# Metalloproteinases in neuroinflammation

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# ABSTRACT

Metalloproteinases (MPs) include the families of matrix metalloproteinases (MMPs) and metalloproteinase-disintegrins (ADAMs). MPs are implicated in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Both MS and EAE involve central nervous system (CNS) infiltration, microglial activation, and expression of chemokines and cytokines including CC chemokine ligand 2 (CCL2) and interferon-γ (IFNγ). MPs mediate cellular infiltration of the CNS parenchyma and regulate activity of chemokines and cytokines. This thesis describes studies of MPs in EAE and other animal models of neuroinflammation.

Gene expression of the majority of MPs was upregulated in the CNS of mice with EAE. In contrast, four of the six membrane-bound MMPs (MT-MMPs) were downregulated. MMP-8, MMP-10, MMP-12, ADAM-12, TIMP-1, and all MT-MMPs were selected for further analysis. Macrophages were identified as a major source of MMP-12 and the tissue inhibitor of MPs-1 (TIMP-1), and granulocytes as a major source of MMP-8. ADAM-12 was expressed primarily by T cells. All but one of the MT-MMPs were expressed by microglia, and three MT-MMPs were downregulated by microglia in EAE. Five of the MT-MMPs were downregulated in transgenic mice overexpressing IFNγ specifically in the CNS.

MPs were also regulated in non-immune models of CNS infiltration that did not involve production of IFNγ. After entorhinal cortex stab lesion, which results in prominent influx of leukocytes to the injured area, three MT-MMPs were significantly downregulated. In transgenic (Tg) mice that overexpress CCL2 specifically in the CNS, leukocytes spontaneously cross the endothelial basement membrane of the blood-brain barrier (BBB) and accumulate in the perivascular space surrounding CNS vessels, but the mice do not show clinical symptoms. Pertussis toxin (PTx) given intraperitoneally induced encephalopathy and weight loss in CCL2 Tg mice. This involved leukocyte migration across the glia limitans

into the brain parenchyma. PTx induced expression of TIMP-1, ADAM-12 and MMPs 8 and 10 in brains of CCL2 Tg mice, whereas there was no significant change in expression of MT-MMPs. Weight loss and parenchymal infiltration induced by PTx were significantly inhibited by the broad-spectrum MP inhibitor BB-94/Batimastat.

These studies identify cellular sources of MPs in neuroinflammation and links stages of cellular CNS infiltration to distinct MP profiles.

# RESUME

Parmi les métalloprotéinases (MPs) on retrouve la famille des métalloprotéinases matricielles (MMPs) et celle des métalloprotéinase-disintégrines (ADAMs). Les MPs sont impliquées dans la sclérose en plaques (SP) et l'encéphalomyélite autoimmune expérimentale (EAE), un modèle animal de la SP. La SP et l'EAE sont caractérisées par l'infiltration du système nerveux central (SNC), l'activation des cellules de la microglie et l'expression de chimiokines (e.g. CCL2) et de cytokines (e.g. interféron (IFN)-gamma). Les MPs régulent la pénétration du parenchyme du SNC par les cellules inflammatoires et l'activité des chimiokines et des cytokines. Cette thèse présente des études sur le rôle des MPs dans l'EAE et neuroinflammation.

L'expression de la majorité des gènes MPs est élevée dans le SNC de souris atteintes d'EAE. Au contraire, l'expression de quatre des six membrane-type MMPs (MT-MMPs) est réduite. MMP-8, MMP-10, MMP-12, ADAM-12, l'inhibiteur de tissu des MPs (TIMP)-1, et toutes les MT-MMPs ont été choisies pour des analyses plus approfondies. Les macrophages ont été identifiés comme source importante de MMP-12 et de TIMP-1 et les granulocytes comme source importante de MMP-8. ADAM-12 est principalement exprimé par les cellules T. Toutes les MT-MMPs sauf une sont exprimées par les cellules de la microglie et l'expression de trois des MT-MMPs est réduite par les cellules de la microglie chez les souris atteintes d'EAE. L'expression de cinq des MT-MMPs est réduite chez les souris transgéniques (Tg) qui expriment l'IFN-gamma spécifiquement dans le SNC.

L'expression des MPs est également régulée dans les modèles d'infiltration non-immunitaires, où les cellules pénètrent le SNC sans produire d'IFN-gamma. Après une lésion du cortex entorhinal, qui provoque l'infiltration de leucocytes dans le tissu endommagé, l'expression de trois des MT-MMPs est réduite de manière significative. Dans les souris Tg qui expriment CCL2 spécifiquement dans le SNC, les leucocytes traversent spontanément la membrane basale des

cellules endothéliales qui forment la barrière hématoméningée (BHM) et s'accumulent dans l'espace périvasculaire entourant les vaisseaux sanguins et ce, sans induire des symptômes cliniques. L'administration intrapéritonéale de toxine de Pertussis (PTx) induit une encéphalopathie et une perte de poids chez les souris CCL2 Tg. Ce traitement cause aussi la migration de leucocytes au travers du glia limitans, dans le parenchyme du cerveau. La PTx induit l'expression de TIMP-1, ADAM-12 et des MMPs 8 et 10 dans le cerveau des souris CCL2 Tg, mais n'altère pas les niveaux d'expression des MT-MMPs. La perte de poids et l'infiltration du parenchyme induites par la PTx sont inhibées par l'inhibiteur général des MPs, BB-94/Batimastat.

Ces études identifient des sources cellulaires de MPs lors du processus de neuroinflammation et associent des étapes d'infiltration cellulaire du SNC à des profils distincts d'expression de MPs.

# CONTRIBUTIONS OF AUTHORS

This thesis consists of a collection of three original manuscripts. I am first author on all three manuscripts, and analyzed the data in them. The interpretation of results and writing of the manuscripts took place in collaboration with my supervisor Professor T Owens, who is senior author on all three manuscripts. Specific contributions of co-authors are listed below.

## Chapter II

**Key Metalloproteinases Are Expressed by Specific Cell Types in Experimental Autoimmune Encephalomyelitis**

Henrik Toft-Hansen, Robert K. Nuttall, Dylan R. Edwards, and Trevor Owens

*The Journal of Immunology, 2004, 173(8):5209-18*

- Dr. RK Nuttall performed the real-time PCR on my RNA samples resulting in Fig. 1 and 5. Dr. RK Nuttall also performed the *in situ* hybridization for TIMP-1 and MMP-10 on my tissue samples (Fig. 8). This took place in the laboratory of Dr. DR Edwards at University of East Anglia, UK.

## Chapter III

**Metalloproteinases Control Brain Inflammation Induced by Pertussis Toxin in Mice Overexpressing the Chemokine CCL2 in the Central Nervous System**

Henrik Toft-Hansen, Richard Buist, Angela Schellenberg, James Peeling, and Trevor Owens

*In revision for The Journal of Immunology*

- A Schellenberg and Dr. R Buist performed magnetic resonance imaging (Fig. 2A-C), and perfusion fixated the mice for further processing. This took place in the laboratory of Dr. J Peeling at The University of Manitoba.

## Chapter IV

**Regulation of Expression of Membrane-Type Matrix Metalloproteinases in Central Nervous System Inflammation**

Henrik Toft-Hansen, Alicia A. Babcock, Jason M. Millward, and Trevor Owens

- Dr. AA Babcock performed entorhinal cortex lesions, and prepared RNA samples used to generate part of Fig. 7. Dr. AA Babcock also did flow cytometric analysis on cultured microglia cells (Fig. 8A).

- JM Millward induced EAE in IFNγ-/- mice, and prepared RNA samples used to generate part of Fig. 9.

# CONTRIBUTIONS TO OTHER MANUSCRIPTS NOT INCLUDED IN THIS THESIS

Trevor Owens, Alicia A. Babcock, Jason M. Millward and Henrik Toft-Hansen
*Cytokine and chemokine inter-regulation in the inflamed or injured CNS*
Brain Research Reviews, 2005;48(2):178-84. Review

Alicia A. Babcock, Martin Wirenfeldt, Henrik Toft-Hansen, Jason Millward, Regine Landmann, Serge Rivest, Bente Finsen and Trevor Owens.
*Toll-like receptor 2 signalling in response to brain injury: an innate bridge to neuroinflammation*
In revision for The Journal of Neuroscience

# LIST OF ABBREVIATIONS

**ADAM**, a disintegrin and metalloproteinase; **APC**, antigen presenting cell; **AT-EAE**, adoptive transfer EAE; **BBB**, blood-brain barrier; **CCL2**, CC chemokine ligand 2; **CD**, cluster of differentiation; **CFA**, complete Freund's adjuvant; **CNS**, central nervous system; **CSF**, cerebrospinal fluid; **CT**, cycle threshold; **DC**, dendritic cell; **EAE**, experimental autoimmune encephalomyelitis; **EC**, entorhinal cortex; **ECL**, EC lesion; **ECM**, extracellular matrix; **FBS**, fetal bovine serum; **GFAP**, glial fibrillary acidic protein; **GPI**, glycosylphosphatidylinositol; **HBSS**, Hanks' balanced salt solution; **IFN**, interferon; **IL**, interleukin; **ip.**, intraperitoneally; **ISH**, in situ hybridization; **iv.**, intravenous; **LPS**, lipopolysaccharide; **MBP**, myelin basic protein; **MCP**, monocyte chemoattractant protein; **MHC**, major histocompatibility complex; **MMP**, matrix metalloproteinase; **MOG**, myelin oligodendrocyte glycoprotein; **MP**, metalloproteinase; **MRI**, magnetic resonance imaging; **MS**, Multiple sclerosis; **MT-MMP**, membrane-type MMP; **PAMP**, pathogen-associated molecular pattern; **PB**, prussian blue; **PBS**, phosphate buffered saline; **PCR**, polymerase chain reaction; **PFA**, paraformaldehyde; **PREMO**, as PTx-induced reversible encephalopathy dependent on MCP-1/CCL2 overexpression; **PRR**, pattern recognition receptor; **PTx**, pertussis toxin; **qPCR**, quantitative real-time PCR; **RECK**, reversion-inducing cysteine-rich protein with kazal motifs; **RNA**, ribonucleic acid; **RT**, reverse transcriptase; **SEM**, standard error of the mean; **sc**, subcutaneous; **SC**, spinal cord; **SCID**, severe combined immunodeficiency; **SPF**, specific pathogen free; **TACE**, TNFα convertase enzyme; **Tg**, transgenic; **TGF**, transforming growth factor, **TIMP**, tissue inhibitor of metalloproteinase; **TNF**, tumor necrosis factor; **TLR**, toll-like receptor; **TREM**, triggering receptor expressed by myeloid cells; **USPIO**, ultra small superparamagnetic iron oxide; **VLA**, very late antigen; **WT**, wild type

# CHAPTER I

# General Introduction

Multiple sclerosis (MS) is a neurological disease involving infiltration of immune cells to the central nervous system (CNS) as part of the neuroinflammatory pathology. Although the etiology of MS is unknown, the neuroinflammation is established as detrimental and is the target for all approved MS therapy. Metalloproteinases (MPs) are proteolytic enzymes with extracellular matrix protein substrates, and are implicated in cellular transmigration across the blood-brain barrier. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS well suited to investigate mechanisms of neuroinflammation.

In this thesis, the expression pattern of MP genes in EAE and other models of neuroinflammation will be described. Also, stages of CNS infiltration are defined and related to chemokine, cytokine and MP expression. Finally, the involvement of a pathogen-derived factor in neuroinflammation is described and related to MP expression.

## 1.1. Multiple Sclerosis

### *1.1.1. A neurological disease with autoimmune characteristics*

Clinical symptoms of MS were first described in the 1830's by Cruveilhier and Carswell, and defined as MS in 1868 By Charcot (1). The initial symptoms can be varied, but often include temporary paralysis, blindness or other neurological impairment. MS is rarely fatal but leads to progressing disability. Despite intensive research, MS is still an enigmatic disease with only limited treatment possibilities. MS is the most common neurological disorder among young adults with a prevalence of about 120 in 100,000 individuals in high-prevalence countries in Northern Europe and North America (2). The disease is characterized

by inflammation of the CNS, demyelination of nerve fibers and neurodegeneration. The cause of MS is unknown and there is no cure.

The prevailing hypothesis of the etiology of MS is that an autoimmune response is triggered by an environmental insult in genetically susceptible individuals and propagated within the CNS (3). The CNS can be considered the victim organ, which is invaded by pathogenic immune cells crossing the blood-brain barrier (BBB). CNS inflammation is a hallmark sign of MS, but it is debated where the immunological reaction that leads to neuroinflammation is initiated (4). Two main possibilities exist: The periphery, by some environmental insult, e.g. infection leading to an autoimmune reaction through molecular mimicry; or as a consequence of an underlying damage to endogenous cells of the CNS. It is generally believed that MS is an autoimmune disease, although it has been proposed that primary oligodendrocyte dysfunction is the precipitating event in MS pathogenesis (5). MS is not a uniform disease; rather it can follow different courses, with the majority of patients presenting with relapsing-remitting MS, and the remaining with primary progressive MS or a milder form of MS described as benign MS (6). Of patients with relapsing-remitting disease, 80% will develop secondary progressive MS within 10 years. (7). The pathology of MS is also heterogeneous (8), so it is possible that MS has several etiologies, all leading to related symptoms which are classified as various forms of MS. Given the involvement of both an inflammatory and neurodegenerative component in MS pathogenesis (9), a logical theory would be that relapsing-remitting MS predominantly is a consequence of neuroinflammation, whereas the progressive pathology is more a consequence of neurodegeneration (10).

### *1.1.2. Genes and environment*

MS is primarily a disease of the temperate areas of the world, with prevalence increasing with the distance from equator. The curious geographical distribution of MS has not been adequately explained, but might have its reason in environmental factors and/or in demographics, with descendants of northern European ancestry having the highest risk presumably for genetic reasons (11,

12). Perhaps exposure to an environmental agent in childhood is involved in the etiology, as children, who move before puberty adopts the prevalence of their new residence, whereas adults, who move after puberty keep the prevalence of their original residence (13). Despite long-standing efforts to identify a causal environmental agent of MS, the search has so far been fruitless (14).

More than 30 years ago, it was demonstrated that there is convincing genetic linkage of MS to regions within the MHC complex (15). In spite of considerable effort, the search for other genetic markers of MS has not yielded significant new information, other than that regions within both MHC class I and class II confer MS risk (16). This does, however, give credence to the hypothesis of an autoimmune etiology of MS, or, at least, a strong immune involvement in the MS pathogenesis. The inability to identify a single, or even a few, factors as critical in MS etiology has led to the consensus that MS is a multifactorial disease, where genes, environment and stochastic factors play a role. Epidemiological studies clearly illustrate the involvement of both genetic and environmental parameters in the etiology of MS. The concordance rate for homozygotic twins was found to be 24% in Denmark (17), considerably higher than for dizygotic twins (3%), showing that genotype is important. However, since genetic risk is significantly less than 100%, genotype cannot account fully for the occurrence of disease; environmental cues must also be important. Those environmental cues must work on a population rather than individual basis, as there is no evidence of higher risk of MS in a step-sibling of an MS patient (18).

## 1.2. Experimental Autoimmune Encephalomyelitis

### *1.2.1. An autoimmune animal disease model of MS*

EAE is an animal model of MS. It is an autoimmune neuroinflammatory disease, which is most commonly studied in rodents. EAE can be generated actively by immunization of animals with myelin antigens in the presence of adjuvant, or passively by adoptive transfer of myelin specific T cells from immunized animals (19, 20). The adjuvants most commonly used are complete Freund's adjuvant

(CFA) and PTx. CFA contains heat-inactivated *Mycobacterium tuberculosis* and is used for immunizations in an emulsion given sc. PTx is a toxin derived from the bacterium *Bordetella pertussis*, the causative agent of whooping cough, and is administered ip. or iv. The adjuvants are thought to substitute for potential critical environmental agents in the disease pathogenesis. These environmental agents are likely microbial pathogens, and exacerbations of MS have been associated with microbial infections (21, 22).

An adaptive immune system in recipient mice after adoptive transfer of myelin specific T cells is not needed, since EAE can be transferred to SCID mice (23). Secondary lymphoid organs in recipient mice are also not needed, when EAE is passively transferred (24). The pathology of EAE resembles MS in many aspects, including cellular infiltration of the CNS, loss of BBB integrity, demyelination and axonal damage, and neurological symptoms including paralysis. However, the value of EAE as a model of MS has been called into question, based on the fact that it is a pure autoimmune disease, and therefore might not represent other aspects of MS pathology (25). Nevertheless, neuroinflammation involving autoreactive T cells is an important part of MS pathogenesis, and that particular aspect is modeled very well by EAE. The fundamental process of leukocyte entry to the CNS is shared by MS and EAE, regardless of the underlying reason.

## 1.3. Neuroinflammation

### *1.3.1. The neurovascular unit*

The BBB was originally defined based on exclusion of macromolecules from the CNS (26). This exclusion is based on the existence of a specialized endothelium with tight junctions in the CNS, and possibly also on paucity of vesicle transport through CNS endothelial cells. The mechanisms behind cell trafficking to the CNS are more complicated than transport of macromolecules, and it is not meaningful to only apply the diffusion-based definition of the BBB with regards to cellular infiltration. Other structures surrounding CNS vessels need to be taken

into account, and in recent years, many investigators have broadened the definition of the BBB to also include the astrocyte endfeet and associated perivascular cells of myeloid origin making up the glia limitans (27-29). The term "neurovascular unit" has been coined to include all these components (30). To prevent confusion, it seems logical to define the BBB simply as the diffusion barrier composed of specialized endothelium, which is meaningful to study in the context of accessibility of molecules, e.g. drugs, to the CNS. In the context of cellular migration into the CNS, all the structures surrounding CNS vessels, i.e. the neurovascular unit, should be considered.

### *1.3.2. Stages of CNS infiltration*

Two separate steps can be identified in CNS infiltration. The leukocytes first cross the endothelial barrier and then the glia limitans (29). Cells associated with the BBB, particularly astrocytes and perivascular antigen presenting cells (APCs), might be actively involved in MS pathogenesis by regulating leukocyte migration across the BBB. In particular, perivascular APCs might regulate the infiltration of T cells across the glia limitans by reactivating them in the perivascular space through presentation of myelin antigens. The tight junctions have long been thought to constitute the main barrier for migrating cells in CNS infiltration. This might not actually be the case since cells crossing CNS venules can do so next to tight junctions, rather than between cells (31). Between the endothelial basal lamina and the glia limitans is the perivascular space, which is usually not distinguishable, but becomes enlarged and filled with infiltrating cells during neuroinflammation; for example in EAE (29). Entering the perivascular space, however, is not equivalent to entering the CNS parenchyma (32, 33). The glia limitans, mainly composed of astrocyte endfeet, must also be crossed. This distinction is important, as the presence of leukocytes in the perivascular space is not associated with clinical symptoms (34-36). An important technical consideration is that flow cytometry analysis is unable to distinguish between cells in the perivascular space and the parenchyma, since the perivascular space is non-perfusable.

### *1.3.3. Regulation of CNS infiltration*

The endothelial cells, pericytes, perivascular myeloid cells and astrocytes are the first cell types an infiltrating cell encounters on its way to the brain parenchyma. These cells are situated on the border between the CNS and the periphery and are therefore ideally situated to regulate CNS infiltration. Microglia are the resident CNS antigen presenting cells (APC) found dispersed throughout the parenchyma. They share myeloid lineage with macrophages. Microglial activation involves a change in morphology, and upregulation of molecules involved with antigen presentation as well chemokines and proinflammatory cytokines, such as TNFα, IL-1β and IFNγ. It has long been discussed whether activation of microglia is advantageous or detrimental in the context of neuroinflammation (37, 38), but recent results favor a detrimental role, as prevention of microglial activation curbed EAE symptoms (39).

There is currently much focus on APCs other than parenchymal microglia of the CNS. These APCs are found in the perivascular space and defined as perivascular microglia, macrophages or dendritic cells (DCs) based on surface markers. The perivascular APCs have a higher turn-over rate than parenchymal microglia, and they can thereby be distinguished in bone marrow chimeras, where the perivascular space is repopulated with APCs of donor origin (40). The perivascular position of these APCs make them ideally suited for interacting with and stimulating infiltrating lymphocytes. Indeed, antigen presentation by APCs in the perivascular space may be necessary for parenchymal infiltration (24, 41-43), and perivascular APCs were also found to be critical for epitope spreading in EAE (44).

### *1.3.4. Blocking cellular entry to the CNS*

*In vitro* models of the BBB have been used experimentally for over 30 years (45). Using such models, it has been shown that transmigration is regulated by various adhesion molecules, which participate in a reorganization of the endothelial cell structure to permit migration (46). Much of the current development in

therapeutic management of MS is focused on targeting the transmigration of leukocytes by blocking adhesion molecules on either the endothelial cells or the leukocytes. One of the most promising new MS drugs (natalizumab) is based on blocking such an adhesion molecule, namely VLA-4 (also called $\alpha 4\beta 1$ integrin), an integrin expressed on lymphocytes, thereby preventing lymphocytes from attaching to and migrating across endothelial cells in the CNS. Blocking VLA-4 efficiently prevented or reversed EAE (47-49), and promoted recovery from symptoms (50). Treatment with natalizumab reduced relapses in MS patients (51, 52), but it was withdrawn from clinical use after three patients developed severe side effects in form of progressive multifocal leukoencephalopathy (PML) (53-55). This could imply that natalizumab had prevented normal immune surveillance of the CNS with detrimental consequences (56), and would stress the need for developing treatment strategies that will only target harmful elements of leukocyte interaction with the BBB. However, alternative explanations of the link between VLA-4 blockade and development of PML have been pointed out, e.g. that VLA-4 blockade can have adverse effects on the development of immune cells in the bone marrow (57).

Despite problems with unforeseen side effects, the remarkable efficacy of natalizumab in reducing MS relapse by 66% is very encouraging (47). It is proof of principle, that limiting access of harmful immune cells to the CNS is of substantial benefit to MS patients. The obvious challenge is to achieve this benefit therapeutically without disrupting normal and essential physiological mechanisms of cellular interaction and traffic. Another strategy to therapeutically manipulate access of leukocytes to the CNS is to target the proteinases which mediate transmigration across the neurovascular unit.

## 1.4. Metalloproteinases

### *1.4.1. A superfamily of proteinases*

The MMPs belong to the group of Metzincin MPs which also include astacins, serralysins and ADAM families (58). In total there are eight groups in the

superfamily of MPs, giving a total number of more than 200 proteinases in the superfamily. There are 29 mammalian ADAMs of which 17 have the consensus MP catalytic site. They all have adhesion and transmembrane domains and are thought to function primarily as membrane-tethered sheddases (59). Some ADAMs are specifically expressed in testis, but many are expressed in the CNS (60). Like MMPs, ADAMs can cleave ECM molecules, but are likely to also directly modulate cellular interactions. Importantly, broad-spectrum inhibitors of MPs, which initially were developed to inhibit MMPs, are just as efficient in inhibiting ADAMS with the consensus catalytic site, since the inhibitors are peptidomimetics which mimic the characteristic substrate sequence recognized by the MP catalytic site.

Genes for MMPs are not only present in mammals, but are conserved in the evolution of a large variety of species. MMP genes are found in flies (61), nematodes (62), and plants (63). The first MMP was described in 1962 as a collagenolytic enzyme present in tadpole tail and involved with tissue morphogenesis (64). Today, 25 vertebrate MMP genes are described and numbered from 1-28, excluding number 4 to 6 (65). The original MMPs 4 to 6 were subsequently found to be identical to MMP-2 or 3. MMP-18 is considered to be specific for Xenopus. MMP-26 is absent in mice, and MMP-22 is absent in both mice and humans. Mice have two forms of MMP-1 and humans have two forms of MMP-23. Thus, the total number of MMP genes is 24 for humans and 23 for mice. It is considered unlikely that more MMP genes will be discovered in mice and humans as both genomes have been extensively searched.

### *1.4.2. MMP substrates*

MMPs are classically known to degrade ECM proteins. This is based on *in vitro* studies where substrate specificities were determined under optimal conditions. This approach has led researchers to focus on ECM substrates, which MMPs are able to cleave *in vitro*, rather than potential other substrates *in vivo*. Only recently has there been an appreciation of the various other roles of MMPs. It may even be that ECM proteolysis is not the main role of MMPs in tissues (66). A very

important and interesting area of MMP research is to identify *in vivo* substrates, which will enable a more insightful interpretation of the consequence of changes in expression level and activity of MMPs (67).

### *1.4.3. Structure of MMPs*

MMPs can be grouped based on substrate specificity determined in *in vitro* studies, or based on domain structure. The latter distinction seems most appropriate, since there is considerable specificity overlap, and substrates may be different *in vivo* (68). MMPs have a common 3-dimensional structure and a common gene arrangement which indicate that they evolved by duplication from a common ancestor gene (69).

There are several characteristic domains in MMPs. All members share a conserved pro-domain and catalytic domain. The catalytic domain contains three histidine residues binding zinc at the catalytic site, and a conserved methionine that is positioned beneath the catalytic site (70). The prodomain ensures that MMPs in general are expressed as inactive zymogens in order to protect against excessive proteolysis. The prodomain contains a cysteine residue that forms a thiol bond with the zinc in the catalytic site of the MMP in a structure called the cysteine-switch (71, 72). Removal of the prodomain by another protease disrupts the cysteine switch and activates the MMP. Removal of the prodomain is not strictly necessary for MMP activation. Breaking the thiol bond in the cysteine switch by other means will also cause activation, and this can be done chemically, for example by oxygenation by leukocyte-derived oxidants (73-75). A hinge-region connects the catalytic domain with the hemopexin-like domain found in most MMPs. The hemopexin-like domain is involved in protein-protein interactions, and is thought to confer substrate specificity to MMPs (76, 77).

Six MMPs are membrane-bound (MMP-14, 15, 16, 17, 24, and 25) and named membrane-type MMPs (MT-MMPs). Four MT-MMPs are type-1 transmembrane proteins with cytoplasmic domains. The remaining two MT-MMPs (MMP-17 and 25) are anchored to the cell surface by a glycosylphosphatidylinositol (GPI) structure. The cytoplasmic domain of

transmembrane MT-MMPs has been proposed to be important for the activity of MT-MMPs by facilitating dimerization, clustering, and internalization (78-81). Binding to intracellular ligands could also be an important function of the cytoplasmic domain of MT-MMPs (82).

### *1.4.4. Regulation of MP activity*

MMP activity is regulated at five points: Gene transcription, activation, compartmentalization, enzyme inhibition and internalization. MMPs are generally not expressed at high levels in normal healthy tissue, but are upregulated in tissue undergoing repair, remodeling, inflammation, or in a diseased state (66). The induction of MMPs is mainly controlled by chemokines and cytokines; chemokines attract MMP-producing cells, and cytokines regulate transcription of MMPs from both infiltrating and endogenous cells (83). Once MMPs are present in a tissue, they are activated in a complicated interplay with other MMPs, other proteinases, or oxidants. In some cases, the need for extracellular activation can be bypassed by intracellular cleavage of the prodomain. A furin recognition domain makes it possible for intracellular proteases like furin to cleave the prodomain off in the trans Golgi network (84). All six MT-MMPs have a furin recognition domain. This is only the case for three other MMPs: MMP-11, 23 and 28. Furin cleavage can bring an MMP to the cell surface in the activated state, as has been reported for MMP-14 (85). To prevent undirected proteolysis, MMPs can be compartmentalized on the cell membrane. This is naturally the case for MT-MMPs, but also secreted MMPs are often confined to the pericellular environment (86-88). For example, MMP-2 binds the $\alpha v\beta 3$ integrin, and MMP-9 binds the hyaluronan receptor CD44 (89). This prevents diffusion of MMPs and ensures that substrates are cleaved in the vicinity of the cell surface, where receptors might bind the cleaved product.

Naturally, proteolysis by activated MPs must to be controlled in order to prevent excessive tissue damage. The activity of all MMPs in tissue is controlled by tissue inhibitors of MPs (TIMPs), of which four are described. Generally, all

secreted MMPs are inhibited to various degrees by all four TIMPs, but TIMP-1 is a poor inhibitor of most MT-MMPs, whereas TIMP-2 and the membrane-associated TIMP-3 are efficient inhibitors of MT-MMPs (90). All TIMPs are expressed in the CNS, where their expression is controlled by cytokines including IFNγ and IL-1β (91). In serum, the main inhibitor of MPs is α2-macroglobulin (α2M), which cause irreversible clearance of a range of proteinases (92). A membrane-bound inhibitor reversion-inducing cysteine-rich protein with kazal motifs (RECK) can also control MP activity (93, 94). Another way of inactivating MMPs is by internalization after binding to scavenger receptors (95).

### *1.4.5. MP-based therapy*

Most of the knowledge about MPs comes from cancer research, where MPs have been investigated since their discovery. MPs are thought to promote tumor development in a variety of ways involving invasiveness, angiogenesis, proliferation and cell survival (96). MT-MMPs may promote tumor growth or spreading in other ways than directly cleaving ECM proteins. MMP-14 can for example cleave cell surface molecules which bind matrix molecules, as has been shown for CD44 (97). This releases cells from attachment to the surrounding matrix and increases migratory capacity. Despite success in animal models, broad-spectrum inhibitors of MPs were not effective in cancer trials (98). Furthermore, they caused significant side-effects, mainly nausea and joint pain. There is hope, however, that more specific MP inhibitors would be beneficial for treatment of cancer patients (99, 100). The only drug approved for clinical use based on its action as an MP-inhibitor is the tetracycline-derivative periostat. It is used to treat periodontitis (101). Other drugs, such as interferon-β (102, 103), used in MS, or minocycline (104) (another tetracycline-derivative) used in treatment of acne, also inhibit MMPs, but that was not the rationale behind their introduction.

MPs are thought to generally be pro-inflammatory given their ability to facilitate migration of inflammatory cells across barriers and within tissues.

Contrary to the general picture, there are indications that some MMPs have divergent actions in arthritis (105, 106). This reinforces the need to define the actions of individual MMPs better in order to design rational therapies aimed at only inhibiting detrimental actions of MMPs in certain disease states.

### *1.4.6. Metalloproteinases in the CNS*

Since 1980, it has been known that proteinase inhibitors can curb EAE (107), and MMPs are increasingly being recognized as important in neuroinflammation (65, 89, 108). The serum and CSF levels of several MMPs are increased in MS patients, and can be correlated to attacks (109, 110). Specifically, the MMP-9/TIMP-1 ratio has been proposed to be diagnostic of attacks (111). MPs can cleave myelin and thereby cause demyelination and axonal injury (112, 113), and possibly also generate encephalitogenic peptides which are involved in the autoimmune response (114, 115). More recently, it has been recognized that actions of MPs in the CNS might also be beneficial (65). Several MPs and TIMPs are expressed in the developing CNS (116), and MMPs are involved in neurogenesis (117), axonal growth (118), as well as neurogenic migration after stroke. Oligodendrocyte process formation also involves MMPs (93, 119), and myelination in ontogeny is impaired in MMP-9 and 12 deficient mice (120). Sprouting following neuronal injury is dependent on MPs (121). In the peripheral nervous system, Wallerian degeneration of transected nerves mediated by MPs is a prerequisite to repair (122).

## 1.5. Cytokines, Growth factors, Chemokines, and the MPs

### *1.5.1. Cleaving bioactive molecules*

Among the recently described non-ECM substrates of MMPs are bioactive molecules, such as cytokines and chemokines, implicating a regulatory role for MMPs (123, 124). Growth factors can also be cleaved or released from the matrix by MMPs (125), and MMPs can via that mechanism be involved in cell survival

by influencing availability of these factors. MMPs are also able to induce apoptosis in an indirect way by a process termed anoikis (homelessness). This implies the detachment of cells from their normal ECM supporting structure leading to cell death, presumably from lack of integrin signaling (126). Other ways for MPs to influence neuronal survival is by cleaving the neurotrophins NGF and BDNF (127), or cleaving FasL (CD178), the ligand for the death receptor Fas (CD95). FasL is important in the protection of the CNS from reactive T cells in a process of astrocyte-induced apoptosis (128). MMP3 can cleave FasL to diminish Fas-FasL interaction on the surface of neurons in culture, thereby protecting them from Fas induced apoptosis (129).

### *1.5.2. Cytokine substrates*

The proinflammatory cytokine IL-1β can be activated in a caspase-1 independent way by three MMPs (130), and also inactivated by MMP action (131). The anti-inflammatory cytokine TGFβ can likewise be activated by MMPs (87, 132), proving the point of there being both pro and anti-inflammatory roles of MMPs. The conversion of membrane-bound TNF to soluble TNF is a well known example of MP activation of a cytokine (133, 134). The conversion is carried out by TACE which is also known as ADAM-17. Mice deficient in ADAM-17 are non-viable, indicating the importance of this specific MP, even though other MMPs (MMP-17, 7 and 12) have been shown to be able to shed TNF, albeit with lower efficiency than ADAM-17 (135-137).

### *1.5.3. Chemokine substrates*

Chemokines are small chemoattractant cytokines with the ability to direct cellular migration. There are more than 50 chemokines described, and more than 18 receptors. The receptors have seven transmembrane domains and are G-protein coupled. Chemokines control leukocyte trafficking into the CNS in neuroinflammation (138). Activated glial cells upregulate chemokine production, and chemokine levels are elevated in both MS and EAE (139). MMPs can control

the activity of chemokines by either activating or inactivating them (140-143), or by cleaving other substrates that interact with chemokines (144-146), such as receptors (147). MMP-9 inhibits activity of several chemokines (143), whereas it increases the activity of IL-8 (142). All four human forms of monocyte chemoattractant protein (MCP): MCP-1 to 4, can be cleaved and inactivated by recombinant MMPs *in vitro*. Of 8 MMPs tested, MMP-1 and 3 could cleave MCP-1 (CCL2 in modern nomenclature) to produce a truncated form with anti-inflammatory capacity *in vivo* (141). By cleaving ECM proteins with attached chemokines, MMPs can create gradients of chemokines which then direct migration of inflammatory cells as has been demonstrated for MMP-7 (145, 148), and MMP-2 (144, 149).

### *1.5.4. CCL2/MCP-1*

Several studies led to the conclusion that CCL2 (formerly MCP-1) is a critical factor in promoting cellular entry to the CNS. Migration of leukocytes to sites of axonal degeneration following transection was significantly curbed in mice deficient for the CCL2 receptor CCR2 (150). Likewise, CCR2 deficient mice are resistant to EAE (151), just as CCL2 deficient mice show significantly milder disease symptoms in EAE (152). CCL2-deficient T cells were capable of transferring EAE to recipient WT mice whereas WT T cells were unable to transfer disease to CCL2 deficient recipients. From another series of adoptive transfer EAE experiments, it was concluded that expression of the CCL2 receptor CCR2 on recipient mononuclear cells is critical for induction of disease (153). Hence, in order to induce EAE, both CCL2 expression in the CNS and expression of the receptor CCR2 on mononuclear cells in the periphery are essential. A recent study showed that transgenic low-level expression of CCL2 in the CNS of mice resulted in protection against EAE (154), apparently due to a weak Th1 autoimmune response.

Transgenic expression of CCL2 under control of a truncated MBP promoter led to perivascular accumulation of leukocytes, without parenchymal infiltration and no clinical pathology (34). In contrast, when the neutrophil-

attracting chemokine CXCL1 was expressed under control of the MBP promoter, the transgenic expression resulted in parenchymal infiltration as well as perivascular accumulation with leukocytes (155). Furthermore, the CXCL1 Tg mice show spontaneous neurological syndromes starting about 40 days after birth. In mice with astrocyte-directed transgenic expression of CXCL10 there was spontaneous accumulation of leukocytes with no evident changes in brain MMP expression (156). The leukocyte accumulation in these CXCL10 Tg mice could be potentiated by administration of CFA and PTx, but no overt pathology was observed.

The transgenic expression of CCL2 in the CNS was found to increase infarction volume as well as cellular brain infiltration following ischemic brain injury (157). Also, CCL2 Tg mice were more sensitive than WT mice to effects of LPS injections. Administration of the broad-spectrum synthetic MP-inhibitor BB-94 reduced ischemic lesion volumes (158). Likewise, in a study of hippocampal neuronal damage following ischemia, MMP-2 and 9 activity was shown to be increased in the hippocampus (159). Treatment of mice with BB-94 protected against damage of hippocampal neurons.

## 1.6. Pertussis Toxin

### *1.6.1. Effects of a microbial product on neuroinflammation*

PTx is used as adjuvant to induce EAE as discussed in section 1.2.1., but the mechanism behind the adjuvant effect of PTx has not been clearly identified. In a study measuring the effect of PTx on BBB permeability *in vitro* it was shown that PTx inhibits T cell migration across brain endothelial cells by blocking G protein signaling (160). The G-protein coupled receptor is likely to be a chemokine receptor, as PTx can inhibit chemokine receptors and is routinely used in *in vitro* assays as a chemokine receptor inhibitor (161). Other studies have proposed that PTx increases the permeability of the BBB *in vivo* (162, 163). It could seem that PTx has opposing effects on the permeability of the BBB. Depending on the time of administration, PTx can have both enhancing and inhibitory effects in the

context of inducing autoimmune disease (164). PTx consists of two subunits, A and B, of which B will bind to cell surfaces followed by insertion of the A subunit into the cell cytoplasm. Su an colleagues found that the A subunit of PTx is responsible for the inhibition of G-protein signaling and the B subunit was the disease-inducing part of PTx. Administration of the B subunit alone was equivalent to whole PTx in inducing autoimmune disease in mice, most likely by promoting a Th1-polarized innate immune response. Possibly, PTx does inhibit migration by inhibiting chemokine receptor signaling, but at the same time promotes migration by upregulating adhesion molecules on brain endothelial cells, such as P-selectin, as has been demonstrated in work by Kubes and colleagues (165). Likely, PTx could also promote brain infiltration through an effect on leukocytes in the blood-stream by causing them to upregulate expression of effector molecules such as MPs. Potentially, PTx interacts with leukocytes by binding to innate immune receptors, e.g. TLR4 as suggested (165).

# HYPOTHESIS AND OBJECTIVES

Some members of the large family of MPs promote neuroinflammation, whereas other MPs are neutral or have protective roles. Gaining detailed knowledge about the expression pattern of individual MPs in neuroinflammation will aid towards designing a rational therapy based on targeting specific MPs at specific stages of CNS infiltration as occurs in both MS and EAE.

Hypothesis: CNS infiltration can be divided in stages based on location of infiltrating cells in relation to different components of the neurovascular unit: the endothelial barrier and the glia limitans. Leukocyte crossing of the endothelial barrier resulting in perivascular infiltration is associated with a different profile of MP expression compared to parenchymal infiltration after crossing of the glia limitans.

The objectives of this thesis are:

Chapter 2: to study expression profiles of MPs in EAE and identify cellular sources of selected MPs.

Chapter 3: to investigate regulation and activity of MP expression in different stages of PTx-driven CNS infiltration.

Chapter 4: to investigate regulation of MT-MMPs in EAE and other models of neuroinflammation.

# CHAPTER II

## Key Metalloproteinases Are Expressed by Specific Cell Types in Experimental Autoimmune Encephalomyelitis

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## 2.1. Abstract

Metalloproteinases (MPs) include matrix metalloproteinases (MMPs) and metalloproteinase-disintegrins (ADAMs). Their physiological inhibitors are tissue inhibitor of metalloproteinases (TIMPs). MPs are thought to be mediators of cellular infiltration in the pathogenesis of Multiple Sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE).

We used real-time RT-PCR to profile expression of all 22 known mouse MMPs, seven ADAMs and all four known TIMPs in spinal cord from SJL/J mice and from mice with adoptively transferred Myelin Basic Protein (MBP)-specific EAE. A significant and greater than 3-fold alteration in expression was observed for MMP-8, MMP-10, MMP-12, ADAM-12 and TIMP-1, which were upregulated, and for MMP-15, which was downregulated. Expression levels correlated with disease course with all but ADAM-12 returning towards control levels in remission.

To examine potential cellular sources of these strongly affected proteins in the inflamed central nervous system (CNS), we isolated macrophages, granulocytes, microglia, and T cells by cell sorting from the CNS of mice with EAE, and analyzed expression by real-time RT-PCR. This identified macrophages as a major source of MMP-12 and TIMP-1. Granulocytes were a major source of MMP-8. ADAM-12 was expressed primarily by T cells. Cellular localization of MMP-10, TIMP-1 and ADAM-12 in perivascular infiltrates was confirmed by immunostaining or *in situ* hybridization (ISH). Microglia from control mice expressed strong signal for MMP-15. Strikingly, the expression of MMP-15 by microglia was significantly downregulated in EAE, which was confirmed by immunostaining.

Our study identifies the cellular sources of key MPs in CNS inflammation.

## 2.2. Introduction

Leukocyte infiltration of the CNS is a hallmark of Multiple Sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), a widely used animal model for MS. To infiltrate the CNS parenchyma, leukocytes must migrate across the blood-brain barrier (BBB), which, under normal circumstances, restricts the entrance of cells and large molecules into the CNS. Disruption of the integrity of the BBB accompanies the extensive cellular infiltration and pathology seen in MS and EAE and is assumed to be a pre-requisite for disease.

The functional BBB includes endothelial cells joined by tight junctions and a basement membrane made up of extra-cellular matrix (ECM) proteins such as laminin, collagen and fibronectin. Leukocyte extravasation to CNS parenchyma is facilitated by proteolysis of ECM proteins carried out by a family of $Zn^{++}$ and $Ca^{++}$ dependent proteinases called matrix metalloproteinases (MMPs) (also designated matrixins). There are at least 25 MMPs (22 identified in mice), which, between them, can degrade virtually all components of the ECM (68). The MMPs have therefore attracted attention as potential critical mediators of diseases involving inflammation of the CNS, including MS (89, 108), as well as in other diseases where degradation of ECM is part of the pathogenesis, such as in cancer metastasis and rheumatoid arthritis (166). Besides their role in ECM proteolysis, MMPs can also act as signaling molecules and regulators of cell fate by shedding or cleaving adhesion molecules, growth factors, chemokines and cytokines (123, 124)

Metalloproteinase-disintegrins (ADAMs) (also called adamalysins) are another family of metalloproteinases, which potentially can act as proteases, signaling molecules and regulators of cell fate. They comprise more than 30 members with diverse actions (167-169). There are indications that ADAMs are involved in regulating inflammatory processes, as exemplified by ADAM-17, which can cleave membrane-bound TNF-α leading to shedding of the more pro-

inflammatory soluble TNF-α (133); hence the synonym for ADAM-17 is TNF-α converting enzyme (TACE).

Tissue inhibitors of metalloproteinases (TIMPs) are physiological inhibitors of metalloproteinases. There are four known TIMPs and they inhibit metalloproteinases by binding to the catalytic site in a 1:1 stoichiometry. No comprehensive study of the binding of all four TIMPs to all MMPs and ADAMs has been undertaken, but some generalizations can be made. TIMPs generally inhibit all MMPs to various degrees, with the exception that TIMP-1 is unable to inhibit MMP-14, 15, 16 and 24 (92). These are four of the 6 membrane-bound-MMPs, the remaining two being MMP-17 and MMP-25. The ADAMs show a more restricted pattern of inhibition by TIMPs with only few ADAMs being inhibited by TIMPs (170).

Administration of synthetic broad-spectrum MP-inhibitors alleviated symptoms of EAE, presumably by curbing leukocyte infiltration (171-173). However, in addition to their detrimental roles, MPs might also have beneficial effects in MS. For example, MMPs are suggested to be involved in repair processes such as remyelination, as indicated by the finding that MMP-9 is necessary for outgrowth of oligodendrocyte processes (93). The use of current generation broad-spectrum MP-inhibitors would inhibit such beneficial actions of MPs as well as their detrimental actions. It is likely that inhibition of specific MPs, or MPs produced by specific cell types at specific times in the pathogenesis of MS, would be of greater benefit than using broad-spectrum inhibitors indiscriminately. Detailed knowledge of the MP and TIMP expression profiles by the cell types involved in CNS infiltration is required to enable such approaches. Until now, only a limited number MMPs, ADAMs and TIMPs have been studied in MS or EAE.

In this study, we profiled expression of all 22 known mouse MMPs, all four known TIMPs as well as seven ADAMs in spinal cord from un-manipulated control mice and from mice with EAE at peak disease, using quantitative real-time RT-PCR. The seven ADAMs chosen all have a putative functional protease domain and are not restricted to expression in the testis unlike other members of

this family (168, 169). We find a significant and greater than three-fold upregulation in expression of MMP-8, MMP-10, MMP-12, ADAM-12 and TIMP-1, and a significant and greater than three-fold downregulation of MMP-15. We have identified macrophages as a major source of MMP-12 and TIMP-1, and granulocytes as a major source of MMP-8. None of the cell types we studied by PCR emerged as a single major source of MMP-10, but low-level expression by multiple infiltrating cell types was observed by ISH. Unmanipulated microglia expressed MMP-15, and this MMP-15 expression by microglia was significantly downregulated in EAE. ADAM-12 was found to be expressed almost exclusively by T cells.

## 2.3. Materials and Methods

Adoptive transfer EAE

Female SJL/J mice (6–8 weeks old) were purchased from Jackson Laboratories, Bar Harbor, ME, USA. EAE was induced by passive transfer of MBP-reactive T cells. Donor mice were immunized sc. at the base of the tail with 100 µl of an emulsion containing 400 µg MBP (Sigma, Oakville, Ontario, Canada) and 100 µg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, Michigan, USA) in Freund's Incomplete Adjuvant (Difco) and boosted in the flanks 7 days later with the same amount. A single cell suspension was prepared from the draining lymph nodes 14 days after the first immunization by passing lymph nodes through a 70 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA), and cells ($4x10^6$/ml) were cultured in the presence of 50 µg/ml MBP in RPMI 1640 (Gibco, Burlington, Ontario, Canada) supplemented with 10% FCS (Sigma), 50 µM µ-mercaptoethanol (Sigma), 2 mM L-glutamine (Gibco) and 100 U/ml Penicillin-Streptomycin (Gibco). After 4 days in culture, cells were collected by centrifugation on Ficoll-Paque (Amersham Biosciences, Baie d'Urfe, Quebec, Canada), and $4x10^6$ lymphoblasts were injected into the tail vein of naive mice. MBP reactivity of the lymph node cells was measured by $^3H$ Thymidine (ICN

Biomedicals, Irvine, CA, USA) incorporation assay. Following transfer of MBP-reactive cells, mice were weighed and monitored daily for clinical signs of EAE, scored as: 1, flaccid tail; 2, hind limb weakness and poor righting ability; 3, one hind limb paralyzed; 4, both hind limbs paralyzed with or without forelimb paralysis and incontinence; 5, moribund. All mice with EAE included in this study were sacrificed at peak disease (grade 4). Mice were kept in an SPF environment. Animal maintenance and all experimental protocols were in accordance with Canadian Council for Animal Care guidelines and approved by McGill University Animal Care Committee.

Isolation of RNA

For spinal cord analysis, mice were anaesthetized with a lethal dose of Somnotol (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and intracardially perfused with ice-cold PBS followed by removal of the spinal cord. Total RNA was purified using Trizol RNA isolation reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol for whole tissue RNA extraction. For sorted cells, Trizol was used according to the protocol for RNA extraction for low amounts of RNA.

Cell sorting

Mice were anaesthetized with a lethal dose of Somnotol (MTC Pharmaceuticals) and intracardially perfused with 20 ml ice-cold PBS. Brain and spinal cord were collected and brought into a single cell suspension by passing through a 70 μm cell strainer (Becton Dickinson). After centrifugation with 37% Percoll (Amersham Biosciences), the myelin was removed and cells resuspended in buffer containing rat IgG to block non-specific antibody binding. The cells were stained with antibodies as indicated in figure legends. CyC-conjugated streptavidin and the antibodies PE-conjugated anti-CD45, biotin-conjugated anti-CD3, FITC-conjugated anti-Mac-1/CD11b and anti-Gr-1 were purchased from BD-Pharmingen, San Diego, CA, USA. Cells were isolated using a Becton-Dickinson FACSVantage cell sorter (Becton Dickinson Immunocytometry

Systems, San Jose, CA, USA) and flow cytometry data were analyzed using CellQuest software.

Reverse transcriptase reaction

Prior to real-time PCR analysis, total RNA was subjected to a reverse transcriptase (RT) protocol. 3 μg RNA from each spinal cord sample was incubated with M-MLV RT (Invitrogen Life Technologies) according to manufacturer's protocol using random hexamer primers. RNA from sorted cells was incubated with SuperScript II RT (Invitrogen Life Technologies) according to manufacturer's protocol using both oligo (dT) and random hexamer primers, and glycogen as carrier.

Quantitative real-time PCR

For spinal cord analysis and for analysis of T cells and myeloid cells, qPCR was done using the ABI Prism 7700 Sequence Detection System according to previously described methods (174). Quantitative real-time PCR (qPCR) was performed for all mouse MMP and TIMP genes (primer and probe sequences in (175) and several mouse ADAMs (see Table I for primer and probe sequences). 18S rRNA (primers and probes from Applied Biosystems, Paisley, UK) was used as an endogenous control to account for differences in the extraction and reverse transcription of total RNA.

For analysis of sorted cells, qPCR was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA, USA). Each reaction was performed in 25 μl with 50% TaqMan 2x PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 100 nM each of the forward and reverse primer, and 200 nM of probe. cDNA was diluted 1:1000 for 18S rRNA analysis, which served as an endogenous control (18S rRNA primers and probes from Applied Biosystems, USA). MMP-8, MMP-10, MMP-12, MMP-15, TIMP-1 and ADAM-12 primers were synthesized by Sigma. Corresponding probes were synthesized at Applied Biosystems, USA. Sequences for these primers and probes (175) are identical to the primers and probes used for spinal cord analysis (Table I). Conditions for the PCR were 2 min

at 50°C, 10 min at 95°C, and then 40 cycles, each consisting of 15 s at 95°C, and 1 min at 60°C. To determine the relative RNA levels within the samples, standard curves for the PCR were prepared by using the cDNA from one sample and making 4-fold serial dilutions (8-fold dilutions for 18S rRNA). Standard curves for CT (cycle threshold; the cycle at which the detected signal became significantly different from background signal) versus arbitrary levels of input RNA were prepared, and relative levels of mRNA in each sample were determined. CT values were verified to be in the linear amplification range on the appropriate standard curves.

*In situ* hybridization

Full length murine TIMP-1 cDNA was cloned into the pBluescript KS(-) plasmid (Stratagene, La Jolla. CA) as previously described (176). The murine MMP-10 cDNA was amplified from mouse brain tissue by RT-PCR using the 5' primer: GCC CAG CTA ACT TCC ACC TTT and the 3' primer: GAG AGT GTG GAT CCC CTT TGG. The resulting 400 bp fragment was cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Paisley, UK). Plasmids were linearized with an appropriate endonuclease, and antisense and sense RNA probes for each gene were transcribed *in vitro* using either a T7 or T3 polymerase (Roche Applied Science, Lewes, U.K.) in the presence of digoxigenin (DIG)-labeled rUTP (Roche) according to the manufacturer's instructions.

Frozen brain tissues were sectioned on a cryostat at 6 μm thick, with the first and fourth sections used for hematoxylin and eosin staining (H&E), the second section for antisense staining, and the third for sense staining. ISHs were performed as described elsewhere (177) with some modifications. Sections were first fixed in 4% (w/v) paraformaldehyde (PFA) for 15 min and then incubated in 0.2M HCl and 0.2% Triton X-100 for 10 min each, and digested in proteinase K (20 μg/ml) at 37°C for 5 min. Sections were refixed in 4% (w/v) PFA for 15 min, acetylated in 0.1 M triethanolamine with 0.5% (v/v) acetic anyhdride, and dehydrated through an alcohol series. Slides were hybridized in 50% (v/v) formamide, 4X SSC, 10% (w/v) dextran sulfate, 1X Denhardt's solution, with

salmon sperm DNA (1 mg/ml) and appropriate riboprobe (100 ng/section) for 20 hours at 50°C in a sealed humidified chamber. Sections were washed in 2X SSC, treated with RNase A (20 μg/ml) and processed for immunological detection of the DIG-labeled cRNA using anti-DIG antibodies at a dilution of 1:500 (Roche). Color development was allowed to continue until a blue-purple precipitate was detected with the antisense probe; the color reaction for slides treated with the sense probe was terminated at the same time as for the antisense slides.

Immunostaining

Mice were anaesthetized with a lethal dose of Somnotol (MTC Pharmaceuticals) and intracardially perfused with 5 ml ice-cold PBS followed by 20 ml 4% PFA (Fisher, FairLawn, NJ, USA). Brain and spinal cord were dissected, post-perfusion fixed 1 hr in 4% PFA, and incubated overnight in PBS with 20% sucrose (EMD Chemicals, Gibbstown, NJ, USA). Tissues were embedded in OCT (EMS, Hatfield, PA, USA) and frozen in a 2-methylbutane (EMD Chemicals) bath immersed in liquid nitrogen. Immunohistochemical staining was performed on 10-μm cryostat sections. Frozen sections were blocked in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS for 1 hr at room temperature followed by incubation with primary rabbit Abs: MMP-15 (Chemicon, Temecula, CA, USA) and ADAM-12 (Chemicon) overnight at 4°C, then with biotinylated goat anti-rabbit Ig (Vector Laboratories) for 1 hr at room temperature. Sections were treated with 3% $H_2O_2$ to quench endogenous peroxidase activity, and then incubated with an avidin-HRP complex (Vector Laboratories) following the manufacturer's instructions. HRP activity was detected by the use of DAB (Lab Vision, Fremont, CA, USA) as chromagen according to the manufacturer's instructions. Control sections were incubated with equal concentration of rabbit immunoglobulin (DAKO, Mississauga, ON, Canada) purified from serum of non-immunized rabbits. For H&E staining, sections were incubated 5 min in Harris hematoxylin (Surgipath, Winnipeg, MB, Canada), followed by 1 min in eosin (Surgipath).

**Table I.** Primer and probe sequences for mouse ADAMs[a]

| Gene | | Sequence |
|---|---|---|
| ADAM-10 | Forward: | GTGCCAGTACAGGCTCTTTGC |
| | Reverse: | CACAGTAGCCTCTGAAGTCATTACATG |
| | Probe: | ACTATCACTCTGCAGCCGGGCTCTCC |
| ADAM-12 | Forward: | ATCAGTGTCTTCGGCGTTCA |
| | Reverse: | GGCAATTCTTCCTGTTGTTACATACC |
| | Probe: | CCATGCAGTGCCACGGCCG |
| ADAM-15 | Forward: | GACCACTCCACAAGCATCTTAGG |
| | Reverse: | GGGAGAATCATGGTCCAAACC |
| | Probe: | TGCCTCCTCGATTGCCCATGAATTG |
| ADAM-17 | Forward: | AAGTGCAAGGCTGGGAAATG |
| | Reverse: | CACACGGGCCAGAAAGGTT |
| | Probe: | CCTGCGCATGCATTGACACTGACAAC |
| ADAM-19 | Forward: | CGGGCCCACCTCGAA |
| | Reverse: | CCGTTTCATTCTGCGAGGTT |
| | Probe: | TGGGCCCTTCAGTTTACACATCAGACCA |
| ADAM-28 | Forward: | TACTGCTTGAAGGGCAAATGTC |
| | Reverse: | TGTCCCACCTTCATTCTGCTT |
| | Probe: | TCCAGGAACCAAGGTTGCAAATACATCATG TTAC |
| ADAM-33 | Forward: | CAGGCACTGTCAGAATGCTACCT |
| | Reverse: | CTATTGCAAACCCCACCGTTA |
| | Probe: | TGGAACGTTGCTTGACTGCCTGCC |

[a] All probes are in the 5'-3' orientation. For the probes, a FAM fluorescent reporter is coupled to the 5' end, while a TAMRA quencher is coupled to the 3' end.

## 2.4. Results

### *2.4.1. MMPs-8, 10, 12 and 15, ADAM-12 and TIMP-1 are the most strongly affected MP genes in severe EAE*

The aim of our study was to identify significant differences in expression levels of MMP, TIMP and ADAM genes as a consequence of inflammation in EAE spinal cord, and then to localize expression of these genes to individual cell populations. Our criteria for genes to be studied in more detail were that they showed a statistically significant and greater than three-fold difference in expression levels. The analysis was done using quantitative real-time RT-PCR and results for spinal cord are shown as fold alteration in expression level between mice with EAE and control mice (Table II). Six genes fulfilled our criteria: MMP-8, MMP-10, MMP-12, MMP-15, ADAM-12 and TIMP-1. These genes are marked with arrows in Fig. 1a, which is a graphical representation of the data in Table II. MMP-15 was the only gene among the six that showed decreased expression. MMP-15 is one of the six membrane-bound MMPs (MMP-14, 15, 16, 17, 24 and 25), of which four (MMP-15, 16, 17 and 24) were downregulated in EAE (Table II and Fig. 1a).

The analysis of alteration in expression levels of genes clearly shows up and downregulation of gene expression but is not informative about the absolute levels of expression of the individual genes. A good estimation of this can be obtained from the delta cycle threshold (ΔCT) values obtained from the quantitative real-time PCR analysis (Fig. 1b). A small ΔCT value corresponds to a high level of expression, and vice versa. It is evident from Fig. 1b that the large fold increase in expression of MMP-8, 10 and 12 in EAE SC reflected a very low level of expression in the un-manipulated SC, rather than a high level of expression in EAE. By contrast, TIMP-1 was expressed at a fairly low level in un-manipulated SC, but, in EAE, became the most highly expressed of the genes studied. MMP-15 fell from an intermediate to a low level of expression. ADAM-12 falls in the low range in both cases.

**Table II.** Analysis of MP and TIMP expression profiles in CNS of mice with EAE at peak disease compared to unmanipulated controls[a]

| Gene | Fold increase in EAE | Fold decrease in EAE | P value |
|---|---|---|---|
| MMP-1a | ND | ND | |
| MMP-1b | ND | ND | |
| MMP-2 | 1.6 | | 0.0642 |
| MMP-3 | 2.2 | | 0.1248 |
| MMP-7 | | 1.5 | 0.3662 |
| MMP-8 | 90.1 | | P<0.0001 |
| MMP-9 | 2.6 | | 0.094 |
| MMP-10 | 70 | | 0.0183 |
| MMP-11 | 1.3 | | 0.3477 |
| MMP-12 | 132.9 | | 0.0134 |
| MMP-13 | 1.2 | | 0.6135 |
| MMP-14 | 2.2 | | 0.0322 |
| MMP-15 | | 5.5 | 0.0026 |
| MMP-16 | | 2.2 | 0.0024 |
| MMP-17 | | 1.9 | 0.0606 |
| MMP-19 | 2.7 | | P<0.0001 |
| MMP-20 | | 1.1 | 0.901 |
| MMP-21 | | 2 | 0.0821 |
| MMP-23 | | 2.8 | 0.0091 |
| MMP-24 | | 2.9 | 0.001 |
| MMP-25 | 1.9 | | 0.0108 |
| MMP-28 | 1.3 | | 0.1868 |
| ADAM-10 | 1.05 | | 0.798 |
| ADAM-12 | 3.87 | | 0.0036 |
| ADAM-15 | 2.3 | | 0.0145 |
| ADAM-17 | 2.8 | | 0.0002 |
| ADAM-19 | 2.14 | | 0.0018 |
| ADAM-28 | ND | ND | |
| ADAM-33 | 1.42 | | 0.1019 |
| TIMP-1 | 115.1 | | 0.0023 |
| TIMP-2 | 1.3 | | 0.1476 |
| TIMP-3 | | 1.9 | 0.0344 |
| TIMP-4 | | 1.2 | 0.1007 |

[a] N=4 for each group. Genes showing a more than three-fold alteration with a P value less than 0.05 in an unpaired t-test were chosen for detailed analysis. ND: not detected.

### *2.4.2. Expression of MP genes correlates with disease course*

Our screen of a wide range of MMPs, ADAMs and TIMPs showed that expression of six genes was altered more than 3-fold in spinal cord of mice with severe (grade 4) EAE (Table II and Fig. 1a). To further characterize the expression of these six genes in EAE, we studied the kinetics of their expression at onset of disease, at severe disease and after the first episode of symptoms. Fig. 2 shows the clinical course of EAE in individual mice after transfer of MBP-reactive T cells. As evident from Fig. 2, mice progressed rapidly from onset to grade 2, most often overnight. Mice at grade 2 EAE were therefore used to represent onset of disease. Mice in each group in the kinetic study of expression were sacrificed at equivalent stage of disease. Fig. 3 reveals a clear correlation between increased expression levels and increased severity of symptoms for MMP-8, 10, 12 and TIMP-1 (Fig. 3a, b, c and f). Notably, expression levels of these genes were strongly reduced after remission from symptoms, probably due to loss of a significant number of the relevant infiltrating cell types (macrophages and granulocytes) expressing these genes after remission. The results for MMP-15 indicate an inverse relationship between expression levels and disease severity (Fig. 3d), with expression in remitted mice returning to levels comparable to controls. There was a clear upregulation of ADAM-12 in mice with severe (grade 4) EAE (Fig. 3e), but in contrast to the other five genes analyzed, expression of ADAM-12 persisted after remission at a level comparable to grade 4 disease.

### *2.4.3. Myeloid cells are the major producer of MPs*

We first screened expression of all 22 MMPs, four TIMPs and seven ADAMs in two cell populations (T cells and myeloid cells) sorted from EAE CNS using flow cytometric sorting. The cells were sorted from brain and spinal cord of individual mice; we included brain to retrieve a greater number of cells. The trends in expression levels of the analyzed genes were similar between SC and brain samples (data not shown). We sorted T cells as $CD45^{high}$ $CD3^{+}$ cells, and a population of $CD45^{high}$ Mac1/$CD11b^{+}$ cells, which includes macrophages and

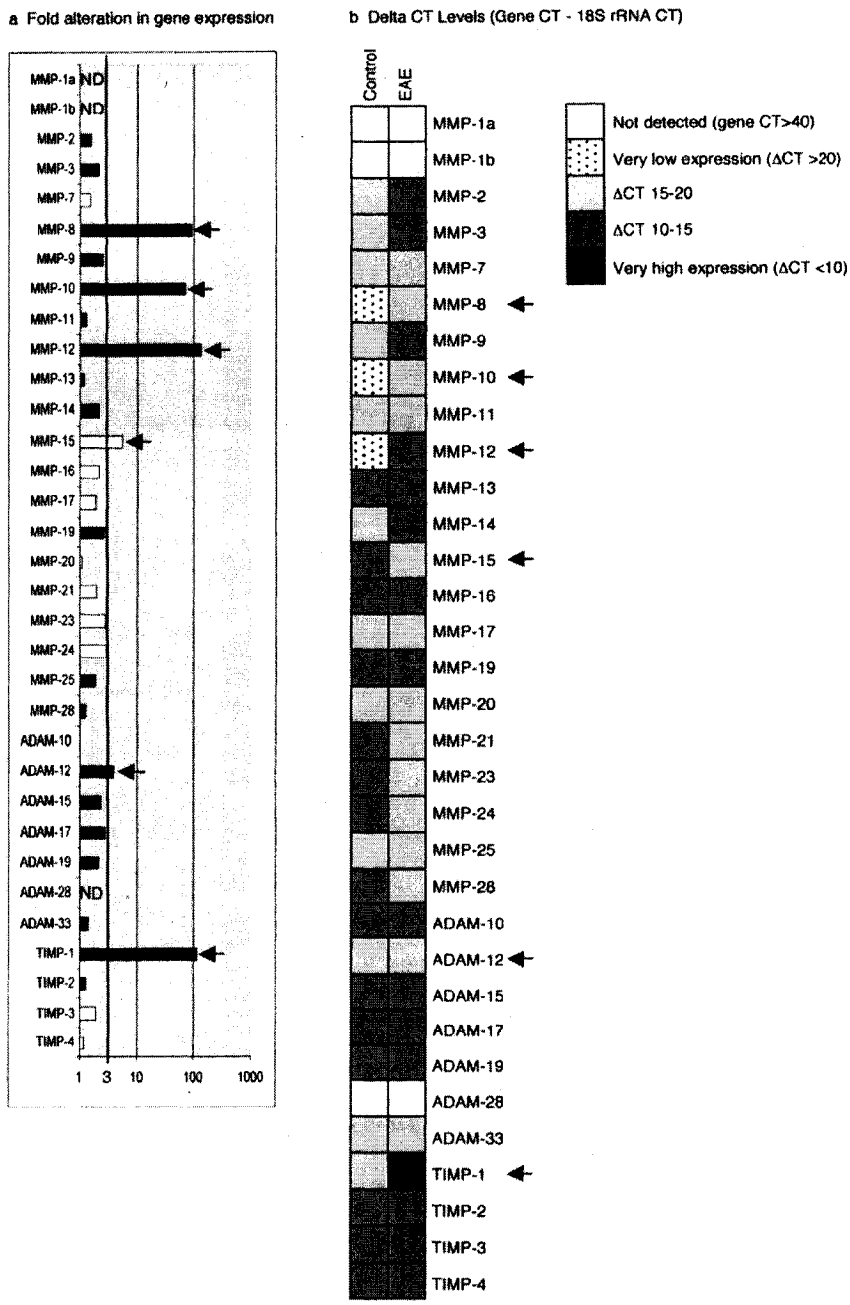


**Figure 1.** a. Graphical representation of the data in Table II. Downregulated expression is represented by open bars. The vertical lines represent specified fold alterations in expression. ΔCT (CT: cycle threshold) values for each of the genes analyzed are shown as ranges indicated by shading of the boxes. ΔCT is the difference in CT between the gene of interest and the internal reference control gene 18S rRNA. Arrows indicate the genes chosen for detailed analysis. ND: not detected.

granulocytes (178) (fig. 4b-c). This allows us to compare expression by infiltrating T cells and myeloid cells. Results of this analysis for genes showing an upregulation in SC EAE of 1.2 fold or more (see Table II) are shown in Fig. 5. Of the 19 genes analyzed this way, most (10) were expressed at significantly higher levels by myeloid cells than by T cells. Six genes (MMP-3, MMP-10, MMP-13, MMP-25, MMP-28 and ADAM-33) were expressed at equivalent levels (no statistically significant difference). Three genes (MMP-11, ADAM-12 and ADAM-19) were expressed at significantly higher levels by T cells than by myeloid cells.

### *2.4.4. MP genes are expressed by specific cell types*

In order to assess expression of the six most strongly affected genes (MMP-8, MMP-10, MMP-12, MMP-15, ADAM-12 and TIMP-1) in more detail by individual cell types, we isolated infiltrating macrophages, granulocytes and T cells as well as resident microglia from the CNS of mice with EAE, using flow cytometric sorting. As mentioned earlier, the population isolated as $CD45^{high}$ $Mac1/CD11b^{+}$ cells included both macrophages and granulocytes (Fig. 4c). To assess expression from granulocytes alone, we isolated $CD45^{high}$ $Gr\text{-}1^{high}$ cells, a population we have shown to consist of polymorphonuclear leukocytes (Zehntner S. P. and Owens T., personal communication) (Fig. 4d). Subtraction of the granulocyte contribution from the total allowed estimation of expression by macrophages alone. T cells were identified as $CD45^{high}$ $CD3^{+}$ cells (Fig. 4b) and microglia as $CD45^{intermediate}$ $Mac1/CD11b^{+}$ cells (179, 180). Unlike infiltrating leukocytes, microglia can be isolated in significant numbers both from un-manipulated and inflamed SC (Fig. 4a and c, respectively).

Fig. 4e-f shows the average proportions of the cell types isolated from CNS that were used in the real-time PCR analysis. The total number of cells in CNS increased by 56% in EAE, mainly due to infiltration of macrophages, granulocytes and T cells. The proportion of microglia remained at 23%, which, given the increase in total cells, actually reflects an increase in the number of microglia in EAE compared to unmanipulated mice.

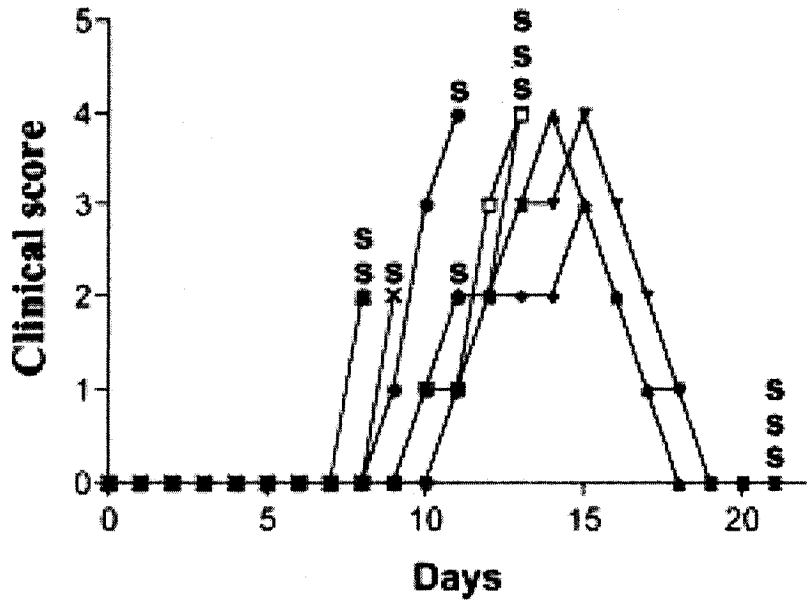


**Figure 2.** Disease progression after adoptive transfer of EAE. MBP-specific T cells were transferred to mice at day 0. Daily clinical scores for 11 individual mice are shown. Mice were sacrificed at different time points (indicated with an S). Mice that remitted (graded 0) had no symptoms except residual tail stiffness when sacrificed at day 21. S: sacrificed.

Fig. 6 shows gene expression levels of the six most strongly affected genes, in the four isolated cell types. It is clear that macrophages were the major source of MMP-12 (Fig. 6c) and TIMP-1 (Fig. 6f), whereas granulocytes were the major source of MMP-8 (Fig. 6a). For MMP-10, expression was seen by microglia and macrophages (and in T cells in only one of four samples) (Fig. 6b). None of the cell types investigated stood out as a major producer of MMP-10. ADAM-12 was expressed almost exclusively by T cells (Fig. 6e). Uniquely among the genes we analyzed, MMP-15 was strongly expressed by resting microglia, and this was reduced 15-fold in EAE (Fig. 6d).

When expression data from cell subpopulations from EAE SC were normalized per relative cell proportions (results not shown), the overall conclusions from Fig. 6 were unchanged. That is, TIMP-1 was expressed mainly by macrophages; MMP-8 mainly by granulocytes with some expression by macrophages and T cells; MMP-10 by microglia and macrophages; MMP-12 mainly by macrophages with some expression by microglia and T cells; MMP-15 was expressed by microglia with a small contribution from macrophages and ADAM-12 was expressed by T cells.

### *2.4.5. ADAM-12 protein is expressed by infiltrating T cells, whereas parenchymal MMP-15 levels decrease in EAE*

To verify that the expression of ADAM-12 message localized by flow cytometry and PCR to T cells (Fig. 6e) was reflected by production of protein, we used immunohistochemistry to analyze production of ADAM-12 in spinal cord white matter of mice with EAE (Fig. 7a, b). By comparison with staining of the same infiltrate in a separate section with control rabbit IgG (Fig. 7e), we found that about 50% of cells in perivascular infiltrates were producing ADAM-12. Analysis of the cell proportions in EAE CNS (Fig. 4f) showed that infiltrating T cells account for about 50% of the infiltrating cells. The fact that about half of the infiltrating cells stained positive for ADAM-12 and had a round morphology suggests that these cells are T cells.

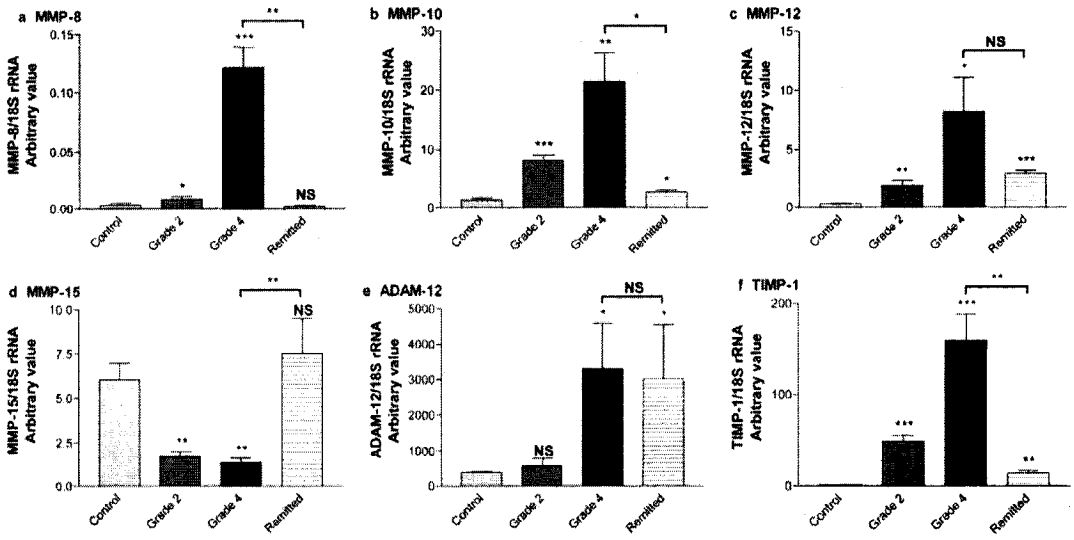


**Figure 3.** Kinetics of MP gene expression during EAE. Expression levels of MMPs 8, 10, 12 and 15, ADAM-12 and TIMP-1 in spinal cord from unmanipulated mice and mice with early onset (Grade 2) and severe, established (Grade 4) EAE, and mice in remission, were measured by real-time PCR. Significance of comparisons to unmanipulated mice was determined using an unpaired t-test. An unpaired t-test was also used to compare Grade 4 mice to the remitted group. N=4 for all groups except remitted mice (N=3). *:$P<0.05$; **:$P<0.01$; ***:$P<0.001$; NS: not significant. Error bars represent SEM.

In whole spinal cord, we found MMP-15 message to be downregulated 5.5-fold in EAE (Table II and Fig. 1a). In contrast to ADAM-12, anti-MMP-15 antibody did not stain cells within infiltrates in EAE spinal cord more intensely than control serum (Fig. 7c, e). This is consistent with cell sorting data that infiltrating cells in EAE express virtually no MMP-15 (Fig. 6d). The analysis of MMP-15 expression by sorted cells pointed to a downregulation of MMP-15 expression by microglia in EAE. Immunostaining showed a general reduction of MMP-15 staining intensity between spinal cord white matter in unmanipulated control mice (Fig. 7d) and a comparable uninfiltrated area in mice with EAE (Fig. 7f). This reduction of staining intensity was evident both on cell bodies and in the stroma, likely corresponding to cell processes, thereby indicating that the level of MMP-15 protein is indeed reduced in spinal cord of mice with EAE.

### *2.4.6. MMP-10 and TIMP-1 expression localizes to perivascular infiltrates*

Expression analysis from sorted cells did not provide clear information whether the increase in MMP-10 expression in CNS was due to infiltrating cells, or whether another cell type was responsible (Fig. 6b). To address this question, we used ISH to analyze MMP-10 expression (Fig. 8). We also analyzed TIMP-1 expression, which had been shown to be expressed by cell-sorted macrophages (Fig. 6f). Brain sections from the same mice whose spinal cords were used for the initial screening of gene expression (Fig. 1) were analyzed by ISH. These mice had severe EAE (grade 4) and numerous cerebellar infiltrates could be identified by H&E staining (Fig. 8a). Sections were hybridized with anti-sense and control (sense) probes for TIMP-1 or MMP-10. A uniformly strong signal for TIMP-1 was detected in about half of the infiltrating cells (Fig. 8b). By contrast, MMP-10 message was expressed by a majority of infiltrating cells, but at variable levels of intensity (Fig. 8d).

For both TIMP-1 and MMP-10, other cellular sources of message, most notably neurons in the granular cell layer and Purkinje cells, were observed in cerebellum (not shown). Cells associated to blood vessels, with morphology

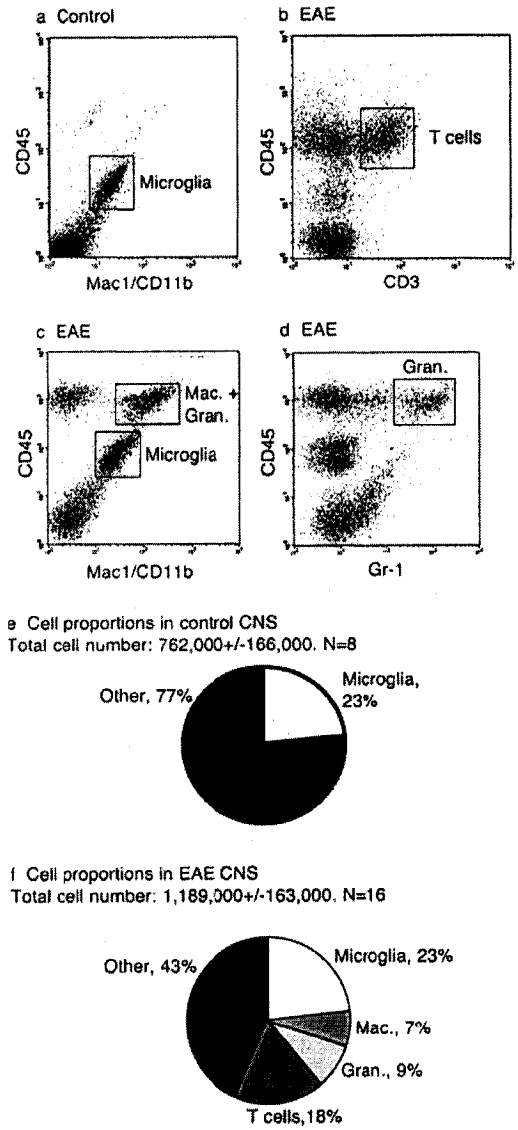


**Figure 4.** Flow cytometric analysis of cell populations in unmanipulated (a) and EAE CNS (b-d). Panels a and c show staining for PE-CD45 versus FITC-Mac1/CD11b. Panel b shows PE-CD45 versus CD3-biotin coupled to streptavidin-CyC. Panel d shows PE-CD45 versus FITC-Gr-1. Boxes are drawn to identify microglia (a, c), T cells (b), macrophages and granulocytes (c) and granulocytes (d). Fluorescence levels are matched between panels a and b and between panels c and d. Panels e-f show average numbers and proportions of cells sorted from CNS preparations, (e): unmanipulated controls (N=8), (f): EAE (N=16 for microglia and macrophages, N=6 for neutrophils and T cells). Mac.: Macrophages; Gran.: granulocytes.

similar to endothelial cells, were also positive for MMP-10 (data not shown). There was no difference in ISH signal intensity for neuronal or endothelial expression between EAE samples and controls. We conclude that the increase in message levels for MMP-10 in EAE was due to combined expression by multiple types of infiltrating cells.

## 2.5. Discussion

To our knowledge, this is the first and most comprehensive study of expression of all known mouse MMPs and TIMPs, as well as seven ADAMs, in CNS tissue from animals with EAE. This is also the first analysis of expression of MMPs, ADAMs and TIMPs in cell populations sorted from the CNS of mice with EAE. Our findings show distinct patterns of expression, which likely reflect functional differences in these genes in CNS inflammation.

Clearly, macrophages are an important source of MP and TIMP expression in EAE. Macrophage infiltration is required for EAE, and, if blocked, prevents disease (181, 182). Our study shows fairly high expression by macrophages of all but two (MMP-10 and ADAM-12) of the six most strongly affected genes. The finding that TIMP-1 was expressed by macrophages suggests a potential regulatory role for macrophages in EAE and MS by controlling the MP/TIMP ratio and thereby controlling the level of MP activity. Also astrocytes would be suspected to play a role in controlling the MP/TIMP ratio, as activated astrocytes have been demonstrated to express TIMP-1 in EAE (183).

The MMP-9/TIMP-1 ratio has received much attention since the finding that serum MMP-9/TIMP-1 ratio is increased in patients with active MS, mainly due to increased MMP-9 levels (110, 184). Hence, MMP-9 has become a candidate in the pathogenesis of MS and EAE. It has been shown that MMP-9 deficient mice younger than 4 weeks were less susceptible to EAE, but that adult mice were unaffected (185). MMP-9 has previously been reported to be upregulated in both mouse and rat EAE (173, 183). In a kinetic study (173), the

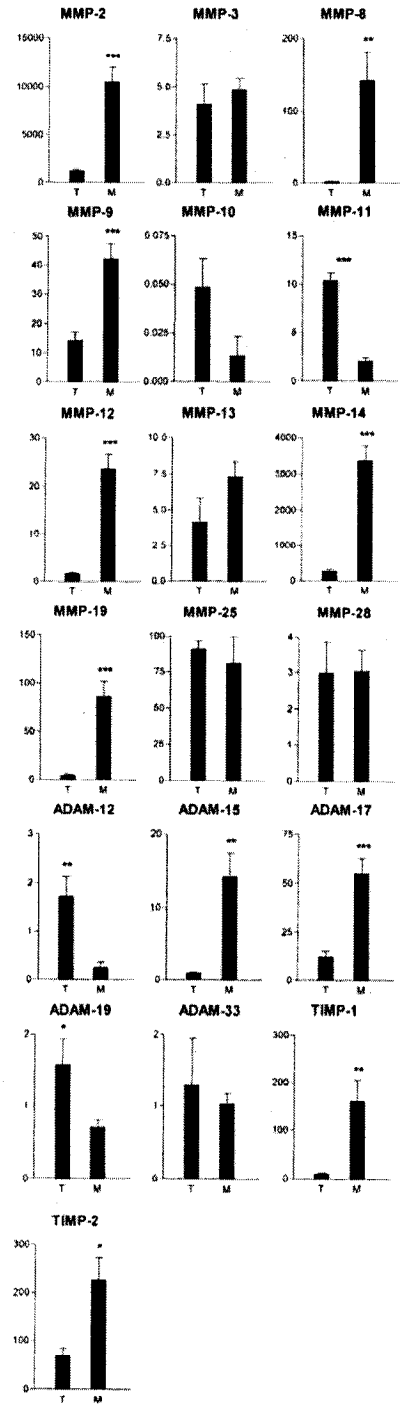


**Figure 5.** Relative expression of selected genes by T cells (T) and myeloid cells (M) (macrophages and granulocytes) from EAE CNS at peak disease. N=6 for each group. Values on the ordinate are relative levels of gene expression compared to expression of 18S rRNA. The values are arbitrary and cannot be compared between different genes.

upregulation of MMP-9 was seen at onset of disease and had virtually disappeared when the rats reached maximum disease. In our study, we found a 2.6 fold upregulation of MMP-9 (Table II), but it was not significant and was not chosen for further study. This relatively small increase in MMP-9 expression is probably due to the fact that the mice analyzed in our study were at an advanced stage in the disease, when an early large rise in MMP-9 expression will have subsided. MMP-7 has previously been found to be upregulated in rat models of EAE (173, 186). We found a non-significant 1.5-fold decrease in MMP-7 expression in grade 4 disease. However, our finding of no upregulation of MMP-7 is consistent with an earlier observation that MMP-7 was not upregulated in mouse EAE (183). This points to a possible species difference between mice and rats with respect to MMP-7 expression in EAE.

We find that, whereas T cells are a minor source of MMP-8 and MMP-12, they are virtually the only source of ADAM-12 among the infiltrating cells (Fig. 6e). The cellular sources of ADAM-12 as well as its functions are not well studied. A study of ADAM-12 deficient mice suggested that ADAM-12 plays a role in adipogenesis and myogenesis (187). To our knowledge, the only previous demonstration of ADAM-12 expression in the CNS identified oligodendrocytes as sources of ADAM-12 (188). These could be the cells responsible for the expression of ADAM-12 we found in the unmanipulated CNS (Fig. 1b). ADAM-12 was the only gene of the six analyzed that was expressed at levels comparable to severe disease after remission from disease (Fig. 3e). One may speculate that this is due to persisting T cells in the CNS after remission. Our demonstration of expression of this gene by T cells could indicate a novel role for ADAM-12 in T cell function.

The differential expression of MPs by infiltrating T cells and myeloid cells is of interest. In a study of MMP expression by subsets of human leukocytes isolated from blood, monocytes were found to express more MMP members than T cells or B cells, and to have higher migratory potential (189). In our study, we find myeloid cells to be the major source of MP message compared to T cells,

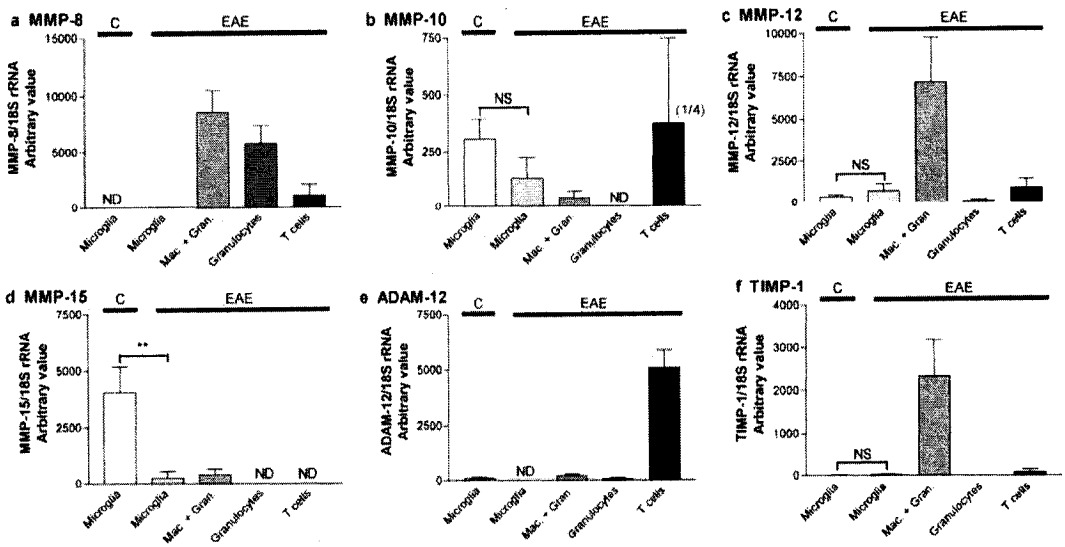


**Figure 6**. Relative expression of selected genes in cell populations sorted from CNS. N=7 for unmanipulated microglia, and N=10 for EAE microglia. N values for macrophages + granulocytes, granulocytes alone and T cells are 8, 6 and 4, respectively. For MMP-10 (b), only one out of four samples showed detectable expression in T cells. Where possible, expression level differences between control microglia and EAE microglia were analyzed using an unpaired t-test. Mac.: Macrophages; Gran.: Granulocytes; NS: not significant; ND: not detected; **: $P<0.01$.

with MMP-11, ADAM-12 and ADAM-19 as exceptions (Fig. 5). However, both cell populations were isolated from the CNS and have therefore demonstrated capacity to infiltrate, and both T cells and myeloid cells co-localize within CNS infiltrates. One could speculate that the differences in MP expression reflect different timings for entry of T cells and myeloid cells into the CNS. If a particular cell type were responsible for extensive breakdown of the BBB at initiation of inflammation, then other cells, with a smaller or different arsenal of MPs, would have an easier route into the CNS. The sequence of cellular infiltration in EAE could be orchestrated by changing chemokine profiles over the course of disease (190).

From Fig. 6a, it is evident that granulocytes were a major source of MMP-8 (also named neutrophil collagenase), consistent with previous findings (191). In addition to the mRNA messages detected in our study, granulocytes may contribute to the MP load by releasing MPs from pre-formed vesicles upon infiltration, without need for *de novo* MP synthesis.

In our analysis, we also included microglia, the resident macrophage-like cells in the CNS. Microglia have been implicated as critical cells in MS/EAE pathogenesis (192, 193), perhaps through production of MMPs (194). We found that microglia expressed MMPs 10, 12 and 15 in EAE. These cells share bone marrow origin and many functional properties with macrophages, so shared expression of MMPs would be expected. The downregulation of membrane-associated MMP-15 by microglia was a striking demonstration of a specific downregulation of an MMP by a specific cell type in EAE. This finding raises questions regarding the function of this membrane-associated MMP. Since it is downregulated in EAE, it would not appear to play a role in facilitating infiltration. Indeed, four out of the six membrane-associated MMPs were downregulated in EAE (Table II and Fig. 1a). It will be interesting to determine how many are expressed by microglia and whether membrane-associated MMPs play a special role in microglial biology. A study of membrane-associated MMPs in gliomas showed them to generally be elevated (174). The functional

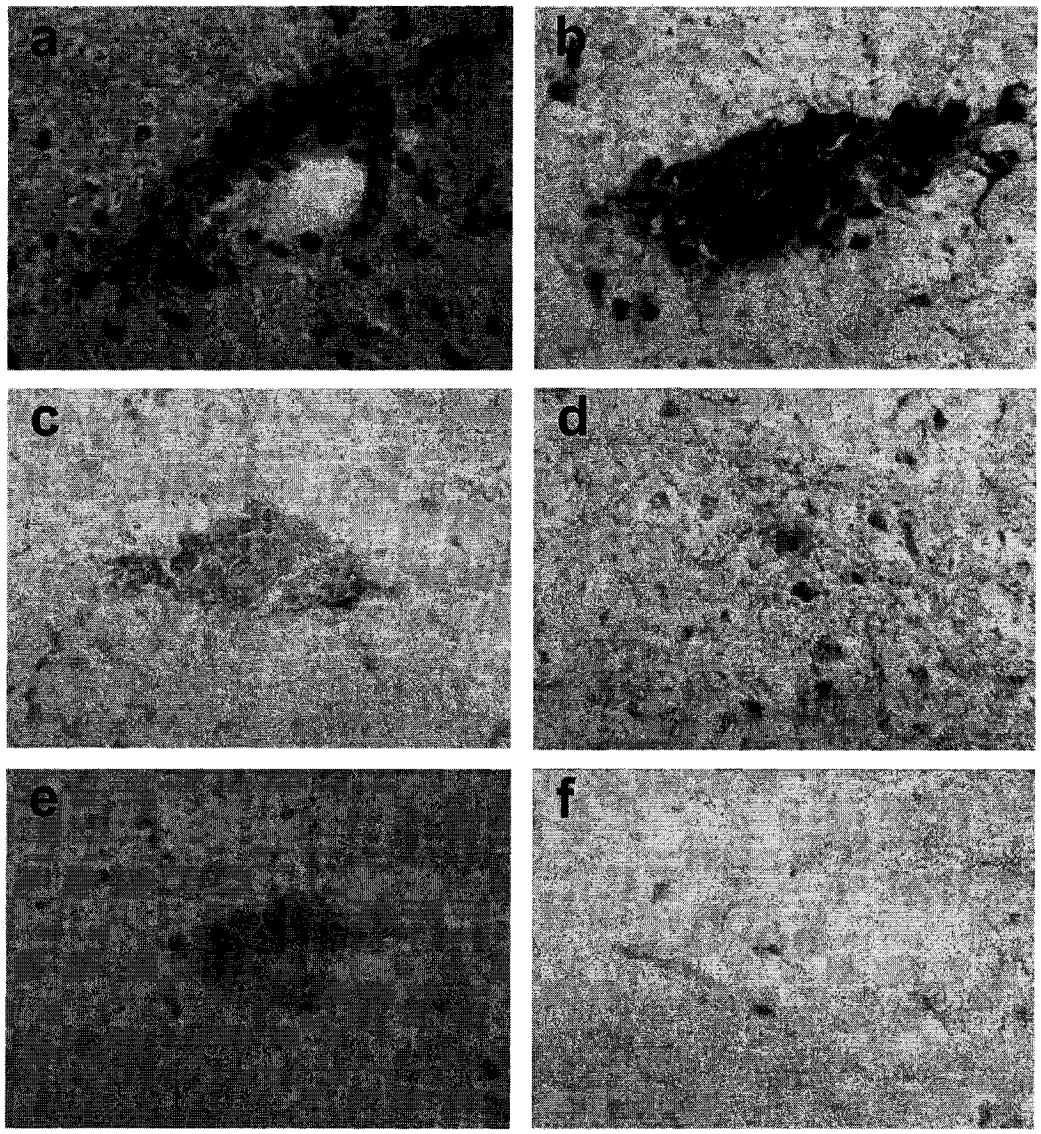


**Figure 7.** Immunostaining for ADAM-12 and MMP-15 in EAE. Sequential sections from a perivascular infiltrate in spinal cord white matter of a mouse with EAE were stained with H&E (a), anti-ADAM-12 antibody (b), anti-MMP-15 antibody (c) and control rabbit immunoglobulin (e). An equivalent area of spinal cord white matter from an unmanipulated mouse was stained with anti-MMP-15 antibody (d). Panel (f) shows MMP-15 staining for an uninfiltrated area adjacent to the infiltrate shown in panels a-c. Results shown are representative of four mice with EAE and four unmanipulated mice. Original magnification: 63X.

consequences of this contrasting response of membrane-associated MMPs in the context of different CNS diseases remain to be elucidated.

We also note an increase in absolute numbers of microglia in the CNS in EAE (Fig. 4e-f), which is consistent with an earlier study (195). This increase is likely due to proliferation of resident microglia as a consequence of activation in the inflammatory milieu, although a contribution from immigrating blood-derived cells cannot be excluded (196, 197). One can speculate whether the change from resting to activated microglia is reflected in their expression profiles of membrane-associated MMPs, including the downregulation of MMP-15. Fig. 3d shows that MMP-15 levels after remission from EAE return to a level comparable to unmanipulated controls. This may indicate a change in the activation status of the microglia that produce MMP-15, returning to a more resting state with higher MMP-15 expression after remission

The MS therapeutic IFNβ is proposed to work in part by inhibiting MMPs, as indicated by a decrease in transcription of MMP-7 and 9 in peripheral blood leukocytes from patients with relapsing-remitting MS receiving IFNβ treatment (198). Likewise, the attenuation of EAE symptoms in mice treated with the antibiotic Minocycline may be mediated in part by the MMP inhibitory effect of this drug, as Minocycline decreases transcription levels of MMPs and inhibits T cell migration across a fibronectin membrane (104). The use of synthetic broad-spectrum MP inhibitors in MS might be problematic given the current lack of knowledge about potential beneficial and detrimental roles of specific MPs in CNS inflammation. The use of such drugs in clinical trials involving cancer patients was terminated due to low efficacy and serious side effects (98). Our study provides comprehensive information about MP and TIMP expression in EAE as well as information about cell type specific expression of MPs and TIMPs. We anticipate that our findings will aid in the development of rational MS therapies based on manipulating specific MP and TIMP action.

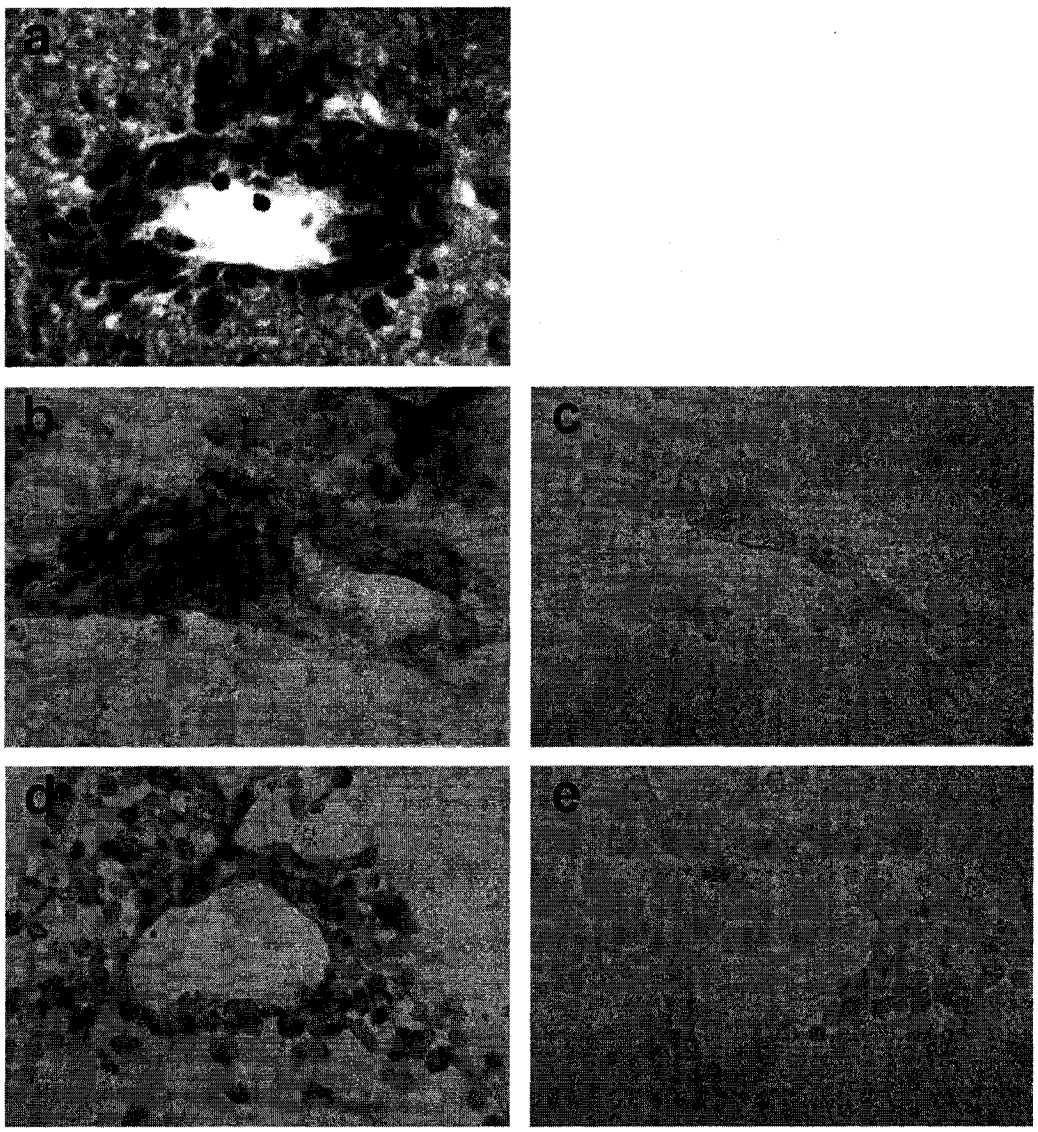


**Figure 8.** *ISH* for TIMP-1 and MMP-10 expression in EAE. Perivascular infiltrates in cerebellar white matter of mice with EAE were identified using H&E staining (a). Infiltrates were hybridized on separate sections with TIMP-1 anti-sense probe (b) and MMP-10 anti-sense probe (d). Panels c and e show lack of hybridization with TIMP-1 and MMP-10 sense probes, respectively. Results shown are representative of three mice with EAE. Original magnification: 63X.

# PREFACE TO CHAPTER III

The following chapter builds on findings from Chapter 2 (199), which identified prominent expression and cellular sources of five MP and one TIMP genes in EAE based on a comprehensive screen of MP expression. It addresses whether these same MPs, strongly implicated in EAE, are also involved in another model of neuroinflammation. Transgenic mice that overexpress the chemokine CCL2 in the CNS have pronounced accumulation of leukocytes in the perivascular space, but are asymptomatic until challenged with PTx. Encephalopathy triggered by PTx is associated with infiltration across the glia limitans. With this model it is thus possible to distinguish processes involved in migration across the endothelium from those involved in crossing the glia limitans. The experiments described in Chapter 3 test the hypothesis that MPs are involved in the PTx-induced leukocyte transmigration across the glia limitans in CCL2 Tg mice, by applying the broad-spectrum MP-inhibitor BB-94. This study also has relevance to the role of microbial involvement in the pathogenesis of MS, by investigating synergy between a pathogenic stimulus and an inflammatory chemokine.

# Chapter III

# Metalloproteinases Control Brain Inflammation Induced by Pertussis Toxin in Mice Overexpressing the Chemokine CCL2 in the Central Nervous System

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## 3.1. Abstract

Leukocytes accumulate spontaneously in the perivascular space in brains of transgenic (Tg) mice that overexpress CC chemokine ligand 2 (CCL2) under control of a CNS-specific promoter. The CCL2-Tg mice show no clinical symptoms, even though leukocytes have crossed the endothelial basement membrane of the blood-brain barrier (BBB). Pertussis toxin (PTx) given intraperitoneally induced encephalopathy and weight loss in CCL2-Tg mice.

We used flow cytometry, USPIO-enhanced magnetic resonance imaging and immunofluorescent staining to show that encephalopathy involved leukocyte migration across the astroglial basement membrane of the BBB into the brain parenchyma, identifying this as the critical step in inducing clinical symptoms.

Metalloproteinase enzymes (MPs) are implicated in migration of leukocytes across the blood-brain barrier. Unmanipulated CCL2-Tg mice had elevated expression of TIMP-1, MMP-10 and 12 mRNA in the brain. PTx further induced expression of TIMP-1, ADAM-12 and MMPs 8 and 10 in brains of CCL2-Tg mice. Levels of microglial-associated MMP-15 were not affected in control or PTx-treated CCL2-Tg mice. PTx also upregulated expression of proinflammatory cytokines IL-1β and TNFα mRNA in CCL2-Tg CNS.

Weight loss and parenchymal infiltration induced by PTx were significantly inhibited by the broad-spectrum MP inhibitor BB-94/Batimastat.

Our finding that MPs mediate PTx-induced parenchymal infiltration to the chemokine-overexpressing CNS has relevance for the pathogenesis of human diseases involving CNS inflammation, such as multiple sclerosis.

## 3.2. Introduction

Multiple sclerosis (MS) is a common neurological disease that is characterized by demyelination and inflammation of the CNS. The cause of MS is unknown, but may be the result of an environmental stimulus in genetically predisposed individuals (2). There is an established link between genotype and disease susceptibility to MS, and microbial infections can precipitate attacks in MS patients (3, 21, 200). Tg mice in which all T cell receptors recognize a myelin basic protein (MBP) epitope only developed inflammatory demyelinating disease if given PTx or kept in a non-sterile environment (201). PTx is derived from *Bordetella pertussis*, the causative agent of whooping cough, and is routinely used as adjuvant to induce experimental autoimmunity, possibly substituting for critical environmental cues in the pathogenesis of autoimmune disease.

Chemokines are small chemoattractant cytokines with the ability to direct cellular migration. CCL2, previously named monocyte chemoattractant protein-1 (MCP-1), is critical for cellular entry to the CNS in MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS (202). Blockade of CCL2 function in systems of non-immune mediated CNS infiltration curbs migration of leukocytes to sites of damage (150, 203, 204). Transgenic expression of CCL2 targeted to oligodendrocytes leads to perivascular accumulation of leukocytes in the brain without clinical symptoms (34). Transgenic mice overexpressing CCL2 in astrocytes are also unaffected until six months of age, after which they develop delayed encephalopathy (205). If PTx is injected into these mice at an early age (8-10 weeks old), it induces a condition described as PTx-induced reversible encephalopathy dependent on MCP-1/CCL2 overexpression (PREMO) (206). Both PREMO and delayed encephalopathy are dependent on a functioning CCR2 (205, 206).

The BBB restricts the entrance of cells and macromolecules into the CNS. It consists of a number of separate barriers. Endothelial tight junctions are responsible for limiting passage of macromolecules such as dyes. There is an

endothelial basement membrane and a separate astroglial basement membrane, the latter associated with the glia limitans (29). In the inflamed CNS, the BBB is crossed by leukocytes, which enter the CNS parenchyma. The actual mechanism behind cellular crossing of the BBB remains to be fully explained (207), but is likely to involve degradation of extracellular matrix proteins in the BBB by MPs. MPs have been implicated in virtually all CNS inflammatory diseases (89, 108).

Twenty two matrix metalloproteinases (MMPs) have been identified in mice, and collectively they can degrade all components of the extracellular matrix (66, 68). Six MMPs are membrane-bound (MT-MMPs). Metalloproteinase-disintegrins (ADAMs) are another family of MPs, which comprise more than 30 members (168). There are four tissue inhibitors of metalloproteinases (TIMPs). A series of papers have demonstrated that administration of synthetic broad-spectrum MP-inhibitors alleviates symptoms of EAE (171-173).

In this study, we examined the role of MMPs in PTx-induced encephalopathy in CNS-specific CCL2 Tg mice. We show that crossing of the astroglial basement membrane, not the endothelial basement membrane, was the critical step inducing clinical symptoms. The parenchymal brain infiltration induced by PTx was associated with changes in expression of MP genes and could be alleviated by treatment with a broad-spectrum MP inhibitor.

## 3.3. Materials and Methods

Mice

Transgenic mice expressing the chemokine CCL2 in the CNS under control of a myelin basic protein (MBP) promoter (34) were obtained from Bristol-Myers Squibb (New Brunswick, New Jersey) and maintained as a colony at the Montreal Neurological Institute. Male and female 8-12 week old transgenics and wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, Maine) were used for all experiments. Mice were bred and maintained in a specific pathogen-free environment. Animal breeding, maintenance and all experimental protocols were performed in

accordance with Canadian Council for Animal Care guidelines as approved by the McGill University Animal Care Committee.

Administration of pertussis toxin and BB-94

Pertussis toxin (10 μg/kg) (Sigma, Oakville, Ontario) or HBSS (Invitrogen Life Technologies, Carlsbad, California) were injected ip. at day 0. Mice were weighed and monitored daily. BB-94 (Vernalis, a kind gift from Dr. Yong, University of Calgary) was injected ip. as a suspension of 3 mg/ml in PBS containing 0.01% Tween-80. To increase solubility, the suspension was sonicated 3x10 seconds on ice. Mice were treated with BB-94 (50 mg/kg per dose) or vehicle at 1 and 4 hours after injection with PTx, and once per day thereafter.

Cell sorting

Mice were anaesthetized with Somnotol (MTC Pharmaceuticals, Cambridge, Ontario) and intracardially perfused with 20 ml ice-cold PBS. Brain and spinal cord were collected and a single cell suspension generated by passing through a 70 μm cell strainer (BD Biosciences Pharmingen, Mississauga, Ontario). After centrifugation on 37% Percoll (Amersham Biosciences, Baie d'Urfe, Quebec), the myelin was removed, cells were washed in HBSS and incubated in supernatant from the anti-FcR 24G2 (anti CD16/32) hybridoma containing an additional 2 % fetal calf serum (FCS) (Sigma) and hamster IgG (50 μg/ml) (Cedarlane, Hornby, Ontario) on ice for 20 min. to block non-specific antibody binding. The cells were stained on ice for 30 min. with antibodies as indicated in figure legends. Cy5-conjugated streptavidin and the antibodies phycoerythrin (PE)-conjugated anti-CD45, biotin-conjugated anti-CD3, fluorescein isothiocyanate (FITC)-conjugated anti-CD11b and anti-Gr-1 were purchased from BD Biosciences Pharmingen. The cells were washed in HBSS with 2% FCS and sorted using a Becton-Dickinson FACSVantage cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, California). Flow cytometry data were analyzed using CellQuest software.

Isolation of RNA

Total RNA was purified using Trizol RNA isolation reagent (Invitrogen Life Technologies) according to the manufacturer's protocol for whole tissue RNA extraction. For sorted cells, Trizol was used according to the manufacturer's protocol for RNA extraction for low amounts of RNA.

Reverse transcriptase reaction

RNA (3 μg) from each brain sample was incubated with M-MLV reverse transcriptase (RT) (Invitrogen Life Technologies) according to manufacturer's protocol using random hexamer primers. RNA from sorted cells was incubated with SuperScript II RT (Invitrogen Life Technologies) according to the manufacturer's protocol using random hexamer primers, and glycogen as carrier.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was done using the ABI Prism 7000 Sequence Detection System according to our previously described method and probe and primer sequences (199). Expression of 18S rRNA (primers and probes from Applied Biosystems, Foster City, California) in cDNA samples diluted 1:1000 was used to control for differences in the extraction and reverse transcription of total RNA. All other primers were synthesized by Sigma. Corresponding probes were synthesized at Applied Biosystems. Each reaction was performed in 25 μl with 50% TaqMan 2x PCR Master Mix (Applied Biosystems), 100 nM each of the forward and reverse primer, and 200 nM of probe. Conditions for the PCR were 2 min at 50°C, 10 min at 95°C, and then 40 cycles, each consisting of 15 s at 95°C, and 1 min at 60°C. To determine the relative RNA levels within the samples, standard curves for the PCR were prepared by making 4-fold serial dilutions (8-fold dilutions for 18S rRNA) of cDNA. Standard curves for CT (cycle threshold; the cycle at which the detected signal became significantly different from background signal) versus arbitrary levels of input cDNA were prepared, and the expression level of mRNA in each sample was determined. CT values were verified to be in the linear amplification range on the appropriate standard curves.

The relative expression level for each sample was calculated by dividing the expression level of the target gene by the expression level of 18S rRNA.

Immunofluorescence staining

Mice were anaesthetized with Somnotol (MTC Pharmaceuticals) and intracardially perfused with 5 ml ice-cold PBS followed by 20 ml 4% PFA (Fisher, Nepean, Ontario) in PBS. Brains were dissected, fixed for 1 hr in 4% PFA in PBS at 4°C, incubated overnight in PBS with 20% sucrose (EMD Chemicals, Gibbstown, New Jersey) at 4°C. Brains were freeze-embedded in OCT (Cedarlane) and cut in twelve micron cryostat sections. The following primary antibodies were used: Rabbit anti-mouse Laminin-1 (Cedarlane) and rat anti-mouse CD45 (Serotec, Hornby, Ontario). Cryostat sections were fixed with 4% PFA followed by 1% Triton X treatment for 20 min. They were then blocked with 20% goat serum in PBS for 1 hr. Endogenous biotin was blocked with a biotin block kit (Vector laboratories, Burlingame, California) according to the manufacturer's instructions. Sections were incubated with primary antibodies 1 hr at room temperature. Biotinylated goat anti-rabbit (Vector Laboratories) and goat anti-rat Alexa555 (Molecular Probes, Eugene, Oregon) were added for 45 min, followed by streptavidin-Alexa488 (Molecular Probes). Nuclei were stained with Hoechst staining reagent (Molecular Probes) for 10 min. As negative controls for primary antibodies, rat gamma globulin (Jackson Immunoresearch, West Grove, Pensylvania) and rabbit immunoglobulin (DAKO, Mississauga, Ontario) were used at equivalent concentrations. All antibodies were used at empirically-determined optimal dilutions. Sections were photographed using a Leica DMIRE2 fluorescent microscope (Leica Microsystems, Richmond Hill, Ontario)

Prussian blue and H&E staining

Mice were anaesthetized with Somnotol (MTC Pharmaceuticals) and intracardially perfused with 5 ml ice-cold PBS followed by 20 ml 4% PFA (Fisher) in PBS. Brains were dissected, fixed for 1 hr in 4% PFA in PBS at 4°C and then embedded in paraffin. Five micron sections of paraffin-embedded brains

were cut on a microtome. The Prussian blue stain was performed to detect ferric iron. Briefly, brain sections were incubated at room temperature for 30 minutes in a solution of 5% potassium ferrocyanide (American Chemicals, Montreal, Quebec) and 5% hydrochloric acid (Anachemia, Montreal, Quebec) followed by counterstaining for 5 minutes in Nuclear Fast Red (Sigma). For H&E staining, sections were incubated 10 min in Harris hematoxylin (Surgipath, Winnipeg, Manitoba), followed by 1 min in eosin (Surgipath).

USPIO-enhanced MRI

T2-weighted images were acquired using a Bruker Biospec 7T/21cm spectrometer with a 2cm diameter quadrature volume coil (National Research Council, Institute for Biodiagnostics, Winnipeg, Manitoba) using a spin-echo multi-slice multi-echo sequence with 12 slices spanning the brain (echo time 26.8ms, repetition time 2540ms, FOV 2.5x2.5 $cm^2$, slice thickness 0.75mm, matrix 256x256, signal averages 4). Anesthesia was induced using 5% halothane in 70% nitrous oxide/30% oxygen and maintained at 1.5-2% halothane during the imaging session. PTx (10 or 20 μg/kg) was injected ip. on day 0 followed by a tail vein injection of 15 mg/kg of the USPIO ferumoxytol (Advanced Magnetics, Cambridge, Massachusetts) that was re-administered every 24 hours. No differences were observed between mice injected with different doses of PTx. The first imaging session took place at least a week before the injection of PTx and subsequent imaging was performed daily post PTx. Control experiments were performed either on Tg mice injected daily with USPIO but not PTx, or on WT mice injected with both PTx and USPIO.

## 3.4. Results

The CCL2 Tg mice used in this study overexpress the chemokine CCL2 specifically in the CNS under control of a truncated MBP promoter (34). CCL2 is produced throughout the white matter and leukocytes accumulate spontaneously in the perivascular space around CNS vessels in white matter areas without parenchymal infiltration (34). No accompanying clinical symptoms are observed.

### *3.4.1. PTx induces encephalopathy and weight loss in CCL2 Tg mice*

We used PTx to induce encephalopathy in CCL2 Tg mice. PTx caused Tg mice to lose weight compared to Tg mice injected with HBSS only. PTx-injected Tg mice showed a statistically significant ($P<0.05$) weight loss starting at day 2 and lasting throughout a 5 day period with maximum weight loss at day 4. The weight loss range was between 5 and 15%. Significant weight loss was not observed in WT control mice injected with PTx or in Tg mice that received HBSS. Clinical symptoms induced by PTx in Tg mice included tremor, inactivity, limb clasping and/or death. These were variably seen in 22% of PTx-treated mice, although this did not correlate to underlying histopathology. Clinical symptoms were not observed in any of the above mentioned controls (a total of 12 WT and 21 Tg controls).

### *3.4.2. $CD45^{high}$ cells are recruited to the brain of PTx-treated Tg mice*

Flow cytometry revealed a large number of leukocytes ($CD45^{high}$) in the CNS of CCL2 Tg mice (Fig. 1A, left panel). The combination of CD45 and CD11b staining makes it possible to distinguish CNS-resident microglia ($CD45^{dim}$ $CD11b^{+}$) and a population consisting of blood-derived macrophages and granulocytes ($CD45^{high}$ $CD11b^{+}$) (180). These flow cytometry profiles did not change dramatically following administration of PTx (Fig. 1A, right panel). However, the total number of cells was significantly higher in the PTx-injected

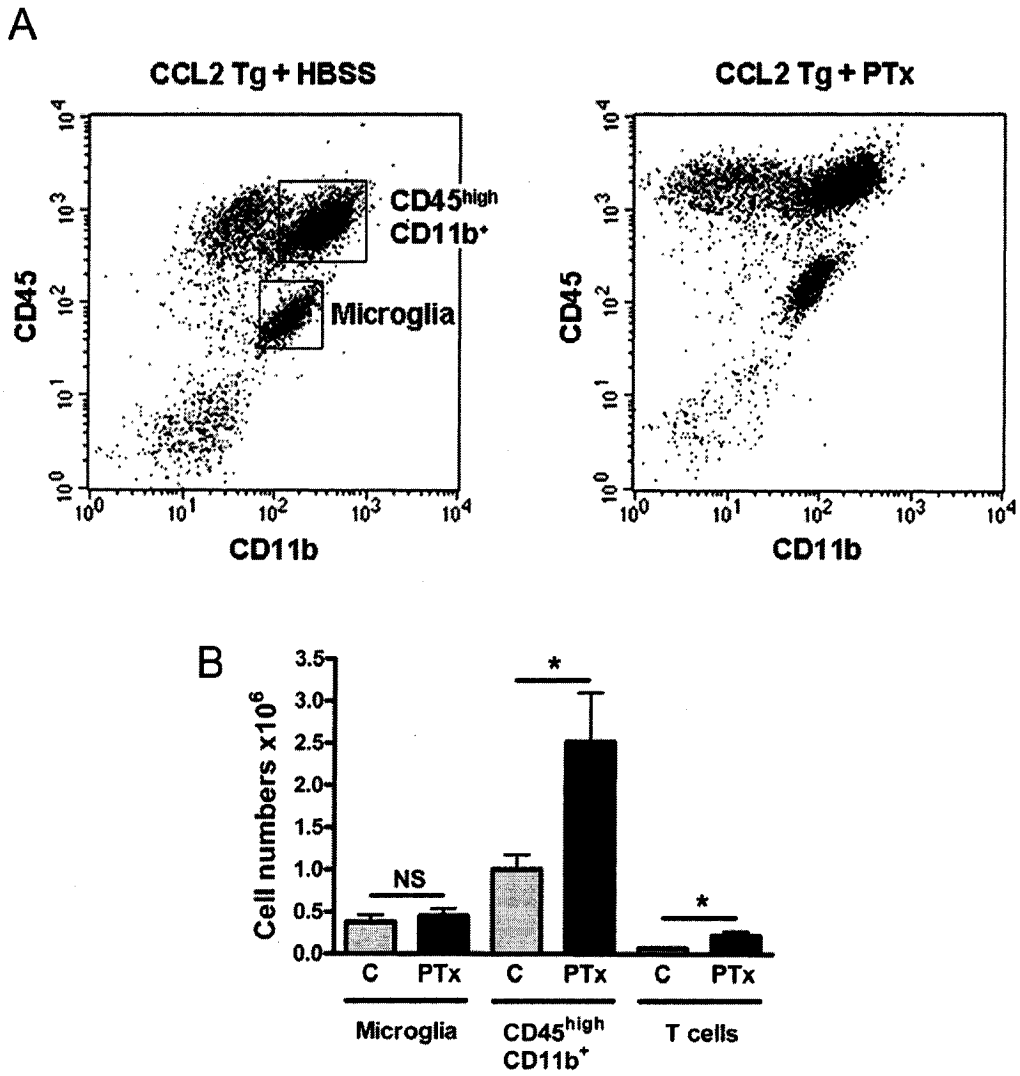


**Figure 1.** Flow cytometric analysis.

A. Cells isolated from CNS of PTx-injected CCL2 Tg mice or HBSS-injected controls were stained as indicated and sorted. B. Quantification of results from flow cytometric analysis. Cell numbers were calculated as proportions multiplied by total number of cells, estimated by counting on a hemocytometer. Mice were sacrificed at day 5 post PTx injection. n = 4-8; *:$P<0.05$; NS: not statistically significant; C: controls. Error bars represent SEM.

mice (3.85±0.77 x $10^6$ compared to 2.38±0.39 x $10^6$ in the HBSS-injected mice, an increase of 62%, $P<0.05$). When numbers of individual cell types were compared (Fig. 1B), it was clear that the increase in total cell number in PTx-treated mice was mainly due to an increase in $CD45^{high}$ $CD11b^+$ cells, which doubled in number. The vast majority of these cells were macrophages, since we observed less than 1% granulocytes, identified by Gr-1 staining (not shown). T cells were isolated as $CD45^{high}$ $CD3^+$ cells (not shown). There was a 3-fold increase in T cells, yet T cells remained a relatively small proportion of the infiltrate. Microglial numbers were unchanged.

### *3.4.3. Brain parenchymal infiltration in PTx-treated Tg mice*

Infiltration into the brain was followed over the course of a week, using ultra small superparamagnetic iron oxide (USPIO)-enhanced magnetic resonance imaging (MRI). The dextran-covered USPIO particles are injected into the blood and engulfed by actively phagocytosing cells. Cellular infiltration of USPIO-loaded cells can be followed on T2-weighted MRI, where the iron in the USPIO particles results in an area with low signal intensity on the scan (208, 209). USPIO was injected on day 0 and every 24 hours thereafter. The mice were imaged every day and results for one mouse are shown in Fig. 2A. There was a clear development of lesion areas on the MRI scans with low signal intensities due to magnetic susceptibility effects representing infiltration of USPIO-loaded cells (Fig. 2A, arrowheads). These areas became noticeable starting on day 2 or 3 and persisted to day 7. The development of lesions was dynamic, with some lesion areas eventually decreasing in size. No lesions were observed in any WT control mice injected with PTx and USPIO (Fig. 2B) or in any CCL2 Tg mice receiving USPIO, but HBSS instead of PTx (Fig. 2C).

Infiltrating USPIO-loaded cells were visualized *in situ* by prussian blue staining, which stains iron (and therefore USPIO) blue in tissue sections. In Tg mice receiving PTx and USPIO, blue stained cells were seen around vessels and in the surrounding parenchyma (Fig. 2D, arrows), corresponding to areas of low signal intensity on MRI scans. The lesion area on the scan appears larger than the

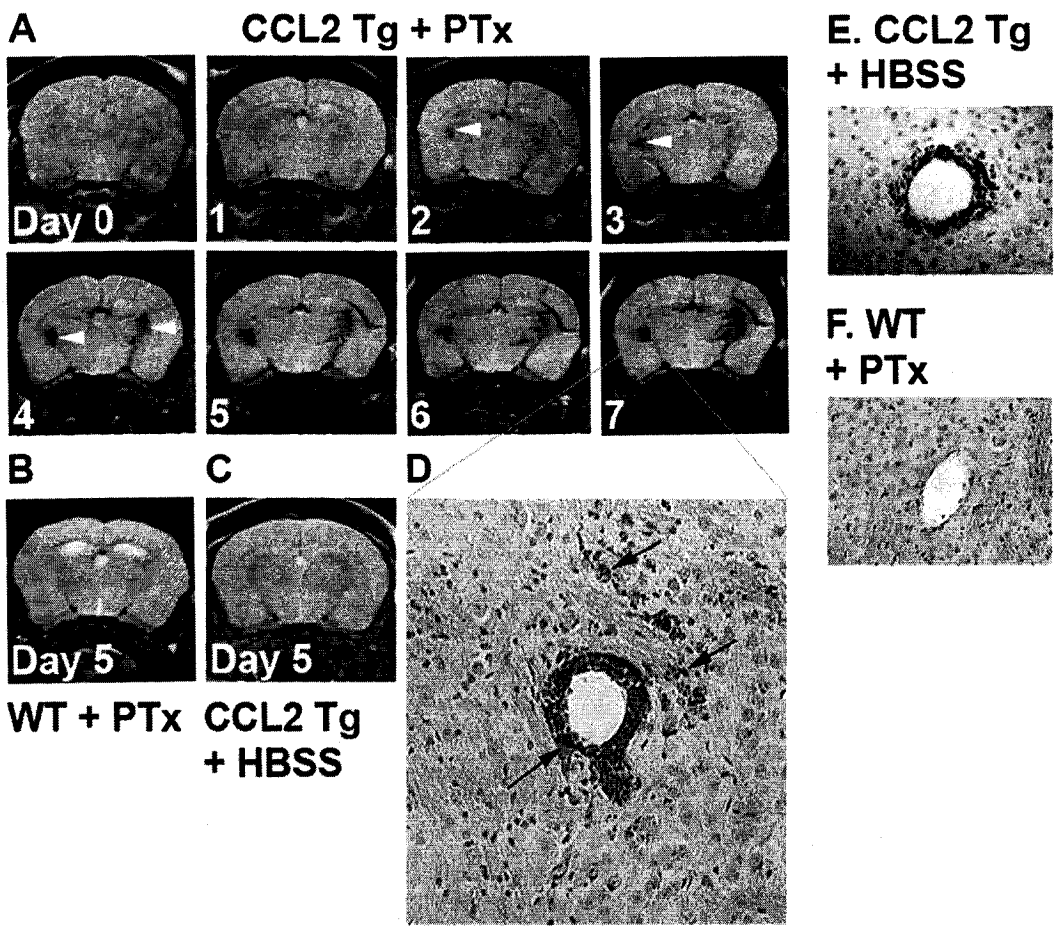


**Figure 2.** USPIO-enhanced MRI.

A. Images from day 0 to 7 of the brain of a CCL2 Tg mouse injected with PTx on day 0. USPIO contrast agent was injected every day starting on day 0. The image from day 0 is taken before administration of USPIO and PTx. B. Day 5 scan of WT control mouse injected with PTx. C. Day 5 scan of Tg mouse injected with HBSS as control. All scans are coronal in position bregma -0.5 mm. D. Prussian blue (PB) staining of a paraffin-embedded section at the same position as scans shown in Panel A. The mouse shown in Panel A was sacrificed at day 7 and the brain used for the staining in Panel D. E. PB staining of a Tg mouse injected with USPIO but with HBSS instead of PTx, sacrificed at day 5. F. PB staining of a WT mouse injected with both PTx and USPIO, sacrificed at day 5. Results shown are representative of at least 4 mice in each treatment group.

actual infiltrated area because of "blooming" of the MRI signal (210). No prussian blue staining was observed in Tg mice receiving USPIO but no PTx (Fig. 2E), or in WT mice receiving both PTx and USPIO (Fig. 2F), indicating that parenchymal infiltration of cells that actively phagocytosed USPIO requires signals from both PTx and CCL2. Based on the observation that mice showed maximal weight loss at day 4, and that USPIO-enhanced MRI showed dynamic lesions increasing up to day 7, we decided to sacrifice animals at day 5 for further analysis. At this time, weight loss and the size of lesions on MRI scans were both prominent.

### *3.4.4. Induction of proinflammatory cytokines and metalloproteinases*

We analyzed expression of two proinflammatory cytokines and 6 MP genes using real-time PCR (Fig. 3). The transgenic expression of CCL2 alone induced IL-1β and TNFα expression compared to WT mice. Both were further upregulated in the PTx-treated Tg mice. The six metalloproteinase genes included ADAM-12, three secreted MMPs: MMP-8, -10 and -12, the membrane-bound MMP-15 as well as the physiological MP inhibitor TIMP-1. These six genes were chosen on the basis of their selective involvement in leukocyte trafficking in EAE, where expression of MMP-8, 10, 12, ADAM-12 and TIMP-1 were markedly induced, whereas MMP-15 was downregulated (199). Expression of MMP-10, -12 and TIMP-1 were inherently significantly higher in the Tg mice compared to WT mice. MMP-8, -10, ADAM-12 and TIMP-1 were significantly upregulated in Tg mice by PTx. MMP-12 was not significantly upregulated by PTx in Tg mice ($P<0.07$). Expression of the membrane-bound MMP-15 was unaffected in all cases.

### *3.4.5. Distinct cell types express specific metalloproteinase genes*

We have previously shown that MMP-8 is primarily expressed by infiltrating $CD45^{high}$ $CD11b^{+}$ cells, that MMP-15 is primarily expressed by microglia, and that ADAM-12 is exclusively expressed by T cells among the infiltrating cells in EAE (199). We analyzed expression of these three MPs in cells sorted from

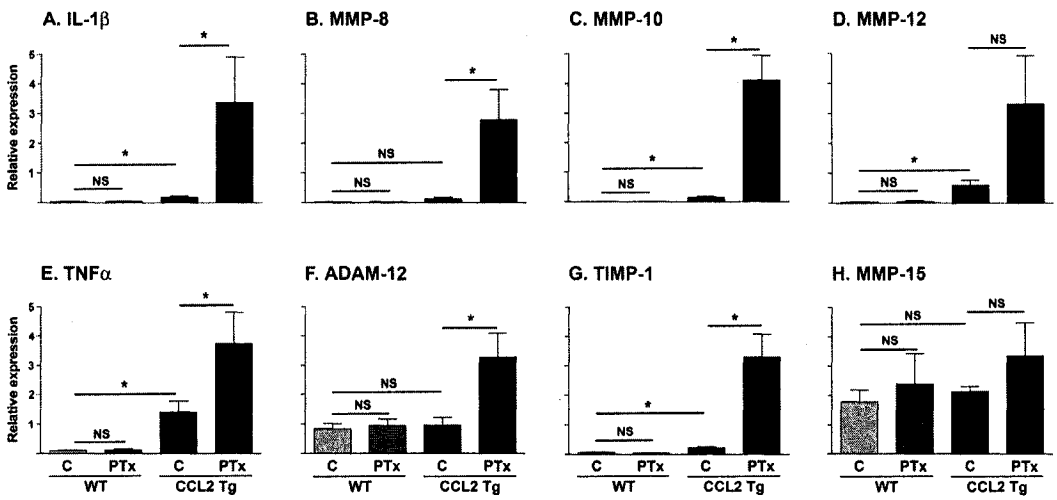


**Figure 3.** Real-time PCR analysis of expression of cytokine and metalloproteinase mRNAs in brain.

Values on the Y-axis, normalized to expression of 18SrRNA, are arbitrary and cannot be compared between panels. All mice were sacrificed at day 5 post PTx injection. n = 5 for all groups. NS: not statistically significant; C: control; *: $P<0.05$. Error bars represent SEM.

CCL2 Tg mice (Fig. 4). We found upregulated expression of MMP-8 in the $CD45^{high}$ $CD11b^{+}$ population. MMP-15 was expressed almost exclusively by microglia and levels were unaffected by PTx injection. ADAM-12 was specifically expressed by T cells among the populations that were sorted, and did not show significant change in expression after PTx injection.

### *3.4.6. The metalloproteinase inhibitor BB-94 curbs PTx-induced weight loss and parenchymal infiltration*

BB-94 is a synthetic broad-spectrum MP inhibitor, which acts by competitive reversible binding to the catalytic site of MPs (211). Treatment of PTx-injected CCL2 Tg mice with BB-94 curbed weight loss, weights of treated animals being significantly higher than the weights of untreated mice starting on day 3 and lasting to day 5 (Fig. 5). To assess the effect of the MP-inhibitor on the PTx-induced parenchymal infiltration, we stained for CD45 and laminin-1 in sections of brains from PTx-injected Tg mice treated with BB-94 or PBS, as well as control Tg mice receiving only PBS (Fig. 6A). Laminin-1 stains both the endothelial basement membrane (Fig. 6A, left panel, thin arrow) and the astroglial basement membrane (Fig. 6A, left panel, thick arrow) of the BBB. CD45 is expressed at high levels on infiltrating leukocytes (Fig. 6A, middle panel, arrowheads, and Figure 1A). Consequently, this staining allows us to identify the location of leukocytes in relation to the BBB. We counted infiltrating cells within a 100 μm distance of the astroglial basement membrane around distinct infiltrated vessels. In control Tg mice, numbers of infiltrating cells were at a basal level (Fig. 6A, left panel). Injection of PTx caused striking parenchymal infiltration associated with infiltrated blood vessels (Fig. 6A, middle panel). Treatment with BB-94 abolished this parenchymal infiltration (Fig. 6A, right panel). Counting CD45-positive cells within a distance of 100 μm from the astroglial basement membrane of infiltrated vessels on each section demonstrated that BB-94-treatment reduced PTx-induced brain infiltration to a level close to basal level (Fig. 6B).

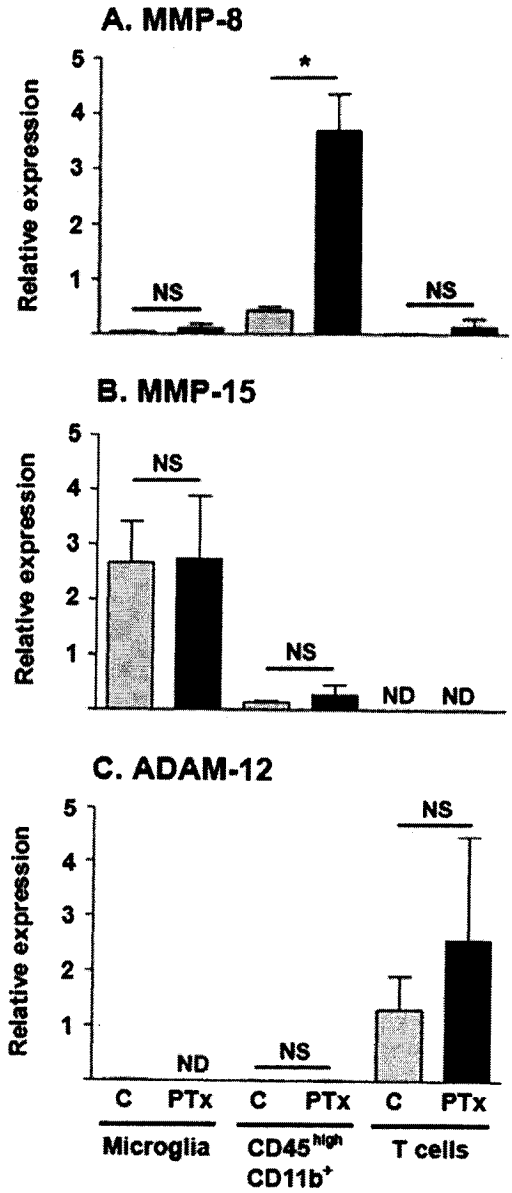


**Figure 4.** Real-time PCR analysis of MP mRNA expression in sorted cell populations.

Grey bars represent cells from mice that received HBSS; black bars represent cells from PTx-injected mice. Values on the Y-axis, normalized to 18SrRNA, are arbitrary and cannot be compared between panels. All mice were sacrificed at day 5 post PTx injection. n = 3-5; C: control; NS: not statistically significant; ND: not detected; *:$P<0.05$. Error bars represent SEM.

## 3.5. Discussion

The process of cellular entry to the inflamed CNS parenchyma involves leukocyte migration across endothelial and astroglial barriers, and the perivascular space between them, driven by multiple stimuli that include chemokines and action of proteases. In the system we have studied, unmanipulated CCL2 Tg mice contain large numbers of $CD45^{high}$ leukocytes in the perivascular space surrounding CNS vessels. The bacterial toxin PTx induced leukocytes to cross the astroglial basement membrane in Tg mice, and this led to weight loss and clinical symptoms. Hence, the combination of a bacterial product and chemokine overexpression resulted in parenchymal infiltration. This demonstrates that exposure of a primed CNS milieu to an environmental stimulus can result in disease, in this case encephalopathy. Gene expression of two MPs was induced by the chemokine CCL2 and one of these and two others were further induced by PTx. Because CNS infiltration could be curbed by treatment with an MP inhibitor, the PTx-induced expression of effector molecules such as metalloproteinases likely promotes brain infiltration. The molecular mechanisms behind this action of PTx remain to be established, but the involvement of innate immune receptors such as Toll-like receptors is an interesting possibility (165, 212).

The symptoms we observed following PTx-injection in CCL2 Tg mice were similar to, but less severe than, the PREMO condition described by Huang et al. to occur in astrocyte-directed CCL2 Tg mice challenged with CFA sc. and twice with 500 ng PTx iv. (206). In the present study, we used a lower dose of PTx (10 μg/kg ip. per mouse once) and no CFA. Consequently, we observed lower incidence, less severe symptoms and a lower mortality. The symptoms and deaths in our experiment occurred in a non-predictable pattern with some mice dying without showing previous symptoms, and some mice recovering from symptoms.

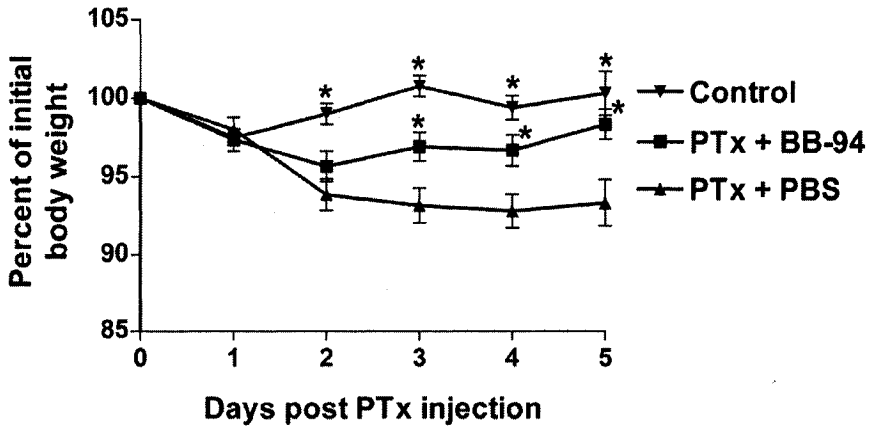


**Figure 5.** Weight loss following PTx injection.

Mice were injected with PTx ip. on day 0. BB-94 or PBS were then injected ip. at 1hr, 4 hr and thereafter every 24 hr post PTx injection. Control mice received appropriate vehicles for PTx and BB-94. Mice were sacrificed on day 5. n = 6-12; *, $P<0.05$. Error bars represent SEM.

We previously demonstrated that expression of a number of MP genes was altered in spinal cord of mice with adoptive transfer EAE (199). Although EAE and the encephalopathy induced by PTx in CCL2 Tg mice are different pathologies, they share the process of parenchymal infiltration and may share fundamental mechanisms of leukocyte migration across the BBB. In the spinal cord of mice with EAE, MMP-8, 10, 12, ADAM-12 and TIMP-1 are all upregulated more than 3-fold, whereas MMP-15 is downregulated more than 3-fold. Comparison of the findings in EAE and in infiltration driven by the synergistic effects of PTx and a chemokine may be instructive for understanding mechanism of CNS inflammation.

MMP-8 is strongly expressed by granulocytes in EAE (199). In CCL2 Tg mice, less than 1% of CNS cells analyzed by flow cytometry are granulocytes ($CD45^{high}$ $Gr1^{+}$). It is more likely $CD45^{high}$ $CD11b^{+}$ macrophages which upregulate MMP-8 mRNA levels following PTx administration. It has elsewhere been demonstrated that monocytes/macrophages can express MMP-8 (213). The upregulation of MMP-10 and ADAM-12 following PTx administration is consistent with EAE studies, reflecting the recruitment of macrophages and T cells. The expression level of the macrophage-specific MMP-12 was inherently higher in Tg mice compared to WT, but neither PTx nor the leukocyte infiltration that it induced, led to significantly increased MMP-12 expression. This may reflect differential regulation of infiltrating macrophages compared to EAE, where MMP-12 is the most highly upregulated MMP (199, 214). Interestingly, Weaver and colleagues found that MMP-12 null mice developed more severe EAE than WT mice, indicating that the role of MMP-12 in CNS infiltration is complex. TIMP-1 is an inhibitor of most MPs (92), and is expressed by macrophages (199) and astrocytes (183) in EAE. The higher level of TIMP-1 in Tg mice compared to control, and the PTx-induced upregulation, probably reflects CCL2- or PTx-driven influx of monocytes/macrophages in the Tg mice and/or a response by astrocytes, which were unavailable to us by flow cytometric analysis.

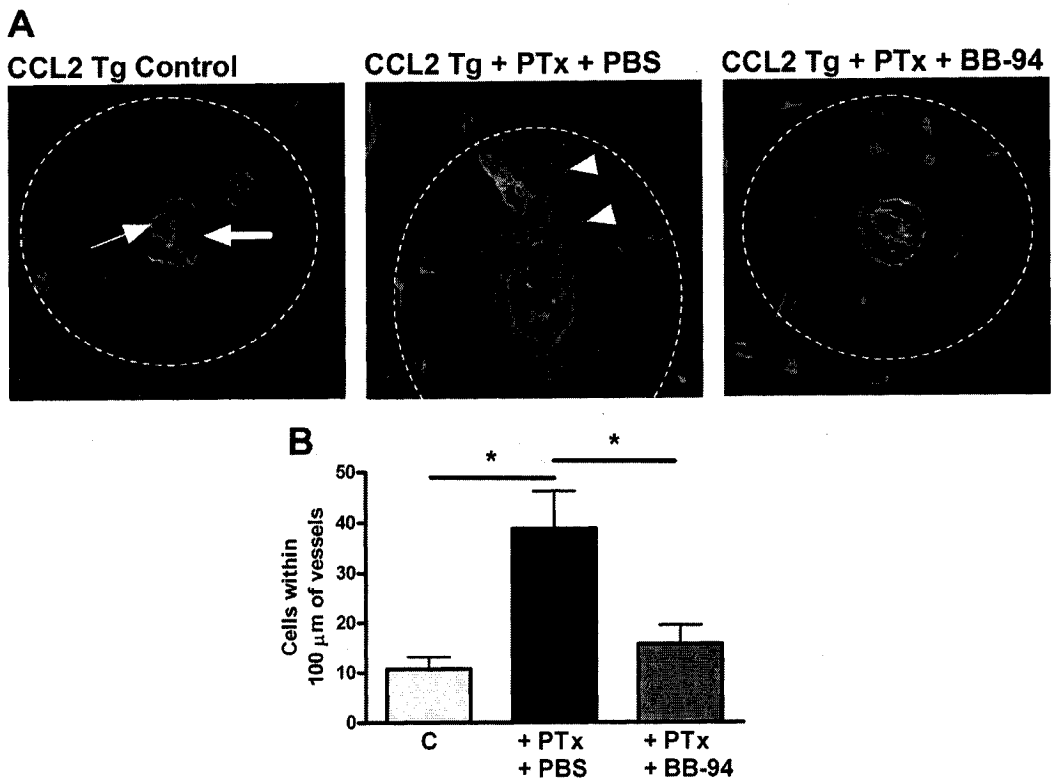


**Figure 6.** Immunofluorescence staining.

A. Laminin-1 (green) and CD45 (red) staining in brain stem white matter of sagittal brain cryosections. No staining was observed with species-matched immunoglobulin (not shown). Mice were sacrificed at day 5 post PTx injection. The dotted line around vessels is drawn to indicate a distance of 100 μm from the astroglial basement membrane. B. Quantification of parenchymal infiltration. $CD45^+$ cells surrounding isolated infiltrates within a 100 μm distance of the outer (astroglial) basement membrane of the BBB were counted in a blinded fashion. Five to seven infiltrated vessels were randomly selected throughout the white matter on at least 4 separate sagittal brain sections from each animal and counted. Counted cells were verified as such by Hoechst staining for nuclei. The results represent 5 mice from each treatment group. *, $P<0.05$. Error bars represent SEM.

Although it is a possibility that glial cells contributed to the production of MPs in response to PTx in Tg mice, we consider this unlikely. The size of PTx (117 kDa) should normally prevent it from readily crossing the BBB to enter the parenchyma and affect glial cells directly. Furthermore, the finding that sorted microglia, in contrast to macrophages, from Tg mice did not upregulate MMP-8 argues against involvement of glial cells in the brain parenchymal infiltration. Also, expression of microglial membrane-bound MMP-15, which we previously found to be downregulated by microglia in EAE (199), was not altered in the Tg mice or by PTx administration. Finally, we observed no change in microglial cell numbers in PTx-treated Tg mice.

PTx upregulated expression of the proinflammatory cytokines TNFα and IL-1β in Tg mice. Both IL-1β and TNFα are associated with weight loss (215) and the increased expression level of these cytokines might contribute to the observed PTx-induced weight loss in Tg mice. In addition, proinflammatory cytokines could increase leukocyte migration by a direct effect on the BBB (216, 217).

Pertussis toxin is classically considered to open the blood-brain barrier (162). However, EAE can be induced in some mouse strains independently of PTx and its role in EAE is likely more complex than increasing vascular permeability (165). This is supported by findings in EAE, where opening of the BBB in mice was found to be independent of PTx, although PTx did enhance severity of disease (218). In our study, administration of PTx did not increase expression of any of the investigated genes in the brains of WT mice. Nor did it induce weight loss or clinical symptoms in WT mice. Only in the case of overexpression of CCL2 in brains of Tg mice did PTx have an effect, presumably either by acting on the already accumulated leukocytes, or on leukocytes in blood, which were then attracted to the brain by CCL2 chemokine. The synergizing effects of a chemokine-rich brain environment and exposure to the environmental stimulus PTx result in dramatic upregulation of proinflammatory and metalloproteinase genes, accompanied by clinical symptoms.

CCL2 Tg mice have large numbers of leukocytes accumulated in the perivascular space, and the fact that perivascular accumulation of leukocytes does not cause disease is of interest. Similar observations were made when EAE was induced in TNFα deficient mice (35), and in mice in which peripheral macrophages have been depleted (36). Perivascular accumulation of leukocytes is therefore not in itself enough to cause pathology. In this context, it is important to take into consideration that the barrier to cellular entry to the CNS actually comprises two separate barriers: endothelial and astroglial (29). The endothelial barrier allows leukocyte transit in states of perivascular accumulation without accompanying clinical symptoms. Migration across the endothelial basement membrane alone is not sufficient to initiate clinical symptoms. The critical event for disease is the PTx-induced migration across the astroglial basement membrane leading to parenchymal infiltration.

Our results show that leukocyte migration across the astroglial basement membrane is dependent on action of MPs, as it could be blocked by treatment with an MP inhibitor. Whether endothelial basement membrane transmigration is also MP-dependent was not addressed in our study, since it had occurred before administration of inhibitor. *In vitro* studies of human leukocyte migration across endothelial basement-like barriers suggest that MPs do play a role in this step (189, 219, 220). Our study emphasizes the need to distinguish between the two basement membranes of the BBB in the context of CNS infiltration. The particular MP profiles needed to cross the endothelial basement membrane and the astroglial basement membrane may not be the same, as the two membranes are structurally different and distinct in their composition of extracellular matrix proteins, especially laminin isoforms (29, 221).

Our results demonstrate that exposure to the environmental agent pertussis toxin in concert with CNS chemokine expression can promote parenchymal brain infiltration through an effect on metalloproteinases. This illustrates potential interplay between infection and inflammation in promoting CNS autoimmune disease. We also demonstrate that it is MP-dependent leukocyte migration across

the astroglial basement membrane of the BBB, not across the endothelial basement membrane, which is the critical event in inducing disease.

# PREFACE TO CHAPTER IV

In Chapter 2 (199) it was established that four out of six MT-MMPs were downregulated in EAE. Chapter 4 extends this finding, by studying the expression of MT-MMPs in detail, both in EAE and in non-immune models of neuroinflammation. These other models include the PTx-driven parenchymal infiltration in CCL2 Tg mice described in Chapter 3, and a model of CNS injury, where the entorhinal cortex is subjected to a stab wound. Furthermore, Chapter 4 addresses the hypothesis that IFNγ may control downregulation of certain MT-MMPs, by examining MT-MMP expression in IFNγ Tg mice, in IFNγ-/- mice with EAE, and in cultured microglia.

# Chapter IV

# Regulation of Expression of Membrane-Type Matrix Metalloproteinases in Central Nervous System Inflammation

**Henrik Toft-Hansen, Alicia A. Babcock, Jason M. Millward, and Trevor Owens**

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## 4.1. Abstract

Matrix metalloproteinases (MMPs) are a large family of proteinases involved in degradation of extracellular matrix and processing of bioactive molecules. We showed previously that four of the membrane type (MT)-MMPs (MMP-15, 16, 17 and 24) were downregulated in spinal cord of mice with severe experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), whereas the two remaining MT-MMPs (MMP-14 and 25) and the majority of secreted MMPs were upregulated.

In this study, we used flow cytometric cell sorting and real-time RT-PCR to demonstrate that all but one of the MT-MMPs are constitutively expressed by microglia, the resident antigen presenting cell in the CNS. Microglia isolated from mice with severe EAE showed statistically significant downregulation of the MMP-15, 17 and 25. We then investigated the relationship of MT-MMP expression with CNS infiltration and IFNγ expression, since these are characteristics of both MS and EAE.

Pertussis toxin-induced parenchymal CNS infiltration in transgenic mice overexpressing CCL2 specifically in the CNS caused no significant change in expression of IFNγ or MT-MMPs. After entorhinal cortex stab lesion, which results in prominent influx of leukocytes to the injured area without IFNγ production, MMP-15, 17 and 25 were significantly downregulated.

Five of the MT-MMPs were downregulated in transgenic mice that specifically overexpress IFNγ in the CNS, whereas MMP-25 was upregulated. We noted no change in MT-MMP expression in IFNγ-/- mice with EAE. IFNγ did not regulate microglial expression of MT-MMPs *in vitro*.

We conclude that expression of MT-MMPs *in vivo* is differentially regulated in models of neuroinflammation, and that presence of IFNγ in some cases correlates with MT-MMP expression.

## 4.2. Introduction

Matrix metalloproteinases (MMPs) are proteinases implicated in all diseases involving neuroinflammation, including multiple sclerosis (MS) (89, 108). MMPs are considered to facilitate cellular infiltration of the central nervous system (CNS) by degrading extracellular proteins, including those present in the neurovascular unit composed of endothelial cells connected by tight junctions, and the glia limitans. Inhibition of MMP activity with broad-spectrum synthetic inhibitors alleviates symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (171-173, 222).

There are 23 matrix metalloproteinases (MMPs) identified in mice (including two forms of MMP-1). Of these, six (MMP-14-17 and 24-25) are membrane-bound and referred to as membrane-type matrix metalloproteinases (MT-MMPs). MMP-17 and 25 are glycosylphosphatidylinositol (GPI)-anchored whereas the other four are type 1 transmembrane proteins with short cytoplasmic domains of about 20 amino acids. MMP-23 is anchored to the cell membrane as a type 2 transmembrane protein. In contrast to the MT-MMPs, it is not active in its bound form, but is secreted and activated in one single proteolytic step (223). Like secreted MMPs, MT-MMPs can cleave extracellular matrix molecules as well as chemokines, cytokines and growth factors (90, 124). MT-MMPs are generally thought to play important regulatory roles because of their ability to cleave substrates in immediate vicinity to the cell membrane, where the cleaved products can influence the cell. In addition, MT-MMPs are known to cleave and activate secreted MMPs, which was first described for activation of MMP-2 by MMP-14 through interaction with tissue inhibitor of metalloproteinases-2 (TIMP-2) (224, 225).

Expression of MMPs can be regulated by cytokines, chemokines, growth factors, and ECM proteins such as collagen (226-228). IFNγ is a prominent cytokine in EAE, and its expression in CNS is reported to increase with severity and decrease with remission of symptoms (179, 229, 230). Expression of other

proinflammatory cytokines such as TNFα and IL-1β also parallel EAE symptoms (231-233). It has previously been determined that the presence of IFNγ alone did not cause parenchymal infiltration or activation of glial cells in the IFNγ Tg mice used in this study (234), where INFγ is expressed in oligodendrocytes under control of the MBP promoter.

In addition to inflammatory conditions, MMPs are important in cancer where they facilitate cellular traffic across basement membranes and within tissues, as well as cleave bioactive molecules (66, 123). It has been recognized for more than 20 years that MT-MMPs could be important in cancer, since they promote angiogenesis and invasion (235, 236). MT-MMPs are in fact expressed in most cancer types and can in many cases be correlated with malignancy (90). Several studies have demonstrated importance of MMPs in animal models of cancer, but MMP inhibitors have so far not been successful in clinical cancer trials (98). This is likely because the broad-spectrum inhibitors used failed to specifically target detrimental effects of MMPs, and also inhibited beneficial effects (100). This illustrates the potential benefit of understanding the differences between MMPs in greater depth.

The particular roles and substrate specificities of MT-MMPs have not been described in detail, and the role of MT-MMPs in neuroinflammation is unclear. We previously showed that the majority of MT-MMPs, with the exceptions of MMP-14 and MMP-25, are downregulated in spinal cord of mice with adoptively transferred EAE (AT-EAE) at peak disease (199). This downregulation is in contrast with the general upregulation of secreted MMPs. The same result was obtained in $MOG^{35-55}$ induced EAE (214). The functional consequence of this specific pattern of MT-MMP expression is unknown.

In this study, we report differential regulation of MT-MMPs in neuroinflammation. In particular, we describe differences in expression of MT-MMPs by microglia and infiltrating cells in EAE. Finally, we show that regulation of MT-MMPs in some cases correlates with expression of IFNγ.

## 4.3. Materials and Methods

Mice

Wild-type (WT) SJL/J (SJL) and C57BL/6J (B6) were obtained from Jackson Laboratory, Bar Harbor, Maine or from Charles River Canada (St. Constant, QC, Canada). Transgenic mice expressing the chemokine CCL2 in the CNS under control of a myelin basic protein (MBP) promoter (34) were obtained from Bristol-Myers Squibb (New Brunswick, New Jersey) and maintained as a colony at the Montreal Neurological Institute. Generation of IFNγ Tg mice of the VT1 line expressing IFNγ in the CNS under control of a truncated MBP promoter was described previously (234). IFNγR-/- mice were originally donated by Dr. David Willenborg (Canberra Hospital, Australia). For EAE, female 6-12 weeks old mice were used. Mice for ECL were females and weighed 20-25g. For other experiments, 6-12 weeks old mice of mixed sex were used. Mice were bred and maintained in a specific pathogen-free environment. Animal breeding, maintenance and all experimental protocols were performed in accordance with Canadian Council for Animal Care guidelines as approved by the McGill University Animal Care Committee.

Actively induced EAE

B6 mice were immunized sc. at the base of the tail with 100 µl of an emulsion containing 100 µg $MOG^{35-55}$ (Sheldon Biotechnology, Montreal, Canada) and 1 mg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, Michigan, USA) in Freund's incomplete adjuvant (Difco) and boosted in the flanks 7 days later with the same amount. 15 µg/kg PTx was administered ip. on day 0 and 2. SJL mice were immunized sc. at the base of the tail with 100 µl of an emulsion containing 100 µg $PLP^{139-151}$ (Sheldon Biotechnology) and 200 µg of *Mycobacterium tuberculosis* H37 RA (Difco) in Freund's incomplete adjuvant (Difco) and boosted in the flanks 7 days later with the same amount. Mice were weighed and monitored daily for clinical signs of EAE, scored as: 1, flaccid tail; 2, hind limb

weakness and poor righting ability; 3, one hind limb paralyzed; 4, both hind limbs paralyzed with or without forelimb paralysis and incontinence; 5, moribund.

Adoptive transfer EAE (AT-EAE)

AT-EAE in SJL mice was induced by passive transfer of MBP-reactive T cells as described previously (199). AT-EAE in B6 mice was induced by passive transfer of $MOG^{35-55}$-reactive T cells. B6 mice were immunized as for active EAE and lymph node cells were cultured in the presence of 10 μg/ml $MOG^{35-55}$ (Sheldon Biotechnology, Montreal, Canada) and 5 U/ml IL-2 (Biosource, Nivelles, Belgium). Otherwise, culture conditions were as described for SJL AT-EAE. $10x10^6$ lymphoblasts were transferred into the tail vein. Recipient mice received 15 μg/kg PTx ip on day 0 and 2 after transfer, and were followed and scored as described for active EAE.

Administration of pertussis toxin to CCL2 Tg mice

Pertussis toxin (10 μg/kg) (Sigma, Oakville, Ontario) in Hank's balanced salt solution (HBSS) (Invitrogen Life Technologies, Carlsbad, California) was injected ip. at day 0. Mice were monitored daily until sacrifice at day 5.

Flow cytometry and cell isolation

Cells from CNS of mice were analyzed using flow cytometry as described (199), except EC samples, which were not subjected to a Percoll gradient in order to make analysis from individual samples possible. For flow cytometric analysis of cultured microglia, cells were washed in HBSS and incubated in supernatant from the anti-FcγR 24G2 (anti CD16/32) hybridoma with hamster IgG (50 μg/ml) (Cedarlane, Hornby, Ontario) and 2% added FBS on ice for 20 min. to block non-specific antibody binding. Cells were stained on ice for 30 min. with phycoerythrin (PE)-conjugated anti-CD45 and fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (BD Biosciences Pharmingen). The cells were washed in HBSS with 2% FCS and analyzed using a Becton-Dickinson FACScan or a

FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, California). Flow cytometry data were analyzed using CellQuest software.

Isolation of RNA

Total RNA was purified using Trizol RNA isolation reagent (Invitrogen Life Technologies) according to the manufacturer's protocol for whole tissue RNA extraction. For sorted cells, Trizol with added glycogen (Invitrogen Life Technologies) was used according to the manufacturer's protocol for RNA extraction for low amounts of RNA.

Reverse transcriptase reaction

RNA (3 μg) from each tissue sample was incubated with M-MLV reverse transcriptase (RT) (Invitrogen Life Technologies) according to the manufacturer's protocol using random hexamer primers. RNA from sorted cells was incubated with SuperScript II RT (Invitrogen Life Technologies) according to the manufacturer's protocol using random hexamer primers.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was done using ABI Prism 7000 or 7300 Sequence Detection Systems according to our previously described method and MMP probe and primer sequences (199). IL-1β probe: AGC TGG AGA GTG TGGAT. IL-1β forward primer: CTT GGG CCT CAA AGG AAA GAA. IL-1β reverse primer: AAG ACA AAC CGT TTT TCC ATC TTC. TNFα probe: CTC ACA CTC AGA TCAT. TNFα forward primer: CCA AAT GGC CTC CCT CTCAT. TNFα reverse primer: TCC TCC ACT TGG TGG TTTGC. IFNγ probe: TCA CCA TCC TTT TGC CAG TTC CTC CAG. IFNγ forward primer: CAT TGA AAG CCT AGA AAG TCT GAA TAAC.

Brain stab lesion

Under anesthesia, B6 mice were placed into a stereotactic apparatus (Kopf, Tujunga, CA) for wireknife transection of axons in the entorhinal cortex (EC), as

previously described (150). This induces a mechanical stab injury of the EC. Twenty-four hours following surgery, mice were perfused with PBS, the EC was dissected, and approximately 2 mm of tissue surrounding the wireknife lesion was collected. For uninjured controls, the EC was dissected from either the contra lateral hemisphere of the brain or else from unmanipulated mice.

Isolation and culturing of microglia

Female SJL mice (8-10 weeks old) were perfused with 20 ml ice-cold PBS, then brains and spinal cords were dissected and collected in HBSS. The tissue was dissociated through a 70 μm cell strainer (BD Biosciences Pharmingen) and suspended in 70% isotonic Percoll (Amersham Biosciences, Baie d'Urfe, Quebec). Myelin was removed after centrifugation through a Percoll gradient, and cells were collected at the interface between 30 and 37% Percoll. Cells were washed and resuspended in RPMI 1640 (Gibco, Burlington, Ontario, Canada) supplemented with 10% FCS (Sigma), 50 μM β-mercaptoethanol (Sigma), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Finally, cells were diluted to $1x10^6$ cells/ml and plated in a 96 well plate at a density of $2x10^5$ cells per well. An aliquot of cells were stained with anti-CD45 and anti-CD11b antibodies for verification as $CD45^{dim}$ CD11b+ microglia. Purity was 95%. Approximately $2.5x10^5$ microglia were obtained per mouse. Microglia were left overnight to settle and thereafter treated with mouse IFNγ (Sigma) or LPS (Sigma) for 16 hours. The supernatant was removed and microglia were lysed in Trizol for RNA isolation.

## 4.4. Results

### *4.4.1. MT-MMP expression during EAE*

Given the previous finding by ourselves (199) and others (214), that four of the six MT-MMPs (MMP-15, 16, 17 and 24) are downregulated in spinal cord of mice with EAE at peak disease, we decided to study the expression of all six MT-MMPs during various stages of relapsing-remitting AT-EAE: early (grade 2),

peak (grade 4), and mice that had remitted from symptoms. The expression profiles for the downregulated MMPs followed disease progression with restored expression after remission of symptoms (Fig.1, panels B-E). Similar results for MMP-15 have been published by us previously (199), and are confirmed here. MMP-14 (Fig. 1A) showed early downregulation in grade 2, but upregulation at peak disease with sustained upregulation after remission. By contrast MMP-25 (Fig. 1F) was upregulated both in early and peak disease, and also with sustained expression after remission. Expression of IFNγ followed disease course (Fig. 1G). In addition, we analyzed expression of MT-MMPs in both actively induced and adoptively transferred $MOG^{35-55}$ EAE in B6 mice at peak disease (Fig. 2). Some differences in fold regulation were observed compared to AT-EAE in SJL mice, but we confirmed detection of IFNγ, statistically significant upregulation of MMP-14 and 25, and downregulation of MMP-15, 16, 17 and 24 in both models. This shows that the pattern of MT-MMP regulation in EAE at peak disease is independent of mouse strain, myelin antigen and method of disease induction.

### *4.4.2. Cellular sources of MT-MMP expression in EAE*

We investigated cellular sources of MT-MMP expression at peak disease, focusing on infiltrating immune cells and microglia (Fig. 3). Microglia were defined as $CD45^{dim}$ CD11b+, macrophages and granulocytes as $CD45^{high}$ CD11b+, and T cells as $CD45^{high}$ CD3+. Microglia are present in both control mice and mice with EAE, whereas infiltrating cells are only available in sufficient numbers from mice with EAE. We observed a significant downregulation by microglia in EAE of MMP-15, 17 and 25 (Fig. 3B, D and E), as well as a contribution from infiltrating cells. This confirms results for MMP-15 published previously (199). Expression of MMP-14 and 16 by microglia (Fig. 3A and C) were not significantly altered in EAE. There was a contribution to whole brain levels of message for both by infiltrating cells, especially MMP-14 expression by

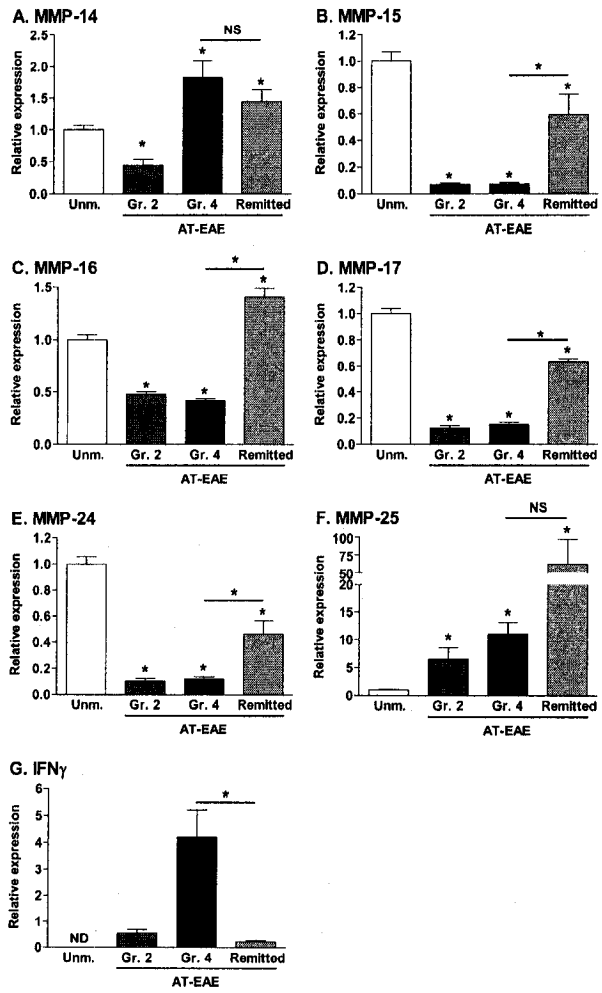


**Figure 1.** MT-MMP and IFNγ gene expression during AT-EAE in SJL mice.

Gene expression levels of MT-MMPs and IFNγ in spinal cord from unmanipulated SJL mice, mice with early onset (grade 2), severe EAE (grade 4), and mice after remission, were measured by real-time PCR. Values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the unmanipulated group (except for IFNγ). The values are arbitrary and cannot be compared between panels. N = 3-6. Significance of comparisons to unmanipulated mice was determined using a two-tailed t-test. A two-tailed t-test was also used to compare mice with severe EAE to the remitted group. *:$P < 0.05$; NS: not significant; ND: not detected; Unm.: unmanipulated; Gr.: grade. Error bars represent SEM.

macrophages and granulocytes. Expression levels of MMP-24 by sorted cells were too low for analysis.

### *4.4.3. Correlations between MT-MMP and IFNγ expression in EAE*

As previously published (179, 229, 230), expression of the proinflammatory cytokine IFNγ, which is expressed by activated T cells in EAE, paralleled disease severity (Fig. 1G). We tested if there was a correlation between expression of MT-MMPs and IFNγ by plotting expression levels of MT-MMPs in individual samples against expression level of IFNγ in the same samples (Fig. 4). There was a significant positive correlation between expression of MMP-14 and IFNγ expression (Fig. 4A). In contrast, there was a significant negative correlation for MMP-15, 16 and 17, which were downregulated at peak disease (Fig. 4B-D). There was no correlation between MMP-24 or MMP-25 and IFNγ (Fig. 4E-F).

### *4.4.4. MT-MMPs expression is unchanged by PTx-induced parenchymal infiltration in CCL2 Tg mice*

Leukocytes spontaneously accumulate in the perivascular space in the CNS of transgenic mice which express the chemokine CCL2 in the CNS under control of a truncated MBP promoter (34). Systemic administration of PTx caused parenchymal infiltration in CCL2 Tg mice, accompanied by weight loss and occasional clinical symptoms (Chapter 3). A similar finding has been described using another CNS-specific CCL2 Tg mouse (206). Contrary to WT, unmanipulated CCL2 Tg mice showed a basal level of IFNγ expression in brain (Fig. 5A), but this was unchanged by systemic administration of PTx. Unmanipulated CCL2 Tg mice also showed a significantly higher expression of MMP-14 and 17 compared to WT (Fig. 5B and D), whereas none of the other MT-MMPs were significantly altered. Administration of PTx to WT mice led to a small but statistically significant upregulation of MMP-24 expression, but there was no change in the remaining MT-MMPs. Importantly, none of the MT-MMPs

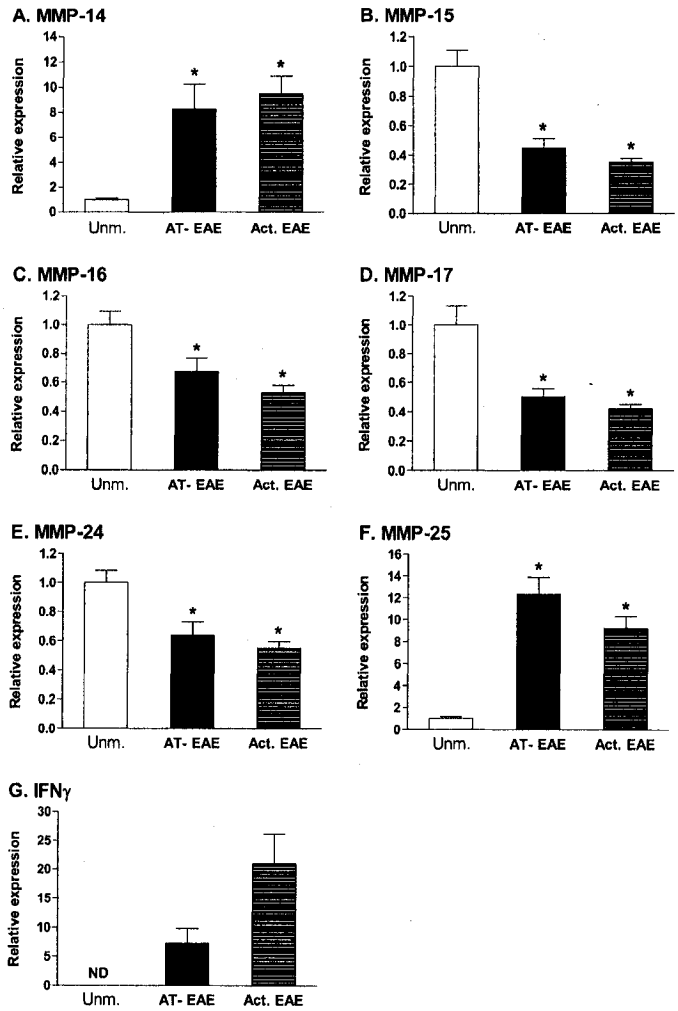


**Figure 2.** MT-MMP gene expression in active and AT-EAE in B6 mice.

Gene expression levels of MT-MMPs in spinal cord from unmanipulated B6 mice and mice with severe (grade 4) EAE were measured by real-time PCR. EAE was induced either by immunization with $MOG^{35-55}$ in CFA (active) or adoptive transfer of $MOG^{35-55}$ specific T cells (AT-EAE). Values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the unmanipulated group (except for IFNγ). The values are arbitrary and cannot be compared between panels. N = 4 or 5. Significance of comparisons to unmanipulated mice was determined using a two-tailed t-test. *:$P < 0.05$; ND: not detected; Unm.: unmanipulated; Gr.: grade; Act.: active. Error bars represent SEM.

were significantly affected by PTx in CCL2 Tg mice, even though this leads to parenchymal infiltration (Chapter 3). Equivalent results for MMP-15 are not shown here, but are reported in Chapter 3; there were no changes in MMP-15 expression in any of the groups. In contrast to the MT-MMPs, a number of secreted MMPs were upregulated upon PTx injection in CCL2 Tg mice (Chapter 3).

### *4.4.5. MT-MMPs after brain stab lesion*

24 hours after stab lesion in the EC, we observed considerable infiltration of $CD45^{high}$ cells (Fig. 6). The majority of the infiltrating cells (3.58±0.29 % of total cells compared to 0.05±0.01 % in control EC, n=4) were macrophages or granulocytes, defined as $CD45^{high}$ CD11b+ (Fig. 6A, upper right quadrants), and a smaller proportion (0.22 ± 0.01 %, compared to 0.02 ± 0.01 % in control EC, n=4) were T cells defined as $CD45^{high}$ TCRβ+ (Fig. 6B, inserted boxes). The proinflammatory cytokines TNFα and IL-1β were upregulated following stab lesion, as well as in EAE (Fig. 7A-B). Apart from a low level in one out of five samples, IFNγ was not detected following lesion, which is in contrast to EAE (Fig. 7C). No change in expression of MMP-14, 16 and 24 was noted following stab lesion, whereas MMP-15, 17 and 25 were significantly downregulated (Fig. 7D-I).

### *4.4.6. IFNγ does not regulate microglial MT-MMP expression* in vitro

We isolated microglia from CNS of adult SJL mice and IFNγR-/- mice on SJL background. Cell preparations were determined by FACS to contain 95% microglia (Fig. 8A). The microglia were then treated with varying concentrations of IFNγ, or with LPS. As expected, the microglia responded to LPS by upregulating expression of IL-1β (data not shown) and TNFα, and to IFNγ by upregulating TNFα, as measured by real-time PCR (Fig. 8B). However, no

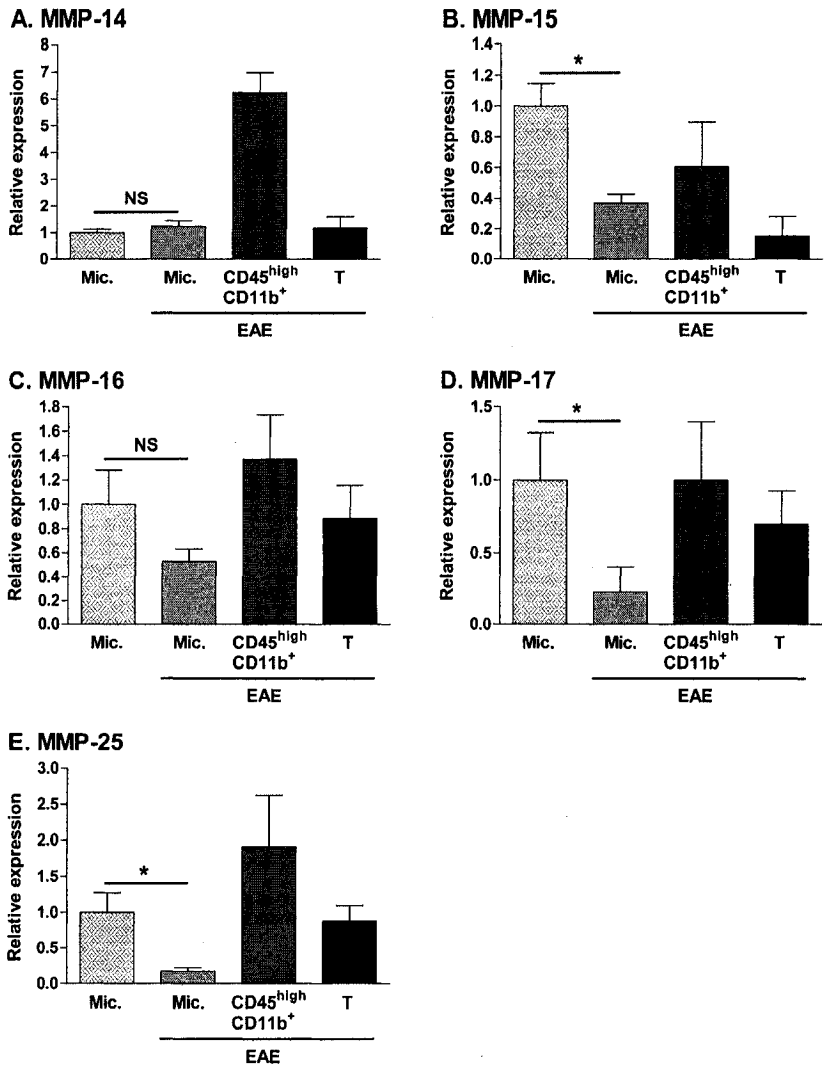


**Figure 3.** MT-MMP mRNA expression in cell populations sorted from CNS.

Cell populations were sorted from whole CNS preparations based on flow cytometric detection of surface markers: Microglia as $CD45^{dim}$ CD11b+, macrophages and granulocytes as $CD45^{high}$ CD11b+, and T cells as $CD45^{high}$ CD3+. Values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the unmanipulated microglia. The values are arbitrary and cannot be compared between panels. N = 4-8. Significance of comparison between microglial groups was determined using a two-tailed t-test. *:$P < 0.05$; NS: not significant; Mic.: microglia; T: T cells. Error bars represent SEM.

statistically significant change in expression of MMP-15 (Fig. 8C) or any other MT-MMP (data not shown) in response to IFNγ or LPS treatment was observed.

#### *4.4.7. MT-MMPs in IFNγ-/- and IFNγ Tg mice*

All MT-MMPs except MMP-25 were significantly downregulated in spinal cord of IFNγ Tg mice expressing IFNγ specifically in the CNS (Fig. 9). No statistically significant difference in expression of any of the MT-MMPs was observed when EAE was actively induced in IFNγ-/- mice (Fig. 9).

## 4.5. Discussion

Our aim was to study the relationship of MT-MMP expression with CNS infiltration and IFNγ expression. The expression patterns of the MT-MMPs from the described *in vivo* models are summarized in Table I. It is clear, that individual MT-MMPs are differentially regulated in neuroinflammation. IFNγ expression correlates with expression of some MT-MMPs, but not all. Other factors must also play a role.

Expression of MT-MMPs can be regulated by growth factors and cytokines (226, 227). Interaction with extracellular matrix might also be important, and it was shown that TNFα only stimulated fibroblasts to upregulate MMP-14 when the fibroblasts were grown in collagen (237). Transcription factors also play a role, as TNFα-mediated upregulation of MMP-14 expression was dependent on NFκB activation; and binding of another transcription factor, Egr-1, to the MT-MMP promoter sequence can enhance MMP-14 transcription (238). However, the promoter sequence of MMP-14 lacks the TATA box and binding sites for AP-1 and ETS transcription factors found in promoter regions of most secreted MMPs. (239-242), so it is possible that differences exist between regulation of secreted and MT-MMPs. To our knowledge, detailed information for promoter sequences for MT-MMPs other than MMP-14 is lacking.

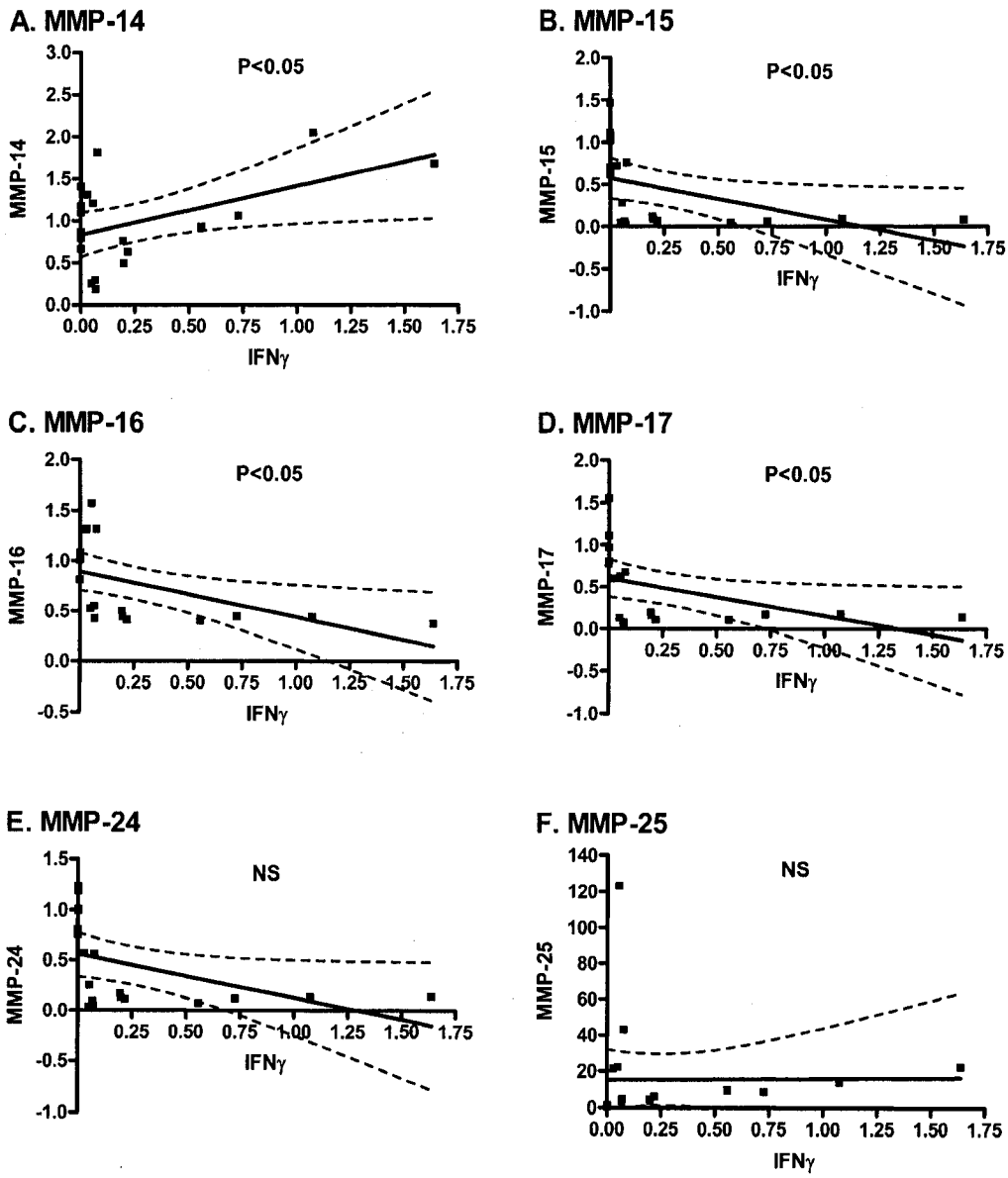


**Figure 4.** Correlations between expression of MT-MMPs and IFNγ in EAE. Spinal cord gene expression levels of MT-MMPs are plotted against expression levels of IFNγ from the samples used to generate Fig. 1 (unmanipulated, early and severe EAE, and after remission). A t test was used to determine if the slope of the linear regression line was different from zero with 95 % probability. $P<0.05$ indicates significant correlation. r: correlation coefficient. NS: not significant.

MMP-14 is the best studied among the MT-MMPs. MMP-14-/- mice have defects in angiogenesis, and severe skeletal disorders resulting in dwarfism and death within 3 months (243, 244). MMP-14 activates MMP-2 via TIMP-2 (224, 225), and activation of MMP-2 is deficient in MMP-14-/- mice. However, lack of activated MMP-2 cannot fully account for the severe phenotype in MMP-14-/- mice, since MMP-2-/- mice develop normally except for a delay in growth (245). However, angiogenesis was suppressed in MMP-2-/- mice. Overexpression of MMP-14 in mammary glands leads to tumor development (246), indicating a deleterious effect of excessive MMP-14 expression. We observed upregulated MMP-14 expression in EAE at peak disease (Fig. 1 and 2), which, at least in part, could be explained by its expression in infiltrating cells, especially macrophages and granulocytes (Fig. 3). We observed basal expression of MMP-14 by microglia which was not altered in EAE. MMP-14 has before been demonstrated to be expressed by white matter microglia in both normal and Alzheimer's disease brain tissues (247). We have not in this study examined MMP-14 expression by other CNS resident cells, but such cells might contribute to the overall level of MMP-14. MMP-14 has for example been shown to be expressed in reactive astrocytes (248). We found that MMP-14 was upregulated in unmanipulated CCL2 Tg mice (Fig. 5), which probably reflects expression in accumulated leukocytes. MMP-14 correlates positively with IFNγ expression in EAE spinal cord samples (Fig. 4). However, MMP-14 was downregulated in two cases where IFNγ was present: In early EAE (Fig. 1) and in IFNγ Tg mice (Fig. 9); and it was not altered in microglia from mice with severe EAE (Fig. 3). A possible explanation could be, that IFNγ downregulates MMP-14 expression by CNS resident cells other than microglia, but the expression level increases on the whole tissue level in neuroinflammation due to the influx of cells expressing MMP-14.

MMP-15 is known to be associated with tumor progression, possibly through anti-apoptotic activity (249), and has also been reported to activate MMP-2 without the involvement of TIMP-2 (250). In general, MMP-15 is considered to be very similar to MMP-14 in structure and action, but it has not been studied to the same degree. We find that MMP-15 is regulated differently than MMP-14 in a

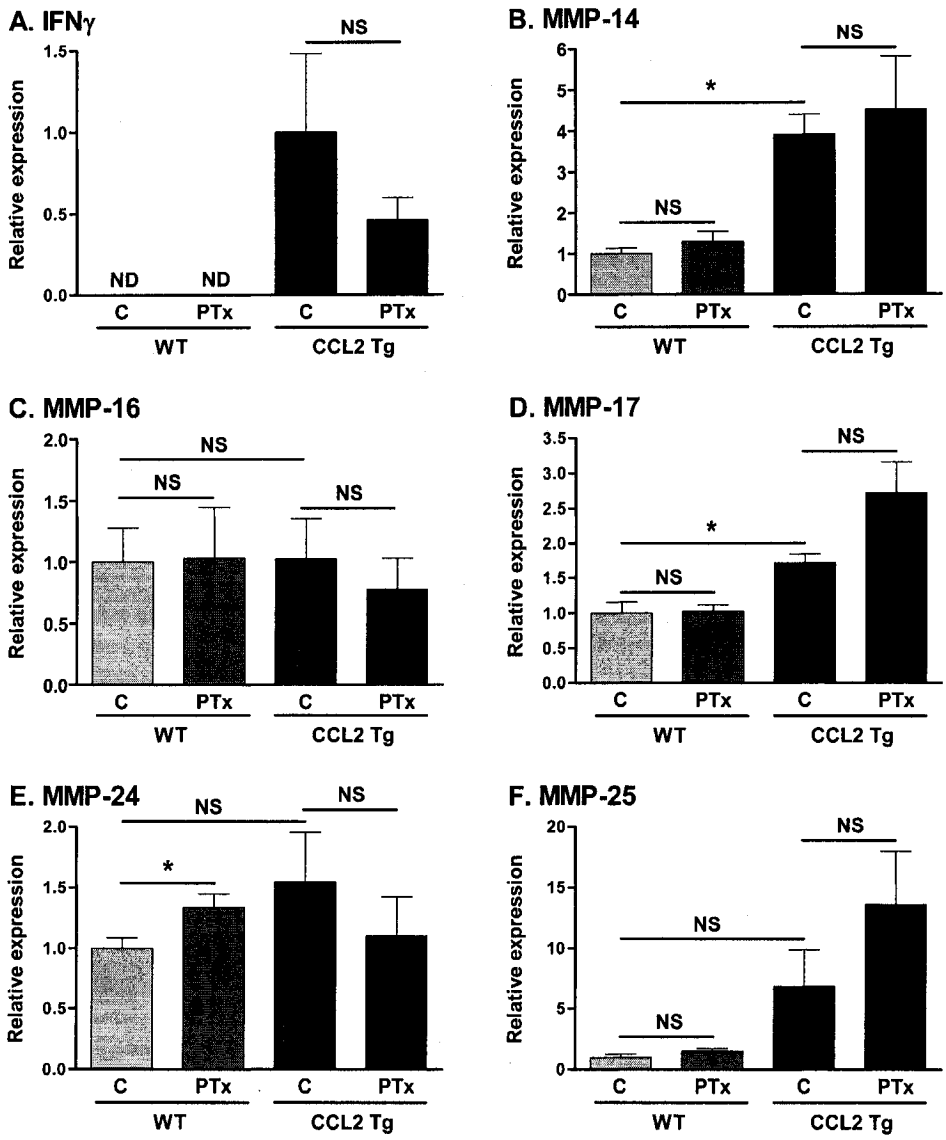


**Figure 5.** Gene expression of IFNγ and MT-MMPs in brain.

WT or CCL2 Tg mice received PTx by iv. injection and were sacrificed 5 days later. Gene expression in brain samples was analyzed using real-time PCR. Values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the control WT group (except for IFNγ). The values are arbitrary and cannot be compared between panels. N = 5 for each group. Significance of comparisons between control and PTX-injected mice was determined using a two-tailed t-test. *:$P < 0.05$; ND: not detected; C: control; NS: not significant. Error bars represent SEM.

number of cases. We and others (214) have noted that MMP-15 is downregulated in EAE. This probably reflects downregulation by microglia and a relatively low level of MMP-15 expression by infiltrating cells (Fig. 3), as stated previously (199). In contrast to MMP-14, MMP-15 is negatively correlated with IFNγ expression in EAE (Fig. 4). It is also downregulated in IFNγ Tg mice (Fig. 9) and after stab lesion in the EC (Fig. 7). In accordance with a putative negative regulatory effect by IFNγ on MMP-15, there was no downregulation of MMP-15 in IFNγ-/- mice with EAE (Fig. 9). However, IFNγ is not the only factor involved in MMP-15 regulation, as we also noted downregulation after stab lesion, where IFNγ is not present. We were not able to demonstrate changes in expression of MMP-15 or other MT-MMPs *in vitro* (Fig. 8), perhaps due to the inherent activation involved with culturing cells, and/or lack of three dimensional matrix structure in culture (237, 251, 252).

MMP-16 has been shown to be expressed by microglia in human brain tissue (253). We also find MMP-16 expressed by mouse microglia (Fig. 3). MMP-16 was not downregulated by microglia in EAE. This and the fact that MMP-16 was not downregulated after stab lesion are the only two major differences from the regulation of MMP-15 expression. Both CNS injury and EAE induce microglial activation (38, 254, 255). Taken together with the observation that MMP-16 is downregulated in many cases with IFNγ present, it could mean that MMP-16 is downregulated by IFNγ, but not by activated microglia.

Expression of MMP-17 follows the pattern for MMP-15 with one exception, which is the significantly increased expression in brains of CCL2 Tg mice. MMP-17 is one of the two GPI-anchored MT-MMPs, the other being MMP-25. MMP-17 is expressed by monocytes (136), analogously to MMP-25 which was found to be expressed by leukocytes (hence the original name for MMP-25: leukolycin), in particular by neutrophils (256, 257). Despite the analogies between MMP-17 and 25, we find them quite differently regulated. The pattern of MMP-17 expression and regulation is more like the pattern of MMP-15 and 16, rather than MMP-25.

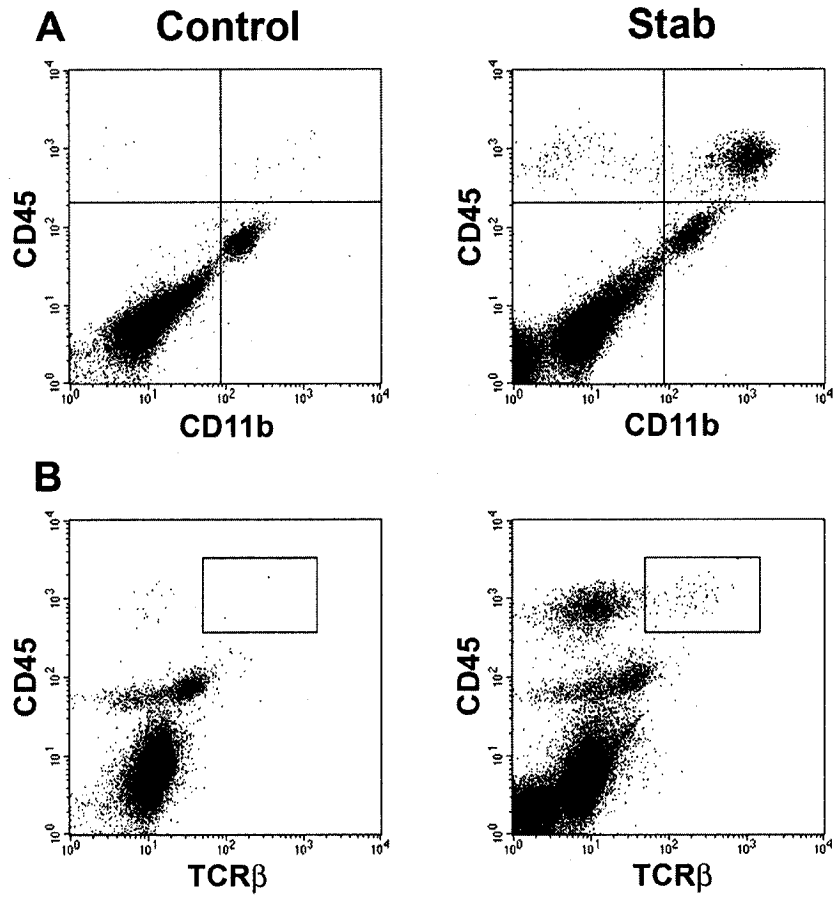


**Figure 6.** Flow cytometric analysis of cells in EC following stab lesion.

A: Flow cytometry profiles showing $CD45^{high}$ CD11b+ macrophages and granulocytes (upper right quadrants) in uninjured control EC (left panel) and 24 hours after stab injury (right panel) in B6 mice. B: Equivalent analysis of $CD45^{high}$ TCRβ+ T cells (inserted boxes).

MMP-25 was upregulated in EAE at peak disease, like MMP-14 (Fig. 1 and 2). In other aspects, however, MMP-25 was regulated differently from MMP-14. Expression of MMP-25 was not correlated with IFNγ expression (Fig. 4), and not significantly altered in IFNγ Tg mice (Fig. 9). MMP-25 was downregulated by microglia in EAE (Fig. 3), and in EC after stab injury (Fig. 7). It is possible that MMP-25 is downregulated by activated microglia in an IFNγ-independent manner. MMP-25 has been proposed to be especially suited to facilitate leukocyte crossing of basement membranes (258), and we also noted expression by infiltrating leukocytes (Fig. 3). In IFNγ-/- mice with EAE there was a trend toward upregulation of MMP-25, although not statistically significant (Fig. 9).

We observed MMP-24 expression in whole spinal cord and brain, but not by microglia or infiltrating cells. This corresponds with previous findings that MMP-24 is CNS specific and expressed by neurons (259, 260). MMP-24 expression might be regulated by IFNγ, since it is downregulated in IFNγ Tg mice (Fig. 9) and in WT EAE (Fig. 1 and 2), whereas it is not affected in IFNγ-/- mice with EAE (Fig. 9). However, the negative correlation with IFNγ expression in EAE was not sufficiently strong to be statistically significant (Fig. 4). The small upregulation by PTx in CCL2 Tg mice (Fig. 5) cannot be explained at this point.

Some caution must be exerted in interpreting a result that shows a small downregulation of a molecule on the whole tissue level in an organ that is undergoing infiltration. We have previously determined the proportion of $CD45^{high}$ infiltrating cells at peak AT-EAE in SJL mice to be 34% in the CNS preparation for flow cytometry (199). If these infiltrating cells do not express a particular gene, which is expressed by endogenous cells in the unmanipulated CNS, then the influx of cells alone will lead to an overall downregulation at the whole tissue level, even if the expression level in individual resident cells did not change. We analyzed microglia from both unmanipulated mice and mice with EAE for direct comparison of this cell type in a disease state with the normal state (Fig. 3).

MMP-15, 17 and 25 were downregulated in EAE microglia, and this corresponds with the change in whole spinal cord for MMP-15 and 17 (Fig. 1 and

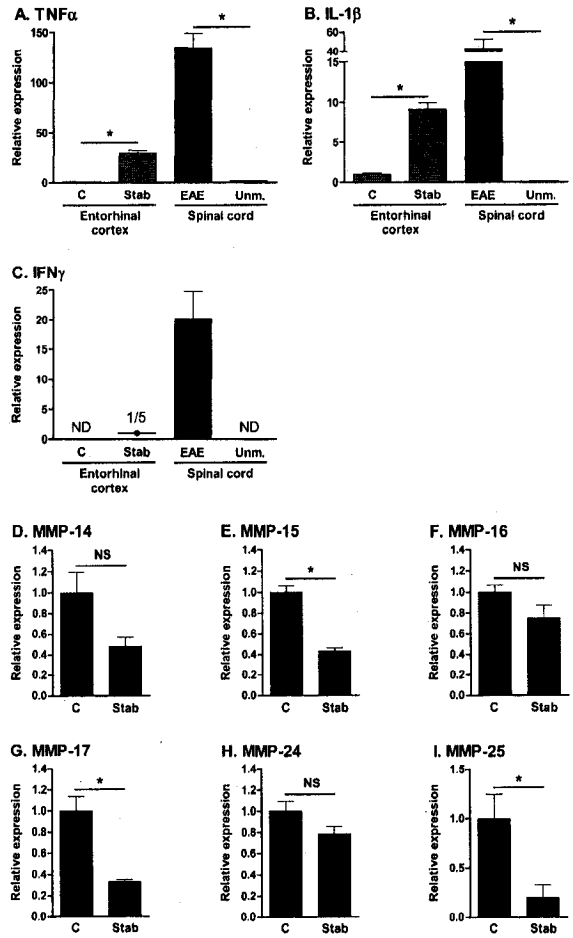


**Figure 7.** Gene expression of cytokines and MT-MMPs after brain stab lesion. Real-time PCR analysis of cytokine and MT-MMP expression. Panels A-C show cytokine expression in EC (left hand side of graphs), and in spinal cord (right hand side). Panels D-I show MT-MMP expression in EC. Spinal cord samples were from B6 mice with grade 4 AT-EAE induced by $MOG^{35-55}$ specific T cells, or from unmanipulated controls. EC samples were from B6 mice 24 after stab lesion, or from control EC. IFNγ was only detected in one of the stab samples (Panel C). Values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the control WT group (except for IFNγ). The values are arbitrary and cannot be compared between panels. Significance of comparisons between groups was determined using a two-tailed t-test. N = 4-6. *:$P < 0.05$; ND: not detected; Unm.: unmanipulated; C: control; NS: not significant. Error bars represent SEM.

2). For MMP-25, the change on the whole spinal cord level was in the opposite direction, which illustrates the point that changes in individual cell types can be masked by changes in cellular composition of the tissue. The same pattern of MT-MMP regulation was apparent in stab injured EC as in EAE microglia. Possibly, this illustrates the same fundamental regulation of MT-MMP expression on microglia in both EAE and after stab lesion, but with a difference in the composition of the infiltrating cells. MMP-25 is expressed by neutrophils, and we determined the proportion of granulocytes in AT-EAE in SJL mice to be 9%, slightly higher than macrophages (7%) (199). We have not investigated the proportion of infiltrating granulocytes relative to macrophages after stab lesion.

The functional consequence of upregulation of MMP-14, MMP-25 or any secreted MMP, in neuroinflammation is straight-forward to explain: expression of proteinases by infiltrating cells enables them to infiltrate the CNS by cleaving extracellular matrix proteins. This is most likely not the complete explanation, since MMPs have many other substrates than matrix proteins, and MMPs are likely also involved in regulation of the inflammatory process by cleaving bioactive molecules (124). The downregulation of certain MMPs in neuroinflammation does not correspond well with the simple notion of MMPs as only matrix degrading proteins. However, at this time, when the specific substrates of individual MT-MMPs are yet ill-defined, it is not possible to clearly explain the consequence of the down-regulation. One can speculate that microglia downregulate certain MT-MMPs in the process of activation, such as is the case for the innate immune receptor TREM-2 (261, 262). Further studies need to be carried out in order to determine whether it would be beneficial for patients with diseases involving neuroinflammation to inhibit or enhance the action of MT-MMPs.

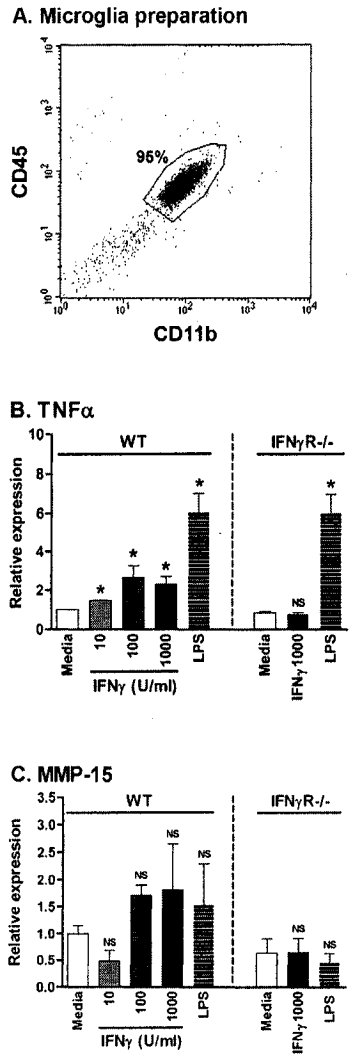


**Figure 8.** Gene expression by microglia stimulated *in vitro*.

A: Microglia were isolated from whole CNS and determined by flow cytometry to be 95% pure. B, C: TNFα and MMP-15 expression by cultured microglia stimulated with IFNγ at varying concentrations, or LPS at 100 ng/ml. Microglia from several WT or IFNγR-/- mice were pooled separately and dispersed in culture wells. Each bar graph represents average expression in three identically treated wells. Panels B and C show values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the control WT group. The values are arbitrary and cannot be compared between panels. Significance of comparisons to the media-treated controls was determined using a two-tailed t-test. *:$P < 0.05$; NS: not significant. Error bars represent SEM.

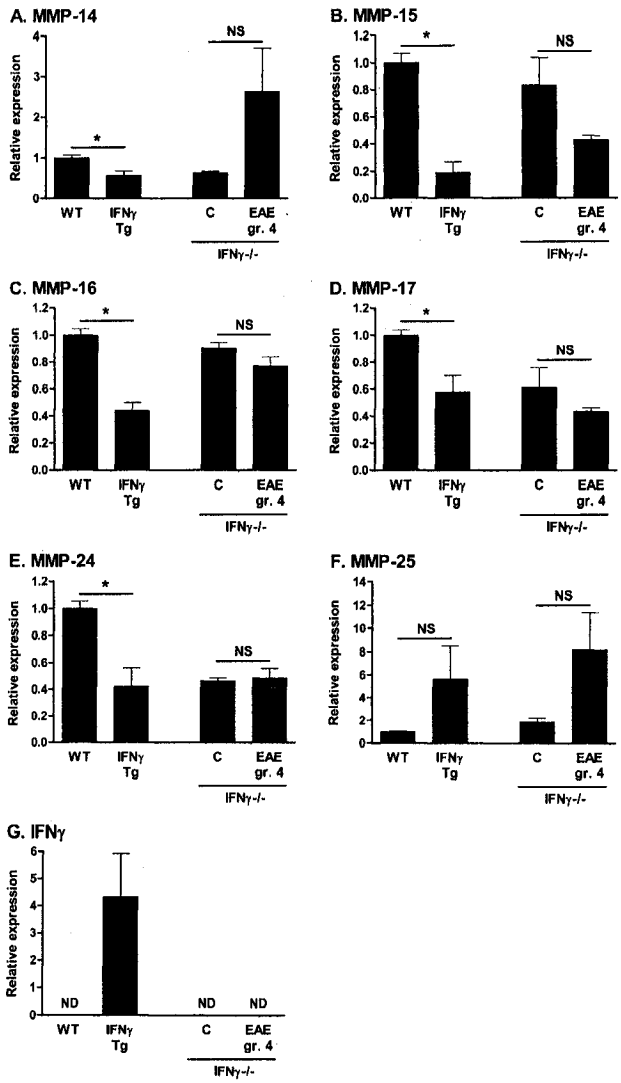


**Figure 9.** Gene expression of MT-MMPs in IFNγ Tg mice, and IFNγ-/- mice with EAE.

Gene expression was analyzed in spinal cord samples using real-time PCR. IFNγ-/- mice were immunized with $PLP^{139-151}$ to induce EAE. Values on the Y-axis are relative to expression of 18S rRNA, and normalized to the expression level of the control WT group (except for IFNγ). The values are arbitrary and cannot be compared between panels. Significance of comparisons to controls was determined using a two-tailed t-test. N = 6 for WT and IFNγ Tg groups, and 3 for IFNγ-/- groups. *:$P < 0.05$; ND: not detected; C: control; NS: not significant; gr.: grade. Error bars represent SEM.

**Table I.** Changes in gene expression of IFNγ and MT-MMPs in *in vivo* models of neuroinflammation[a]

| | IFNγ | MMP-14 | MMP-15 | MMP-16 | MMP-17 | MMP-24 | MMP-25 | Parenchymal infiltration |
|---|---|---|---|---|---|---|---|---|
| SJL AT-EAE | Yes | Up | Down | down | down | down | Up | Yes |
| B6 active EAE | Yes | Up | Down | down | down | down | Up | Yes |
| B6 AT-EAE | Yes | Up | Down | down | down | down | Up | Yes |
| IFNγ Tg | Yes | Down | Down | down | down | down | - | No |
| CCL2 Tg | Yes | Up | - | - | up | - | - | No |
| CCL2 Tg + PTx | Yes | - | - | - | - | - | - | Yes |
| IFNγ -/- active EAE | No | - | - | - | - | - | - | Yes |
| Brain stab lesion | No | - | Down | - | down | - | Down | Yes |

[a] Only statistically significant ($P<0.05$) changes in gene expression compared to the appropriate control group are reported as up or down-regulation. EAE results listed are for peak disease. IFNγ is either present or not.

# CHAPTER V

# Discussion

## 5.1. Regulation of MPs in Neuroinflammation

### *5.1.1. Gene transcription*

This study provides details about gene transcription of individual MPs in neuroinflammation. Protein expression was confirmed for MMP-15 and ADAM-12 in Chapter 2. Gene transcription studies are instructive with regards to the response to inflammatory stimuli. However, it is important to consider that MP gene transcription need not always correlate exactly with enzyme activity. The fact that MPs are synthesized as zymogens adds a level of regulation by proteolytic or chemical activation. Furthermore, MPs need not necessarily be synthesized *de novo* in an inflammatory setting. In studies of cancer and spinal cord injury, it was demonstrated that inflammatory cells, especially neutrophils, can carry preformed MMPs to a site of inflammation and release them there from granules rather than producing them *in situ* (263, 264). The analysis of MMP message in a tissue undergoing inflammation might therefore underestimate the actual proteinase activity. In this light it is interesting to note, that we did not see a statistically significant upregulation of MMP-9 message in EAE (Chapter 2), which seems at odds with reports of increased levels of MMP-9 protein in CSF and serum in MS patients (110, 265). However, as described in Chapter 2, a large proportion of infiltrating cells were granulocytes. The granulocytes, including neutrophils, could increase the MMP-9 proteolysis level by degranulation. Indeed, increased MMP-9 enzyme activity in spinal cord of rats with EAE has been demonstrated with gel zymography (186).

### *5.1.2. Regulatory cytokines*

Cytokines are known to be potent inducers of MPs (242, 266). In neuroinflammation, a plethora of cytokines are expressed by a variety of both endogenous and infiltrating cells (267, 268), which likely leads to the regulation of several MP genes, as seen in Chapter 2. It seems probable that mechanisms are in place to counteract this great increase of proteolytic activity. Among those counteracting mechanisms is the upregulation of TIMP-1, which became the most highly expressed among the genes analyzed. Both infiltrating macrophages (Chapter 2) and resident astrocytes (183) contribute to the upregulated TIMP-1 expression in EAE. IL-1β was strongly upregulated in EAE (Chapter 4), and this cytokine has the capacity to induce expression of MMPs, ADAMs and TIMPs (91, 269-271). As is the case for MPs, TIMP expression can be regulated by a number of proinflammatory cytokines, including IL-1β, TNFα IFNγ and Il-6 (91, 272). Interestingly, Bugno and colleagues found that TNFα and IFNγ upregulated TIMP-1, but downregulated TIMP-3 in cultured murine brain endothelial cells and astrocytes. In accordance with this, TIMP-1 was upregulated and TIMP-3 downregulated in spinal cord of mice with EAE (Chapter 2). Generally, it seems plausible that cytokines regulate both proteinases and their inhibitors concomitantly in neuroinflammation.

### *5.1.3. Astrocytes*

In the present study, the potential for MP production by astrocytes was not studied. The role of astrocytes in neuroinflammation needs to be investigated further, specifically their potential role in regulating cell entry to the CNS parenchyma across the glia limitans, of which astrocyte endfeet make up a substantial part. Astrocytes have been shown to produce MPs both *in vitro* and *in vivo* (248, 270, 273, 274), but there is still little known about the contribution of MPs by astrocytes *in vivo*. Activation of astrocytes occurs in EAE and MS. It is thought that astrocytes contribute to pathogenesis by producing proinflammatory cytokines and chemokines, and potentially by activating T cells (275). Astrocytes

form networks in CNS with both neurons and glial cells, and are increasingly recognized to have a regulatory role, e.g. by regulating blood flow (276).

## 5.2. Regulation of MT-MMPs

### *5.2.1. MT-MMPs in IFNγ-/- mice*

Some MT-MMPs were downregulated in spinal cord in EAE, specifically in microglia from WT mice (Chapter 2). No statistically significant alteration of MT-MMP expression was observed in spinal cord of IFNγ-/- mice with EAE, albeit with low n-values (Chapter 4). IFNγ-/- mice develop a more severe and often lethal disease course when challenged with EAE (277-279). This is associated with striking infiltration of neutrophils (280, 281). An interesting hypothesis emerging from these findings could be that the downregulation of certain MT-MMPs in WT mice is protective against the lethal EAE phenotype seen in the IFNγ-/- mice. In contrast to WT mice, IFNγ-/- mice with EAE do not have F4/80 immunoreactive microglia in spinal cord parenchyma (280). Also in contrast with WT mice, IFNγ-/- mice with EAE show no MHC II immunoreactivity in spinal cord (280). These observations suggest that microglia in IFNγ-/- mice with EAE are not activated in the same way as microglia in WT mice are. Perhaps this is the reason no downregulation of MT-MMPs is noted in INFγ-/- mice with EAE (Chapter 4), although it remains to be verified directly that microglia from IFNγ-/- mice with EAE do not downregulate MT-MMPs. Further studies should elucidate whether downregulation of particular MT-MMPs is a cause or effect of microglial activation.

### *5.2.2. MT-MMPs in IFNγ Tg mice*

Overexpression of IFNγ specifically in the CNS did not lead to parenchymal infiltration or activation of glial cells in the IFNγ Tg mice used in Chapter 4 (234), yet the present study shows significant downregulation of five of the six MT-

MMPs. Also, MT-MMPs were downregulated in EAE (Chapter 2). Both these *in vivo* models share the fact that IFNγ was present in the spinal cord, where it is normally absent, and can lead to the straight forward conclusion that IFNγ causes downregulation of certain MT-MMPs. However, MT-MMPs were also downregulated after ECL, where IFNγ was not present, so other factors must be involved. CNS injury in general leads to activation of microglia (38, 282-284), and this is also the case in the EC after lesion (285).

### *5.2.3. MT-MMPs in CCL2 Tg mice*

In the CCL2 Tg mice, no significant change in MT-MMP expression was detected after injection of PTx (Chapter 3). IFNγ is present in the CNS of unmanipulated CCL2 Tg mice, but they do not show downregulation of any MT-MMPs compared to WT. At this point, it has not been established if the IFNγ in unmanipulated CCL2 Tg mice is localized in the perivascular space or also in the parenchyma. Also, it is not established whether microglia are activated in this model. It seems most likely that IFNγ is expressed by the accumulated leukocytes, of which about 7% are T cells (Chapter 3). If this is the case, perivascular microglia might be activated due to influence by IFNγ and maybe other cytokines produced by the accumulated leukocytes. Parenchymal microglia, on the other hand, are less likely to be affected by cytokines in the perivascular space, and therefore not likely to be activated.

### *5.2.4. MT-MMPs in cultured microglia*

*In vitro* stimulation of microglia with IFNγ did not lead to downregulation of any MT-MMPs. It is not clear yet if this lack of effect in vitro is due to an artifact of isolating and culturing microglia, or actually represents the situation *in vivo*. IFNγ has been reported to inhibit MMP expression *in vitro* (270). In that study, the cytokines IL-1β and TNFα, and the bacterial product LPS were shown to induce

expression of MMP-2, 3 and 9 by astrocytes and microglia in culture, but IFNγ inhibited the response to LPS.

### *5.2.5. Downregulation of MT-MMPs and the activation of microglia*

Downregulation of certain MT-MMPs might be involved in activation of microglia and a switch from antigen uptake to antigen presentation. This perhaps parallels the situation for the innate immune receptor TREM-2. TREM-2 mediates clearance of apoptotic neurons and downregulation of TREM-2 leads to increased production of inflammatory mediators (262). Dendritic cells also downregulate TREM-2 upon activation (286). TREM-2 is downregulated on microglia as part of activation, e.g. by IFNγ (261). Analogously, IFNγ-mediated activation of microglia could also cause downregulation of certain MMPs.

## 5.3. Stages of Neuroinflammation

### *5.3.1. The endothelium vs. the glia limitans*

Leukocyte migration across the glia limitans likely has different requirements than migration across the endothelial basement membrane. A role for cytokine-induced chemokines in CNS infiltration has been proposed based on studies in cytokine-deficient mice. If EAE is induced in TNFα-/- mice, there is an initial asymptomatic period, in which leukocytes accumulate in the perivascular space (35), showing that TNF is not required for trans-endothelial migration, but facilitates parenchymal infiltration. The TNFα normally produced in WT mice by infiltrating leukocytes was shown to stimulate production of chemokines by CNS resident cells, thereby facilitating leukocyte infiltration (190). The TNFα-/- mice do eventually develop EAE symptoms, which coincides with a transition from perivascular accumulation to parenchymal infiltration, i.e. crossing the glia limitans, showing that the TNFα-induced chemokine production is facilitatory, but not essential for parenchymal infiltration.

As further support for distinct mechanisms involved in migration across the two barriers, the endothelial basement membrane and the glia limitans contain different ECM proteins (29). MPs are potential candidates to be among the required factors needed by leukocytes to traverse the individual barriers. It is a possibility that particular MPs needed for a cell to infiltrate the parenchyma are not actually expressed by that cell type. For example macrophages could aid the infiltration of T cells by expressing MPs that clear the way for T cells by degrading ECM proteins in the barriers the T cells need to cross to enter the parenchyma (discussed in section 5.3.2.). Macrophages are important in the development of EAE, as macrophage-depletion leads to perivascular accumulation, but not parenchymal infiltration, when mice are challenged with EAE (36).

### *5.3.2. Macrophage MPs could aid T cell infiltration*

Depletion of macrophages markedly decreases the disease severity of EAE (36, 287, 288). From these observations, it appears that macrophages control infiltration of myelin-specific T cells into the CNS. As discussed previously, macrophages may clear the way for T cells by expressing proteinases that are required for parenchymal infiltration. It is evident from Chapter 2, and from a study using human cells (189) that macrophages are the dominant producers of MMPs compared to T cells. Strongly upregulated MMPs in EAE would be logical candidates for a "macrophage-factor", that is required to aid CNS infiltration. A prime candidate is MMP-12 which is expressed by macrophages in MS lesions (289), and is the most upregulated MMP in spinal cord of mice with EAE (199, 214). Also, MMP-12 mediated macrophage infiltration of lung tissue in a model of smoke-induced emphysema (290). However, the role of MMP-12 in EAE turns out to be controversial, as MMP-12 null mice were recently shown to develop more severe EAE than WT mice (214). MMP-12 null mice showed a Th1 cytokine shift and this may be the reason for the more severe EAE. It would be interesting to test if MMP-12 influences cellular infiltration in a non-immune mediated model of neuroinflammation.

### *5.3.3. Migration of transferred T cells in AT-EAE*

Two phases of infiltration follow AT-EAE (291). Initially, the cells enter peripheral lymphoid organs, where they are shown to develop a more migratory phenotype, e.g. by upregulating chemokine receptors. After about 60 hours, the cells enter the spinal cord, get reactivated, and upregulate pro-infiltrating molecules further. The activation state of T cells determines their infiltration of the CNS parenchyma, and CNS antigen specificity is required for persisting parenchymal infiltration. Upregulated MMPs could be part of the activated phenotype that determines infiltration. MMPs have not been investigated in this context yet. It could be that transferred cells upregulate their MP expression profile in peripheral organs, which enables them to traverse the endothelial barrier, and again after reactivation in perivascular space, which enables the cells to enter the CNS parenchyma.

### *5.3.4. CCL2 Tg mice*

Fuentes and colleagues showed that leukocytes accumulate spontaneously in the perivascular space in mice expressing the chemokine CCL2 in the CNS (34). The accumulation was potentiated by LPS challenge, although no overt pathology was observed. In Tg mice which overexpress CCL2 in alveolar epithelial cells, an equivalent phenomenon was observed (292). Transgenic CCL2 expression in the lungs leads to increased numbers of monocytes and lymphocytes recovered by bronchoalveolar lavage. CCL2 expression alone was not sufficient to cause activation of the accumulated leukocytes or inflammation in the lungs. However, when stimulated with LPS, the transgenic mice developed pulmonary infiltrates as opposed to WT mice. Mice expressing CCL2 under control of the glial fibrillary acidic protein (GFAP) promoter, which is specific for astrocytes, also develop neuroinflammation following LPS challenge (293). These GFAP/CCL2 Tg mice are more susceptible to Theiler's murine encephalomyelitis virus-induced demyelination. Bennett and colleagues reported diffuse infiltration of mononuclear cells throughout the CNS tissue in unmanipulated GFAP/CCL2 Tg

mice, whereas leukocyte infiltration is restricted to the perivascular space in unmanipulated MBP/CCL2 Tg mice (34) used in the present study. In both GFAP/CCL2 and MBP/CCL2 Tg mice, LPS potentiates accumulation of leukocytes in the CNS, and in both types of Tg mice, PTx induces encephalopathy (Chapter 3 and (206).

PTx increases the incidence of EAE in B6 mice immunized with $MOG^{35-55}$ in CFA (294). In that study, it was shown that TLR9 activating CpG oligonucleotides injected directly into the brain could substitute for PTx, in inducing encephalopathy although the symptoms were different from EAE, probably due to different localization of the lesions. Other studies have also identified TLR9 as important in EAE (295, 296). These studies support the theory that PAMP stimulation of APCs can lead to relapse of organ-specific autoimmune diseases, such as MS. Both PTX and LPS are pathogen-derived molecules, and in these studies cause pathology in synergy with chemokine overexpression. Likewise, in patients with chronic inflammatory disease, infection might augment the disease.

## 5.4. Environmental Influence on CNS Infiltration

### *5.4.1. The role of PTx in EAE*

PTx has been proposed to act through TLR4 (165). Clearly, PTx must also have other effects, as it promotes disease in C3H/HeJ mice (297), which are TLR4-defective because of a point mutation that renders TLR4 nonfunctional (298). 10/10 C3H/HeJ mice got EAE, when they were given PTx as adjuvant, whereas it was only 4/10 without PTx. Another important point is that TLR4 signaling can be bypassed entirely, given that EAE can be induced in C3H/HeJ mice. PTx was originally proposed to open the BBB and facilitate transmigration of pathogenic cells in EAE (162). Recent results go against this dogma by reporting that PTx does not enhance BBB permeability (218, 297). It seems likely that PTx promotes EAE through other mechanisms. PTx may enhance EAE susceptibility by overriding genetic checkpoints in the disease pathogenesis, rather than increasing

BBB permeability (299). Results from the present study show that PTx promotes parenchymal infiltration, but not necessarily through a direct effect on the BBB. PTx was shown to cause upregulation of MPs by leukocytes, thereby promoting their transmigratory capability (Chapter 3). This can subsequently lead to breakdown of ECM barriers of the neurovascular unit, and indirectly to increased permeability.

### *5.4.2. Innate immune receptors in neuroinflammation*

Given the need for adjuvants in EAE and the correlation between infections and relapse in MS patients, it is reasonable to assume that innate immune receptors are involved in MS pathogenesis. As mentioned in section 5.3.4., TLRs have emerged as an important family of innate immune receptors which are proposed to link innate and adaptive immunity (300). TLR signaling leads to activation of transcription factors, particularly NFκB, most often through the common adaptor molecule MyD88, which is also used by IL-1 and IL-18 signaling pathways. Most TLRs are expressed in human CNS, and upregulation of several of them was shown in the CNS of MS patients (301). It was recently shown that many TLRs as well as MyD88 are upregulated in EAE, and that MyD88-/- mice were resistant to EAE (295).

It would be interesting to study at which level in neuroinflammation PTx, TLRs and other PRRs exert their disease-promoting effect. In actively induced EAE, they would be expected to participate in the generation of the autoimmune response by activating APCs. However, PTx also promotes EAE after adoptive transfer (302). One hypothesis could be that perivascular APCs are actively involved in MS pathogenesis by regulating CNS infiltration. As discussed in section 1.3.3., recognition of myelin antigen presented on perivascular APCs by infiltrating T cells promotes CNS infiltration, in which MPs are likely involved. This process could be enhanced by activation of perivascular APCs through binding of microbial products to innate immune receptors.

## 5.5. Therapy of Neuroinflammation

### *5.5.1. Are MPs good or bad?*

It is not meaningful to simply describe MP activity, or even activity of a single MMP, as either good or bad during disease. A particular MP expressed by a certain cell type under inflammatory circumstances can do tissue damage, whereas the same MP expressed in a different location in the same organ at the same time, might be involved in repair. Some generalizations can be made, though. In the early stages of inflammation, MP expression seems overall to be detrimental, whereas in later phases of chronic disease, MPs perform beneficial functions including repair, making overall MP activity beneficial (65). From this follows that inhibition of MPs would be beneficial in treatment of patients with acute inflammation as in spinal cord injury, stroke, or during an MS attack, whereas patients in the chronic stage of MS would not benefit from MP inhibition. In a rat model of stroke, it was shown that treatment with MMP inhibitors in the acute phase was beneficial, whereas continued treatment in the delayed response phase inhibited beneficial effects of MP action and thereby impaired neurovascular remodeling and functional recovery (303).

In the present study four of the six MT-MMPs were downregulated in EAE (Chapter 4). It seems unlikely that these four MT-MMPs are promoting transmigration. The other two (MMP-14 and 25) might promote migration, as they are upregulated in situations with parenchymal infiltration, and they could potentially be targets to inhibit in neuroinflammation. This should be tested in knock-out mice.

The TIMPs are likely upregulated in a specific spatial and temporal manner to prevent excessive proteolysis by all the upregulated MPs. More insight into the sources and pattern of expression of both TIMPs and MPs is needed to understand the interplay between the proteinases and their inhibitors, with the aim of therapeutically manipulate this.

### *5.5.2. Inhibition of MPs*

The antibiotic minocycline attenuated EAE symptoms in mice, and decreased leukocyte traffic across an *in vitro* fibronectin barrier (104). Minocycline decreased both expression and activity of MMPs in that study. Minocycline has also proven to be protective against EAE in rats, where it decreased inflammation, demyelination, microglial activation as well as MMP-2 expression (304). Treatment of mice with minocycline improved the clinical outcome following spinal cord injury (305), where MMPs appears to have a detrimental role, as demonstrated for MMP-12 (175). In addition to inhibiting MPs, minocycline is also an inhibitor of microglial activation (306). Preliminary results from a clinical trial of minocycline in MS patients show encouraging results (307).

In terms of MS therapy, it is not known exactly which proteases are most critical to inhibit. In order to optimize treatment and prevent side effects in patients, it is necessary to use selective MP inhibitors. However, developing such selective MMP inhibitors has proven difficult, possibly because of an inherent conformational flexibility in the structure of MPs (308). However, some recent attempts at developing specific MMP inhibitors show encouraging results in cancer research. The MMP inhibitor Ro-28-2653, with high selectivity for MMP-2, 9 and 14 was a more potent antitumor and antiangiogenic agent than the broad-spectrum inhibitor BB-94 (309). The crystal structures of some MT-MMPs have been determined, and it might be possible to design specific inhibitors against those based on unique features (310). The crystal structure of secreted MPs binding substrates or inhibitors have been elucidated, including the structure of MMPs binding to BB-94 (311, 312).

### *5.5.3. Alternative therapeutic strategies*

Perhaps a more strategic therapeutic approach would be to target the stimulus leading to excessive MP action, rather than the MPs themselves. For example could cytokines that cause upregulation of detrimental MPs be inhibited, or the action of cytokines leading to upregulation of beneficial MPs could be augmented

Signaling molecules or transcription factors leading to MP expression could also be targeted. Finally, the substrates cleaved by MPs could be targeted for therapeutic purposes, i.e. if a particular product of MP cleavage is detrimental, then the substrate could be sequestered; and if the product is beneficial, the substrate could be supplemented.

# CONCLUSION AND PERSPECTIVES

The results described in this thesis provide novel information about the regulation of MPs in neuroinflammation, and how MPs are involved in discrete stages of CNS infiltration. Numerous MPs take part in the very complex process of neuroinflammation, where they interact with other large families of inflammatory mediators: cytokines and chemokines. In this study, correlations between certain MPs and IFNγ are described, as well as the interaction between MPs, the microbial product PTx, and the CCL2 chemokine. Furthermore, the distinction between endothelial transmigration and parenchymal infiltration has been more clearly illustrated. Obtaining more detailed knowledge about the interplay between these large families of inflammatory mediators and how they affect CNS infiltration would be of great benefit in relation to therapeutic management of diseases involving neuroinflammation.

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# APPENDICES

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Research compliance certificates

As co-author on the manuscript

"Regulation of expression of membrane-type matrix metalloproteinases in central nervous system inflammation",

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01187-2-08.5

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Licence Number / Numéro de permis

## I) LICENSEE

Pursuant to section 24 of the Nuclear Safety and Control Act, this licence is issued to:

Montreal Neurological Institute and Hospital/Institut et Hôpital neurologiques de Montréal
3801 University Street
Montreal, QC
H3A 2B4
Canada

hereinafter «the licensee».

This licence replaces licence 01187-2-08.4 .

## II) LICENCE PERIOD

This licence is valid from: November 30, 2005 to April 30, 2008 unless otherwise suspended, amended, revoked or replaced.

## III) LICENSED ACTIVITIES

This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances and the prescribed equipment listed in section IV) of this licence.

This licence is issued for: consolidated uses of nuclear substances (815)

## IV) NUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

| ITEM | NUCLEAR SUBSTANCE | UNSEALED SOURCE MAXIMUM QUANTITY | SEALED SOURCE MAXIMUM QUANTITY | EQUIPMENT MAKE AND MODEL |
|---|---|---|---|---|
| 1 | Carbon 11 | 2 GBq | n/a | n/a |
| 2 | Carbon 14 | 11 GBq | n/a | n/a |
| 3 | Calcium 45 | 800 MBq | n/a | n/a |
| 4 | Chromium 51 | 2 GBq | n/a | n/a |
| 5 | Copper 64 | 925 MBq | n/a | n/a |
| 6 | Fluorine 18 | 2 GBq | n/a | n/a |
| 7 | Iron 55 | 100 MBq | n/a | n/a |
| 8 | Gallium 68 | 400 MBq | n/a | n/a |
| 9 | Hydrogen 3 | 33 GBq | n/a | n/a |
| 10 | Iodine 124 | 185 MBq | n/a | n/a |
| 11 | Iodine 125 | 4 GBq | n/a | n/a |
| 12 | Iodine 131 | 2 GBq | n/a | n/a |
| 13 | Krypton 85 | 74 GBq | n/a | n/a |
| 14 | Oxygen 15 | 2 GBq | n/a | n/a |
| 15 | Phosphorus 32 | 11 GBq | n/a | n/a |
| 16 | Phosphorus 33 | 2 GBq | n/a | n/a |
| 17 | Sulfur 35 | 22 GBq | n/a | n/a |
| 18 | Technetium 99m | 11 GBq | n/a | n/a |
| 19 | Cobalt 57 | n/a | 370 MBq | n/a |
| 20 | Cesium 137 | n/a | 1 GBq | n/a |
| 21 | Germanium 68/ Gallium 68 | n/a | 1 GBq | n/a |
| 22 | Sodium 22 | n/a | 4 MBq | n/a |
| 23 | Cesium 137 | n/a | 1480 kBq | Beckman LS series |
| 24 | Radium 226 | n/a | 370 kBq | Wallac 1200 Series |
| 25 | Europium 152 | n/a | 740 kBq | Wallac 1400 series |

The total quantity of an unsealed nuclear substance in possession shall not exceed the corresponding listed unsealed source maximum quantity. The total quantity of nuclear substance per sealed source shall not exceed its corresponding listed sealed source maximum quantity. Sealed sources shall only be used in the corresponding listed equipment.

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*V) LOCATION(S) OF LICENSED ACTIVITIES*

3801 University Street
Montréal, QC

*VI) CONDITIONS*

1. Internal Authorization
The licensee shall ensure that
(a) internal authorizations are issued in accordance with the licensee's internal authorization policies and procedures approved by the Commission or a person authorized by the Commission; and
(b) internal authorization forms are posted in a readily visible location in or near each room, area or enclosure where nuclear substances and radiation devices are used or stored.
(c) the licensed activity is conducted in accordance with the terms and conditions of the internal authorization.
(2215-2)

2. Location Notification
The licensee shall, for any site where licensed activities are to be conducted for more than 90 consecutive days, notify the Commission in writing of the site within 7 days of starting to conduct the activities at the site. The licensee shall notify the Commission in writing within 7 days of the discontinuance of licensed activities at any site. The continuity of consecutive days is not broken during off site use or off site temporary storage.
(2300-2)

3. Project Approval
The licensee shall obtain written approval from the Commission or a person authorized by the Commission before starting any work requiring the use of more than 10,000 exemption quantities of a nuclear substance at a single time.
(2214-0)

4. Prohibition of Human Use
This licence does not authorize the use of nuclear substances in or on human beings.
(2696-0)

5. Emergency Contact - Radiation Device
The licensee shall post and keep posted, in readily visible location at the place where any radiation device listed in section IV) of this license is used, a durable and legible sign that indicates the name or job title and the telephone number of a person who can initiate the accident procedure that pertains to the radiation device and who can be contacted 24 hours a day.
(2559-2)

6. Area Classification
The licensee shall classify each room, area or enclosure where more than one exemption quantity of an unsealed nuclear substance is used at a single time as:

(a) basic-level if the quantity does not exceed 5 ALI,
(b) intermediate-level if the quantity used does not exceed 50 ALI,
(c) high-level if the quantity does not exceed 500 ALI,
(d) containment-level if the quantity exceeds 500 ALI; or
(e) special purpose if approved in writing by the Commission or a person authorized by the Commission.

Except for the basic-level classification, the licensee shall not use unsealed nuclear substances in these rooms, areas or enclosures without written approval of the Commission or a person authorized by the Commission.



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(2108-2)

7. Laboratory Lists
The licensee shall maintain a list of all areas, rooms and enclosures in which more than one exemption quantity of a nuclear substance is used or stored.
(2569-1)

8. Laboratory Procedures
The licensee shall post and keep posted, in a readily visible location in areas, rooms or enclosures where nuclear substances are handled, a radioisotope safety poster approved by the Commission or a person authorized by the Commission, which corresponds to the classification of the area, room or enclosure.
(2570-1)

9. Thyroid Monitoring
Every person who
(a) uses at a single time a quantity of volatile iodine-125 or iodine-131 exceeding;
(i) 5 MBq in an open room;
(ii) 50 MBq in a fume hood;
(iii) 500 MBq in a glove box;
(iv) any other quantity in other containment approved in writing by the Commission or a person authorized by the Commission; or
(b) is involved in a spill of greater than 5 MBq of volatile iodine-125 or iodine-131;
(c) or on whom iodine-125 or iodine-131 external contamination is detected; and shall, undergo thyroid screening within five days following the exposure to iodine-125 or iodine-131.
(2046-7)

10. Thyroid Screening
Screening for internal iodine-125 and iodine-131 shall be performed using:
(a) a direct measurement of the thyroid with an instrument that can detect 1 kBq of iodine-125 or iodine-131; or
(b) a bioassay procedure approved by the Commission or a person authorized by the Commission.
(2600-1)

11. Thyroid Bioassay
If thyroid screening detects more than 10 kBq of iodine-125 or iodine-131 in the thyroid, the licensee shall immediately make a preliminary report to the Commission or a person authorized by the Commission and have bioassay performed within 24 hours by a person licensed by the Commission to provide internal dosimetry.

(2601-4)

12. Extremity Dosimetry
The licensee shall ensure that any person who handles a container which contains more than 50 MBq of phosphorus 32, strontium 89, yttrium 90, samarium 153 or rhenium 186 wears a ring dosimeter. The dosimeters must be supplied and read by a dosimetry service licensed by the Commission.
(2578-0)

13. Survey Meter Requirements
The licensee shall provide at all times where nuclear substances, except for hydrogen-3 and nickel-63, are handled or stored a radiation survey meter.
(2058-0)

14. Contamination Meter Requirements
The licensee shall make available to workers at all times at the site of the licensed activity a properly functioning portable contamination meter.
(2572-1)

15. Contamination Criteria
The licensee shall ensure that for nuclear substances listed in the licence application guide table titled "Classification of



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Licence Number / Numéro de permis



Radionuclides";
(a) non-fixed contamination in all areas, rooms or enclosures where unsealed nuclear substances are used or stored does not exceed:
(i) 3 becquerels per square centimetre for all Class A radionuclides;
(ii) 30 becquerels per square centimetre for all Class B radionuclides; or
(iii) 300 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres; and
(b) non-fixed contamination in all other areas does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; or
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres.
(2642-2)

16. Decommissioning
The licensee shall ensure that prior to decommissioning any area, room or enclosure where the licensed activity has been conducted;
(a) the non-fixed contamination for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides" does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; and
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres;
(b) the release of any area, room or enclosure containing fixed contamination, is approved in writing by the Commission or person authorized by the Commission;
(c) all nuclear substances and radiation devices have been transferred in accordance with the conditions of this licence; and
(d) all radiation warning signs have been removed or defaced.
(2571-2)

17. Storage
The licensee shall:
(a) ensure that when in storage radioactive nuclear substances or radiation devices are accessible only to persons authorized by the licensee;
(b) ensure that the dose rate at any occupied location outside the storage area, room or enclosure resulting from the substances or devices in storage does not exceed 2.5 microSv/h; and
(c) have measures in place to ensure that the dose limits in the Radiation Protection Regulations are not exceeded as a result of the substances or devices in storage.
(2575-0)

18. Disposal (Consolidated)
When disposing of unsealed nuclear substances to municipal garbage or sewer systems or to atmosphere, the licensee shall ensure that the following limits are not exceeded:

| COLUMN 1 - Nuclear Substance - - - | COLUMN 2(a) LIMITS solids to municipal garbage system (quantity per kilogram) | COLUMN 3(b) LIMITS liquids(water soluble)to municipal sewer system (quantity per year) | COLUMN 4(c) LIMITS gases to atmosphere (quantity per cubic metre) |
|---|---|---|---|
| Argon 41 | | | 0.037 kBq |
| Carbon 14 | 3.7 MBq | 10 000 MBq | |
| Chromium 51 | 3.7 MBq | 100 MBq | |

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| | | | |
|---|---|---|---|
| Cobalt 57 | 0.37 MBq | 1000 MBq | |
| Cobalt 58 | 0.37 MBq | 100 MBq | |
| Gallium 67 | 0.037 MBq | 100 MBq | |
| Hydrogen 3 | 37 MBq | 1 000 000 MBq | 37 kBq |
| Indium 111 | 0.037 MBq | 100 MBq | |
| Iodine 123 | 3.7 MBq | 1000 MBq | |
| Iodine 125 | 0.037 MBq | 100 MBq | |
| Iodine 131 | 0.037 MBq | 10 MBq | 0.175 kBq |
| Krypton 79 | | | 0.37 kBq |
| Krypton 85 | | | 3.7 kBq |
| Phosphorus 32 | 0.37 MBq | 1 MBq | |
| Phosphorus 33 | 1 MBq | 10 MBq | |
| Sulfur 35 | 0.37 MBq | 1000 MBq | |
| Technetium 99m | 3.7 MBq | 1000 MBq | |
| Thallium 201 | 0.037 MBq | 100 MBq | |
| Xenon 133 | | | 3.7 kBq |

(a) The limits in column 2 apply to quantities of solid waste of less than three tonnes per year per facility. Nuclear substances released to the municipal garbage system must be in solid form and uniformly distributed in the waste with a concentration that is less than the limits in column 2. Where more than one nuclear substance is disposed of at one time, the quotients obtained by dividing the quantity of each substance by its corresponding limit in column 2 shall not exceed one.

(b) The limits in Column 3 apply to the water soluble liquid form of each nuclear substance which may be disposed of per year per building.

(c) The limits in Column 4 may be averaged over a one-week period. These limits apply to releases of less than 3 million cubic metres per year. Where more than one nuclear substance is disposed of at one time, the quotients obtained by dividing the quantity of each substance by its corresponding limit in column 4 shall not exceed one.
(2160-3)

19. Export Restrictions
This licence does not authorize the licensee to export for the valid period of this licence:
(a) any quantity in any form of:
    (i) Deuterium;
    (ii) Plutonium;
    (iii) Thorium;
    (iv) Tritium;
    (v) Uranium; or
(b) any quantity of the elemental form, or any quantity of a compound or mixture greater than or equal to 37 GBq/kg, or any sealed source or device greater than or equal to 3.7 GBq of:
    (i) Actinium 225, 227;
    (ii) Californium 248, 250, 252, 253, 254;
    (iii) Curium 240, 241, 242, 243, 244;
    (iv) Einsteinium 252, 253, 254, 255;
    (v) Fermium 257;
    (vi) Gadolinium 148;
    (vii) Mendelevium 258;





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(iix) Neptunium 235;
(ix) Polonium 208, 209, 210; or
(x) Radium 223;

(c) any quantity of Americium 241 or 243, except in a compound or mixture, or any sealed source or device;
(d) any quantity of Neptunium 237, except in a compound or mixture, or any sealed source or device;
(e) any quantity of Radium 226, or any quantity of a compound or mixture of Radium 226;
(f) any sealed source or device (except for medical applicators) of more than 0.37 GBq of Radium 226; and
(g) any neutron generator system (including tubes) designed for operation without an external vacuum system and utilizing electrostatic acceleration to induce a tritium-deuterium nuclear reaction.
(2403-2)

20. Annual Compliance Report
The licensee shall, by February 28 of each year, submit to the Commission a written annual compliance report in a form acceptable to the Commission.
(2916-6)

Designated Officer pursuant to subsection 37(2)(c) of the Nuclear Safety and Control Act

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## I) TITULAIRE DE PERMIS

Conformément à l'article 24 de la Loi sur la sûreté et la réglementation nucléaires, le présent permis est délivré à:

Montreal Neurological Institute and Hospital/Institut et Hôpital neurologiques de Montréal
3801, rue University
Montreal (Québec)
H3A 2B4
Canada

Ci-après désigné sous le nom de «titulaire de permis»

Le présent permis remplace le permis 01187-2-08.4.

## II) DURÉE DU PERMIS

Ce permis est valide du 30 novembre 2005 au 30 avril 2008, sauf si le permis est suspendu, modifié, révoqué ou remplacé.

## III) ACTIVITÉS AUTORISÉES

. Le présent permis autorise le titulaire à avoir en sa possession, transférer, importer, exporter, utiliser et stocker les substances nucléaires et les équipements autorisés qui sont énumérés dans la section IV) du présent permis.

Le présent permis est délivré pour le type d'utilisation: utilisation globale de substances nucléaires (815)

## IV) SUBSTANCES NUCLÉAIRES ET ÉQUIPEMENT AUTORISÉ

| ARTICLE | SUBSTANCE NUCLÉAIRE | SOURCE NON SCELLÉE QUANTITÉ MAXIMALE | ASSEMBLAGE DE LA SOURCE SCELLÉE QUANTITÉ MAXIMALE | ÉQUIPEMENT - FABRICANT ET MODÈLE |
|---|---|---|---|---|
| 1 | Carbone 11 | 2 GBq | s/o | s/o |
| 2 | Carbone 14 | 11 GBq | s/o | s/o |
| 3 | Calcium 45 | 800 MBq | s/o | s/o |
| 4 | Chrome 51 | 2 GBq | s/o | s/o |
| 5 | Cuivre 64 | 925 MBq | s/o | s/o |
| 6 | Fluor 18 | 2 GBq | s/o | s/o |
| 7 | Fer 55 | 100 MBq | s/o | s/o |
| 8 | Gallium 68 | 400 MBq | s/o | s/o |
| 9 | Hydrogène 3 | 33 GBq | s/o | s/o |
| 10 | Iode 124 | 185 MBq | s/o | s/o |
| 11 | Iode 125 | 4 GBq | s/o | s/o |
| 12 | Iode 131 | 2 GBq | s/o | s/o |
| 13 | Krypton 85 | 74 GBq | s/o | s/o |
| 14 | Oxygène 15 | 2 GBq | s/o | s/o |
| 15 | Phosphore 32 | 11 GBq | s/o | s/o |
| 16 | Phosphore 33 | 2 GBq | s/o | s/o |
| 17 | Soufre 35 | 22 GBq | s/o | s/o |
| 18 | Technétium 99m | 11 GBq | s/o | s/o |
| 19 | Cobalt 57 | s/o | 370 MBq | s/o |
| 20 | Césium 137 | s/o | 1 GBq | s/o |
| 21 | Germanium 68/ Gallium 68 | s/o | 1 GBq | s/o |
| 22 | Sodium 22 | s/o | 4 MBq | s/o |
| 23 | Césium 137 | s/o | 1480 kBq | Beckman LS series |
| 24 | Radium 226 | s/o | 370 kBq | Wallac Série 1200 |
| 25 | Europium 152 | s/o | 740 kBq | Wallac série 1400 |

La quantité totale d'une substance nucléaire non scellée possédée ne doit pas excéder la quantité maximale qui est indiquée pour une source non scellée correspondante. La quantité de substance nucléaire par source scellée ne doit pas excéder la quantité maximale indiquée



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par source scellée correspondante. Les sources scellées doivent être utilisées seulement dans l'équipement indiqué correspondant.

### V) ENDROIT(S) OÙ LES ACTIVITÉS AUTORISÉES PEUVENT ÊTRE EXERCÉES

3801, rue University
Montréal (Québec)

### VI) CONDITIONS

1. Exigences d'autorisation interne
Le titulaire veille à ce que :
a) des autorisations internes soient délivrées conformément aux politiques et procédures d'autorisation interne du titulaire de permis, qui doivent être approuvées par la Commission ou une personne autorisée par la Commission;
b) les formulaires d'autorisation interne soient affichés bien en évidence dans chaque salle, zone ou enceinte où des substances nucléaires et des appareils à rayonnement sont utilisés ou stockés, ou à proximité de ces salles, zones ou enceintes.
c) l'activité autorisée est exercée conformément aux conditions de l'autorisation interne.

(2215-2)

2. Avis d'emplacement
Pour tout endroit où se dérouleront des activités autorisées pendant plus de 90 jours consécutifs - sans égard à tout autre lieu d'utilisation ou à toute période de stockage temporaire, le titulaire de permis signale l'endroit par écrit à la Commission dans les sept jours après le début des activités. Il l'avise par écrit dans les sept jours de la cessation des activités autorisées à cet endroit.
(2300-2)

3. Approbation du projet
Le titulaire de permis obtient l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci avant d'entreprendre tout projet exigeant plus de 10 000 quantités d'exemption d'une substance nucléaire à un moment donné.
(2214-0)

4. Interdiction visant l'utilisation chez les humains
Le permis n'autorise pas l'utilisation des substances nucléaires dans ou sur le corps d'une personne.
(2696-0)

5. Contact en cas d'urgence - appareil
Le titulaire affiche en permanence et bien en évidence, sur les lieux où un appareil à rayonnement figurant à la section IV) du permis est utilisé, un panneau durable et lisible indiquant le nom ou le titre ainsi que le numéro de téléphone de la personne qui peut être contactée jour et nuit pour lancer la procédure à suivre en cas d'accident pour cet appareil.
(2559-2)

6. Classification des zones, pièces et enceintes
Le titulaire de permis désigne chaque zone, pièce ou enceinte où on utilise plus d'une quantité d'exemption d'une substance nucléaire non scellée à un moment donné selon la classification suivante :

(a) de niveau élémentaire si la quantité ne dépasse pas 5 LAI,
(b) de niveau intermédiaire si la quantité utilisée ne dépasse pas 50 LAI,
(c) de niveau supérieur si la quantité ne dépasse pas 500 LAI,
(d) de confinement si la quantité dépasse 500 LAI;
(e) à vocation spéciale, avec l'autorisation écrite de la Commission

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travailleurs sur les lieux de l'activité autorisée un contaminamètre portatif en bon état de fonctionnement.
(2572-1)

15. Critères de contamination
En ce qui a trait aux substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis, le titulaire de permis veille à ce que :
(a) la contamination non fixée dans toutes les zones, pièces ou enceintes où on utilise ou stocke des substances nucléaires non scellées ne dépasse pas :
(i) 3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 30 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 300 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la contamination non fixée pour toutes les autres zones ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2.
(2642-2)

16. Déclassement
Avant le déclassement d'une zone, d'une pièce ou d'une enceinte où s'est déroulée l'activité autorisée, le titulaire de permis veille à ce que :
(a) la contamination non fixée pour les substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la mise en disponibilité de toute zone, pièce ou enceinte contenant une contamination fixée soit approuvée par la Commission ou une personne autorisée par celle-ci;
(c) toutes les substances nucléaires et tous les appareils à rayonnement ont été transférés conformément aux conditions du permis;
(d) tous les panneaux de mise en garde contre les rayonnements ont été retirés ou ont été rendus illisible.
(2571-2)

17. Stockage
Le titulaire :
a) veille à ce que seules les personnes autorisées par lui aient accès aux substances nucléaires radioactives ou aux appareils à rayonnement stockés;
b) veille à ce qu'à tout endroit occupé à l'extérieur de la zone, de la salle ou de l'enceinte de stockage le débit de dose provenant des substances ou appareils stockés ne dépasse pas 2,5 microSv/h;
c) a des mesures en place pour assurer que les limites de dose indiquées dans le Règlement sur la radioprotection ne sont pas dépassées en raison du stockage de ces substances ou appareils.
(2575-0)

18. Évacuation (permis global)
Lorsqu'il évacue des substances nucléaires non scellées dans une décharge municipale, un réseau d'égouts ou l'atmosphère, le titulaire de permis veille à ce que les limites suivantes ne soient pas dépassées :

| COLONNE 1 | COLONNE 2(a) | COLONNE 3(b) | COLONNE 4(c) |
|---|---|---|---|
| - | LIMITES | LIMITES | LIMITES |
| Substance nucléaire | solides à la décharge municipale | liquides (hydrosolubles) à l'égout municipal | gaz dans l'atmosphère quantité au |
| - | (quantité au kg) | (quantité par an) | mètre cube) |
| Argon 41 | | | 0,037 kBq |
| Carbone 14 | 3,7 MBq | 10 000 MBq | |

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ou d'une personne autorisée par celle-ci.

À l'exception du niveau élémentaire, le titulaire de permis n'utilise pas de substances nucléaires non scellées dans ces zones, pièces ou enceintes sans l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.
(2108-2)

7. Liste des laboratoires
Le titulaire de permis tient à jour une liste de toutes les zones, salles et enceintes dans lesquelles plus d'une quantité d'exemption d'une substance nucléaire est utilisée ou stockée.
(2569-1)

8. Procédures de laboratoire
Le titulaire affiche en tout temps et bien en évidence dans les zones, les salles ou les enceintes où des substances nucléaires sont manipulées une affiche sur la radioprotection qui a été approuvée par la Commission ou une personne autorisée par la Commission et qui correspond à la classification de la zone, de la salle ou de l'enceinte.
(2570-1)

9. Surveillance thyroïdienne
La personne
a) qui utilise à un moment donné une quantité d'iode 125 ou d'iode 131 volatiles dépassant :
(i) 5 MBq dans une pièce ouverte,
(ii) 50 MBq dans une hotte,
(iii) 500 MBq dans une boîte à gants,
(iv) toute autre quantité dans une enceinte de confinement approuvée par écrit par la Commission ou une personne autorisée par celle-ci;
(b) qui est impliqué dans un déversement mettant en cause plus de 5 MBq d'iode 125 ou d'iode 131 volatiles;
(c) chez laquelle on détecte une contamination externe à l'iode 125 ou l'iode 131;
doit se prêter à un dépistage thyroïdien dans les cinq jours suivant l'exposition.
(2046-7)

10. Dépistage thyroïdien
Le dépistage de l'iode 125 et de l'iode 131 internes se fait :
(a) par mesure directe à l'aide d'un instrument capable de détecter 1 kBq d'iode 125 ou d'iode 131;
(b) par essai biologique approuvé par la Commission ou une personne autorisée par celle-ci.
(2600-1)

11. Essai biologique thyroïdien
Si la charge thyroïdienne dans une personne dépasse 10 kBq d'iode 125 ou d'iode 131, le titulaire de permis doit présenter immédiatement un rapport préliminaire à la Commission ou à une personne autorisée par celle-ci. Dans un délai de 24 heures, la personne en question doit subir des essais biologiques par une personne autorisée par la Commission à offrir un service de dosimétrie interne.
(2601-4)

12. Dosimétrie des extrémités
Le titulaire de permis veille à ce que toute personne qui manipule un contenant renfermant plus de 50 MBq de phosphore 32, de strontium 89, d'yttrium 90, de samarium 153 ou de rhénium 186 porte une bague dosimètre. Le dosimètre est fourni et lu par un service de dosimétrie autorisé par la Commission.
(2578-0)

13. Radiamètres
Le titulaire de permis fournit en tout temps un radiamètre là où des substances nucléaires (à l'exclusion de l'hydrogène 3 et du nickel 63) sont manipulées ou stockées.
(2058-0)

14. Exigences concernant les contaminamètres
Le titulaire de permis met en tout temps à la disposition des

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| | | | |
|---|---|---|---|
| Chrome 51 | 3,7 MBq | 100 Mbq | |
| Cobalt 57 | 0,37 MBq | 1000 MBq | |
| Cobalt 58 | 0,37 MBq | 100 MBq | |
| Gallium 67 | 0,037 MBq | 100 MBq | |
| Hydrogène 3 | 37 MBq | 1 000 000 MBq | 37 kBq |
| Indium 111 | 0,037 MBq | 100 MBq | |
| Iode 123 | 3,7 MBq | 1000 MBq | |
| Iode 125 | 0,037 MBq | 100 MBq | |
| Iode 131 | 0,037 MBq | 10 Mbq | 0,175 kBq |
| Krypton 79 | | | 0,37 kBq |
| Krypton 85 | | | 3,7 kBq |
| Phosphore 32 | 0,37 MBq | 1 MBq | |
| Phosphore 33 | 1 MBq | 10 Mbq | |
| Soufre 35 | 0,37 MBq | 1000 MBq | |
| Technétium 99m | 3,7 MBq | 1000 MBq | |
| Thallium 201 | 0,037 MBq | 100 MBq | |
| Xénon 133 | | | 3,7 kBq |

(a) Les limites indiquées à la colonne 2 s'appliquent aux quantités de déchets solides de moins de trois tonnes par an. Les substances nucléaires évacuées dans la décharge municipale doivent être sous forme solide et distribuées uniformément dans les déchets; la concentration doit être inférieure aux limites indiquées à la colonne 2. Lorsqu'on évacue plus d'une substance nucléaire à la fois, le quotient obtenu en divisant la quantité de chaque substance par sa limite correspondante de la colonne 2 ne doit pas dépasser un.

(b) Les limites indiquées à la colonne 3 s'appliquent à la forme liquide (hydrosoluble) de chaque substance nucléaire qui peut être évacuée par an et par bâtiment.

(c) On peut faire la moyenne sur une semaine des limites indiquées à la colonne 4. Ces limites s'appliquent aux rejets de moins de 3 millions de mètres cubes par an. Lorsqu'on évacue plus d'une substance nucléaire à la fois, le quotient obtenu en divisant la quantité de chaque substance par sa limite correspondante de la colonne 4 ne doit pas dépasser un.

(2160-3)

19. Restrictions à l'exportation
Au cours de sa période de validité, le permis n'autorise pas le titulaire de permis à exporter :
(a) toute quantité, sous toute forme :
    (i) de deutérium;
    (ii) de plutonium;
    (iii) de thorium;
    (iv) de tritium;
    (v) d'uranium;
(b) toute quantité, sous forme élémentaire, ou toute quantité d'un composé ou d'un mélange contenant l'un ou plusieurs des radio nucléides suivants, avec une activité égale ou supérieure à 37





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GBq/kg, ou toute source scellée ou dispositif dont l'activité est égale ou supérieure à 3,7 GBq :
- (i) d'actinium 225, 227;
- (ii) de californium 248, 250, 252, 253, 254;
- (iii) de curium 240, 241, 242, 243, 244;
- (iv) d'einsténium 252, 253, 254, 255;
- (v) de fermium 257;
- (vi) de gadolinium 148;
- (vii) de mendélévium 258;
- (iix) de neptunium 235;
- (ix) de polonium 208, 209, 210;
- (x) de radium 223;

(c) toute quantité d'américium 241 ou 243, sauf sous forme de composé ou de mélange, ou toute source scellée ou dispositif;
(d) toute quantité de neptunium 237, sauf sous forme de composé ou de mélange, ou toute source scellée ou dispositif;
(e) toute quantité de radium 226, ou toute quantité d'un composé ou d'un mélange de radium 226;
(f) toute source scellée ou dispositif (à l'exception des applicateurs médicaux) contenant plus de 0,37 GBq de radium 226;
(g) tout système générateur de neutrons, y compris les tubes, conçu pour fonctionner sans installation de vide extérieure et utilisant l'accélération électrostatique pour déclencher une réaction nucléaire tritium-deutérium.
(2403-2)

20. Rapport annuel de conformité
Le titulaire de permis soumet par écrit à la Commission, au plus tard le 28 février de chaque année, un rapport annuel de conformité sous une forme acceptable à la Commission.
(2916-6)

Fonctionnaire désigné en vertu du paragraphe 37(2)(c) de la Loi sur la sûreté et la réglementation nucléaires

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