Extracellular matrix and oligodendrocyte regulators in different types of multiple sclerosis lesions: Implications for lesion development and regulation of remyelination

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften (Dr. rer. nat)
der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

vorgelegt von
Hema Mohan
India

Munich, February 2010

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit Ausnahme einer immunhistochemischen Färbung sowie der in vitro Myelinisierung, die von unseren Kollaborationspartnern durchgeführt wurde, selbständig und ohne unerlaubte Hilfe angefertigt habe.

Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen beziehungsweise einer Prüfungskommission vorzulegen, noch eine Doktorprüfung durchzuführen.

Dissertation eingereicht: 02.02.2010
Tag der mündlichen Prüfung: 26.07.2010
Erstgutachter: Prof. Elisabeth Weiß
Zweitgutachter: Prof. Frank Bradke

Dedicated to my parents

ACKNOWLEDGEMENTS

I owe my deepest gratitude to Prof. Dr. Hartmut Wekerle for offering me a graduate fellowship to work in his department. I am grateful to Prof. Dr. Reinhard Hohlfeld to accept me in his group.

I owe my deep appreciation and gratitude to my supervisor Prof. Dr. Edgar Meinl to accept me as a graduate student in his group. I thank him for his constant guidance, encouragement and stimulating scientific discussions. I also appreciate his support to develop my own ideas and work as an independent scientist.

I express my gratitude to Prof. Elisabeth Weiß for accepting to act as my principal supervisor at the Faculty of Biology of the Ludwig Maximilian University here in Munich. I am also thankful for her comments during my annual presentations. I wish to extend my thanks to the members of my thesis examination board.

I am also thankful to the members of my thesis advisory committee, Dr Michael Sixt and Dr. Klaus Dornmair, for their helpful discussions and ideas. I greatly appreciate Michael Sixt for sharing his knowledge about extracellular matrix with me and Edgar.

I would like to thank Prof. Dr. Hans Lassmann for his excellent help and teaching me the basics of neuropathology. I also thank Rakhi Sharma for her co-operation.

I thank my co-operation partners Prof. Chris Linington and Christina Elliot.

I am also grateful to Prof. Larry Fisher, NIH, US for providing biglycan and decorin anti sera.

I would like to thank all my lab members Andreas, Anne-Katrin, Cora, Florian, Johann, Korcan, Markus, Petra, Robert, Sacha, Sylvia and Verena for all their help and also for maintaining a relaxed atmosphere in the lab. I would also like to thank all other members of the department, especially Guru, Kerstin, Marsilius, Sofia, and Vijay for their help in various ways.

I thank all my friends specially Bhargavi, Hari, Sonia, and Visu. I am indebted to Sonia and Visu for their endless support and encouragement.

Last but not the least I thank my family. No words can describe the love, concern, guidance, understanding, patience and support they have extended.

TABLE OF CONTENTS

1 Summary

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) accompanied by demyelination and axonal loss. Only a minority of the demyelinated MS lesions gets remyelinated. In animal models, however, remyelination is the default program following immune-mediated or toxic demyelination. This thesis aims to find factors regulating remyelination in MS.

The starting point was autoptic tissue from MS patients. First, different types of MS lesions were macrodissected namely, chronic inactive demyelinated lesions that had failed to remyelinate, lesions undergoing active demyelination and remyelinated lesions. Healthy white matter was used as control tissue. Gene expression profiles of these lesions were established using quantitative PCR low density arrays. Thereby the focus was on the extracellular matrix (ECM) and on factors known to regulate the biology of oligodendrocytes.

ECM components can regulate oligodendrocyte differentiation and modify immune reactions in multiple ways, e.g., by sequestering or displaying growth factors and by directly interacting with immune cells and glial cells. The expression of 50 ECM components and 34 ECM degrading enzymes was measured by qPCR. *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* chains were strongly induced in active lesions and even more in chronic inactive lesions. These collagen polypeptide chains interact to form collagen types I, III and V, which are grouped as fibrillar collagens. Furthermore, two Small Leucine Rich Proteoglycans (SLRPs), biglycan and decorin, which can decorate fibrillar collagens, were also strongly induced. Immunostaining localized the fibrillar collagens, biglycan, and decorin around blood vessels. These ECM molecules were largely seen in the perivascular space closely associated with infiltrating immune cells forming a mesh between the endothelium and the astrocytic glia limitans. In active lesions collagen V was also seen in the heavily infiltrated brain parenchyma. Since these ECM molecules were found largely in the perivascular space close to immune cells and hardly in the surrounding parenchyma where

oligodendrocyte differentiation takes place, the interaction of these ECM components with immune cells was further analysed.

In vitro experiments revealed that the fibrillar collagens I and III inhibited the monocytic production of CCL2 (MCP-1), an inflammatory chemokine thought to be involved in the recruitment of immune cells to the inflamed brain. This suggests that the induced fibrillar collagens may contribute to the limitation of MS lesions expansion by inhibition of the CCL2 production by monocytes.

The second set of analysed genes comprised 32 factors regulating survival, proliferation and/or differentiation of oligodendrocytes and 18 receptors of these genes. The key factors for oligodendrocyte differentiation (IGF1, IGF2 and CNTF) and oligodendrocyte proliferation (FGF2 and PDGFAA) were still present in demyelinated lesions, although their expression ratio was altered. The most striking result was the up-regulation of FGF9 in a subset of chronically demyelinated lesions, but in none of the remyelinated shadow plaques. The potential functional role of this observation was investigated by treating myelinating rat central nervous system cultures with exogenous FGF9. In this experimental setting, FGF9 inhibited the ability of mature oligodendrocytes to myelinate and ensheath axons. All these data suggests that the induction of FGF9 in some chronic MS lesions is one of the inhibitory mechanisms accounting for the failure of remyelination.

Together, this thesis has two main findings: A) Fibrillar collagens, biglycan and decorin form a perivascular fibrosis and the fibrillar collagens I and III inhibit production of CCL2 by monocytes. Inhibition of CCL2 production by fibrillar collagens might contribute to lesion confinement. B) Expression profiles of remyelinated MS lesions were established for the first time and thereby, up-regulation of FGF9 in demyelinated, but not in remyelinated lesions was revealed. The inhibition of myelination *in vitro* by FGF9 suggests that this is one potential mechanism to explain why demyelinated lesions expressing higher FGF9 level fail to remyelinate.

2 Introduction

Autoimmune diseases are caused when the body starts producing an inappropriate immune response against itself. 5-10% of the general population is affected and the pathogenesis is multifactorial, with both inherited and environmental components [Invernizzi *et al.* 2009].

Autoimmune diseases can be classified in to two types: those that damage many organs (systemic autoimmune disease, e.g., systemic lupus erythematosus) and those where only a single organ or tissue is damaged by the autoimmune processes (localised autoimmune diseases, e.g., multiple sclerosis, type I diabetes and Hashimoto's thyroiditis).

2.1 Multiple sclerosis

2.1.1 Prominent features

MS is a chronic inflammatory demyelinating disease of the CNS, which typically begins in the second or third decade of life and has a female preponderance of 2:1 [Noseworthy *et al.* 2000]. MS is considered as an autoimmune disease of the CNS where the infiltrating immune cells are directed against various components of myelin and/or gray matter. MS is a heterogeneous disease with a broad spectrum of interindividual differences in clinical presentation, location and frequency of lesions, and response to immunosuppressive treatments [Breij *et al.* 2008]. Typical disabilities caused by MS include paralysis, loss of co-ordination, sensory disturbances, and impairment of vision and cognitive function [Sospedra *et al.* 2005].

2.1.2 Clinical course

The clinical course of MS is highly diverse and can be divided primarily into four types (Figure 2.1). *Relapsing-remitting MS (RRMS)*: 80-85% of MS patients begin with recurrent and reversible neurological deficits. Females are twice likely to suffer from RRMS than males. *Secondary progressive MS (SPMS)*: approximately

50% of RRMS patients transit into the SP form. SPMS is characterised by disease progression with or without relapse and minor remission. *Primary progressive MS (PPMS)*: 15% of MS patients have a PP disease course with continuous worsening of neurological deficits without clear relapses. *Progressive-relapsing MS (PRMS)*: this is the least common form with continuous progression of neurological deficits and superimposed acute relapse but the period between relapses is characterised by continuous progression of symptoms [Trapp *et al.* 2008; Kieseier *et al.* 2003].

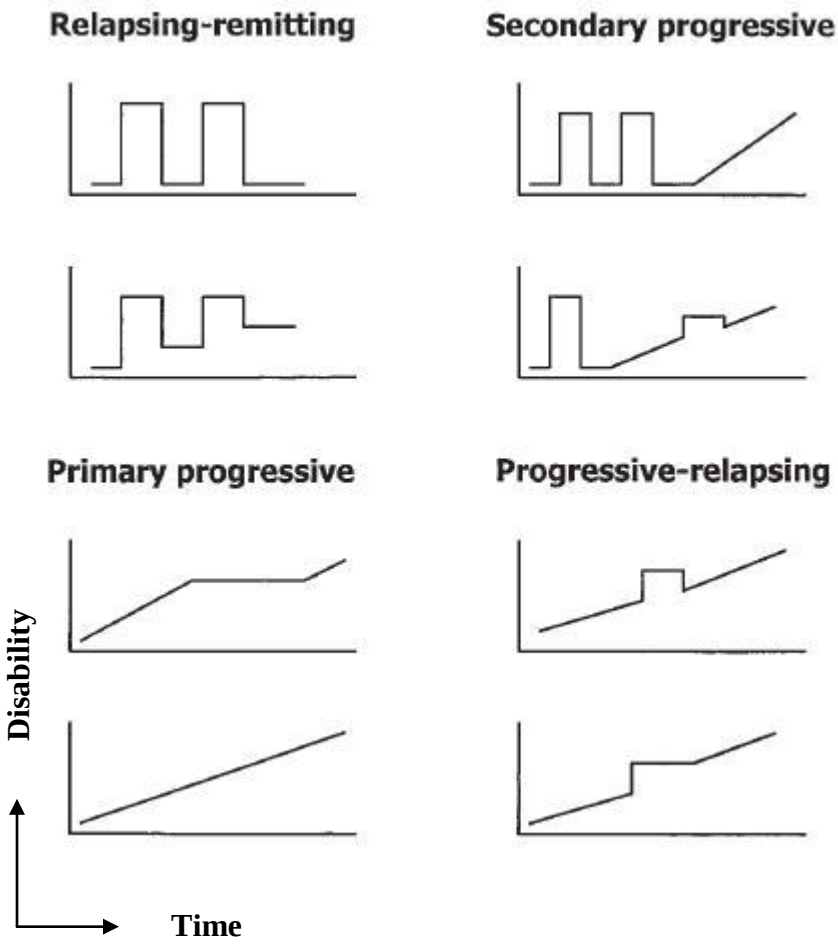


Figure 2.1: Clinical courses of multiple sclerosis (modified from Kieseier and Hartung, 2003).

2.1.3 Pathology

MS lesions have three main pathological characteristics, namely, inflammation, demyelination with limited remyelination and axonal injury. Histopathologically MS is characterized by focal demyelination of white matter with a well demarcated hypocellular area characterized by loss of myelin and the formation of astrocytic scar and axonal loss. In addition to the classical white matter lesion, recent histopathological analysis and modern imaging techniques showed that grey matter is also heavily involved in MS. Predilection sites are the optic nerve, the periventricular white matter, the cerebellum, the brain stem and the cervical spinal cord [Breij *et al.* 2008]. Lesions are often found around one or several medium sized vessels. Inflammatory cells are typically perivascular in location but they also diffuse through the damaged BBB and infiltrate the parenchyma. The dominant inflammatory cells in MS lesions are T cells and macrophages with lower and variable contribution from B cells and plasma cells [Lassmann 2005]. Both CD4 and CD8 positive T cells have been observed in active lesions [Traugott *et al.* 1983], and clonal expansion of CD8 positive T cells has also been documented in MS lesions [Babbe *et al.* 2000; Junker *et al.* 2007]. Axonal damage is an integral and early pathological feature of MS lesions [Ferguson *et al.* 1997]. Axonal transection was also observed in demyelinating lesions and was more in inactive lesions compared to the active lesions [Trapp *et al.* 1998].

MS lesions can be divided into different stages based on different parameters, including inflammatory cells, glial cells, axonal loss and myelin staining. These stages are *Active demyelinating lesions* which contain abundant macrophages with myelin degradation products (luxol fast blue or Oil Red O positive) either throughout the lesion or as a broad rim around the lesion edge. *Inactive demyelinated lesions* which can be sharply demarcated from the normal appearing white matter and are without a rim of microglial activation and do not contain any luxol fast blue or Oil Red O positive macrophages.

The active demyelinating lesions can be further divided into four different patterns, based on the distribution of myelin loss, the plaque geography and extension, the pattern of oligodendrocyte destruction, immunoglobulin deposition and complement activation [Lucchinetti *et al.* 1999]. One common hallmark among all the four patterns of demyelination is the presence of inflammatory infiltrates like T cells, macrophages and microglia. It should be noted that these patterns of classification are controversial.

The four patterns are: ***Pattern I (macrophage-associated demyelination)*****:** lesions are perivenously distributed and are characterised by a sharply demarcated lesion edge. There is a T cell mediated inflammation with macrophage and microglia activation. Activated macrophages and microglia are associated with degenerating myelin and demyelination assumably induced by macrophage toxins. This pattern closely resembles the myelin destruction in mouse models of experimental autoimmune encephalomyelitis (EAE), where toxic products of activated macrophages are responsible for myelin destruction. ***Pattern II (antibody-mediated demyelination)*****:** similar to pattern I. There is T cell mediated inflammation with macrophage and microglia activation and deposition of immunoglobulin's and activated complements at sites of active myelin destruction. Demyelination might be induced by complement mediated lysis of antibody targeted myelin. This pattern is reflected in models of EAE where the disease is induced by sensitization with myelin oligodendrocyte glycoprotein (MOG) and where demyelination is induced by encephalitogenic T cells and demyelinating anti-MOG antibodies. ***Pattern III (distal oligodendrogliopathy associated demyelination)*****:** these lesions are not centered by veins and venules. The lesion border is ill defined, with a diffused spread into the surrounding white matter. There is a T cell mediated inflammation with macrophage and microglia activation along with small vessel vasculitis and secondary ischemic damage of the white matter. Preservation of a rim of myelin is frequently observed around inflamed blood vessels with in a demyelinated plaque. The striking feature in pattern III lesions is the preferential loss of myelin associated glycoprotein (MAG), while other myelin proteins like proteolipid protein (PLP), myelin basic protein

(MBP) and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) are still present within the partially damaged myelin sheaths. Demyelination is caused by degeneration of distal oligodendrocyte processes followed by oligodendrocyte apoptosis. This pattern is not reflected in animal models of MS. ***Pattern IV (primary oligodendrocyte degeneration mediated demyelination)*: these** lesions are also similar to pattern I. there is a T cell mediated inflammation with macrophage and microglia activation. Demyelination is induced by macrophage toxins on the background of metabolically impaired oligodendrocytes. Oligodendrocyte degeneration is observed in a small rim of periplaque white matter, adjacent to the zone of active myelin destruction. This pattern of demyelination is very infrequent in the MS population and is restricted to a subset of patients with PPMS [Lassmann *et al.* 2001; Lucchinetti *et al.* 2000].

2.2 Remyelination

Besides the destructive mechanism in MS, endogenous repair mechanisms which contribute to axonal protection called 'remyelination' occurs [Irvine *et al.* 2008]. Remyelination has been described as a frequent phenomenon in acute or early MS lesions [Lassmann 1983; Raine *et al.* 1993]. Remyelinated lesions are also present in chronic MS lesions [Prineas *et al.* 1979]. Frequently, remyelination is found as a rim at the border of chronic MS lesions. Completely remyelinated lesions called *shadow plaques* are less frequently observed (Figure 2.2). It has been suggested that remyelination is a transient phenomenon [Frohman *et al.* 2006] and that remyelinated shadow plaques might become affected with the new bouts of demyelination [Prineas *et al.* 1993]. Extensive remyelination is found not only in patients with RRMS, but also in a subset of patients with progressive disease. Older age at death and longer disease duration is associated with significantly more remyelinated lesions (shadow plaques) or remyelinated lesion rim (lesion areas) [Patrikios *et al.* 2006]. Location of the lesion also influences the likelihood of remyelination, 44.4% of cortical lesions in contrast to 22% of periventricular lesions showed marked remyelination [Goldschmidt *et al.* 2009].

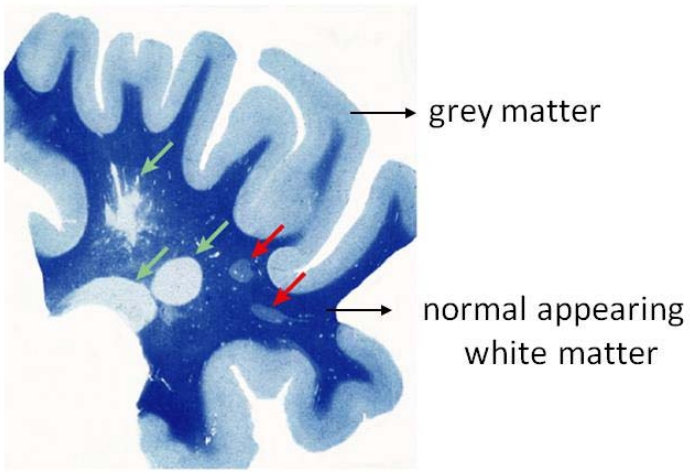


Figure 2.2: Pathology of MS lesion: MS brain stained with Luxol Fast Blue (LFB), which stains myelin blue. Green arrows show demyelinated lesions and red arrows indicate shadow plaques (completely remyelinated lesions) (modified from Adams C.W.M, 1989, Wolfe Medical Publications, London.).

2.2.1 Why is remyelination important?

In the first place, remyelination is important for restoring saltatory action potential. The second key function of remyelination in MS is axonal survival [Irvine *et al.* 2008]. Remyelination protects neurons by providing trophic support to promote survival of underlying neurons [Wilkins *et al.* 2003; Franklin *et al.* 2008b].

Demyelination undoubtedly represents a major part of the pathology of MS, but in the recent years, it has become increasingly apparent that there is also a substantial axonal and neuronal loss. Hence, permanent neurological dysfunction observed in MS has been attributed to demyelination not to axonal injury, and is the major cause of the chronic progressive disease. Demyelinated axons are especially vulnerable to damage during the acute phase and possibly also prone to damage and atrophy during the chronic phase of MS [Ferguson *et al.* 1997; Trapp *et al.* 1998]. The cause for such axonal atrophy is unclear, but demyelination may predispose axons to secondary injury by (a) multiple molecular cytotoxicity mechanisms, including ion channel dysfunction and mitochondrial damage, or (b) failure of local target derived neurotrophic support for myelinating oligodendrocytes.

2.2.2 How does remyelination occur?

Remyelination involves the generation of new mature oligodendrocytes. Data based largely on rodent studies show that remyelination is mediated by new oligodendrocytes derived from a population of adult CNS stem/precursor cells, referred to as adult oligodendrocyte precursors (OPCs) [Ffrench-Constant *et al.* 1986; Dawson *et al.* 2003]. OPCs are proliferating cells, which are widely spread through the CNS and are found in both white matter and grey matter at a density similar to that of microglia (5-8% of the adult CNS cell population) [Polito *et al.* 2005; Levine *et al.* 2001]. These OPCs are NG2 (a chondroitin sulfate proteoglycan) and PDGFRα (platelet-derived growth factor receptor alpha) positive. The process of remyelination occurs in two phases (a) *Recruitment phase*, during which OPCs are recruited to the demyelinated area and proliferate. (b) *Differentiation phase*, in which the newly recruited OPCs differentiate into premyelinating oligodendrocytes which contact demyelinated axons and differentiate into mature myelin producing oligodendrocytes [Chari 2007]. Remyelination results in a thinner myelin sheath and shorter internodes than it would be expected for a given diameter of the axon [Franklin *et al.* 2008a].

2.2.3 Why does remyelination fail frequently?

Remyelination is a default process in most animal models but fails frequently in MS. Remyelination could fail because of a primary deficiency in the precursor cells, a failure of precursor cell recruitment, or a failure of precursor cell differentiation and maturation [Franklin 2002]. Some of the MS lesions fail to remyelinate not because of the shortage of available precursor cells but because of a failure of OPC recruitment, involving proliferation, migration to and repopulation of demyelinated areas. This might be explained by either the fact that some MS patients generate antibodies against the OPC expressed antigen NG2 [Niehaus *et al.* 2000],or by disturbances in the local expression of the OPC migration guidance cues semaphorin 3A and 3F [Williams *et al.* 2007]. Several sets of observations indicated that differentiation and maturation of oligodendrocytes is most vulnerable to the

failure of remyelination in MS. Analyses of MS lesions show presence of premyelinating oligodendrocytes in chronic MS lesions [Chang *et al.* 2002]. Even though the density of OPCs in the chronic lesions on average is lower than in normal white matter, it also can be as high as in normal white matter and in remyelinated lesions. This adds to the view that the availability of OPCs is not a limiting factor for remyelination, but rather the differentiation of the OPCs is disturbed [Kuhlmann *et al.* 2008]. Several factors can be attributed to this failure of differentiation. Namely, re-expression of the Notch-jagged signalling pathway [Wang *et al.* 1998; John *et al.* 2002], up-regulation of the extracellular matrix protein hyaluronan [Back *et al.* 2005] in chronic demyelinated MS lesions and expression of polysialylated neural cell adhesion molecule (PSA-NCAM) by demyelinated axons, by which these axons send negative signals to oligodendrocytes and thereby inhibit remyelination [Charles *et al.* 2002; Charles *et al.* 2000].

2.2.4 How can remyelination be enhanced?

Currently there are no specific clinical therapies available, which promote remyelination. However, there are two therapies in development, which are preceding towards phase I clinical trials. One is anti-LINGO therapy by Biogen Idec (http://www.biogenidec.com/site/pipeline.html) [Rudick *et al.* 2008; Mi *et al.* 2007] and the other is the application of recombinant human IgM which is currently developed at the Mayo clinic (http://www.mayoclinic.org/medicalprofs/remyelination.html) [Warrington *et al.* 2000; Warrington *et al.* 2008; Warrington *et al.* 2007]. Both of these potential therapies have been shown to promote remyelination in various animal models.

Furthermore, there are two therapeutic approaches currently being tested in animal models of Demyelination. These are cell replacement by transplantation *(exogenous therapy)* and promotion of repair by the resident stem- and precursor- cell populations in the adult CNS *(endogenous repair)*. The effectiveness of any therapy

promoting remyelination will depends on the ability to suppress the effect of any ongoing disease process on the new oligodendrocytes.

Different cell types including primary OPCs, Schwann cells, olfactory ensheathing cells, neural stem cell and embryonic stem cell derived glial precursors have been shown to generate myelinating cells following transplantation [Franklin *et al.* 2008a]. As remyelination can be completed and as the cells that are responsible for remyelination are abundant throughout the adult CNS, an attractive approach is to target the endogenous cells for remyelination. This approach is completely based on the understanding of the mechanisms of remyelination and failure of remyelination. The pro-recruitment therapies may not promote remyelination where the primary problem is OPC differentiation and vice versa [Franklin 2002; Franklin *et al.* 2008a].

2.3 Extra cellular matrix in multiple sclerosis

The ECM in MS lesions potentially affects myelination/remyelination and immune cell function. Hyaluronan, a proteoglycan is up–regulated in inactive demyelinated lesions and inhibits oligodendrocyte maturation, thereby inhibiting remyelination of the chronic demyelinated lesions [Back *et al.* 2005]. In addition, it was recently shown in EAE models that laminin α5 directly inhibited integrin mediated migration of T lymphocytes, resulting in marked and selective reduction of T lymphocyte infiltration into the brain, reduced disease susceptibility and severity [Wu *et al.* 2009]. In MS the interactions between infiltrating immune cells and the CNS environment determine the fate of the MS lesion.

ECM is referred to as the ground substance found in the interstitial spaces of all organs and organ systems providing support to the cells. A substantial volume ($1/5^{th}$) of the brain is filled with ECM [Bignami *et al.* 1993; Rutka *et al.* 1988]. Under normal conditions, the ECM of the CNS is unique in its composition, with smaller amounts of fibrous proteins (collagens, laminin-1 and fibronectin), and higher amounts of linear polysaccharides (glycosaminoglycans (GAGs) such as hyaluronan, chondroitin sulphate and heparin sulfate) [Novak *et al.* 2000; Rauch 2004; Viapiano

et al. 2006]. Brain endothelial cells, astrocytes, neurons and microglia can synthesise and secrete several ECM proteins [Tilling *et al.* 2002; Van der Laan *et al.* 1997a].

The ECM has traditionally been considered to play predominantly a structural role, but in the past few years various other features of the ECM in the CNS have emerged. During the development ,the ECM is involved in the migration, maturation, differentiation and survival of neurons [Oohira *et al.* 2000]. In adults, the ECM provides physical support for the brain resident cells and regulates ionic and nutritional homeostasis [Bandtlow *et al.* 2000; Rauch 2004; Yamaguchi 2000]. Furthermore, the ECM binds to both growth promoting and growth inhibitory factors and many times acts as a reservoir of these factors [Saksela *et al.* 1990; Deepa *et al.* 2004; Deepa *et al.* 2002; Kawashima *et al.* 2002; Kantor *et al.* 2004] .

A complex alteration in the CNS ECM occurs during the disease course of MS, expression of both the parenchymal and the basement membrane related ECM proteins are altered. The expression of the chondroitin and/or dermatan sulphate proteoglycans in the active MS lesions is altered; in the same lesions foamy macrophages accumulated these proteoglycans along with myelin breakdown products [Sobel *et al.* 2001]. Loss of tenascin-C and –R immunoreactivity is seen in acute MS lesions [Gutowski *et al.* 1999]. Among the basement membrane proteins vitronectin immunoreactivity was enhanced in the blood vessel walls of active MS lesions, at the border of chronic active lesions and on few hypertrophic astrocytes [Sobel *et al.* 1995]. Alterations in the expression of different laminin isoforms in the basement membrane of inflamed blood vessels and increased immunoreactivity for fibronectin and collagen IV was observed on immune cells [Esiri *et al.* 1991; Sobel *et al.* 1989; Van *et al.* 2005]. EAE experiments have elaborated decisive roles of the basement membranes and their laminins for immune cell entry into the CNS [Agrawal *et al.* 2006; Sixt *et al.* 2001; Wu *et al.* 2009].

2.3.1 Collagens

In addition to the ECM components mentioned above collagens and SLRPs are also important components of the ECM. Collagens are a large family of triple

helical proteins that are widespread throughout the body. They are important for a broad range of functions, including tissue scaffolding, cell adhesion, cell migration, tissue morphogenesis and tissue repair. Collagens are the best known as the principle tensile element of vertebrate tissues such as tendon, cartilage, bone, and skin where collagens are present as elongated fibrils in the ECM. Collagens contain three polypeptide chains. Each polypeptide chain contains a repeating Gly-X-Y triplet in which glycine residue occupies every third position and the position X and Y are occupied by proline and 4-hydroxyproline, respectively. The three polypeptide chains are held together by interchain hydrogen bonds [Kadler *et al.* 2007]. The human genome codes for at least 43 distinct collagen chains that are assembled in to 28 collagen types [Myllyharju *et al.* 2004]. Collagens can be mainly divided into two groups, the fibril forming and the non fibril collagens. Fibrillar collagens include collagen I, II, III, V, XI, XXIV, and XXVII. Fibrillar collagen chains are associated as homotrimers (e.g. collagen II, III), heterotrimers (e.g. collagen XI) or both (e.g. collagen I, V) [Ricard-Blum *et al.* 2005].

2.3.2 Small leucine rich proteoglycans

Small leucine rich proteoglycans (SLRPs) belongs to a gene class whose structural hallmark is the presence of tandem arrays of leucine-rich repeats flanked by two cysteine-rich regions with highly conserved spacings. The SLRP gene family includes 17 genes which can be classified into five distinct groups based on several parameters including conservation and homology at the protein and genomic levels, the presence of characteristic N-terminal Cys-rich clusters with defined spacing and chromosomal organization. Members of SLRPs are decorin, biglycan, fibromodulin and lumican. These proteoglycans are tissue organizers, orienting and ordering collagen fibrils during development and in pathological processes such as wound healing and tissue repair. They can also interact with soluble growth factors, modulate their functional activity and availability, and bind to cell surface receptors, thereby affecting different cellular processes [Schaefer *et al.* 2008; Iozzo 1997; Iozzo 1999].

2.4 Factors regulating remyelination

The procurement of growth factor–mediated trophic support for oligodendrocytes and their progenitors is one of the potential approaches to stimulate endogenous remyelination. A number of growth factors have been identified which alone or in combination, promote proliferation, survival and differentiation of oligodendrocyte progenitor cells. These include members of the neurotrophin family, insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), cytokines of interleukin-6 (IL-6) family and neuregulins (NRGs).

2.4.1 Neurotrophins

Neurotrophins are a family of growth factors critical for the development and functioning of the nervous system. They include: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. These neurotrophins bind to specific tyrosine kinase receptors (Trks): NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB and NT-3 to TrkC. NT-3 regulates the survival proliferation and differentiation of OPCs *in vitro*, while NGF and BDNF have been shown to protect oligodendrocytes from death induced *in vitro* by the proinflammatory cytokine TNFα and *in vivo* by spinal cord injury, respectively [Heinrich *et al.* 1999; Bertollini *et al.* 1997; Barres *et al.* 1994; Kerschensteiner *et al.* 2003]. BDNF and its high affinity receptor TrkB are markedly increased in MS lesions [Stadelmann *et al.* 2002].

2.4.2 Insulin-like growth factor-I

A role for IGF-I in the proliferation and differentiation of OPCs has been proposed based on its effects on developmental myelination and its enhanced expression in experimental model of demyelination and MS lesions [Gveric *et al.* 1999; Yao *et al.* 1995]. IGF-I has been shown to protect oligodendrocytes from TNFα

induced injury [Ye *et al.* 1999]. Administration of IGF-I in EAE reduced lesion extension and promoted remyelination in one study [Liu *et al.* 1995], but others did not support the protective role of IGF-I in EAE models [Genoud *et al.* 2005; Cannella *et al.* 2000]. A recent study in humans has shown that there is no statistical significant difference in the levels of IGF-I in serum and CSF of MS patients and controls [Poljakovic *et al.* 2006].

2.4.3 Platelet-derived growth factor

Extensive studies have been done on the effects of PDGFs on CNS myelination during development. The PDGF family of growth factor is composed of four different polypeptide chains encoded by four different genes. The four PDGF chains assemble into disulphide bonded dimers via homo or heterodimerization and five different dimeric isoforms have been described; PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, PDGF-DD. PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC can bind to and activate PDGFRα, while PDGF-BB and PDGF-DD can specifically bind to and activate PDGFRβ [Fredriksson *et al.* 2004]. PDGF-AA can act as a survival factor during oligodendrocyte development [Gard *et al.* 1995]. PDGF-AA also has an important pro-migratory and mitogenic effect on oligodendrocyte progenitors. A correlation between cerebral PDGF levels and accumulation of OPCs following experimental demyelination has been established, supporting a role for PDGF-A in the recruitment of these cells into lesion area [Franklin 2002; Woodruff *et al.* 2004].

2.4.4 IL-6 family

Neuropoietins comprise a family of pleiotropic cytokines with implications in development, inflammation, tissue injury and healing processes. Well studied members of this group are IL-6, ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF) for their role in oligodendrocyte development and inflammatory demyelinating disease [Aloisi 2003].In addition to their effects on neuronal survival and differentiation, LIF and CNTF also act on OPCs by promoting

their survival, proliferation and maturation. *In vitro* studies showed that both LIF and CNTF protect oligodendrocytes from death induced by the proinflammatory cytokines TNFα and IFNγ [Vanderlocht *et al.* 2006; Linker *et al.* 2002]. In an EAE model, exogenously administered LIF ameliorated disease and endogenous LIF had a role in limiting oligodendrocyte loss during the disease [Butzkueven *et al.* 2002].

2.4.5 Fibroblast growth factors

FGFs constitute one of the largest family of growth and differentiation factors for cells of mesodermal and neuroectodermal origin. They participate in many developmental and repair processes. This family comprises of 23 FGF members, of which 10 have been identified in brain. The prototypic members are acidic FGF (aFGF or FGF1) and basic FGF (bFGF or FGF2). In the CNS, FGFs are widely expressed. FGF2 is primarily synthesized by astrocytes and FGF5, -8 and -9 are primarily synthesized by neurons [Reuss *et al.* 2003]. Experimental results suggest a putative role for FGF2 in the pathogenesis of demyelination and remyelination in EAE [Messersmith *et al.* 2000]. FGF2, expressed by astrocytes and microglias, has been shown to be a potent mitogen for OPCs [Frost *et al.* 2003].

Recent *in vitro* findings indicate that FGF2 has differential effects on cells of the early and late oligodendrocyte lineage: at first dramatically expanding their population throughout premyelinating and myelinating cells, but subsequently causing the loss of these previously generated cells [Butt *et al.* 2005]. Recently it has been shown that FGF2 levels are elevated in the CSF of MS patients [Sarchielli *et al.* 2008].

3 Objectives and strategy

Focal demyelination with limited remyelination is one of the hallmarks of multiple sclerosis. Why most lesions fail to remyelinate is unclear, as the CNS contains an endogenous repair mechanism that normally acts to ensure rapid and essentially complete remyelination. The aim of this thesis was to get insight into possible mechanisms explaining the frequent failure of remyelination in MS lesions. This failure might be due to alterations of the extracellular matrix and/or alterations of factors mediating growth, survival and differentiation of oligodendrocytes.

This thesis involved the following steps:

1. Identification of different types of MS lesions: demyelinated active, demyelinated chronic inactive and remyelinated lesions in autoptic tissue (in collaboration with Prof. Hans Lassmann).
2. Dissection of these lesions, and preparation of lesion specific RNA and cDNA, and testing for usefulness of transcript quantification.
3. Determining the expression profiles of MS lesions by quantitative PCR, with focus on ECM components, ECM degrading enzymes, growth and/or survival factors for oligodendrocytes and their receptors.
4. Localization of the up-regulated ECM molecules by immunohistochemistry in MS lesions.
5. Performing *in vitro* experiments based on the results of 4. Studying effects of induced ECM molecules on immune cells.
6. Studying the effects of potential inhibitors of remyelination in an *in vitro* myelination assay (co-operation with Prof. Chris. Linington).

4 Materials and Methods

4.1 Materials

4.1.1 Antibodies

Primary antibodies

Antigen	Specificity	Species	Clonality	Source	Catalogue No.
Biglycan	human	rabbit	polyclonal	kind gift from Prof. Larry Fisher	
CD31	human	sheep	polyclonal	R&D	AF806
CD68	human	mouse	monoclonal	DAKO	N1576
Collagen I	human	mouse	monoclonal	Abcam	ab6308
Collagen III	human	mouse	monoclonal	Abcam	Ab6310
Collagen V	human	rabbit	polyclonal	AbD serotec	2150-0180
Decorin	human	mouse	monoclonal	R&D	MAB143
Decorin	human	rabbit	polyclonal	kind gift from Prof. Larry Fisher	
FGF9	human	mouse	monoclonal	Santa Cruz	sc-8413
FGF9	human	goat	polyclonal	R&D	AF-273-NA
FGFR2	human	rabbit	polyclonal	Santa Cruz	ab5476
FGFR3	human	rabbit	polyclonal	Santa Cruz	sc-123
FGFR3	human	mouse	monoclonal	Santa Cruz	sc-13121
GFAP	human	mouse	monoclonal	Molecular Probes	131-17719

Secondary antibodies

Specificity	Species	Label	Source	Catalogue No.
mouse	donkey	alexa-488	Molecular Probes	A21202
rabbit	donkey	alexa-594	Molecular Probes	A21207
sheep	donkey	alexa-488	Molecular Probes	A11015
mouse	rabbit	none	DAKO	Z0259
rabbit	swine	none	DAKO	Z0196

Tertiary antibodies

PAP (peroxidase anti peroxidase) rabbit -DAKO Z0113
PAP (peroxidase anti peroxidase) mouse -DAKO P0850

4.1.2 Buffers and reagents

4% Paraformaldehyde (PFA)

4% PFA in PBS pH 7.4

Cell culture medium

RPMI 1640 or DMEM

1% Pen-Strep (10,000 U/ml penicillin G and 10,000 µg/ml streptomycin)

10% fetal calf serum (FCS)

Citrate buffer

Solution A: 0.1 M citric acid solution in PBS pH 7.4

Solution B: 0.1 M sodium citrate solution in PBS pH 7.4

Mix 9 ml of Sol A + 41 ml of Sol B + 450 ml PBS pH 6

Lithium carbonate

0.1% lithium carbonate in distilled water. (Light sensitive, store in brown bottle).

Luxol fast blue (LFB 0.1%)

0.1 grams of LFB in 100 ml 96% ethanol

0.5 ml 10% acetic acid added to the above solution

MACS buffer

0.2 mM EDTA, 2% FCS in PBS pH 7.4, 0.2 µm filter sterilized

Reagent diluent (ELISA)

1% BSA in PBS pH 7.4, 0.2 µm filter sterilized

Schiff's reagent

10 ml HCl

5 ml 10% sodium pyrosulfite

85 ml distilled water

0.2 gm pararosanilin

Allow the solution to stand O/N and then filter through 0.2-0.3 grams of activated charcoal

Schiff's wash solution

10 ml 10% sodium pyrosulfite

10 ml HCl

200 ml distilled water

Wash buffer (ELISA)

0.05% Tween 20 in PBS pH 7.4

4.1.3 Patients and tissue samples

A total of 19 snap-frozen MS lesions and 6 control white matter from 13 MS subjects and 4 control subjects without clinical or histological evidence of brain disease were analysed for transcript expression (Table 4.1). Three of the MS specimens were obtained from the Department of Forensic Medicine of Ludwig Maximilians University, Munich, Germany; four specimens from the Netherlands Brain Bank; and five specimens from Institute of Neurology, London (courtesy J. Newcombe) and one specimen from UK MS tissue bank, London. Of the control specimens three were obtained from the Department of Forensic medicine of Ludwig Maximilians University, Munich, Germany; and one from the Institute of Neurology, London (courtesy J. Newcombe). In addition to these frozen blocks, 19 formalin fixed paraffin embedded (FFPE) and 8 additional frozen blocks were used for immunohistochemistry.

Tissue blocks containing grossly visible lesions were dissected from anatomically distinct areas and were snap frozen in isopentane cooled in liquid nitrogen and later stored at -80^{o}C. The study was approved by the ethical committee of the Medical Faculty of Ludwig Maximilians University, Munich, Germany.

Table 4.1. Tissue samples of patients and controls.

patients and control	blocks	lesion type	age (Y)	sex	disease duration	MS classification
00-327	2	inactive	50	F	17Y	SPMS
00-43	1	active	52	F	11 Y	SPMS
01-018	1	inactive	48	F	8Y	SPMS
01-090	1	inactive	65	M	25 Y	SPMS
01-130	4	active	53	F	16 Y	SPMS
1191-05	1	inactive	77	F	50 Y	RRMS
1854-05	4	active and inactive	42	M	6 Y	PPMS
403-06	1	inactive	49	F	28	mild relapsing
121	2	active	27	M	9 Y	SPMS
01-135	1	active	43	M	15 Y	SPMS
06-054	1	active	66	M	40 Y	chronic progressive
03-074	1	inactive	59	M	32 Y	SPMS
06-041	1	inactive	63	M	25 Y	PPMS
02-046	1	inactive	71	F	23 Y	chronic progressive
1MK2	1	active	59	F	20 Y	SPMS
1MK4&5	2	active	47	F	20 Y	SPMS
1MK6	1	active	29	F	8 Y	SPMS
1MK7	2	active	65	M	25 Y	SPMS
1	2	active	45	F	0.2 Mo	acute MS
2	2	active	51	F	5 Mo	acute MS
3	1	progressive	55	F	5 Y	PPMS
4	1	progressive	46	F	37 Y	PPMS
5	1	progressive	71	F	22 Y	PPMS
6	1	inactive	64	F	28 Y	SPMS
7	1	inactive	50	F	17 Y	SPMS
8	1		47	M		control
1MK1	1		47	F		control
1345-06	1		48	M		control
696-01	3		46	F		control
976-06	1		49	M		control
1152	2		59	M		control

Y: years; Mo: months; SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis.

4.2 Methods

4.2.1 Morphology

a) Basic stainings: LFB-PAS, H&E, ORO

For histochemical analysis of the frozen tissue: 10-30 µm serial sections were mounted on to the super frost glass slides. The extent of demyelination and inflammation was evaluated by the following stainings: LFB – *PAS (Luxol Fast Blue-Periodic Acid Schiff's)*: 30 µm tissue sections were used for stainings, sections were stained first with LFB and then with PAS. LFB stains the lipoproteins in the myelin blue, hence the myelinated area stained blue and the demyelinated area is unstained. PAS stains the glycolipids pink; it is mainly used to identify macrophages which have phagocytised degraded myelin products. *H&E (Hematoxylin & Eosin)*: 10 µm tissue sections were used for stainings. Hematoxylin stains the nucleus blue and eosin stains the cytoplasm pink. This staining helps in the identification of the inflamed tissue. *ORO (Oil Red O)*: 10 µm tissue sections were used for stainings. ORO stains neutral lipids red, the staining is used to stain the macrophages which have phagocytised degraded myelin products.

b) Immunohistochemistry

Immunohistochemistry of brain sections: immunohistochemistry was performed on both frozen (10 µm) and paraffin (4 µm) sections using Mouse PAP or Rabbit PAP system (DAKO, Hamburg, Germany). Cryosections were fixed with 4% PFA and endogenous peroxidase activity blocked using 1.5% methanolic hydrogen peroxide. In case of paraffin sections, the sections were first deparaffinised twice for 10 min in xylol, 5 min in 100% ethanol, 5 min in 90% ethanol, 5 min in 70% ethanol and antigen retrieval was performed by boiling the slides for 20 min in fresh citrate buffer pH 6.0 and then cooling down to RT. The slides were then washed once in distilled water and thrice in PBS followed by blocking endogenous peroxidase activity with 1.5% methanolic hydrogen peroxide. Later the sections were blocked

with 10% serum (serum of the species of secondary antibody). *Primary* antibodies used were specific for: CD68 (mAb, DAKO), collagen I (mAb, Abcam, Cambridge, UK), collagen III (mAb, Abcam), collagen V (pAb, AbD SeroTec, Düsseldorf, Germany), biglycan (rabbit serum, a kind gift from Prof. Larry Fisher, NIH, US), decorin (mAb, R&D, Wiesbaden-Nordenstadt, Germany and rabbit serum, a kind gift from Prof. Larry Fisher), FGF9 (pAb, R&D), FGFR3 (mAb and pAb, Santa Cruz, Heidelberg, Germany), FGFR2 (pAb, Santa Cruz). Sections were incubated O/N at 4^{o}C followed by *secondary*: polyclonal rabbit anti-mouse (DAKO) or polyclonal swine anti-rabbit Ig (DAKO), for 1hr at RT and *tertiary:* mouse PAP (mAb, DAKO) or rabbit PAP (pAb, DAKO), for 30 min at RT. Bound antibodies were detected with diaminobenzidine (DAB) and sections were counter stained with hematoxylin. For immunofluorescence, *primary* antibodies: CD31 (mAb, R&D), collagen V (pAb, AbD SeroTec), GFAP-alexa 488 (mAb, Molecular Probes, Karlsruhe, Germany), and *secondary:* donkey anti-mouse alexa 488, donkey anti- sheep alexa 488 and donkey anti-rabbit alexa 594 (all from Molecular Probes) were used. Confocal images were taken from Leica SP2UV microscope. Negative controls included omission of primary antibody.

Immunocytochemistry of cultured cells: Cells were fixed with 4% PFA for 15 min at RT, washed in PBS, permeablised in 0.5% Triton X-100 and 0.5% porcine gelatine for 20 min at RT. Primary antibodies used are: SMI-31 (mAb, Abcam) and MOG (monoclonal, Z2 hybridoma). Cultures were incubated O/N at 4^{o}C with primary antibody diluted in blocking buffer, followed by appropriate secondary antibody for 45 min at RT. The cultures were then washed with PBS followed by distilled water and mounted with Vectashied (Vector laboratories).

c) Lesion classification

Tissue blocks were classified according to defined criteria: *Active demyelinated lesions* contained abundant macrophages with myelin degradation products (LFB or ORO positive) either throughout the lesion (early active) or as a broad rim around the lesion edge (chronic active). *Progressive lesions* revealed mild to moderate microglia activation at the lesion margins without any macrophages. *Chronic inactive*

demyelinated lesions were sharply demarcated from the normal appearing white matter (NAWM) and without a rim of microglial activation and without any LFB or ORO positive macrophages.

d) Lesion dissection

7 demyelinated chronic inactive and 4 demyelinated active lesions were macro dissected manually. 4 additional active lesions were included, which were not macro dissected, i.e., whole tissue block with lesion and NAWM was used. To this end, cryosections (20 µM) from the tissue samples were mounted on PEN slides (P.A.L.M. Microlaser, Bernried, Germany). Every 6th section (30 µM) was stained with LFB to identify the demyelinated areas; the unstained sections were superimposed on stained LFB section and lesion area marked and then manually macro dissected. The macro dissected sections were then stained with LFB to counter check the dissected area. Control tissue samples used for qPCR (quantitative PCR) exclusively contained white matter.

4.2.2 Molecular biology

a) RNA extraction and cDNA synthesis

Tissue was lysed in 1 ml Trizol (TRI® Reagent, SIGMA, Munich, Germany) and 0.1 volume of chloroform was added to the lysate and shaken vigorously. Lysate was incubated for 15 min on ice and later centrifuged at 4^{o}C for 15 min at 14,000 rpm. The aqueous phase was separated and mixed with equal volume of isopropanol and centrifuged at 4^{o}C for 15 min at 14,000 rpm. The pellet was dissolved in 600 µl of Trizol and 0.1 volume of chloroform. The above mentioned steps were repeated till we got the pellet again. Pellet was washed once with 75% RNase free ethanol and then dissolved in 25-30 µl of RNase free water (Invitrogen, Karlsruhe, Germany).
cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied Biosystems (ABI), Darmstadt, Germany). 1 µg of total RNA was taken for cDNA synthesis with random hexamers and the procedure was followed as mentioned in the data sheet.

b) qPCR

Gene specific primers and probes labelled with FAM-MGB or FAM-TAMRA or VIC-TAMRA available from ABI were used. Each reaction had 10-25 ng of RNA converted into cDNA, 1X primer-probe and 1X reaction buffer (Eurogentec, Köln, Germany) in a reaction volume of 25 µl, and was run in duplicates on an ABI 7900 machine (Applied Biosystems). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and PPIA (cyclophilin A) were used as internal controls. Primary data was analysed on SDS 2.3 software or RQ manager (ABI).

Primers used were for *COL1A1*, *COL3A1*, *COL5A1*, *COL4A1*, *COL4A2*, *COL4A3*, *COL4A4*, *COL4A5*, *COL4A6*, *BGN*, *DCN*, *CCL2*, *CXCL9*, *CXCL10*, *CXCL11*, *FGF9*, *GAPDH*, and *PPIA* genes.

c) Low Density Array (LDA)

qPCR of dissected MS lesions was performed for 528 genes using custom made Low Density Arrays (LDA) (ABI). In the thesis only the genes belonging to ECM components, ECM modifying enzymes and factors required for oligodendrocyte survival, proliferation and/or differentiation are mentioned (Table 5.1, 5.2, 5.4a and 4b). These LDAs enable us to perform several qPCR reactions simultaneously. Each reaction had 1-8 ng of RNA converted in to cDNA. The qPCR are run for 40 cycles. Data analysis was done using RQ Manager 1.2 software (ABI), taking GAPDH, βactin and PPIA as internal controls.

The genes for monocyte LDA expression analysis included *CD80*, *PDL1-B7-H1*, *PDL2*, *CD69*, *CD200R*, *SIRPα*, *CD206*, *ADORA2A*, *IL-1R2*, *SLAM*, *IL-1*, *IL-6*, *IL-10*, *TNF*, *TGFβ*, *IL-12p40*, *CXCL10*, *CCL18*, *MPO*, *MMP9*, *CCR2*, *CX3CR1*, *CD62L*, *CD204*, *MT-2A*, *BDNF*, *NGF*, *NT-3*, *NT-4*, *NRTN*, *LIF*, *IGF-1*, *IL-4*, *IL-5*, *IFNγ*, *CD36*, *CD163*, *MFGE8*, *MERTK*, *CXCL8*, *CXCL2*, *CCL2*, *CCL5*, *CCL3*, *CCL4*, *BAFF*, *GAPDH* and *PPIA*.

4.2.3 Cell culture

a) Astrocytes and fibroblasts

Human primary astrocytes of embryonic origin [Aloisi *et al.* 1992] and primary human foreskin fibroblast (kindly provided by the Virology department of the University of Erlangen) were grown in RPMI with 10% FCS till they reached confluency before being used for the final experiments. 50000-75000 astrocytes were seeded in each well of a 24 well plate and grown in the presence or absence of ECM components (collagen I, III, V, biglycan and decorin) for two days and were stimulated on the third day for 24 hrs with 10 ng/ml TNFα (R&D) and 100 U/ml IFNγ (R&D). The ECM components were coated on culture dish. In addition, a similar number of astrocytes and fibroblasts were grown in the absence of ECM components for two days. The supernatant was collected and stored at -80^{o}C and the cells were lysed with TRIZOL for RNA extraction.

b) In vitro myelination

Neurosphere derived astrocyte monolayers: Neurospheres were prepared using methods modified form [Zhang *et al.* 1998; Reynolds *et al.* 1996]. Neurospheres were differentiated into astrocytes as described in [Reynolds *et al.* 1996]. Briefly, decapitated Sprague Dawley rat pups and dissected corpus striata from the forebrain. Transferred the tissue into neurosphere medium consisting of DMDM/F12 (1:1, DMEM containing 4,500 mg/l glucose), and supplemented with 0.105% $NaHCO_3$, 2 mM glutamine, 5,000 U/ml penicillin, 5 µg/ml streptomycin, 5 mM HEPES, 10^{-4}% BSA (bovine serum albumin) (all from Invitrogen), 25 µg/ml insulin, 100 µg/ml apotransferrin, 60 µM putrescine, 20 nM progesterone, and 30 nM sodium selenite (all from Sigma), and mechanically dissociated with a Pasteur pipette. The suspension was centrifuged at 4°C for 5 min at 800 rpm. The cell pellet was resuspended in neurosphere medium and plated into an uncoated T75 tissue culture flask. The neurosphere medium was further supplemented with 20 ng/ml mouse submaxillary gland epidermal growth factor (R&D systems). The cultures were incubated at 37°C in a humidified atmosphere of 7% CO_2 and 93% air and two

thirds of the media was changed every 2–3 days. After 7–10 days *in vitro*, the spheres were triturated using a Pasteur pipette to produce smaller spheres/cell clusters. Spheres were differentiated into astrocytes 1–2 days after trituration by plating spheres onto Poly-L-lysine coated coverslips in low glucose DMEM supplemented with 10% FBS and 2 mM L-glutamine (DMEM-FBS). After 7–10 days in culture, a confluent layer of astrocytes formed and dissociated embryonic spinal cord cells were plated onto these as described below.

Myelinating Spinal Cord Cultures: Myelinating spinal cord cultures were prepared according to the method described previously by [Reynolds *et al.* 1996b; Sorensen *et al.* 2007]. Sprague Dawley (SD) rats were time-mated, with the day of plugging denoted as embryonic day 0.5 (E0.5), and embryos were collected on day E15.5. The cranial 5 to 6 mm sections of the spinal cord were dissected, stripped of meninges, minced with a scalpel blade, and placed in a dish containing 1 ml of Hank's balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (Invitrogen). The tissue was enzymatically dissociated by the addition of 2.5% trypsin (100 µl) (Invitrogen) and 1.33% collagenase (100 µl) (MP Biomedicals) for 15 min at 37°C. Enzymatic activity was stopped by adding 2 ml of SD solution containing 0.52 mg/ml soybean trypsin inhibitor, 3.0 mg/ml bovine serum albumin, and 0.04 mg/mL DNase I (all from Sigma) to prevent cell clumping. The cells were triturated through a glass pipette and spun for 5 min at 800 rpm (136 RCF). The cell pellet was resuspended in 5 ml plating medium (PM) [Thomson *et al.* 2006] containing 50% Dulbecco's modified Eagle's medium (DMEM), 25% horse serum, 25% Hanks balanced salt solution with Ca^{++} and Mg^{++}, and 2 mM L-glutamine (Invitrogen). Two coverslips supporting a monolayer of neurosphere derived astrocytes or Poly-L-lysine only (13 µg/ml) (Sigma) were placed in a 35 mm Petri dish, and the dissociated spinal cord cells were plated onto each coverslip at a density of 150,000 cells/100 µl. The cells were left to attach for 2 hrs in the incubator after which 300 µl of PM and 500 µl of differentiation medium [Thomson *et al.* 2006] which contained DMEM (4,500 mg/mL glucose), 10 ng/ml biotin, 0.5% hormone mixture (1 mg/ml apotransferrin, 20

mM putrescine, 4 µM progesterone, and 6 µM selenium (formulation based on N2 mix [Bottenstein *et al.* 1979]), 50 nM hydrocortisone, and 0.5 mg/ml insulin (all from Sigma) was added. The cultures were fed thrice a week with differentiation media by removing half of the media and replacing it with fresh media. After 12 days in culture, insulin was omitted from the differentiation media. The cultures were maintained for 24–30 days in a humidified atmosphere of 7% CO_2 and 93% air at 37°C. In our *in vitro* myelinating culture system, myelination starts on day 20 and concludes 8-10 days later. Effect of FGF9 (human FGF9, R&D) on the *in vitro* myelinating culture was tested by applying FGF9 on day 18 (i.e. before the start of myelination) for 8 days. Fresh FGF9 was substituted during media change, half the media volume was replaced thrice a week with fresh media. Three different concentrations of FGF9 were tested in our experiments: 100 ng/ml, 50 ng/ml, 5 ng/ml. After additional 8 days of culture, the cultures were fixed with 4% PFA and stained for axonal marker SMI-31 and myelination marker MOG and the degree of myelination analysed.

c) Immune cells

Peripheral blood mononuclear cells (PBMCs): were isolated from the blood of healthy donors by density gradient centrifugation. Human blood diluted with PBS in 1:2 ratios was over laid on 15 ml PANCOL (PAN biotech) and centrifuged for 20 min at 1400 rpm without any break at RT. The cells at interface were collected and washed twice with ice cold PBS for 10 min at 2000 rpm at 4^oC. The pellet consists of purified human PBMCs which was either used directly for experiments or was used for monocyte isolation.

Monocytes: monocytes were isolated from PBMCs by positive selection (CD14 MicroBeads, Miltenyi Biotech). These monocytes were plated at a density of 250,000 cells per well of 24 well plate and were grown in presence or absence of ECM components (collagen I, III and V, biglycan and decorin) for 1 day. The ECM components were coated on the wells of 24 well plated (see below). On the following

day cell supernatant was collected and the cells lysed with TRIZOL for subsequent RNA extraction.

Macrophages: positively isolated monocytes were allowed to differentiate into macrophages for 9-10 days in presence of 20 ng/ml M-CSF (R&D). These macrophages were plated at a density of 250,00 cells per well of 24 welled plate and were grown in presence or absence of ECM components (collagen I, III and V, biglycan and decorin) for 1 day. The ECM components were coated on the wells of 24 well plated (see below). On the following day cell supernatant was collected and the cell lysed with TRIZOL for subsequent RNA extraction.

T cells: 20,000 MBP specific $CD4^+$ T cell clone ES-BP8 [Meinl *et al.* 1995] in a volume of 100 µl and 200,000 irradiated antigen presenting cells in a volume of 100 µl were mixed and stimulated with 20 µg/ml human MBP (Biogenesis, Berlin, Germany) or 10 µg/ml MBP 29-48 peptide (human). These cells were grown in wells of 96 well plate coated with indicated ECM components or in uncoated wells.

Coating of the tissue culture plates with ECM proteins.

Tissue culture dishes were coated with: Collagen I, 2 µg/cm^2 (BD Biosciences, Heidelberg, Germany), collagen III, 2 µg/cm^2 (BD Biosciences), collagen V, 2 µg/cm^2 (BD Biosciences), biglycan, 10 µg/ml (R&D), decorin, 10 µg/ml (R&D Systems). All the ECM proteins were diluted to the final concentration with Ca^{++}, Mg^{++} free PBS (Invitrogen) and the cell culture dishes were incubated at RT for 2 hrs, and then washed with distilled water (Invitrogen) and air dried. The ECM proteins were tested negative for LPS contamination (Biowhittaker™ LAL kit, Walkersville, US).

4.2.4 Proliferation assay

The proliferation assay was performed in 96 well flat bottom plates in triplicates. MBP specific T cells (in presence of antigen presenting cell) were grown

in presence of 20 µg/ml human MBP or 10 µg/ml MOG 29-48 peptide in a final volume of 200 µl cell culture medium. After 2 days incubation, cultures were pulsed with ^{3}H-thymidine (specific activity: 2 Ci/mmol; 1 µCi/well) (GE Healthcare, Freiburg, Germany). Samples were harvested after 24 hrs incubation, and the radioactivity was measured by the beta counter (MatrixTM 9600, Packard).

4.2.5 ELISA

Supernatants form monocytes were used for this experiment. 96-well flat bottomed Maxisorb plates (Nunc, Langenselbold, Germany) were coated with 100 µl of 1-4 µg/ml of capture antibody (mouse anti human) O/N. Wells were washed thrice with wash buffer and blocked with 300 µl of reagent diluent for 1 hr. After washing the wells, incubated 100 µl of samples or standard diluted in reagent diluent for 2 hrs. Cytokine standards were serially diluted starting from 1000/2000 pg/ml to make a seven point standard curve. Again wells were washed and incubated 100 µl of 50-300 ng/ml of detection antibody (biotinylated goat anti human) for 2 hrs. After extensive washing 100 µl of streptavidin-HRP (1:200) was added and incubated for 20 min. The wells were washed and added 100 µl of substrate solution (tetramethyl benzidine activated with hydrogen peroxide). The reaction was stopped with 50 µl of 2N H_2SO_4, and the optical density was measured at 405/450 nm in ELISA reader (Victor2™ 1420 mutilabel counter; Perkin Elmer life sciences). All the antibodies and streptavidin-HRP were supplied with the ELISA kit.

4.2.6 Imaging and quantitative analysis

Zeiss Axioplan 2 fluorescent microscope and ISIS software (metasystems) were used for capturing images for myelin analysis. For quantitative analysis of axonal density and myelination, ten images (X10 magnification) from each coverslip were captured. Each experiment was performed in triplicate; therefore, 30 images per treatment per experiment were obtained to quantify axonal density and myelination.

Axonal density was analysed using Image J software (NIH systems, version 1.40g). Using this software, values were obtained for the area of SMI-31 immunoreactivity and the total field of the image. On the basis of the area of SMI-31^{+}

structures approximate axon density was calculated. To calculate the percentage of myelinated axons immunoreactivity of MOG^+ myelin sheath was also measured using image J. Percentage of myelinated axons was obtained by dividing the MOG^+ area by area obtained for axonal density.

5 Results

5.1 Extracellular matrix

5.1.1 Altered expression of ECM components in MS lesions.

We wanted to quantify ECM molecules and ECM modifying enzymes in MS lesions. To this end, LDA was used for determining the gene expression profile of three groups namely, active demyelinated, chronic inactive demyelinated MS lesions and control brain white matter. 528 genes were analysed in a total of 15 MS lesions. In the first part of the thesis I focus on ECM components and ECM modifying enzymes. The up-regulated ECM components localized largely in the perivascular space in association with infiltrating immune cells and not in the parenchyma. This indicates that these ECM components might not directly affect the differentiation of oligodendrocytes, but rather modulate the immune response in the CNS. Hence, only demyelinated inactive and demyelinated active lesions are described in the results part.

The expression of each gene was related to GAPDH. Each gene was calculated for each lesion type (Table 5.1). The table gives the absolute and relative values. Of the tested 50 ECM genes, 21 (42%) were up-regulated more than two-fold in chronic inactive lesions and 22 (44%) in active demyelinated lesions. 15 ECM components *COL1A1*, *COL3A1*, *COL5A1*, *COL5A2*, *COL4A1*, *LAMA5*, *LAMB1*, *LAMB2*, *LAMC1*, *BGN*, *FMOD*, *HAPLN3*, *HSPG2*, *THBS1* and *THBS3* were induced in both chronic inactive and active lesions. 23 (46%) genes in chronic inactive lesions and 25 (50%) in active lesions remained unchanged, i.e. they have a fold change between 0.51-1.99. These up-regulated ECM components belonged to fibrillar collagens, basement membrane collagen, laminins, SLRPs, hyaluronan link proteins, perlecan and thrombospondins.

Table 5.1. Expression level and fold change values of 50 ECM genes

	absolute expression in % GAPDH			fold change	
Genes	**CB**	**De.in**	**De.act**	**De.in:CB**	**De.act:CB**
Fibrillar Collagens (COL)					
COL1A1	0.08	0.87	0.45	10.86	5.63
COL1A2	0.34	0.40	0.74	1.18	2.18
COL3A1	0.06	0.72	0.32	12.00	5.33
COL5A1	0.06	1.57	0.15	26.17	2.50
COL5A2	0.05	0.10	0.15	2.00	3.00
COL5A3	0.44	0.79	0.68	1.80	1.55
Basement membrane collagens (COL)					
COL4A1	0.70	2.03	1.90	2.90	2.71
COL4A2	0.12	0.16	0.28	1.33	2.33
COL4A3	0.20	0.06	0.13	0.30	0.65
COL4A4	0.10	-nd-	0.11	*	1.10
COL4A5	0.65	1.07	0.84	1.65	1.30
COL4A6	0.15	0.12	0.20	0.80	1.33
Anchoring collagen (COL)					
COL7A1	0.51	0.60	0.71	1.18	1.39
Nidogens (NID)					
NID1	0.60	1.11	1.32	1.86	2.20
NID2	0.40	0.31	0.60	0.76	1.50
Laminins (LAMA)					
LAMA1	0.68	2.09	0.36	3.07	0.53
LAMA2	0.64	1.30	0.57	2.03	0.89
LAMA3	0.18	0.22	0.36	1.22	2.00
LAMA4	0.71	1.25	1.24	1.76	1.75
LAMA5	0.13	0.99	0.93	7.62	7.15
LAMB1	0.11	1.06	0.40	9.64	3.64
LAMB2	2.58	5.83	7.89	2.26	3.06
LAMB3	0.14	0.11	0.13	0.79	0.93
LAMC1	0.34	0.83	0.79	2.44	2.32
LAMC2	-nd-	-nd-	-nd-	*	*
Lecticans					
AGC1 (Aggrecan)	0.05	0.03	0.41	0.60	8.20
BCAN (Brevican)	8.00	7.22	8.61	0.90	1.08
CSPG3 (Neurocan)	13.89	7.40	8.80	0.53	0.63
CSPG2 (Versican)	0.01	-nd-	0.01	*	1.00

Table 5.1. continued

	absolute expression in % GAPDH			fold change	
Genes	**CB**	**De.in**	**De.act**	**De.in:CB**	**De.act:CB**
Small leucine rich proteoglycans (SLRPs)					
BGN (Biglycan)	4.83	11.17	11.41	2.31	2.36
DCN (Decorin)	2.99	6.27	5.41	2.10	1.81
FMOD (Fibromodulin)	0.03	0.07	0.09	2.33	3.00
LUM (Lumican)	0.09	0.15	0.24	1.67	2.67
Hyaluronan and proteoglycan link proteins (HAPLNs)					
HAPLN1	0.09	-nd-	0.02	*	0.22
HAPLN2	29.85	5.08	25.74	0.17	0.86
HAPLN3	0.05	0.29	0.36	5.80	7.20
HAPLN4	0.03	0.17	0.03	5.67	1.00
Heparan sulphate proteoglycan (HSPG)					
HSPG2 (Perlecan)	0.63	2.75	1.51	4.37	2.40
Tenascins (TNs)					
TNC	3.68	9.46	3.49	2.57	0.95
TNR	5.05	2.78	3.98	0.55	0.79
Thrombospondins (THBSs)					
THBS1	0.12	0.98	0.41	8.17	3.42
THBS2	2.75	4.22	4.05	1.53	1.47
THBS3	0.01	0.02	0.03	2.00	3.00
THBS4	0.83	1.08	1.09	1.30	1.31
Fibrillins (FBNs)					
FBN1	2.59	3.12	1.46	1.20	0.56
FBN2	0.04	0.03	0.09	0.75	2.25
FBN3	0.11	0.10	0.13	0.91	1.18
Others					
FN1 (Fibronectin)	2.60	4.86	3.94	1.87	1.52
RELN (Reelin)	0.08	0.63	0.04	7.86	0.50
VTN (Vitronectin)	0.03	0.03	0.03	1.00	1.00

CB: control brain, **De.in:** demyelinated inactive lesion, **De.act:** demyelinated active lesion, **De.in:CB-** demyelinated inactive *vs* control brain, **De.act:CB-** demyelinated active *vs* control brain. **nd:** not detectable due to low expression level. In total 6 control white matter from 4 subjects, 7 demyelinated inactive lesions from 5 subjects and 8 demyelinated active lesions from 7 subjects were used for qPCR analysis. ***** Calculation not possible because the values are under detection limit. The values below 0.01% GAPDH are not reliable.

5.1.2 Fibrillar collagens and small leucine rich proteoglycans form perivascular fibrosis in MS lesions

Our expression profiling identified a total of 22 components up-regulated in active MS lesions and 21 in chronic inactive MS lesions. When considering the relative induction in MS lesions, the absolute expression level and possible interactions with each other, our interest was directed to the fibrillar collagens and the SLRPs.

We noted a strong induction of *COL1A1*, *COL3A1, COL5A1* and *COL5A2* chains, both in active and chronic inactive demyelinated lesions (Table 5.1). These collagen chains interact to form collagen type I, III and V, which are grouped as fibrillar collagens known to act as structural proteins. The different chains of collagen I and V interact to form homotrimeric or heterotrimeric collagens, whereas collagen III chain forms only homotrimeric collagen. Two SLRPs biglycan and decorin were also strongly induced in active and inactive demyelinated lesions (Table 5.1). Decorin and biglycan can interact (decorate) with fibrillar collagens and participate in fibril formation, but decorin alone can inhibit fibril formation *in vitro.* To further confirm the induction of these ECM components and to determine their localization we performed immunohistochemistry on chronic inactive, active demyelinated lesions and control brain.

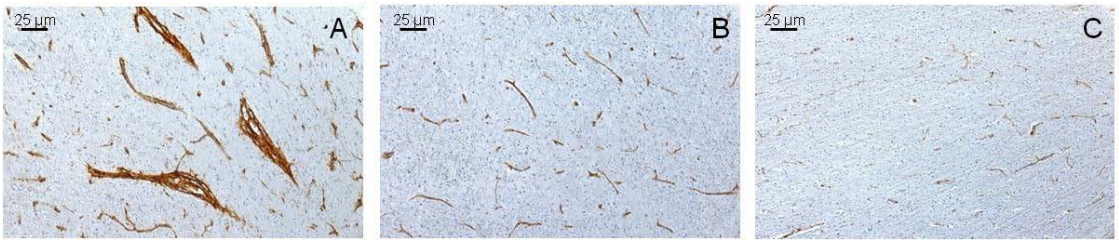


Fig. 5.1 Overview of the extent of induction of fibrillar collagens in MS lesions. Immunostaining for collagen V is shown in **A-C**. Specific antibody binding was visualized with DAB as a substrate resulting in a brown colour. Nuclei were counterstained blue with hematoxylin. Collagen V is deposited around small and big blood vessels in a chronic inactive MS lesion. The strong expression of collagen V in the lesion core decreases to the lesion edge and in the normal appearing white matter (NAWM) it becomes similar to that of control brain.

To determine the localization of fibrillar collagens, biglycan, and decorin in MS lesions, immunostaining was performed with a total of 6 different primary antibodies. We noted that antibodies to three fibrillar collagens, decorin and biglycan gave a similar staining pattern. In the control brain we saw faint staining around blood vessels, which is similar to the staining observed in the NAWM (Figure 5.1C). In contrast, in both active and chronic inactive MS lesions an intense staining of fibrillar collagens, decorin, and biglycan was seen (Figures 5.2A-D, J, K), which was localized around small, medium and large blood vessels (Figure 5.1A). In the larger blood vessels with an extended perivascular (Virchow Robin) space (space between the endothelial basement membrane and the astrocytic glia limitans) a mesh of fibrillar collagen, biglycan and decorin could be seen (Figure 5.2). In chronic active and also early active lesions the infiltrating immune cells in the perivascular space were in close contact with fibrillar collagens, biglycan and decorin (Figure 5.2). Staining for collagens I, III, and V showed that the up-regulated fibrillar collagens were seen in the perivascular space also in early active MS lesions (Figure 5.2G). The up-regulation of ECM molecules seen in the lesion area decreased with the distance from the lesion core and became similar to the expression seen in the control brain (Figure 5.1A-C).

We noted that the deposition of collagen V extended beyond the perivascular space; Collagen V was seen in the brain parenchyma of 6/8 early active lesions (Figure 5.2F-H) and was expressed most likely in astrocytes. Infiltrating immune cells in the parenchyma of such an active lesion could be positively stained with biglycan (Figure 5.2I).

Double staining of the lesions with the endothelial cell marker CD31+collagen V and GFAP+collagen V revealed that the collagens are located between the endothelial cells and the astrocytic glial limitans (Figure 5.3).

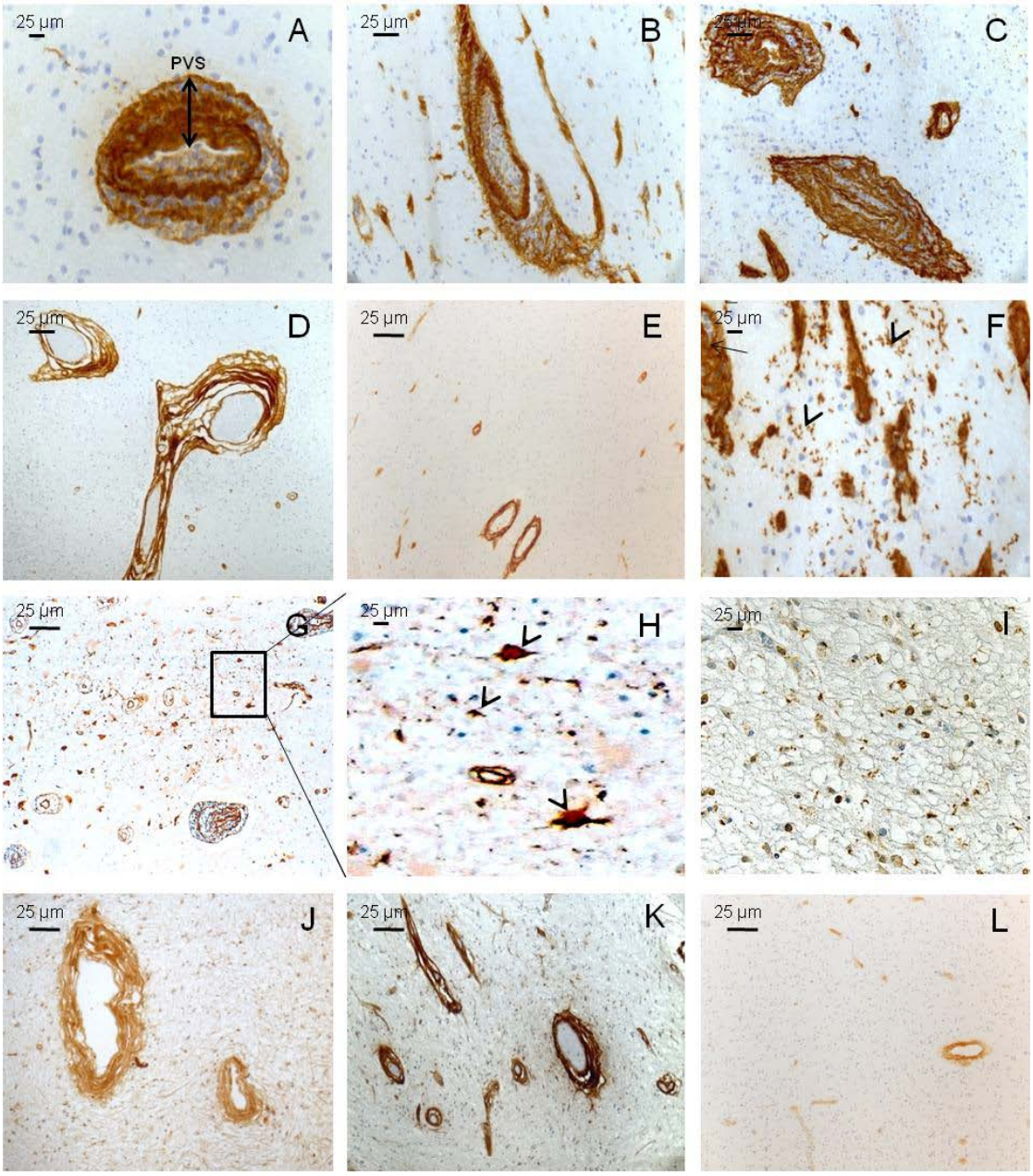


Fig. 5.2 Localization of ECM components up-regulated in MS lesions. Sections of active lesions **(A-C, F-I)**, chronic inactive lesions **(D, J-K) and** control white matter **(E, L)** were immunostained for indicated antibody. Specific antibody binding was visualized with DAB as a substrate resulting in a brown colour. Nuclei were counterstained blue with hematoxylin. Collagen I**(A)**, collagen III **(B,D)** and collagen V **(C)** form a mesh in the perivascular space in MS lesions. In the control brain fibrillar collagens can be detected around blood vessels at lower level **(**collagen I in **E)**. **(F-H)** In active lesions collagen V was found around blood vessels **(arrow in F)** and also outside of the perivascular space **(arrowhead in F and H)**. Biglycan is detected in infiltrating immune cells in active lesions **(I)** and in chronic inactive lesions the fibrotic mesh in the perivascular space also contains biglycan **(J)** and decorin **(K)**. In the control brain biglycan **(L)** and decorin (not shown) are seen around blood vessels at a lower level **(L)**. **PVS** = perivascular space

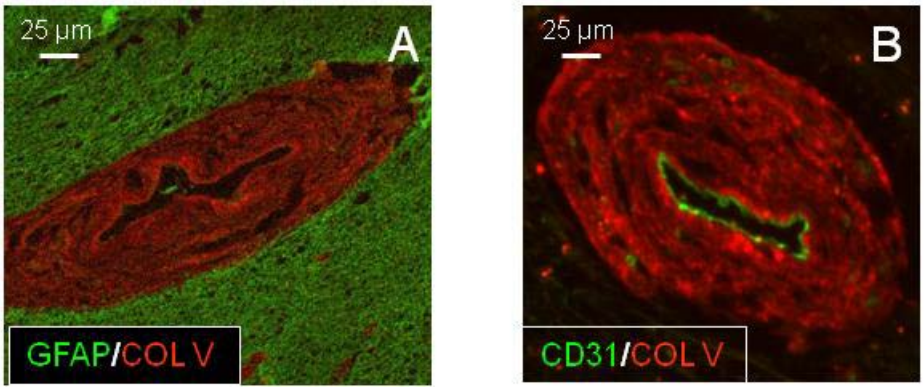


Fig. 5.3 Fibrillar collagens in the perivascular space. Immunofluorescence of inactive lesion **A)** GFAP (green) and collagen V (red) **B)** CD31 (green) and collagen V (red) .Fibrillar collagens form a mesh in the perivascular space of the blood vessel. The mesh formed by fibrillar collagens is strictly limited to the perivascular space between endothelial cells (CD31 positive) and the astrocytic scar (GFAP positive) in the parenchyma.

5.1.3 ECM modifying enzymes in control brain and MS lesions

The ECM modifying enzymes were also tested, because the absolute levels or the turnover of the ECM components depend upon the expression of these enzymes. In this regard 34 enzymes were analysed. In the control white matter, out of 26 matrix metalloproteinases (MMPs), 7 MMPs, namely MMP2, MMP14-17, MMP24, and MMP28 were clearly expressed (>0.1% GAPDH). In chronic inactive lesions 10 (29%) and in active lesions 7 (21%) of all the analysed ECM modifying enzymes were up-regulated at least two-fold (Table 5.2). Five of them, namely MMP9, MMP11, MMP14, MMP19 and tissue inhibitor of matrix metalloproteinase (TIMP) 1 were induced in both chronic inactive and active lesions. We also noted that other ECM modulating enzymes were differentially regulated. MMP2, MMP9 and MMP19 were stronger induced in active lesions; MMP17, MMP24, and MMP28 were stronger up regulated in chronic inactive lesions. Of the four TIMPs analysed, TIMP1 was the one being most strongly regulated, it was induced by about 6-7 fold in both active and chronic inactive lesions. While TIMP3 was up-regulated in chronic inactive lesions and only slightly in active lesions. Expression of other two TIMPs, TIMP2and -4 was similar to that of the control brain (Table 5.2). The expression of 9 (26%) and 14 (41%) genes from chronic inactive and active demyelinated lesions

respectively remained unchanged, i.e., they have a fold change between 0.51-1.99. We also tested the expression of three out of 4 collagenases (degrading fibrillar collagen), namely MMP1, -8 and -13. All the three collagenases were not detected in our study, either their expression was lower than the detection limit or else they were not expressed in the lesions.

Table 5.2. Expression level and fold change values of 34 ECM modifying enzymes.

	absolute expression in % GAPDH			fold change	
Genes	**CB**	**De.in**	**De.act**	**De.in:CB**	**De.act:CB**
A disintegrin and metalloproteinase domains (ADAMs)					
ADAM8	0.11	0.05	0.31	0.45	2.82
ADAM10	16.40	13.58	13.23	0.83	0.81
ADAM12	0.30	0.29	0.40	0.97	1.33
ADAM17	1.29	0.85	1.32	0.66	1.02
A disintegrin like and metalloproteinase with thrombospondin type 1 motifs (ADAMTs)					
ADAMTS1	7.38	7.88	3.92	1.07	0.53
ADAMTS4	16.04	3.24	15.15	0.20	0.94
ADAMTS5	0.04	0.17	0.06	4.25	1.50
Matrix metalloproteinases (MMPs)					
MMP1	-nd-	-nd-	-nd-	*	*
MMP2	0.61	1.09	3.36	1.79	5.50
MMP3	-nd-	-nd-	-nd-	*	*
MMP7	-nd-	-nd-	0.03	*	*
MMP8	-nd-	-nd-	-nd-	*	*
MMP9	0.01	0.05	0.13	5.00	13.00
MMP10	-nd-	-nd-	-nd-	*	*
MMP11	0.05	0.30	0.30	6.00	6.00
MMP12	-nd-	-nd-	-nd-	*	*
MMP13	-nd-	-nd-	-nd-	*	*
MMP14	1.00	5.71	4.61	5.71	4.61
MMP15	0.71	0.83	0.36	1.17	0.51
MMP16	0.75	0.97	1.03	1.29	1.37
MMP17	0.39	5.05	0.36	12.95	0.92
MMP19	0.01	0.04	0.08	4.00	8.00
MMP20	-nd-	-nd-	-nd-	*	*
MMP21	0.07	-nd-	0.07	*	1.00
MMP23	0.02	0.01	0.01	0.50	0.50
MMP24	0.37	1.21	0.34	3.27	0.92
MMP25	-nd-	-nd-	0.01	*	*
MMP26	-nd-	-nd-	-nd-	*	*
MMP27	-nd-	-nd-	-nd-	*	*
MMP28	0.24	0.75	0.08	3.13	0.33

Table 5.2. Continued

	absolute expression in % GAPDH			fold change	
Genes	**CB**	**De.in**	**De.act**	**De.in:CB**	**De.act:CB**
Tissue inhibitor of metalloproteinases (TIMPs)					
TIMP1	0.97	5.96	7.00	6.14	7.22
TIMP2	13.78	9.56	13.82	0.69	1.00
TIMP3	9.21	22.98	15.51	2.50	1.68
TIMP4	0.88	0.69	0.98	0.78	1.11

CB: control brain, **De.in:** demyelinated inactive lesion, **De.act:** demyelinated active lesion, **De.in:CB-** demyelinated inactive *vs* control brain, **De.act:CB-** demyelinated active *vs* control brain. **nd:** not detectable due to low expression level. In total 6 control white matter from 4 subjects, 7 demyelinated inactive lesions from 5 subjects and 8 demyelinated active lesions from 7 subjects were used for qPCR analysis. ***** Calculation not possible because the values are under detection limit. The values below 0.01% GAPDH are not reliable.

5.1.4 Fibrillar collagens up-regulated in MS lesions do not modulate T cell proliferation.

As we observed a close association of infiltrating immune cells with fibrillar collagens, decorin, and biglycan in MS lesions, we tested the effect of these up-regulated ECM proteins on the activation of MBP-specific $CD4^+$ T cells.

To this end, MBP-specific $CD4^+$ T cells were cultured in presence of fibrillar collagens (I, III, V) and stimulated with human MBP or the relevant peptide for this clone (MBP 29-48) and PBMC as antigen presenting cells. The antigen specific proliferation was not modified by the presence of either collagen I, III or V (Figure 5.4).

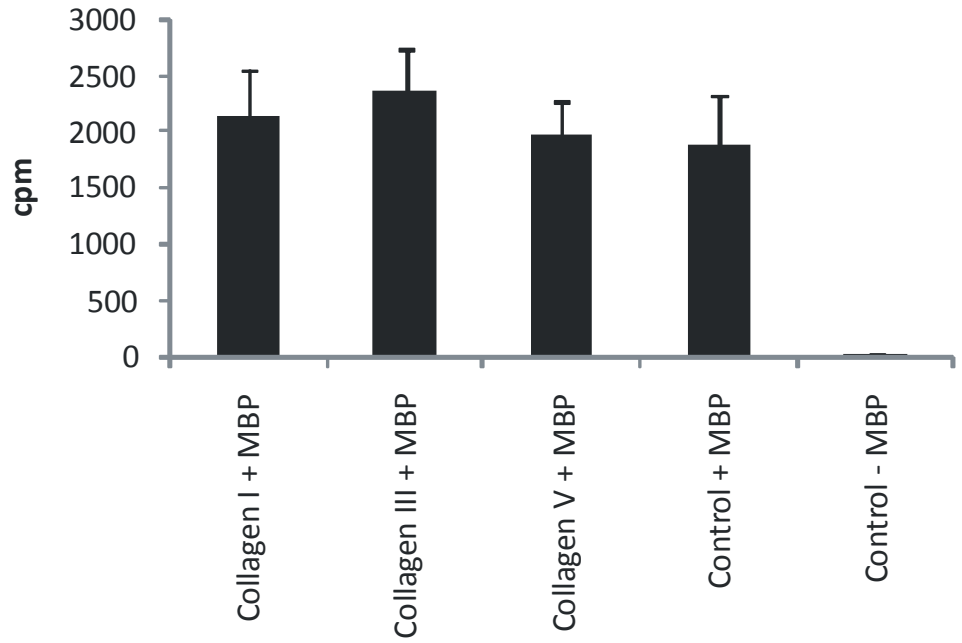


Fig. 5.4 Unaltered antigen specific proliferation of lymphocytes in presence of fibrillar collagens. MBP specific human $CD4^+$ T cells (ES-BP8) were grown in presence of antigen presenting cells and antigen (human MBP or human MBP 29-48 peptide) for 2 days and pulsed with ^{3}H-thymidine for additional 24 hrs. Cells incorporated ^{3}H-thymidine, which indicates proliferation. Proliferation was analysed by measuring radioactivity in a beta counter and shown as counts per minute (cpm). These cells were grown in dishes coated with the indicated collagen or on uncoated dishes (control). Error bars indicate the standard deviation of triplicates. Shown data is a representative of two experiments.

5.1.5 Fibrillar collagens up-regulated in MS lesions modulate chemokine production of monocytes.

Purified monocytes and macrophages were cultured in the presence or absence of fibrillar collagens and SLRPs found to be up-regulated in MS lesions. Subsequently the expression of 46 immune related genes (mentioned in materials and methods) was quantified by qPCR.

Collagen I reduced the expression level of CCL2, CCL4, IL-10 and IL-1β in monocytes by about 50%, whereas collagen III reduced them by 20-40%. In contrast, collagen V, biglycan and decorin did not modulate the production of any of the 46 genes tested (Figure 5.5 A-D).

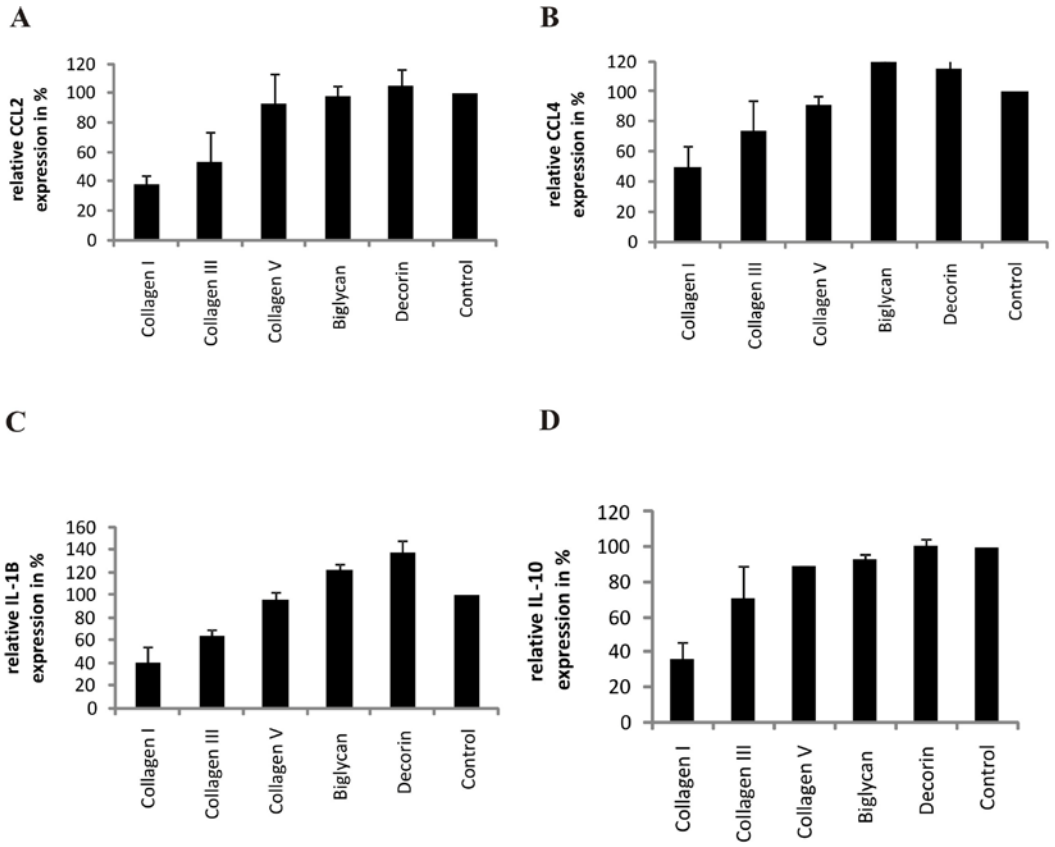


Fig. 5.5 Collagen I and III reduced the expression of monocytic chemokine-cytokine. Human monocytes were isolated by positive selection (CD14 MicroBeads) and cultured in dishes coated with the indicated ECM component for 24 hours. Monocytes grown in uncoated dishes were taken as control. Expression of **A)** CCL2, **B)** CCL4, **C)** IL-1B, **D)** IL-10 was determined by qPCR in relation to housekeeping gene GAPDH and is indicated as percentage of the control dish. Error bars indicate the SEM of three individual experiments.

We went on to test whether the reduced transcript levels detected in our qPCR screening were mirrored at the protein level. The presence of CCL2, CCL4, IL-10 and IL-1β in the supernatant of monocytes was detected by ELISA in three individual experiments. While the amount of released CCL4, IL-10, and IL-1β was not significantly reduced in these experiments (Figure 5.6B), reduction of CCL2 that mirrored the transcript levels was noted. Collagen I reduced the production of CCL2 protein by 60% and collagen III by 25% (Figure 5.6A). This decrease in the production of CCL2 by monocytes is not due to the absorption of CCL2 by the

collagens. In a parallel experiment 1730 pg/ml CCL2, the spontaneous level produced by unstimulated monocytes, was added to the medium in the collagen coated culture dishes and >95% of CCL2 could be recovered from the supernatant after 24 hrs. This indicates that the reduced amount of CCL2 in the supernatant is due to reduced release by monocytes and not due to an absorption by collagen.

Macrophages grown on collagen I, III, V, biglycan and decorin did not show significant regulation in the expression of any of the 46 genes tested.

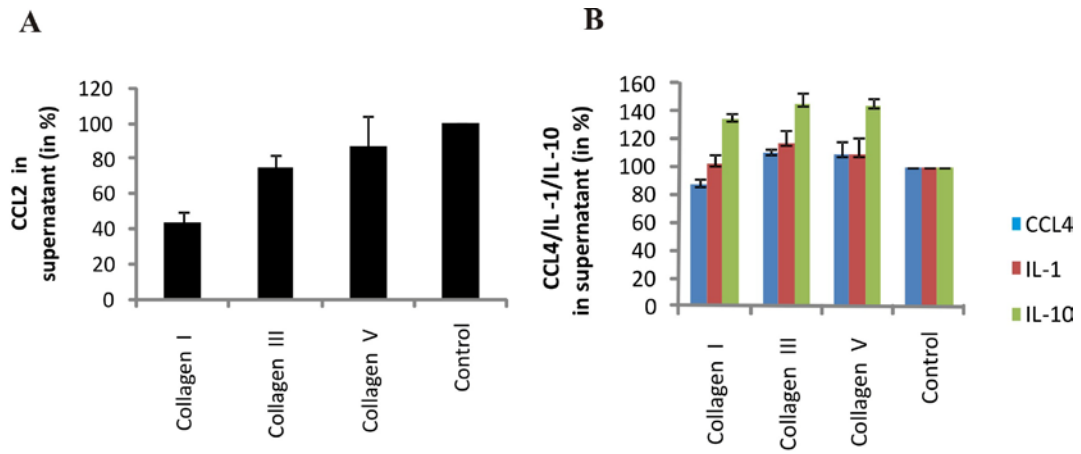


Fig. 5.6 Collagen I decreased CCL2 production by monocytes. Human monocytes were isolated by positive selection (CD14 MicroBeads) and cultured in dishes coated with the indicated ECM component for 24 hours. Monocytes grown in uncoated dishes were taken as control. The release of **A)** CCL2 **B)** CCL4, IL-1 and IL-10 in the culture supernatant was measured by ELISA and is indicated as percent of amount produced in control dish without ECM component. Error bars indicates the SEM of three individual experiments.

5.1.6 *In vitro* production of ECM proteins by astrocytes and fibroblasts

Possible sources of the ECM molecules up-regulated in the perivascular space are fibroblasts and astrocytes. Therefore the *in vitro* production of ECM components by human foreskin fibroblast (HFFs) and human primary astrocytes was analysed by qPCR. Both HFFs and astrocytes produced fibrillar collagens, biglycan and decorin; albeit with varying, expression levels (Table 5.3).

Table 5.3. Expression levels of ECM components produced by HFF and astrocytes *in vitro*.

Genes	Cell types	
	HFF*	Astrocytes*
COL1A1	816	84.4
COL1A3	949	3.4
COL1A5	0.33	2.2
BGN	2.08	2.9
DCN	18.4	0.7

Human primary astrocytes and human primary foreskin fibroblasts were grown for 2 days. RNA was extracted with TRIZOL and expression of collagen I, III and V, biglycan and decorin quantified by qPCR. * Expression values in % GAPDH.

5.2 Factors involved in proliferation, maturation and/or survival of oligodendrocytes

5.2.1 Altered expression of proliferation, maturation and/or survival factors for oligodendrocytes in MS lesions

Expression profiling of remyelinated MS lesions have not been done till date. We were interested to screen factors which promote some lesions to undergo remyelination and factors which might inhibit others form undergoing remyelination. To address this question LDA was used for determining the gene expression profile of four groups namely, remyelinating/ed lesions (n=4), active lesions (n=8), chronic inactive demyelinated lesions that failed to remyelinate (n=7) and control brain white matter (n=6). The expression levels of 32 factors regulating oligodendrocyte development and 18 receptor genes were quantified (Table 5.4a and 5.4b) and the gene regulation was calculated in terms of median value of the group with respect to GAPDH. The analysed oligodendrocyte regulating factors belonged to following groups: neurotrophins (NTs), fibroblast growth factors (FGFs), neuregulins, platelet-derived growth factors (PDGFs), insulin like growth factors (IGFs), interleukin 6 family (IL-6 family), transforming growth factor (TGF) and others. Of the tested 32 factors regulating oligodendrocyte development 12 (38%) factors were up-regulated in remyelinated lesions, 12 (38%) in chronic inactive lesions and 13 (40%) in active

demyelinating lesions. TGF-β, CTGF (connective tissue growth factor) and HGF (hepatocyte growth factor) were up-regulated in all the three lesion types. Four of the up-regulated genes namely NGFB, NT4/5, FGF5 and PDGFB, were up-regulated in only chronic inactive and active demyelinated MS lesions. None of the genes detected was down-regulated in any of the lesion types. 30-45% of genes in each group remained unchanged; they had a fold change between 0.51-1.99. Surprisingly 4 members of IL-6 family were up-regulated in the remyelinated lesions. Five of the analysed genes; namely FGF1, FGF2, PDGFA, PDGFB, TGF-β, CTGF and GRN were abundantly expressed in all the lesion types and in the control brain. FGF1 and PDGFA were the most abundant genes detected with more than 20% GAPDH expression value.

Of the tested 18 receptors for oligodendrocyte regulating factor, 8 (44%) genes were up-regulated in remyelinated and 4 (22%) in both chronic inactive and active demyelinated MS lesions. One (6%) gene was down-regulated in chronic inactive and 2 (11%) genes in active demyelinated lesions. 45-67% genes in each group remained unchanged. ERBB3, NTRK2, FGFR2 and IL6ST were abundantly expressed in all the lesion types and in the control brain, with an expression value greater than 10% GAPDH. These findings indicate that alteration of proliferation, maturation and/or survival factors for oligodendrocytes and their receptors occurs during the development of different stages of MS lesions.

Table 5.4a. Expression value and fold change values of 32 oligodendrocyte regulating factors.

	values in % GAPDH				fold change		
Genes	**CB**	**Re**	**De.in**	**De.act**	**Re:CB**	**De.in:CB**	**De.act: CB**
Neurotrophins							
ARTN	0.005	-nd-	-nd-	0.011	*	*	2.20
BDNF	0.003	-nd-	-nd-	0.004	*	*	1.33
GDNF	0.017	0.034	-nd-	0.019	2.00		1.12
NGFB	0.023	-nd-	0.186	0.07	*	8.09	3.04
NRTN	0.182	-nd-	-nd-	-nd-	*	*	*
NT4/5	0.005	-nd-	0.011	0.025	*	2.20	5.00
PSPN	-nd-	-nd-	-nd-	-nd-	*	*	*

Table 5.4a. Continued

	values in % GAPDH				fold change		
Genes	**CB**	**Re**	**De.in**	**De.act**	**Re:CB**	**De.in:CB**	**De.act: CB**
FGFs							
FGF1	40.945	76.346	26.551	35.728	1.86	0.65	0.87
FGF2	3.575	9.158	5.671	4.957	2.56	1.59	1.39
FGF5	0.0001	-nd-	0.005	0.002	*	50.00	20.00
FGF8	0.004	0.021	-nd-	0.011	5.25	*	2.75
FGF9	0.016	0.016	0.118	0.02	1.00	7.38	1.25
Neuregulins							
NRG1	0.011	0.007	0.027	0.007	0.64	2.45	0.64
NRG2	0.641	1.194	5.817	1.143	1.86	9.07	1.78
NRG4	0.562	0.851	0.437	0.378	1.51	0.78	0.67
PDGFs							
PDGFA	21.271	28.217	14.704	21.003	1.33	0.69	0.99
PDGFB	1.178	1.589	3.899	2.772	1.35	3.31	2.35
PDGFC	0.446	1.171	0.578	0.988	2.63	1.30	2.22
PDGFD	0.086	0.294	0.249	0.111	3.42	2.90	1.29
IL-6 family							
CNTF	0.167	0.467	0.119	0.264	2.80	0.71	1.58
CLCF1	0.022	0.051	-nd-	0.208	2.32	*	9.45
CTF1	0.161	0.464	0.455	0.224	2.88	2.83	1.39
IL-6	0.035	0.072	-nd-	0.097	2.06	*	2.77
LIF	-nd-	0.017	-nd-	0.146	*	*	*
IGFs							
IGF1	0.341	0.232	0.486	0.283	0.68	1.43	0.83
IGF2	0.225	0.372	0.385	0.579	1.65	1.71	2.57
Others							
CTGF	1.467	4.506	9.609	5.154	3.07	6.55	3.51
EPO	-nd-	-nd-	-nd-	-nd-	*	*	*
FIGF	0.214	0.289	0.165	0.323	1.35	0.77	1.51
GRN	1.026	1.294	1.186	1.676	1.26	1.16	1.63
HGF	0.12	0.459	0.314	1.247	3.83	2.62	10.39
TGF	5.213	11.559	11.489	14.57	2.22	2.20	2.79

Table 5.4b. Expression level and fold change value of 18 receptors for oligodendrocyte regulating factors.

	values in % GAPDH				fold change		
Genes	**CB**	**Re**	**De.in**	**De.act**	**Re:CB**	**De.in:CB**	**De.act: CB**
EGFR	1.551	2.814	1.982	1.196	1.81	1.28	0.77
ERBB3	33.819	48.079	9.239	13.988	1.42	0.27	0.41
ERBB4	2.908	4.667	4.536	2.196	1.60	1.56	0.76
NTRK1	0.029	0.088	0.284	0.078	3.03	9.79	2.69
NTRK2	15.913	39.239	22.653	19.537	2.47	1.42	1.23
NTRK3	2.458	4.042	9.164	3.649	1.64	3.73	1.48
FGFR1	3.037	5.365	6.055	7.203	1.77	1.99	2.37
FGFR2	11.713	18.302	10.593	14.032	1.56	0.90	1.20
FGFR3	3.583	11.570	6.692	0.695	3.23	1.87	0.19
FGFR4	0.051	0.127	0.118	0.089	2.49	2.32	1.75
PDGFRA	4.777	10.874	3.620	3.104	2.28	0.76	0.65
PDGFRB	1.426	3.213	2.720	2.431	2.25	1.91	1.71
IGFR1	3.440	7.262	7.789	6.406	2.11	2.26	1.86
IGFR2	0.884	1.456	0.999	1.976	1.65	1.13	2.24
IL6ST	23.770	57.686	24.335	27.040	2.43	1.02	1.14
LIFR	0.003	-nd-	-nd-	0.002	*	*	0.85
CNTFR	2.918	2.646	4.538	2.529	0.91	1.55	0.87
RET	0.011	-nd-	-nd-	0.023	*	*	2.03

CB: control brain, **De.in:** demyelinated inactive lesion, **De.act:** demyelinated active lesion, **Re:** remyelinated lesion, **De.in:CB-** demyelinated inactive *vs* control brain, **De.act:CB-** demyelinated active *vs* control brain, **Re:CB-**remyelinated *vs* control brain, **nd:** not detectable due to low expression level. In total 6 control white matter from 4 subjects, 7 demyelinated inactive lesions from 5 subjects, 8 demyelinated active lesions from 7 subjects and 4 remyelinated lesions from 3 subjects were used for qPCR analysis. ***** Calculation not possible because the values are under detection limit. The values below 0.01% GAPDH are not reliable.

5.2.2 FGF9 and its receptor expression in MS lesions

Of the 32 proliferation, maturation and/or survival factors analysed, 4 of them; namely FGF9 (fibroblast growth factor 9), NRG1 (neuregulin1), NRG2 and PDGFB were up-regulated (Table 5.4a) in chronic inactive demyelinated lesions which failed to remyelinate. PDGFB is a proliferation and survival factor for oligodendrocytes

[Dell'Albani *et al.* 1998; Baron *et al.* 2002]. Its presence in the demyelinated inactive lesions states that these lesions have factors promoting oligodendrocyte proliferation, but their effect might be masked by the presence of certain inhibitory factors in the lesions. NRG1 has been shown to influence peripheral nervous system myelination, but its role in CNS myelination is unclear [Brinkmann *et al.* 2008]. Hence we tried to understand the role of FGF9 and NRG2 in myelination/remyelination. To further confirm the up-regulation of FGF9 and NRG2 we analysed their expression in 6 additional demyelinated chronic inactive lesions and 3 control white matter by qPCR. Surprisingly we could not reproduce NRG2 up-regulation after increasing the sample size for chronic inactive demyelinated lesions and control brain. Hence, we discontinued NRG2 for the follow up studies. In contrast, FGF9 was found to be up-regulated even after the increase of sample size. Closer look at individual chronic inactive demyelinated lesions revealed that some of the lesions had FGF9 expression levels similar to that of the control brain and only a subset of the lesions had higher expression levels (Figure 5.7). FGF9 levels in the remyelinated and active demyelinated lesions were similar to that of the control brain (Table 5.4a). Thereby suggesting that FGF9 could be a probable inhibitory factor for a subset of demyelinated lesions to recover from the demyelinating insult.

The two main receptors for FGF9 are FGFR2 and FGFR3. These receptors were expressed in all the lesion type as examined by LDA. In the demyelinated chronic inactive lesions where FGF9 expression was up-regulated, the expression of FGFR2 is similar to control brain and FGFR3 expression was up-regulated approximately by 2 fold (Table 5.4b). FGFR2 and FGFR3 can also act as a receptor for other FGFs.

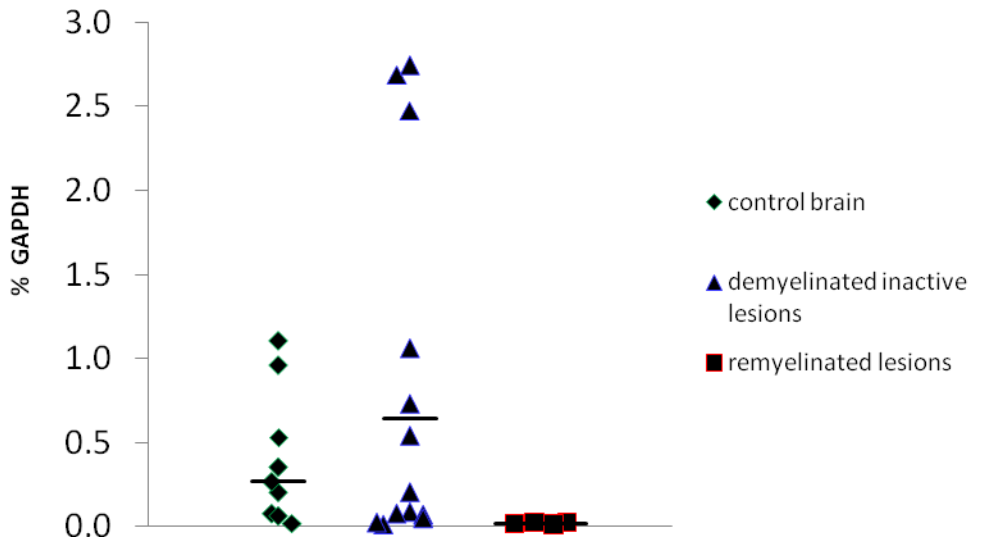


Fig. 5.7 FGF9 up-regulation in a subset of demyelinated inactive lesions. The demyelinated inactive lesions and remyelinated lesions were macro dissected and the RNA was extracted with TRIZOL. FGF9 expression in 9 control white matter (from 8 subjects), 13 demyelinated inactive lesions (from 9 subjects) and 4 remyelinated lesions (from 3 subjects) was determined by qPCR. The values represented here are in relation to housekeeping gene GAPDH and were obtained from individual qPCR. Each data point is an individual lesion/control white matter.

To understand which cells in the lesions could produce FGF9, we performed immunohistochemistry of the demyelinated chronic inactive lesions and control white matter with the antibody directed against FGF9. The results of the immunostaining were not convincing as we sometimes observed nuclear staining (data not shown). Similar staining pattern was observed with the FGF9 receptors (FGFR2 and FGFR3). These stainings were discussed with neuropathologist, Prof. Lassmann for its specificity and later regarded as unspecific.

FGF9 is known to be produced largely by neurons [Kanda *et al.* 1999; Todo *et al.* 1998; Nakamura *et al.* 1997]. Hence, the neuronal presence in the demyelinated chronic inactive lesions and control white matter was analysed by qPCR for NFL (neurofilament protein, light polypeptide) and NFM (neurofilament protein, medium

polypeptide). All the three demyelinated chronic inactive lesions with higher expression of FGF9 showed higher expression of NFM and NFL (Figure 5.8A and 5.8B). Thereby, stating that in these lesions neuronal contribution for FGF9 exists. This also confirms yet another source of FGF9 in the lesions which have moderate expression of FGF9 but low expression of NFL and NFM. In such lesion astrocytes, fibroblasts or oligodendrocytes might contribute for higher FGF9 levels. To this end primary human fibroblast and astrocytes were tested for their *in vitro* production of FGF9. *In vitro* human primary fibroblast produce higher amounts of FGF9 compared to human primary astrocytes, as shown by qPCR. Stimulation with TNFα, IFNγ, TNFα+IFNγ, IL1 or IFNβ of fibroblasts did not significantly alter the expression level of FGF9 (data not shown). The level of expression by astrocytes was near the detection limit and not clearly modulated by the applied cytokines.

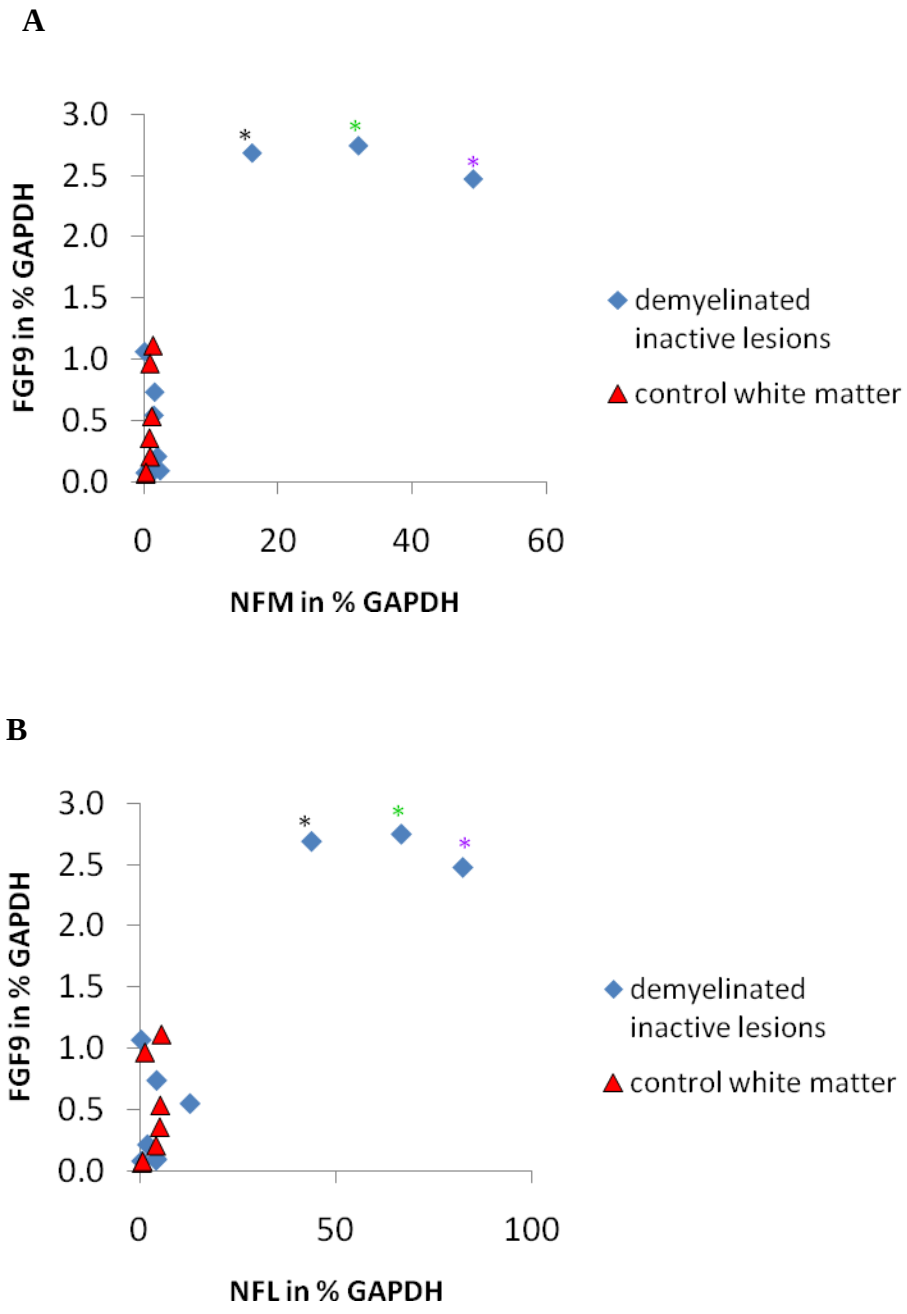


Fig. 5.8 Neuronal contribution to high FGF9 levels in a subset of FGF9 high lesions. The demyelinated inactive lesions were macro dissected as mentioned before. **A)** NFL and **B)** NFM expression in 9 control white matter (from 8 subjects) and 10 demyelinated inactive lesions (from 9 subjects) was determined by qPCR. The values represented here are in relation to housekeeping gene GAPDH and each data point is an individual lesion/control white matter. Lesions marked with * are same in figure A and B

5.2.3 FGF9 blocks *in vitro* myelination

To address the question whether FGF9 affects myelination, an *in vitro* system of myelination was applied in collaboration with Prof. C. Linington. The below mentioned experiments were done in his lab.

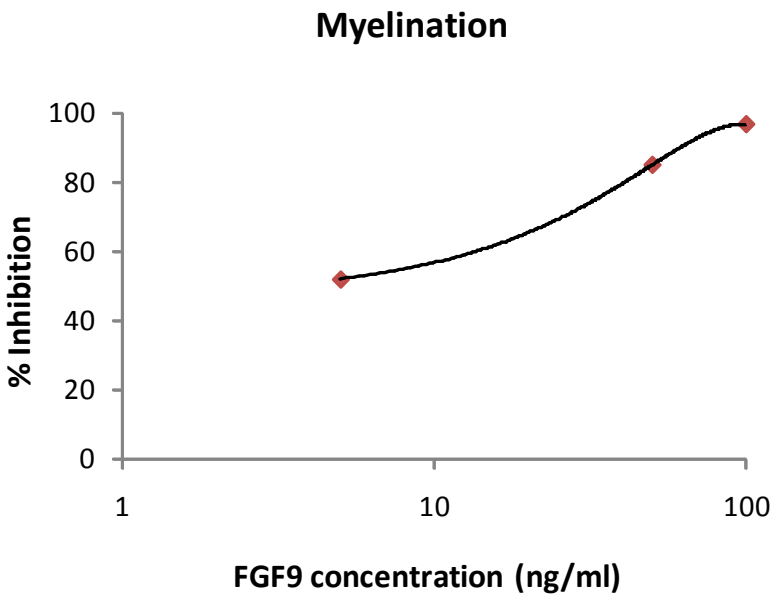
FGF9 inhibits myelination and maturation of oligodendrocytes

In order to deduce the effect of FGF9, the degree of myelination in the cultures was analysed by calculating the number of myelin positive axons (double positive for SMI-31 and MOG) among total number of axons (single positive for SMI-31). A dose dependent decrease in the myelination was observed. Myelination in the culture was almost completely abolished at 100 ng/ml of FGF9, compared to the untreated cultures (Figure 5.9A). Next we tested if this decrease in myelination was because of the effect on oligodendrocyte or on the axons. To this end the number of MOG^+ (mature) oligodendrocytes in the myelinating cultures was analysed. At the highest concentration (100 ng/ml) of FGF9 used ~60% decrease in the number of MOG^+ cells per field of vision was observed compared to the untreated cultures. FGF9 showed effect on oligodendrocytes, particularly on the maturation of oligodendrocytes, thereby inhibiting their myelin production (Figure 5.9B).

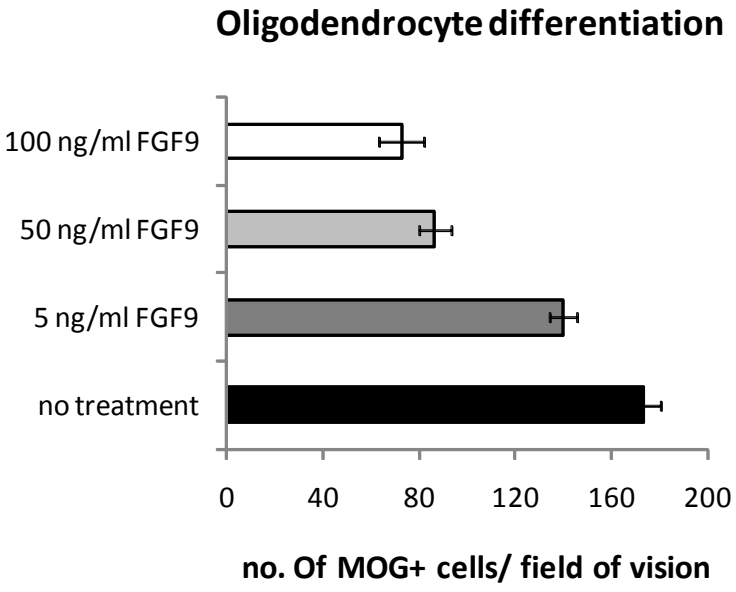
No effect of FGF9 on axonal density in the cultures

In our experiments FGF9 did not influence axonal density in the myelinating cultures at any of the above mentioned FGF9 concentration used. The axonal density which was calculated as percentage of $SMI\text{-}31^+$ axons in a particular field remained unaltered through the experiment (Figure 5.9C).

A


Myelination
100
80
60
40
20
0
% Inhibition
1
10
100
FGF9 concentration (ng/ml)

B


Oligodendrocyte differentiation
100 ng/ml FGF9
50 ng/ml FGF9
5 ng/ml FGF9
no treatment
0
40
80
120
160
200
no. Of MOG+ cells/ field of vision

C

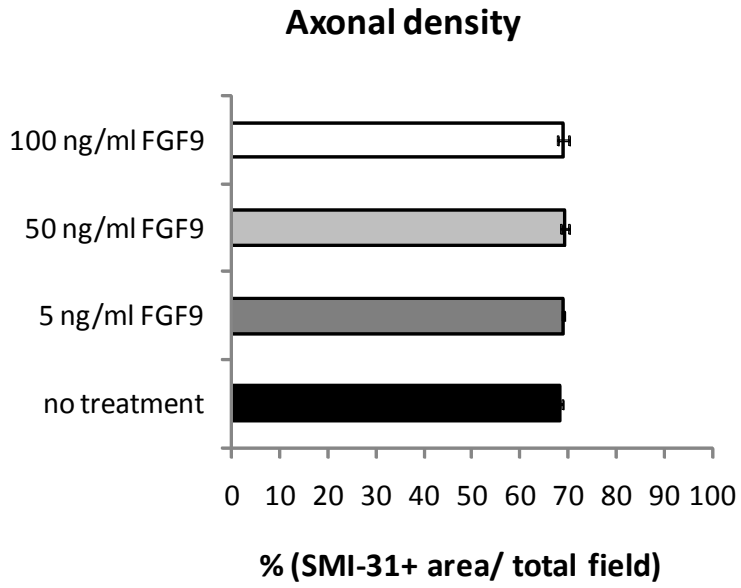


Fig. 5.9 FGF9 selectively blocks myelination *in vitro*. The mixed cultures from E15.5 rat were grown on astrocytic monolayer (derived from rat) for 12 days in presence of insulin, and after 12 days the insulin was omitted from the culture media to promote differentiation of oligodendrocytes. FGF9 was applied to the cultures on day 18 for 8 days, half of the media was changed every third day with fresh media supplemented with FGF9. On day 26 the cultures were fixed and immunostained and analysed by fluorescent microscopy. **A)** FGF9 inhibits in vitro myelination in a dose dependent manner. Myelination is almost completely inhibited at 100 ng/ml of FGF9. **B)** FGF9 reduces the number of MOG^+ cells in the myelinating culture. **C)** FGF9 does not alter axonal density in the myelinating cultures. Axonal density is calculated as the percentage of SMI-31^+ area in the total field. Error bars indicate the SEM of three individual experiments.

6 Discussion

Focal demyelination with limited remyelination is one of the hallmarks of MS. Why most lesions fail to remyelinate is unclear, as the CNS contains an endogenous repair mechanism that normally acts to ensure rapid and essentially complete remyelination. The aim of this thesis was to get insight into possible mechanisms explaining the frequent failure of remyelination in MS lesions. This failure might be due to alterations of the extracellular matrix and/or alterations of factors mediating growth, survival and differentiation of oligodendrocytes.

Alterations of the ECM in MS lesions

In this study, we have quantified alterations of ECM components in MS lesions. The most strongly altered ECM components were the chains forming fibrillar collagens, namely *COL1A1, COL3A1, COL5A1, COL5A2,* and the SLRPs biglycan and decorin. Decorin and biglycan can interact with and decorate fibrillar collagens [Danielson *et al.* 1997]. The up-regulation of some of these fibrillar collagen chains and biglycan is in parallel with previous proteomic results of demyelinated MS lesions (supplementary table1 in [Han *et al.* 2008]). Immunostaining showed that these fibrillar collagens and SLRPs were localized largely in the perivascular space associated with immune cells and not in the parenchyma. This indicates that these ECM components might not directly affect the differentiation of oligodendrocytes, but rather modulate the immune response in the CNS.

Collagens maintain the structure of various tissues and also have various other functions. The most important fibrillar collagens are type I, II, III, V, XI, XXIV and XXVII form collagen fibrils and provide the stiff, resilient characteristics of many tissues like tendon, ligament, cartilage, skin, cornea etc [Okada *et al.* 2007; Van der Rest *et al.* 1991]. Collagens have a characteristic triple helical conformation and the triple helix is formed by the homotrimeric or heterotrimeric interaction of three individual collagen polypeptide chains [Van der Rest *et al.* 1991].

These fibrillar collagens are the molecular components of the perivascular fibrosis in MS lesions as elaborated in this thesis. The presence of fibrous structures around blood vessels in chronic CNS inflammation had been noted by neuropathologists [Speilmeyer 1922; Levine 1970].Modern imaging techniques which combine three MRI sequence modalities, suggest that the perivascular space regulates inflammation in the brain of MS patients [Wuerfel *et al.* 2008]. These perivascular deposits are rather a feature of chronicity, since such a fibrosis (based on BGN and DCN staining) was not mirrored in the analysed EAE lesions. This view is also supported by the finding in this study that the induction of fibrillar collagens is more prominent in chronic inactive lesions than in active lesions. Perivascular fibrosis is also observed in liver cirrhosis, hypertensive heart failure and atherosclerosis [Lopez *et al.* 2006], where collagens I and III form the perivascular deposits, which is similar to that observed in chronic MS lesions. The up-regulation of some of these fibrillar collagen chains and of biglycan observed by us is in harmony with previous proteomic study on demyelinated MS lesions (Supplementary Table 1 in [Han *et al.* 2008]).

The ratio of synthesis and degradation determines the final protein level. Therefore we analysed in parallel to the ECM components also the potential degrading enzymes. The most important collagenases which degrade fibrillar collagens are MMP1, MMP8 and MMP13 [Yong *et al.* 2001], all of which were not detected in our MS lesions.

In addition to the MMPs above mentioned, we observed a strong induction of MMP2, -9, -11, -14 and -19 in both active and chronic inactive lesions. MMP2 and -9 which were strongly induced in active lesions, are known to participate in the BBB leakage [Anthony *et al.* 1997; Cuzner *et al.* 1996; Maeda *et al.* 1996]. A possible involvement of MMP19 in the pathogenesis of MS by remodelling the ECM of the CNS has been shown recently by *Van Horssen et al*. In active MS lesions strong MMP19 immunoreactivity was observed in parenchymal and perivascular macrophages. In the normal brain white matter MMP19 is constitutively expressed by microglia throughout the brain parenchyma, thereby suggesting a physiological role

for MMP19 in the CNS [Van Horssen *et al.* 2006]. We also observed strong induction of MMP11, -17, -24, and -28 in the chronic inactive lesions, the involvement of these MMPs in MS has not been described yet.

The exact source of up regulated ECM components is not very clear. In our study we saw induction of TGFβ1 in the chronic inactive and active MS lesions, and this is in harmony with previously published data [Baranzini *et al.* 2000]. TGFβ1 stimulates the production of ECM genes/proteins, thereby suggesting that increase in TGFβ1 levels in MS patients might induce the expression of ECM components involved in the formation of perivascular fibrosis.

In chronic lesions, the fibrillar collagens were located in the PVS between endothelial cells and astrocytic glia limitans. Potential candidates for the production of fibrillar collagens are fibroblasts and astrocytes, since at least *in vitro* astrocytes were found to produce fibrillar collagens [Heck *et al.* 2003]. *In vitro* we compared the ability of fibroblasts and astrocytes to produce fibrillar collagens. The fibroblasts were found to be much stronger producer of fibrillar collagens than astrocytes and in particular, COL1A1 and COL3A1, which we found to be induced in MS lesions.

It has been shown that endothelial cells can produce decorin [Nelimarkka *et al.* 2001], hence it might be possible that decorin in the PVS is being produced by the endothelial cells of the vascular basement membrane. We found the up-regulated SLRP, biglycan in the perivascular space and also in infiltrating cells in the brain parenchyma of active lesions. Macrophages, known producer of biglycan [Schaefer *et al.* 2005] might contribute to this.

Since immunostaining results indicated a close interaction between the components of the perivascular fibrosis and the infiltrating immune cells, we went on to perform *in vitro* experiments to learn about possible consequences of this interaction. Thereby we found that fibrillar collagens reduced the monocytic production of CCL2. CCL2 is a potent attractant for monocytes, dendritic cells, memory T cells, and basophils and is considered to be a major inflammatory chemokine in autoimmune CNS inflammation [Mahad *et al.* 2003; Huang *et al.* 2001]. Since collagen I and III inhibited the production of CCL2 by monocytes, this

mechanism might contribute to lesion confinement. This interpretation is supported by transfer EAE experiments [Levine 1970], suggesting that reticulin fibers in the perivascular space might be linked to vascular blockade and thereby limiting infiltration and lesion development. Additional effects of the altered ECM on immune cells are expected. For example, biglycan has been reported to be a ligand for TLR2 and TLR4 in the mouse [Schaefer *et al.* 2005]. In our study, however, with human monocytes biglycan did not stimulate cytokine production (data not shown). It is unclear whether the discrepancy of our results and the ones published by *Schaefer et al* are based on species differences or on the exact source of biglycan. Decorin absorbs TGFβ and neutralizes its activity thereby acting as a TGFβ scavenger [Iozzo 1999]. Since TGFβ is a major modulator of autoimmune CNS inflammation, the herein described induction of decorin in MS might therefore contribute to lesion dynamics [Luo *et al.* 2007].

In summary, we have quantified changes in the ECM of MS lesions, identified the components of perivascular fibrosis in chronic demyelinated MS lesions, and shown that fibrillar collagen I and III inhibit the production of CCL2 by human monocytes. There by implying that collagen, I and III in the PVS might function as both, a physical and biological barrier limiting immune cell recruitment and lesion progression.

Myelination/remyelination might further be influenced by ECM components deposited in the parenchyma. Thrombospondin-1 and -3 might be potential candidates. The role of thrombospondins in MS is yet to be established.

Alterations of mediators regulating oligodendrocyte biology in MS lesions

ECM provides a substratum upon which cells migrate, proliferate and differentiate. Apart from the ECM, growth factors also provide necessary signals for cell migration, proliferation, differentiation and survival. In our study we quantified different factors known to play a role in the proliferation, differentiation and/or

survival of oligodendrocytes, the major myelinating cells of the CNS. The composition of these factors might influence the success or failure of remyelination.

In our experiments, the key factors for differentiation (IGF1, IGF2 and CNTF) and proliferation of oligodendrocytes (FGF2 and PDGFAA) were present in the demyelinated inactive lesions, which failed to remyelinate. Among all the regulated factors, we were more interested in factors, which were up-regulated in demyelinated chronic inactive lesions which failed to remyelinate, because such factors might inhibit remyelination. Most striking was the up-regulation of FGF9 in a subset of chronically demyelinated lesions, but in none of the remyelinated shadow plaques. An alteration of FGF9 in MS lesions has not been described before.

Neurons can synthesize FGF9 [Garces *et al.* 2000; Kanda *et al.* 1999; Kanda *et al.* 2000]. Hence, one reason for the high expression of FGF9 in subset of lesions might be the presence of neuronal cell bodies in these particular lesions. Although neuronal cell bodies are largely found in the gray matter, they can also occasionally be seen in the normal white matter and in white matter MS lesions [Chang *et al.* 2008]. Indeed, qPCR showed that those demyelinated lesions with a high FGF9 content also had a high level of the neuron-specific genes NFL (neurofilament protein, light polypeptide) and NFM (neurofilament protein, medium polypeptide). To further elucidate whether this is a patient specific or lesion specific phenomenon, we analysed additional lesions form one patient expressing higher FGF9 levels. In these additional lesions, the expression of FGF9 was similar to control brain, further confirming that the increase in FGF9 levels in a subset of demyelinated lesions is a lesion specific phenomenon.

To learn about potential functional implications of the up-regulated FGF9 in demyelinated MS lesions, we analysed the effect of exogenous FGF9 on *in vitro* myelination. FGF9 was added to embryonal spinal cord cultures grown on astrocytic monolayer. FGF9 inhibited *in vitro* myelination in a dose dependent manner. This decrease in myelination could be due to two reasons: a) decrease in axonal the density b) decrease in the number of MOG^+ oligodendrocytes (myelinating oligodendrocytes). In our experiments, FGF9 did not modulate axonal density, but

triggered a decrease in the number of MOG^+ cells. This shows that FGF9 inhibited oligodendrocyte maturation. This effect of FGF9 on oligodendrocytes can be a direct effect or an indirect effect. The myelinating cultures are grown on a monolayer of astrocytes, so it might be possible that FGF9 directly effects astrocytes, producing secondary factors, possibly inhibiting oligodendrocytes maturation. The observed inhibition of *in vitro* myelination is in harmony with previous study [Cohen *et al.* 2000], where FGF9 was shown to alter myelin protein levels during oligodendrocyte differentiation. However, another study reported that FGF9 increased the process growth in differentiated oligodendrocytes [Fortin *et al.* 2005]. Future studies analyzing effects of FGF9 on pure oligodendrocyte and astrocyte cultures will provide further insight into the mechanism of FGF9 mediated inhibition of myelination. The presence of FGF9 receptors-FGFR2 and FGFR3 on both oligodendrocytes and astrocytes [Fortin *et al.* 2005] make both a direct and an indirect effect of FGF9 possible. Both FGFR2 and FGFR3 were detected by qPCR in MS lesions and in normal brain. In the remyelinated lesions the receptors were ~2 fold more expressed compared to the demyelinated chronic inactive lesions. This increase in transcript levels of the receptors might be contributed by the newly recruited OPCs which are involved in the remyelination of the demyelinated lesion.

Till now only two direct inhibitors of oligodendrocytes are known in relation to remyelination failure in MS. These are hyaluronan, a proteoglycan and LINGO1. Both hyaluronan and LINGO1 negatively regulate oligodendrocyte maturation and hence myelination [Back *et al.* 2005; Mi *et al.* 2005].

In summary, this thesis established expression profiles of de- and remyelinated MS lesions. This showed the failure of remyelination might not be attributed to the absence of one single factor, since factors essential for oligodendrocyte maturation, such as IGF1, IGF2, CNTF, FGF2 and PDGFAA were still found in demyelinated chronic inactive lesions, which failed to remyelinate. The transcript profiling identified an up-regulation of FGF9 in a subset of inactive demyelinated lesions. Together, this suggests that up-regulation of FGF9 is one of the mechanism contributing to the failure of remyelination in MS.

7 References

Agrawal S, Anderson P, Durbeej M, van RN, Ivars F, Opdenakker G, Sorokin LM. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. J Exp Med 2006; 203: 1007-19.

Aloisi F. Growth factors. Neurol Sci 2003; 24: S291-S294.

Aloisi F, Borsellino G, Samoggia P, Testa U, Chelucci C, Russo G, Peschle C, Levi G. Astrocyte cultures from human embryonic brain: characterization and modulation of surface molecules by inflammatory cytokines. J Neurosci Res 1992; 32: 494-506.

Anthony DC, Ferguson B, Matyzak MK, Miller KM, Esiri MM, Perry VH. Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke. Neuropathol Appl Neurobiol 1997; 23: 406-15.

Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schroder R, Deckert M, Schmidt S, Ravid R, Rajewsky K. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med 2000; 192: 393-404.

Back SA, Tuohy TM, Chen H, Wallingford N, Craig A, Struve J, Luo NL, Banine F, Liu Y, Chang A, Trapp BD, Bebo BF, Jr., Rao MS, Sherman LS. Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. Nat Med 2005; 11: 966-72.

Bandtlow CE, Zimmermann DR. Proteoglycans in the developing brain: new conceptual insights for old proteins. Physiol Rev 2000; 80: 1267-90.

Baranzini SE, Elfstrom C, Chang SY, Butunoi C, Murray R, Higuchi R, Oksenberg JR. Transcriptional analysis of multiple sclerosis brain lesions reveals a complex pattern of cytokine expression. J Immunol 2000; 165: 6576-82.

Baron W, Shattil SJ, Ffrench-Constant C. The oligodendrocyte precursor mitogen PDGF stimulates proliferation by activation of alpha(v)beta3 integrins. EMBO J 2002; 21: 1957-66.

Barres BA, Raff MC, Gaese F, Bartke I, Dechant G, Barde YA. A crucial role for neurotrophin-3 in oligodendrocyte development. Nature 1994; 367: 371-5.

Bertollini L, Ciotti MT, Cherubini E, Cattaneo A. Neurotrophin-3 promotes the survival of oligodendrocyte precursors in embryonic hippocampal cultures under chemically defined conditions. Brain Res 1997; 746: 19-24.

Bignami A, Hosley M, Dahl D. Hyaluronic acid and hyaluronic acid-binding proteins in brain extracellular matrix. Anat Embryol (Berl) 1993; 188: 419-33.

Bottenstein JE, Sato GH. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc Natl Acad Sci U S A 1979; 76: 514-7.

Breij EC, Brink BP, Veerhuis R, van den Berg C, Vloet R, Yan R, Dijkstra CD, van d, V, Bo L. Homogeneity of active demyelinating lesions in established multiple sclerosis. Ann Neurol 2008; 63: 16-25.

Brinkmann BG, Agarwal A, Sereda MW, Garratt AN, Muller T, Wende H, Stassart RM, Nawaz S, Humml C, Velanac V, Radyushkin K, Goebbels S, Fischer TM, Franklin RJ, Lai C, Ehrenreich H, Birchmeier C, Schwab MH, Nave KA. Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. Neuron 2008; 59: 581-95.

Butt AM, Dinsdale J. Fibroblast growth factor 2 induces loss of adult oligodendrocytes and myelin in vivo. Exp Neurol 2005; 192: 125-33.

Butzkueven H, Zhang JG, Soilu-Hanninen M, Hochrein H, Chionh F, Shipham KA, Emery B, Turnley AM, Petratos S, Ernst M, Bartlett PF, Kilpatrick TJ. LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival. Nat Med 2002; 8: 613-9.

Cannella B, Pitt D, Capello E, Raine CS. Insulin-like growth factor-1 fails to enhance central nervous system myelin repair during autoimmune demyelination. Am J Pathol 2000; 157: 933-43.

Chang A, Smith MC, Yin X, Fox RJ, Staugaitis SM, Trapp BD. Neurogenesis in the chronic lesions of multiple sclerosis. Brain 2008; 131: 2366-75.

Chang A, Tourtellotte WW, Rudick R, Trapp BD. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. N Engl J Med 2002; 346: 165-73.

Chari DM. Remyelination in multiple sclerosis. Int Rev Neurobiol 2007; 79: 589-620.

Charles P, Hernandez MP, Stankoff B, Aigrot MS, Colin C, Rougon G, Zalc B, Lubetzki C. Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule. Proc Natl Acad Sci U S A 2000; 97: 7585-90.

Charles P, Reynolds R, Seilhean D, Rougon G, Aigrot MS, Niezgoda A, Zalc B, Lubetzki C. Re-expression of PSA-NCAM by demyelinated axons: an inhibitor of remyelination in multiple sclerosis? Brain 2002; 125: 1972-9.

Cohen RI, Chandross KJ. Fibroblast growth factor-9 modulates the expression of myelin related proteins and multiple fibroblast growth factor receptors in developing oligodendrocytes. J Neurosci Res 2000; 61: 273-87.

Cuzner ML, Gveric D, Strand C, Loughlin AJ, Paemen L, Opdenakker G, Newcombe J. The expression of tissue-type plasminogen activator, matrix metalloproteases and endogenous inhibitors in the central nervous system in multiple sclerosis: comparison of stages in lesion evolution. J Neuropathol Exp Neurol 1996; 55: 1194-204.

Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J Cell Biol 1997; 136: 729-43.

Dawson MR, Polito A, Levine JM, Reynolds R. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol Cell Neurosci 2003; 24: 476-88.

Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. J Biol Chem 2002; 277: 43707-16.

Deepa SS, Yamada S, Zako M, Goldberger O, Sugahara K. Chondroitin sulfate chains on syndecan-1 and syndecan-4 from normal murine mammary gland epithelial cells are structurally and functionally distinct and cooperate with heparan sulfate chains to bind growth factors. A novel function to control binding of midkine, pleiotrophin, and basic fibroblast growth factor. J Biol Chem 2004; 279: 37368-76.

Dell'Albani P, Kahn MA, Cole R, Condorelli DF, Giuffrida-Stella AM, de VJ. Oligodendroglial survival factors, PDGF-AA and CNTF, activate similar JAK/STAT signaling pathways. J Neurosci Res 1998; 54: 191-205.

Dr.W.Speilmeyer. Histopathologie des nervous system. 1922; berlin, Verlag von Julius Springer 1922.

Esiri MM, Morris CS. Immunocytochemical study of macrophages and microglial cells and extracellular matrix components in human CNS disease. 2. Non-neoplastic diseases. J Neurol Sci 1991; 101: 59-72.

Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. Brain 1997; 120: 393-9.

Ffrench-Constant C, Raff MC. Proliferating bipotential glial progenitor cells in adult rat optic nerve. Nature 1986; 319: 499-502.

Fortin D, Rom E, Sun H, Yayon A, Bansal R. Distinct fibroblast growth factor (FGF)/FGF receptor signaling pairs initiate diverse cellular responses in the oligodendrocyte lineage. J Neurosci 2005; 25: 7470-9.

Franklin RJ. Why does remyelination fail in multiple sclerosis? Nat Rev Neurosci 2002; 3: 705-14.

Franklin RJ, Ffrench-Constant C. Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci 2008a; 9: 839-55.

Franklin RJ, Kotter MR. The biology of CNS remyelination: the key to therapeutic advances. J Neurol 2008b; 255 Suppl 1:19-25.

Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev 2004; 15: 197-204.

Frohman EM, Racke MK, Raine CS. Multiple sclerosis--the plaque and its pathogenesis. N Engl J Med 2006; 354: 942-55.

Frost EE, Nielsen JA, Le TQ, Armstrong RC. PDGF and FGF2 regulate oligodendrocyte progenitor responses to demyelination. J Neurobiol 2003; 54: 457-72.

Garces A, Nishimune H, Philippe JM, Pettmann B, deLapeyriere O. FGF9: a motoneuron survival factor expressed by medial thoracic and sacral motoneurons. J Neurosci Res 2000; 60: 1-9.

Gard AL, Burrell MR, Pfeiffer SE, Rudge JS, Williams WC. Astroglial control of oligodendrocyte survival mediated by PDGF and leukemia inhibitory factor-like protein. Development 1995; 121: 2187-97.

Genoud S, Maricic I, Kumar V, Gage FH. Targeted expression of IGF-1 in the central nervous system fails to protect mice from experimental autoimmune encephalomyelitis. J Neuroimmunol 2005; 168: 40-5.

Goldschmidt T, Antel J, Konig FB, Bruck W, Kuhlmann T. Remyelination capacity of the MS brain decreases with disease chronicity. Neurology 2009; 72: 1914-21.

Gutowski NJ, Newcombe J, Cuzner ML. Tenascin-R and C in multiple sclerosis lesions: relevance to extracellular matrix remodelling. Neuropathol Appl Neurobiol 1999; 25: 207-14.

Gveric D, Cuzner ML, Newcombe J. Insulin-like growth factors and binding proteins in multiple sclerosis plaques. Neuropathol Appl Neurobiol 1999; 25: 215-25.

Han MH, Hwang SI, Roy DB, Lundgren DH, Price JV, Ousman SS, Fernald GH, Gerlitz B, Robinson WH, Baranzini SE, Grinnell BW, Raine CS, Sobel RA, Han DK, Steinman L. Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. Nature 2008; 451: 1076-81.

Heck N, Garwood J, Schutte K, Fawcett J, Faissner A. Astrocytes in culture express fibrillar collagen. Glia 2003; 41: 382-92.

Heinrich M, Gorath M, Richter-Landsberg C. Neurotrophin-3 (NT-3) modulates early differentiation of oligodendrocytes in rat brain cortical cultures. Glia 1999; 28: 244-55.

Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. J Exp Med 2001; 193: 713-26.

Invernizzi P, Gershwin ME. The genetics of human autoimmune disease. J Autoimmun 2009.

Iozzo RV. The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. Crit Rev Biochem Mol Biol 1997; 32: 141-74.

Iozzo RV. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. J Biol Chem 1999; 274: 18843-6.

Irvine KA, Blakemore WF. Remyelination protects axons from demyelination-associated axon degeneration. Brain 2008; 131: 1464-77.

John GR, Shankar SL, Shafit-Zagardo B, Massimi A, Lee SC, Raine CS, Brosnan CF. Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. Nat Med 2002; 8: 1115-21.

Junker A, Ivanidze J, Malotka J, Eiglmeier I, Lassmann H, Wekerle H, Meinl E, Hohlfeld R, Dornmair K. Multiple sclerosis: T-cell receptor expression in distinct brain regions. Brain 2007; 130: 2789-99.

Kadler KE, Baldock C, Bella J, Boot-Handford RP. Collagens at a glance. J Cell Sci 2007; 120: 1955-8.

Kanda T, Iwasaki T, Nakamura S, Kurokawa T, Ikeda K, Mizusawa H. Self-secretion of fibroblast growth factor-9 supports basal forebrain cholinergic neurons in an autocrine/paracrine manner. Brain Res 2000; 876: 22-30.

Kanda T, Iwasaki T, Nakamura S, Ueki A, Kurokawa T, Ikeda K, Mizusawa H. FGF-9 is an autocrine/paracrine neurotrophic substance for spinal motoneurons. Int J Dev Neurosci 1999; 17: 191-200.

Kantor DB, Chivatakarn O, Peer KL, Oster SF, Inatani M, Hansen MJ, Flanagan JG, Yamaguchi Y, Sretavan DW, Giger RJ, Kolodkin AL. Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. Neuron 2004; 44: 961-75.

Kawashima H, Atarashi K, Hirose M, Hirose J, Yamada S, Sugahara K, Miyasaka M. Oversulfated chondroitin/dermatan sulfates containing GlcAbeta1/IdoAalpha1-3GalNAc(4,6-O-disulfate) interact with L- and P-selectin and chemokines. J Biol Chem 2002; 277: 12921-30.

Kerschensteiner M, Stadelmann C, Dechant G, Wekerle H, Hohlfeld R. Neurotrophic cross-talk between the nervous and immune systems: implications for neurological diseases. Ann Neurol 2003; 53: 292-304.

Kieseier BC, Hartung HP. Multiple paradigm shifts in multiple sclerosis. Curr Opin Neurol 2003; 16: 247-52.

Kuhlmann T, Miron V, Cuo Q, Wegner C, Antel J, Bruck W. Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. Brain 2008; 131: 1749-58.

Lassmann H. Comparative neuropathology of chronic experimental allergic encephalomyelitis and multiple sclerosis. Schriftenr Neurol 1983; 25: 1-135.

Lassmann H. Multiple sclerosis pathology: evolution of pathogenetic concepts. Brain Pathol 2005; 15: 217-22.

Lassmann H, Bruck W, Lucchinetti C. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. Trends Mol Med 2001; 7: 115-21.

Levine JM, Reynolds R, Fawcett JW. The oligodendrocyte precursor cell in health and disease. Trends Neurosci 2001; 24: 39-47.

Levine S. Allergic encepahlomyelitis: cellular transformation and vascular blockade. J Neuropathol Exp Neurol 1970; 29: 6-20.

Linker RA, Maurer M, Gaupp S, Martini R, Holtmann B, Giess R, Rieckmann P, Lassmann H, Toyka KV, Sendtner M, Gold R. CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. Nat Med 2002; 8: 620-4.

Liu X, Yao DL, Webster H. Insulin-like growth factor I treatment reduces clinical deficits and lesion severity in acute demyelinating experimental autoimmune encephalomyelitis. Mult Scler 1995; 1: 2-9.

Lopez SB, Ravassa AS, Arias GT, Gonzalez MA, Querejeta R, Diez MJ. [Altered fibrillar collagen metabolism in hypertensive heart failure. Current understanding and future prospects]. Rev Esp Cardiol 2006; 59: 1047-57.

Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. Brain 1999; 122: 2279-95.

Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol 2000; 47: 707-17.

Luo J, Ho PP, Buckwalter MS, Hsu T, Lee LY, Zhang H, Kim DK, Kim SJ, Gambhir SS, Steinman L, Wyss-Coray T. Glia-dependent TGF-beta signaling, acting independently of the TH17 pathway, is critical for initiation of murine autoimmune encephalomyelitis. J Clin Invest 2007; 117: 3306-15.

Maeda A, Sobel RA. Matrix metalloproteinases in the normal human central nervous system, microglial nodules, and multiple sclerosis lesions. J Neuropathol Exp Neurol 1996; 55: 300-9.

Mahad DJ, Ransohoff RM. The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Semin Immunol 2003; 15: 23-32.

Meinl E, Hart BA, Bontrop RE, Hoch RM, Iglesias A, de Waal MR, Fickenscher H, Muller-Fleckenstein I, Fleckenstein B, Wekerle H, Hohlfeld R, Jonker M. Activation of a myelin basic protein-specific human T cell clone by antigen-presenting cells from rhesus monkeys. Int Immunol 1995; 7: 1489-95.

Messersmith DJ, Murtie JC, Le TQ, Frost EE, Armstrong RC. Fibroblast growth factor 2 (FGF2) and FGF receptor expression in an experimental demyelinating disease with extensive remyelination. J Neurosci Res 2000; 62: 241-56.

Mi S, Hu B, Hahm K, Luo Y, Kam Hui ES, Yuan Q, Wong WM, Wang L, Su H, Chu TH, Guo J, Zhang W, So KF, Pepinsky B, Shao Z, Graff C, Garber E, Jung V, Wu EX, Wu W. LINGO-1 antagonist promotes spinal cord remyelination and axonal integrity in MOG-induced experimental autoimmune encephalomyelitis. Nat Med 2007; 13: 1228-33.

Mi S, Miller RH, Lee X, Scott ML, Shulag-Morskaya S, Shao Z, Chang J, Thill G, Levesque M, Zhang M, Hession C, Sah D, Trapp B, He Z, Jung V, McCoy JM, Pepinsky RB. LINGO-1 negatively regulates myelination by oligodendrocytes. Nat Neurosci 2005; 8: 745-51.

Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004; 20: 33-43.

Nakamura S, Todo T, Haga S, Aizawa T, Motoi Y, Ueki A, Kurokawa T, Ikeda K. Motor neurons in human and rat spinal cord synthesize fibroblast growth factor-9. Neurosci Lett 1997; 221: 181-4.

Nelimarkka L, Salminen H, Kuopio T, Nikkari S, Ekfors T, Laine J, Pelliniemi L, Jarvelainen H. Decorin is produced by capillary endothelial cells in inflammation-associated angiogenesis. Am J Pathol 2001; 158: 345-53.

Niehaus A, Shi J, Grzenkowski M, Diers-Fenger M, Archelos J, Hartung HP, Toyka K, Bruck W, Trotter J. Patients with active relapsing-remitting multiple sclerosis synthesize antibodies recognizing oligodendrocyte progenitor cell surface protein: implications for remyelination. Ann Neurol 2000; 48: 362-71.

Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med 2000; 343: 938-52.

Novak U, Kaye AH. Extracellular matrix and the brain: components and function. J Clin Neurosci 2000; 7: 280-90.

Okada M, Miyamoto O, Shibuya S, Zhang X, Yamamoto T, Itano T. Expression and role of type I collagen in a rat spinal cord contusion injury model. Neurosci Res 2007; 58: 371-7.

Oohira A, Matsui F, Tokita Y, Yamauchi S, Aono S. Molecular interactions of neural chondroitin sulfate proteoglycans in the brain development. Arch Biochem Biophys 2000; 374: 24-34.

Patrikios P, Stadelmann C, Kutzelnigg A, Rauschka H, Schmidbauer M, Laursen H, Sorensen PS, Bruck W, Lucchinetti C, Lassmann H. Remyelination is extensive in a subset of multiple sclerosis patients. Brain 2006; 129: 3165-72.

Polito A, Reynolds R. NG2-expressing cells as oligodendrocyte progenitors in the normal and demyelinated adult central nervous system. J Anat 2005; 207: 707-16.

Poljakovic Z, Zurak N, Brinar V, Korsic M, Basic S, Hajnsek S. Growth hormone and insulin growth factor-I levels in plasma and cerebrospinal fluid of patients with multiple sclerosis. Clin Neurol Neurosurg 2006; 108: 255-8.

Prineas JW, Barnard RO, Revesz T, Kwon EE, Sharer L, Cho ES. Multiple sclerosis. Pathology of recurrent lesions. Brain 1993; 116: 681-93.

Prineas JW, Connell F. Remyelination in multiple sclerosis. Ann Neurol 1979; 5: 22-31.

Raine CS, Wu E. Multiple sclerosis: remyelination in acute lesions. J Neuropathol Exp Neurol 1993; 52: 199-204.

Rauch U. Extracellular matrix components associated with remodeling processes in brain. Cell Mol Life Sci 2004; 61: 2031-45.

Reuss B, von Bohlen und HO. Fibroblast growth factors and their receptors in the central nervous system. Cell Tissue Res 2003; 313: 139-57.

Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 1996; 175: 1-13.

Ricard-Blum S, Ruggiero F. The collagen superfamily: from the extracellular matrix to the cell membrane. Pathol Biol (Paris) 2005; 53: 430-42.

Rudick RA, Mi S, Sandrock AW, Jr. LINGO-1 antagonists as therapy for multiple sclerosis: in vitro and in vivo evidence. Expert Opin Biol Ther 2008; 8: 1561-70.

Rutka JT, Apodaca G, Stern R, Rosenblum M. The extracellular matrix of the central and peripheral nervous systems: structure and function. J Neurosurg 1988; 69: 155-70.

Saksela O, Rifkin DB. Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. J Cell Biol 1990; 110: 767-75.

Sarchielli P, Di FM, Ercolani MV, Chiasserini D, Mattioni A, Bonucci M, Tenaglia S, Eusebi P, Calabresi P. Fibroblast growth factor-2 levels are elevated in the cerebrospinal fluid of multiple sclerosis patients. Neurosci Lett 2008; 435: 223-8.

Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Gotte M, Malle E, Schaefer RM, Grone HJ. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest 2005; 115: 2223-33.

Schaefer L, Iozzo RV. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. J Biol Chem 2008; 283: 21305-9.

Sixt M, Engelhardt B, Pausch F, Hallmann R, Wendler O, Sorokin LM. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. J Cell Biol 2001; 153: 933-46.

Sobel RA, Ahmed AS. White matter extracellular matrix chondroitin sulfate/dermatan sulfate proteoglycans in multiple sclerosis. J Neuropathol Exp Neurol 2001; 60: 1198-207.

Sobel RA, Chen M, Maeda A, Hinojoza JR. Vitronectin and integrin vitronectin receptor localization in multiple sclerosis lesions. J Neuropathol Exp Neurol 1995; 54: 202-13.

Sobel RA, Mitchell ME. Fibronectin in multiple sclerosis lesions. Am J Pathol 1989; 135: 161-8.

Sorensen A, Alekseeva T, Katechia K, Robertson M, Riehle MO, Barnett SC. Long-term neurite orientation on astrocyte monolayers aligned by microtopography. Biomaterials 2007; 28: 5498-508.

Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol 2005; 23:683-747.

Stadelmann C, Kerschensteiner M, Misgeld T, Bruck W, Hohlfeld R, Lassmann H. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? Brain 2002; 125: 75-85.

Thomson CE, Hunter AM, Griffiths IR, Edgar JM, McCulloch MC. Murine spinal cord explants: a model for evaluating axonal growth and myelination in vitro. J Neurosci Res 2006; 84: 1703-15.

Tilling T, Engelbertz C, Decker S, Korte D, Huwel S, Galla HJ. Expression and adhesive properties of basement membrane proteins in cerebral capillary endothelial cell cultures. Cell Tissue Res 2002; 310: 19-29.

Todo T, Kondo T, Nakamura S, Kirino T, Kurokawa T, Ikeda K. Neuronal localization of fibroblast growth factor-9 immunoreactivity in human and rat brain. Brain Res 1998; 783: 179-87.

Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? Annu Rev Neurosci 2008; 31: 247-69.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. N Engl J Med 1998; 338: 278-85.

Traugott U, Reinherz EL, Raine CS. Multiple sclerosis: distribution of T cell subsets within active chronic lesions. Science 1983; 219: 308-10.

Van der Laan LJ, De Groot CJ, Elices MJ, Dijkstra CD. Extracellular matrix proteins expressed by human adult astrocytes in vivo and in vitro: an astrocyte surface protein containing the CS1 domain contributes to binding of lymphoblasts. J Neurosci Res 1997; 50: 539-48.

Van der Rest M, Garrone R. Collagen family of proteins. FASEB J 1991; 5: 2814-23.

Van Horssen J, Vos CM, Admiraal L, van Haastert ES, Montagne L, van d, V, de Vries HE. Matrix metalloproteinase-19 is highly expressed in active multiple sclerosis lesions. Neuropathol Appl Neurobiol 2006; 32: 585-93.

Van HJ, Bo L, Vos CM, Virtanen I, de Vries HE. Basement membrane proteins in multiple sclerosis-associated inflammatory cuffs: potential role in influx and transport of leukocytes. J Neuropathol Exp Neurol 2005; 64: 722-9.

Vanderlocht J, Hellings N, Hendriks JJ, Vandenabeele F, Moreels M, Buntinx M, Hoekstra D, Antel JP, Stinissen P. Leukemia inhibitory factor is produced by myelin-reactive T cells from multiple sclerosis patients and protects against tumor necrosis factor-alpha-induced oligodendrocyte apoptosis. J Neurosci Res 2006; 83: 763-74.

Viapiano MS, Matthews RT. From barriers to bridges: chondroitin sulfate proteoglycans in neuropathology. Trends Mol Med 2006; 12: 488-96.

Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA. Notch receptor activation inhibits oligodendrocyte differentiation. Neuron 1998; 21: 63-75.

Warrington AE, Asakura K, Bieber AJ, Ciric B, Van K, V, Kaveri SV, Kyle RA, Pease LR, Rodriguez M. Human monoclonal antibodies reactive to oligodendrocytes promote remyelination in a model of multiple sclerosis. Proc Natl Acad Sci U S A 2000; 97: 6820-5.

Warrington AE, Bieber AJ, Ciric B, Pease LR, Van K, V, Rodriguez M. A recombinant human IgM promotes myelin repair after a single, very low dose. J Neurosci Res 2007; 85: 967-76.

Warrington AE, Rodriguez M. Remyelination-promoting human IgMs: developing a therapeutic reagent for demyelinating disease. Curr Top Microbiol Immunol 2008; 318: 213-39.

Wilkins A, Majed H, Layfield R, Compston A, Chandran S. Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. J Neurosci 2003; 23: 4967-74.

Woodruff RH, Fruttiger M, Richardson WD, Franklin RJ. Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. Mol Cell Neurosci 2004; 25: 252-62.

Wu C, Ivars F, Anderson P, Hallmann R, Vestweber D, Nilsson P, Robenek H, Tryggvason K, Song J, Korpos E, Loser K, Beissert S, Georges-Labouesse E, Sorokin LM. Endothelial basement membrane laminin alpha5 selectively inhibits T lymphocyte extravasation into the brain. Nat Med 2009; 15: 519-27.

Wuerfel J, Haertle M, Waiczies H, Tysiak E, Bechmann I, Wernecke KD, Zipp F, Paul F. Perivascular spaces--MRI marker of inflammatory activity in the brain? Brain 2008; 131: 2332-40.

Yamaguchi Y. Lecticans: organizers of the brain extracellular matrix. Cell Mol Life Sci 2000; 57: 276-89.

Yao DL, Liu X, Hudson LD, Webster HD. Insulin-like growth factor I treatment reduces demyelination and up-regulates gene expression of myelin-related proteins in experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 1995; 92: 6190-4.

Ye P, D'Ercole AJ. Insulin-like growth factor I protects oligodendrocytes from tumor necrosis factor-alpha-induced injury. Endocrinology 1999; 140: 3063-72.

Yong VW, Power C, Forsyth P, Edwards DR. Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2001; 2: 502-11.

Zhang SC, Lundberg C, Lipsitz D, O'Connor LT, Duncan ID. Generation of oligodendroglial progenitors from neural stem cells. J Neurocytol 1998; 27: 475-89.

8 Abbreviations

BDNF	Brain derived neurotrophic factor
CNP	2’3’-cyclic nucleotide 3’-phosphodiesterase
CNTF	Ciliary neurotrophic factor
DAB	3,3’ -diaminobenzidine
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
H&E	Hematoxylin & Eosin
HFF	Human foreskin fibroblast
IGF	Insulin like growth factor
IL-6	Interleukin-6
LDA	Low density array
LFB-PAS	Luxol fast blue- periodic acid schiffs
LIF	Leukemia inhibitory factor
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MMP	Matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NAWM	Normal appearing white matter
NFL	Neurofilament protein, light polypeptide
NFM	Neurofilament protein, medium polypeptide
NGF	Nerve growth factor
NRG	Neuregulins
NT-3	Neurotrophin-3
O/N	Over night
OPC	Oligodendrocyte glycoprotein
ORO	Oil red O
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PLP	Proteolipid protein
PPIA	Cyclophilin A
PRMS	Progressive-relapsing multiple sclerosis
PSA-NCAM	Polysialylated neural cell adhesion molecule
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SLRP	Small leucine rich proteoglycan

SMI-31	Neurofilament phosphorylated epitope
SPMS	Secondary progressive multiple sclerosis
TIMP	Tissue inhibitor of matrix metalloproteinases
cpm	Counts per minute
h	Hours
min	Minutes
qPCR	Quantative polymerase chain reaction
rpm	Rotations per minute

9 Curriculum Vitae

Name	Hema Mohan
Date of birth	17th October 1979
Place of Birth	Chennai
Nationality	Indian

Education

Since September 2005	PhD student (Biology Faculty – LMU, Munich) Department of Neuroimmunology, Max-Planck Institute of Neurobiology, Martinsried, Germany. Supervisor: Prof. Dr. Edgar Meinl
2000-2002	Masters (Biotechnology) Calicut University, Kerala, India.
1997-2000	Bachelors (Zoology, Botany, Chemistry) Kanpur University Uttar Pradesh, India.

Publications

Mohan H, Krumbholz M, Sharma R, Eisele S, Junker A, Sixt M, Newcombe J, Wekerle H, Hohlfeld R, Lassmann H, Meinl E. Extracellular matrix in multiple sclerosis lesions: fibrillar collagens, biglycan and decorin are up-regulated and associated with infiltrating immune cells. Submitted.

Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, Bittner R, Lassmann H, Wekerle H, Hohlfeld R, Meinl E. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. Brain. 2009 Dec; 132(Pt 12):3342-52.

Mohan H, Elliott C, Krumbholz M, Hohlfeld R, Wekerle H, Lassmann H,
Linington C, Meinl E. FGF9 is up-regulated in chronically demyelinated MS lesions and inhibits myelination. In preparation.

Meetings

Mohan H, Krumbholz M, Eisele S, Sixt M, Newcombe J, Wekerle H, Hohlfeld R, Lassmann H, Meinl E. Extracellular matrix in multiple sclerosis lesions: Induction of fibrillar collagens, biglycan, and decorin in association with infiltrating immune cells. Poster presentation at the 9th International Congress of Neuroimmunology. October 26th-29th, 2008, Fort Worth, USA.

Mohan H, Krumbholz M, Eisele S, Sixt M, Newcombe J, Wekerle H, Hohlfeld R, Lassmann H, Meinl E. Extracellular matrix in multiple sclerosis lesions: Induction of fibrillar collagens, biglycan, and decorin in association with infiltrating immune cells. Poster presentation at BrainNet Europe, 2nd International conference on Human Brain Tissue Research. December 10th-12th, 2008, Munich, Germany.

Mohan H, Elliott C, Krumbholz M, Hohlfeld R, Wekerle H, Lassmann H,
Linington C, Meinl E. FGF9 is up-regulated in chronically demyelinated MS lesions and inhibits myelination. Poster presentation at Myelin Meeting, Gordon Research Conference. February 14th-19th, 2010, Ventura, USA.