. In addition, the findings that CX3CR1 is also expressed by other cells than microglia within brain lesions, indicating a possible role for FKN in controlling invasion of peripheral leukocytes to the brain (paper II).

III. Members of β-subfamily of CkR CCR1, 2 and 5 are prominently expressed within the inflammatory brain lesions in EAE by cells of macrophage and or/microglia lineage, suggesting a pathogenic role for these receptors at differing stages of chronic EAE (paper III).

IV. Treatment of EAE rats with a CCR1 selective antagonist is a very effective means of preventing onset of the inflammatory cascades within the CNS that most likely are the cause of the ensuing functional deficits in MS-like rat model (paper IV).

7 FUTURE PERSPECTIVES

The thesis shed light on some of CkRs and provided new information about mechanisms controlling Ck mRNA expression during immune-mediated inflammation in EAE, consistent with a role for Cks as amplifiers of CNS inflammatory reactions. It also provided several potential CkRs targets for therapeutics that affect the disease at different times and provide the knowledge base needed to modulate tissue inflammation. Despite the large number of CkRs, the challenge is to find molecules that are relatively selective for one CkR compared with another, to turn it to potent and selective molecules as a drug candidate in MS. Whether such selectivity is needed *in vivo* is still not clear. However, in the future, the availability of antibodies that will allow immunohistochemical characterization of human disease tissue and fluids would permit correlation between receptor expression levels in human samples, and animal models. Future studies in search for answers to the following questions might further clarify the immunopathogenesis of EAE and MS.

My studies have provided answer to some questions raised but have also led to the generation of new questions. I will here briefly outline what I think would be interesting to explore in the future.

If combined receptors antagonist (CCR1/CCR2) would be better in blocking macrophage-mediated disease

The activity of CCR1 antagonist compound *in vivo* in animal model of disease has been emerged here, and the expression profile of CCR2 at different stages of disease development was been described with their cellular sources also. Whether combined CCR1/CCR2 antagonist would be better in blocking macrophage-mediated disease is still unknown, and would be hunting ground in the pharmaceutical industry for drug candidate

If CX3CR1 antagonist could locally modulate the inflammation of the CNS *in vivo*

With regard to the elevated levels of CX3CR1 mRNA detected within microglia-like cells in the brain. Inhibition of CNS specific glial activation can also ameliorate the effects of neuronal dysfunction and destruction. The development of CX3CR1 antagonist may allow reductions in the doses of immunosuppressive drugs used in MS patients. Such work is a prerequisite for the development of novel, efficacious and safe drugs to treat CNS inflammation.

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