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Autoantibodies in Multiple Sclerosis

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To my parents

Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) in which myelin and axons are damaged. While T cells have most widely been regarded as key players in MS pathogenesis, there is increasing evidence that B cells and autoantibodies are involved in disease development in at least a subset of patients. Defining potential autoantibody targets has proven challenging due to the complexity and heterogeneity of MS and - despite extensive research efforts - they hitherto largely remain unknown.

Neurofascin had previously been identified as a potential target for autoantibodies in MS patients using glycoproteins in a two-dimensional Western blot analysis, followed by mass spectrometry. Neurofascin is an axoglial protein displayed at the node of Ranvier and exists in two isoforms, the axonal NF186 directly at the node and the glial NF155 in the adjacent paranodes. In an animal model of MS, experimental autoimmune encephalomyelitis (EAE), a monoclonal antibody (mAb) to neurofascin bound to the node and induced axonal injury.

We hypothesized that the node of Ranvier, as an unmyelinated region along the nerve fiber, might be an area prone to antibody mediated damage due to its accessibility and therefore the potential „Achilles heel“ of the CNS.

This thesis addressed two key points:

(i.) Whether monoclonal antibodies directed against the node exhibit pathogenic features in the EAE animal model of MS, and

(ii.) Whether antibodies recognizing components at and around the node of Ranvier are present in the serum of MS patients.

To investigate the first point, two different mAbs (targeting neurofascin and contactin-1) were used. We initially showed their ability to bind the node both *in vitro* and *in vivo* by applying a double staining with the paranodal protein Caspr. However, in the *in vivo* transfer experiment only the neurofascin mAb resulted in enhancement and prolongation of disease, while the contactin-1 mAb induced no effects, although both antibodies were of a complement-activating isotype. Within the limits of this investigation it was therefore concluded that node-targeting antibodies alone are not sufficient in order to exacerbate disease in this MS model system.

The second part of the study focused on the identification of autoantibodies to nodal or paranodal proteins in the serum of MS patients via an immunohistochemical approach, combining the use of longitudinal rat spinal cord sections and a double-staining with paranodal Caspr to increase sensitivity. By these means no reactivity against the

node or the paranodes could be detected in any of the tested serum samples. It was possible, however, to identify MS patients harbouring autoantibodies against axons and oligodendrocytes with so far unknown specificity. Further investigation of the occurrence of the oligodendrocyte pattern revealed a significant difference between MS patients and control individuals. Moreover, using CNS tissue from transgenic mice expressing the green fluorescent protein under the promoter for the myelin protein PLP, one MS serum could be identified that exhibited oligodendrocyte surface as well as process staining.

Taken together this thesis has two main findings:

(i.) Targeting the node of Ranvier does not represent a sufficient condition for the induction of antibody-mediated damage in an animal model of MS.

(ii.) Antibodies to axons and oligodendrocytes, but not to the node of Ranvier, could be identified in MS patients' sera.

The outstanding MS serum binding to oligodendrocytes represents a very promising candidate for further investigation. In the future, the identification of specific autoantibody targets will contribute to the understanding of MS pathology, and as a consequence thereof the development of optimized therapeutic interventions.

Zusammenfassung

Multiple Sklerose (MS) ist eine chronisch entzündliche Erkrankung des zentralen Nervensystems (ZNS), in dem Myelin und Axone beschädigt werden. Während T-Zellen seit jeher als wichtige Akteure in der MS-Pathogenese angesehen wurden, gibt es zunehmend Hinweise dafür, dass B-Zellen und Autoantikörper zumindest in einer Untergruppe von Patienten an der Krankheitsentstehung beteiligt sind. Die Identifikation potenzieller Angriffsziele für Autoantikörper hat sich aufgrund der Komplexität und Heterogenität der MS als Herausforderung erwiesen, so dass diese trotz intensiver Forschungsbemühungen bisher weitgehend unbekannt blieben.

Ein potenzielles Ziel für Autoantikörper in MS-Patienten stellt Neurofascin dar, welches identifiziert wurde, indem man die Nutzung von Myelinglykoproteinen in einem zweidimensionalen Western Blot mit anschließender Massenspekrometrie kombinierte. Neurofascin ist ein axogliales Protein am Ranvier'schen Schnürring, welches in zwei Isoformen existiert, dem axonalen NF186 direkt am Schnürring und dem glialen NF155 in den angrenzenden Paranoden. In einem Tiermodell der MS, der experimentellen autoimmunen Enzephalomyelitis (EAE), band ein monoklonaler Antikörper (mAk) gegen Neurofascin den Ranvier'schen Schnürring und induzierte axonale Schäden.

Zu Beginn der Studie wurde angenommen, dass der Ranvier'sche Schnürring als eine unmyelinisierte Region entlang der Nervenfaser wegen seiner guten Erreichbarkeit ein für Antikörper-vermittelte Schäden anfälliges Gebiet und somit die potenzielle „Achillesferse" des ZNS darstellen könnte.

Dabei sollten in dieser Arbeit zwei wichtige Punkte untersucht werden:

1. Ob monoklonale Antikörper, die an den Ranvier'schen Schnürring binden, pathogene Eigenschaften im EAE Tiermodell für MS aufweisen und

2. Ob Antikörper gegen Komponenten des Ranvier'schen Schnürringes im Serum von MS-Patienten vorhanden sind.

Im ersten Teil wurden zwei verschiedene mAk (anti-Neurofascin und anti-Contactin-1) untersucht. Mit Hilfe einer Doppelfärbung mit dem paranodalen Protein Caspr konnte eingangs gezeigt werden, dass beide mAk den Ranvier'schen Schnürring *in vitro* und *in vivo* binden. Allerdings verursachte nur der mAk gegen Neurofascin eine Verschlechterung und Verlängerung der Erkrankung im *in vivo* Transferexperiment. Der mAk gegen Contactin-1 rief im Gegensatz dazu keinerlei Effekte hervor, obwohl beide Antikörper einen komplementaktivierenden Isotypen aufweisen. Innerhalb der Grenzen der vorliegenden Untersuchung liegt der Schluss nahe, dass Antikörper gegen den Schnürring allein nicht ausreichen, um in diesem MS-Modell für eine Krankheitsverstärkung zu sorgen.

Der zweite Teil der Studie untersuchte die Präsenz von Autoantikörpern gegen nodale oder angrenzende (paranodale) Proteine im Serum von MS-Patienten mittels eines immunhistochemischen Ansatzes. Dieser kombinierte die Verwendung von Rattenrückenmarks-Längsschnitten mit einer paranodalen Doppelfärbung mit Caspr, um die Sensitivität der Methode zu erhöhen. Dennoch konnte in keinem der getesteten Seren eine Reaktivität gegen den Schnürring oder die Paranoden entdeckt werden. Allerdings war es möglich, Autoantikörper gegen Axone und Oligodendrozyten mit bislang unbekannter Spezifität zu identifizieren. Weitere Untersuchungen der Oligodendrozytenfärbung zeigten einen signifikanten Unterschied in deren Häufigkeit zwischen MS-Patienten und Kontrollpersonen.

Darüber hinaus konnte unter Zuhilfenahme von ZNS-Gewebe aus transgenen Mäusen, die das grün fluoreszierende Protein unter dem Promotor für das Myelinprotein PLP exprimieren, ein MS-Serum identifiziert werden, welches Antikörper gegen die Oberfläche von Oligodendrozyten sowie deren Fortsätze aufwies.

Zusammengenommen liefert diese Arbeit zwei Erkenntnisse:

1. Targeting des Ranvier'schen Schnürringes allein stellt keine hinreichende Bedingung für die Induktion von Antikörper-vermittelten Schäden in einem Tiermodell der MS dar.

2. In dieser Studie konnten Antikörper gegen Axone und Oligodendrozyten, nicht aber gegen den Ranvier'schen Schnürring in MS-Patientenproben gefunden werden.

Das auffallende, an Oligodendrozyten bindende MS-Serum stellt einen äußerst vielversprechenden Kandidaten für weitere Untersuchungen dar. In der Zukunft sollte die Identifizierung spezifischer Autoantikörper und deren Ziele maßgeblich zum besseren Verständnis der MS-Pathologie und daher zur Entwicklung optimierter Therapien beitragen.

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List of Abbreviations

ADEM	Acute disseminated encephalomyelitis
AIDP	Acute inflammatory demyelinating polyneuropathy
ALS	Amyotrophic lateral sclerosis
ANA	Anti-nuclear antibody
APP	Amyloid precursor protein
AQP4	Aquaporin 4
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
Caspr	Contactin-associated protein
CD	Cluster of differentiation
CIDP	Chronic inflammatory demyelinating polyneuropathy
CIS	Clinically isolated syndrome
CNP	2′,3′-Cyclic-nucleotide 3′-phosphodiesterase
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
CST	Corticospinal tract
DAB	3,3′-diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
(d)H_2O	(Distilled) water
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F(ab′)$_2$	Fragment antigen binding
Fc	Fragment crystallizable
FCS	Fetal calf serum
FPLC	Fast-protein liquid chromatography
$GABA_B$	γ-aminobutyric acid receptor-B
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBS	Guillain-Barré syndrome
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
gp	Glycoprotein
GM	Grey matter
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride

HE	Hematoxylin-eosin
HRP	Horseradish peroxidase
Ig(G)	Immunoglobulin (G)
IHC	Immunohistochemistry
IL	Interleukin
kDa	kilo Dalton
$KHCO_3$	Potassium hydrogen carbonate
Lg|1	Leucine-rich, glioma inactivated 1
LT	Lymphotoxin
mAb	Monoclonal antibody
MBP	Myelin basic protein
min	Minute
(m)M	(milli)molar
$MgSO_4$	Magnesium sulfate
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple sclerosis
(NA)WM	(Normal-appearing) white matter
n/a	not applicable
NF (155 or 186)	Neurofascin (155 or 186)
NMO	Neuromyelitis optica
NMDAR	N-methyl-D-aspartate receptor
NrCAM	Neuronal cell adhesion molecule
OCB	Oligoclonal band
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PC	Plasma cell
PFA	Paraformaldehyd
PLP	Proteolipid protein
PNS	Peripheral nervous system
PPMS	Primary-progressive multiple sclerosis
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
RRMS	Relapsing-remitting multiple sclerosis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD1	Superoxide dismutase
SPMS	Secondary-progressive multiple sclerosis
TBS	Tris-buffered saline
TNF	Tumor necrosis factor

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1 Introduction

1.1 Multiple sclerosis

1.1.1 General remarks

One critical function of the immune system is to discriminate normal self (the individual's healthy cells) from infectious non-self (pathogens) as well as abnormal self (tumor cells). If the respective regulatory mechanisms fail and reactions to self-tissues occur, a variety of syndromes called autoimmune diseases can be caused (reviewed in [Medzhitov and Janeway, 2002]). Approximately 5 % of the Western population is affected by such diseases, varying in their severity as well as the tissues affected. The latter is used to classify autoimmune diseases from a clinical perspective. „Systemic" autoimmune diseases affect multiple organs simultaneously with tissues as diverse as the skin, kidney and brain like in systemic lupus erythematosus or primary Sjögren's syndrome. In contrast, „organ-specific" autoimmune diseases are restricted to one organ of the body, examples being type I diabetes in the pancreas or multiple sclerosis (MS) which only affects the central nervous system (CNS).

MS is an inflammatory disease of the CNS that was first described in 1868 by Jean-Martin Charcot. The disorder manifests as acute focal inflammatory demyelination and axonal loss with limited remyelination and gets its name from the resulting chronic multifocal sclerotic plaques preferentially located in the periventricular regions, brainstem, cerebellum, spinal cord and optic nerves.

With about 2.5 million people affected worldwide (thereof 130,000 in Germany) and a peak age of onset around 30 years, MS is considered the second most frequent neurological disease affecting young adults after epilepsy [Noseworthy et al., 2000]. As typical for autoimmune diseases, women have an approximately two times higher risk of developing MS.

1.1.2 Clinical features

Patients with MS develop a variety of neurological deficits, merely reflecting the functional anatomy of impaired saltatory conduction at affected sites. Symptoms include, but are not limited to, changes in sensation (hypoesthesia or paresthesia), difficulties with coordination and balance (ataxia), problems in speech (dysarthria) or swallowing (dysphagia), but also optic neuritis, fatigue, pain, as well as bladder and bowel difficulties. Cognitive impairment and emotional symptoms (depression or unstable mood) can also occur [Compston and Coles, 2008].

An individual's first episode of brain demyelination is called a clinically isolated syndrome (CIS), which can be indicative of MS. In 2001, revised guidelines for the clinically definite diagnosis of MS in persons who presented with a single demyelinating event, the McDonald criteria, were issued [McDonald et al., 2001]. The basic requirement, dissemination in time and space, is complemented with guidelines for the interpretation of findings from magnetic resonance imaging (MRI) as well as cerebrospinal fluid (CSF) analysis.

MS disease progression is highly variable in individual patients and can basically be divided into three subtypes, namely:

(i.) Relapsing-remitting RRMS,

(ii.) Secondary-progressive SPMS and

(iii.) Primary-progressive PPMS.

Approximately 85 % of MS patients begin with RRMS, a course of recurrent but reversible neurological deficits where relapses can be followed by periods of months to years with no new signs of disease activity. SPMS is regarded as a common late stage of disease that follows RRMS after 8 – 20 years and is characterized by continuous and irreversible neurological disability unassociated with relapses. PPMS is a less common form, only present in about 15 % of the patients, in which the disease progresses continuously from the beginning without any remission (reviewed in [Trapp and Nave, 2008]).

1.1.3 Pathological features

The pathology of MS is characterized by inflammation, demyelination with limited remyelination, axonal injury and astrocytic scar formation (sclerosis).

In addition to the classical white matter lesions, recent methodological advances also point to a strong contribution of cortical grey matter damage to MS pathology ([Geurts et al., 2005]; [Lucchinetti et al., 2011]). Evaluation of MS brain material revealed the existence of three distinct types of cortical lesions which can be simultaneously found in a given patient. When compared to lesions in the white matter, cortical lesions found at autopsy typically show less inflammatory cell infiltrates and decreased complement activation [Peterson et al., 2001]. Nevertheless, cortical inflammation can be a prominent feature also in early MS stages [Lucchinetti et al., 2011].

A detailed analysis of the white matter pathology reveals four different lesion patterns [Lucchinetti et al., 2000], all of them harboring macrophages, T cells and some B cells. Type I and II lesions are distributed perivenously and possess a sharp lesion edge. In contrast to type I there is immunoglobulin G (IgG) as well as active complement deposition present in the demyelinated areas of lesion type II. Also, remyelination is common. Type III lesions are localized around blood vessels and are not sharply defined. They are free of complement deposits and demyelination is a result of oligodendrocyte apoptosis. In contrast, type IV lesions do have a sharp rim and oligodendrocyte degeneration does not seem to be due to apoptosis. This last pattern, however, is only infrequently seen in a subset of patients with PPMS [Lucchinetti et al., 2000].

These subtypes are supposed to be mutually exclusive, suggesting that patients can be separated according to disparate pathogenic pathways although they present with clinically indistinguishable symptoms. However, it should be mentioned that this classification remains controversially discussed ([Barnett and Prineas, 2004]; [Barnett et al., 2009a]). The heterogeneity of MS lesions has recently been questioned by the finding of a uniform pre-phagocytic pathology. In addition, an overlap of the afore-mentioned lesion subtypes in individual RRMS patients has been reported, which might reflect an evolution of one single pathophysiological process as the disease progresses (reviewed in [Barnett et al., 2009b]). Importantly, the lesion subtypes can only be defined via analysis of the brain tissue. As brain biopsies are not usually performed in MS patients, lesion subtypes can only be performed post-mortem. For this reason, lesion types remain unidentified for most MS patients.

1.1.4 Other factors

The initial cause of MS remains disputed but certainly involves environmental exposure and genetic susceptibility. Genome-wide genetic association studies revealed multiple disease-associated genes involved in immune system function, including the

major histocompatibility complex (MHC). A recent study assessing genetic risk factors carried out by the International Multiple Sclerosis Genetics Consortium confirmed that genetic variations within the MHC have the greatest individual effect on risk [Sawcer et al., 2011]. In addition, genes coding for cytokine pathways, immunologically relevant co-stimulatory and signal transduction molecules, but also environmental risk factors such as vitamin D were reported to have an impact [Sawcer et al., 2011]. Interestingly, a beneficial role of vitamin D has recently been suggested and there is accumulating evidence that it reduces the risk for conversion to MS and MS progression. The role of infections in MS, for example with Epstein-Barr virus (EBV), remains controversially discussed. Another environmental factor potentially involved in MS is smoking. There are recent studies highlighting that smoking may increase the incidence of MS, promote lesion development and accelerate secondary progression (reviewed in [Ascherio et al., 2012]).

1.2 B cell involvement and autoantibodies

1.2.1 Rationale for the involvement of B cells and autoantibodies

While T cells have always been regarded as key players in the pathogenesis of MS, the important role of B cells has only recently been increasingly recognized. The reasons for this shift of focus are reviewed in [Meinl et al., 2006] and include:

(i.) An intrathecal Ig production with oligoclonal Ig („oligoclonal bands“);

(ii.) The clonal expansion of B cells in the CSF, and

(iii.) Follicle-like aggregates in the meninges of some patients with a progressive disease course.

B cells can have pro- as well anti-inflammatory functions. Their classical role is to develop into antibody secreting cells via the generation of short-lived plasmablasts. Short-lived plasmablasts may become long-lived plasma cells, if they find survival niches, which are typically found in the bone marrow, but to some extent also at sites of inflammation. Persisting IgG found in the plasma arises from those long-lived plasma cells. The effector functions of IgG comprise complement activation as well as Fc (fragment crystallizable) receptor binding and are dependent on the specific isotype and on the glycosylation of the Fc part. Apart from their role as antibody producing cells, B cells influence the inflammatory environment in general (Figure 1.1 on page 5). They

produce both pro-inflammatory and anti-inflammatory cytokines and are also very efficient in presenting antigen to T cells (reviewed in [Krumbholz et al., 2012]). Results from clinical trials with rituximab and ocrelizumab, monoclonal antibodies (mAb) that deplete cells of the CD20 positive B cell lineage, indicate that the pro-inflammatory features of B cells dominate in most of the MS patients. However, as those antibodies spare long-lived plasma cells (which lack CD20) and therefore should hardly remove the amount of circulating Ig, their effect on autoantibodies is more complex and suggests that the depletion of B cells is beneficial in a manner independent of Ig [Hauser et al., 2008].

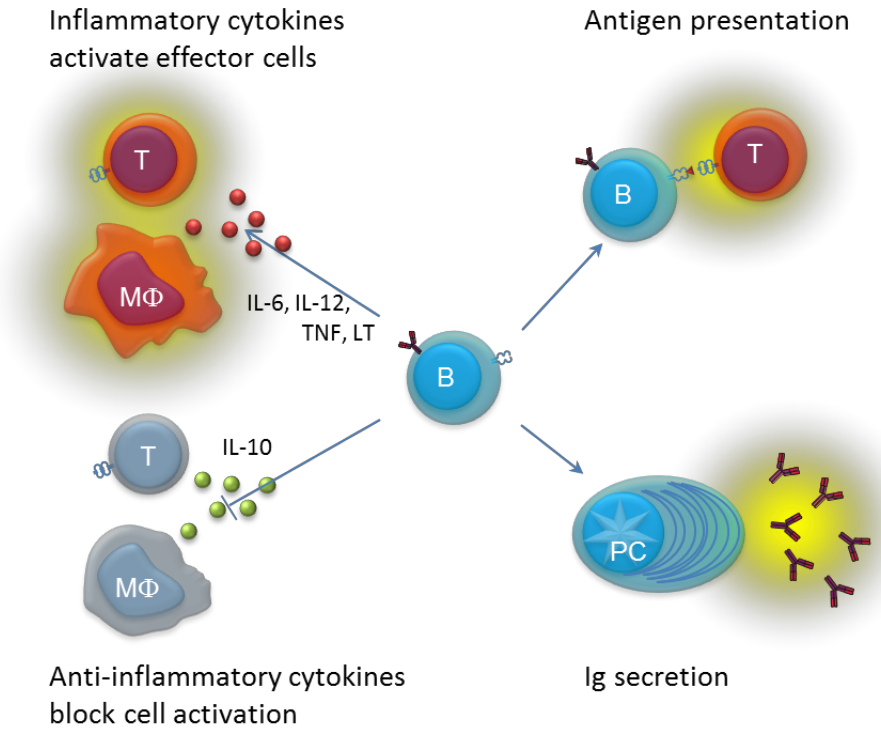

Figure 1.1: Pro-inflammatory and regulatory effects of B cells

Figure 1.1: *B cells possess double-edged functions: They can develop into Ig-secreting plasma cells (PC) and act as antigen-presenting cells to T cells. Furthermore they can activate T cells and macrophages (MΦ) by the secretion of inflammatory cytokines (e.g. interleukin-[IL]-6, IL-12, tumor necrosis factor [TNF], lymphotoxin [LT]), but also secrete regulatory cytokines (e.g. IL-10) that later lead to the blockage of T cell and macrophage activation (adapted from [Krumbholz et al., 2012]).*

Another strong indicator of the B cell involvement in MS are oligoclonal bands (OCB), the most prominent immunodiagnostic abnormality detected in body fluids of patients. The Ig transcriptome of CSF B cells overlaps with the Ig proteome of OCBs, suggesting that the B lineage cells in the CSF are representative of those pro-

ducing the OCBs [Obermeier et al., 2008]. OCBs are present already at the first relapse in about 90 % of patients eventually diagnosed with MS, their long-term persistence going along with the idea of a B cell fostering environment in the CNS of MS patients.

Additionally, there are several lines of evidence suggesting that IgG plays a role in MS pathology in at least a subset of patients, namely:

(i.) Therapeutic success of plasma exchange in a proportion of patients [Keegan et al., 2005],

(ii.) The recent demonstration of demyelinating and axopathic IgG in MS patient serum [Elliott et al., 2012], and

(iii.) The Ig deposition and complement activation in a subset of MS patients [Lucchinetti et al., 2000].

1.2.2 Candidate targets of autoantibodies in multiple sclerosis

The actual target of autoantibodies remains unknown for most MS patient cases. The best studied candidate autoantigen so far is the myelin oligodendrocyte glycoprotein (MOG), because it had been shown that MOG-specific antibodies cause demyelination in several variants of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (reviewed in [Mayer and Meinl, 2012]). Using anti-MOG antibodies, a two-hit model for CNS inflammation was developed, where antibodies enhance pathology as a „second hit“ only after a T cell mediated inflammation with concomitant breach of the blood-brain barrier (BBB), the „first hit“ ([Schluesener et al., 1987]; [Linington et al., 1988]).

Nevertheless, the occurrence of MOG autoantibodies in MS patients is still controversially discussed. One study reports that IgG isolated from the CNS parenchyma of autopsy samples bound to linear MOG in a subgroup of MS patients. This was neither the case for MS CSF and serum nor for non-inflamed control individuals [O'Connor et al., 2005].

However, the picture is increasingly emerging that antibodies recognizing the denatured antigen, meaning linear epitopes, are present not only in MS patients but also in many healthy controls, whereas antibodies binding to native MOG are absent in the vast majority of adult MS patients (reviewed in [Meinl et al., 2010]). Notwithstanding the above, recent studies described antibodies to conformationally correct MOG in cases of pediatric acquired demyelination, i.e. acute disseminated encephalomyelitis (ADEM) and MS ([McLaughlin et al., 2009]; [Brilot et al., 2009]; [Probstel et al., 2011]).

The paucity of MOG-specific antibodies in adult MS puts the identification and validation of new autoantigens recognized by subgroups of MS patients into focus of current research. Different strategies can be employed in order to identify autoantigens, as reviewed in [Derfuss and Meinl, 2012], namely:

(i.) Immunostaining,

(ii.) Blotting and mass spectrometry,

(iii.) Arrays and multiplex approaches,

(iv.) Expression and peptide libraries,

(v.) Recombinant IgG generated from single or clonally expanded cells, and

(vi.) B cell immortalization with EBV to obtain IgG from cultured B cell lines.

Combining strategies (i.) and (ii.), very recently the potassium channel inward rectifier KIR4.1 was found as a novel autoantigen in an unbiased proteomic approach focusing on membrane proteins [Srivastava et al., 2012]. In the brain, KIR4.1 is exclusively expressed by glial cells, especially oligodendrocytes and astrocytes. Patients with MS or a CIS showed higher levels of KIR4.1 compared to healthy donors and patients with other neurological diseases as quantified in an enzyme-linked immunosorbent assay (ELISA) using serum samples. The intriguing finding is the existence of those antibodies in almost half of the MS patients as compared to about 1 % of other neurological diseases and none of the healthy donors. In addition, when anti-KIR4.1 positive patient IgG was transferred into mouse brain, it was able to deplete KIR4.1 on glial cells, altered the expression of GFAP (glial fibrillary acidic protein) in astrocytes and induced antibody-dependent, cell-mediated cytotoxicity, possibly through complement activation at the sites of KIR4.1 expression [Srivastava et al., 2012].

Another proteomic approach with purified glycoproteins put a different CNS structure into the spotlight, when axoglial proteins around the node of Ranvier, namely neurofascin (NF) and contactin-2, were identified as potential targets for autoantibodies in MS ([Mathey et al., 2007]; [Derfuss et al., 2009]).

1.3 The node of Ranvier as a possible target of an autoimmune response

1.3.1 The special architecture of the node of Ranvier

Nodes of Ranvier are the unmyelinated sites of action potential propagation in myelinated fibers, and their formation is essential for the switch to rapid nerve impulse transmission in the developing vertebrate nervous system. Paranodal and juxtaparanodal regions lie adjacent to the node of Ranvier (Figure 1.2 on page 8).

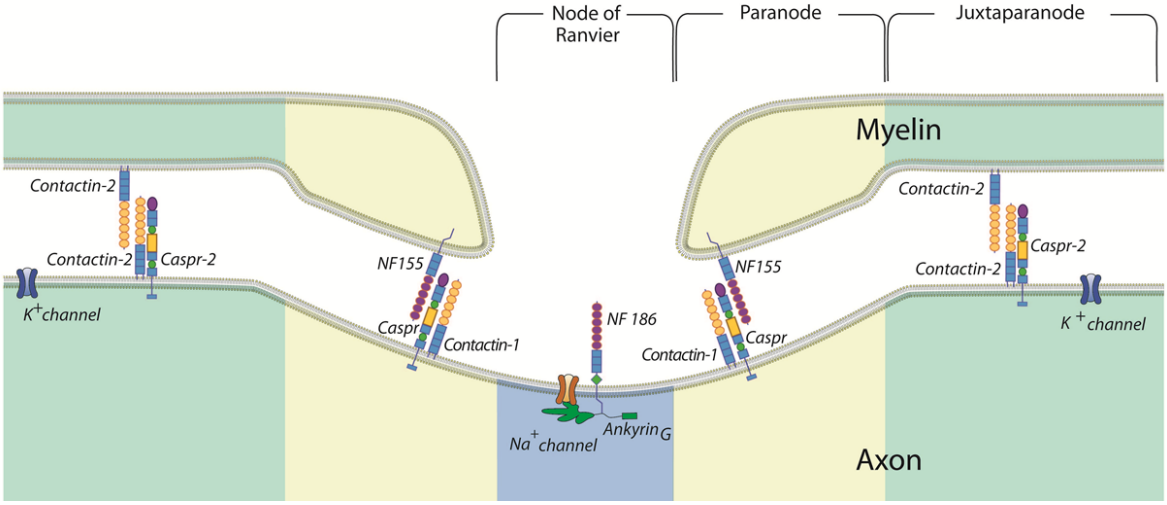

Figure 1.2: Specialized architecture around the node of Ranvier

Figure 1.2: *Distribution of CNS proteins at and around the node of Ranvier: Myelin enwraps an axon, omitting a small area termed the node of Ranvier. Paranodal and juxtaparanodal areas are affiliated, each possessing unique protein compositions. At the node NF186 assists in the clustering of sodium (Na+) channels. In the adjacent paranodes a complex is formed between NF155 and Caspr/contactin-1. Clustered potassium (K+) channels are associated with a Caspr-2/contactin-2 complex in the juxtaparanodal region (adapted from [Meinl et al., 2010]).*

Axonal protein complexes containing voltage-gated sodium channels are assembled at nodes of Ranvier in response to myelination. Components of the nodal complex include anchor proteins like ankyrinG and the neuronal isoform of neurofascin, NF186, that is needed for the clustering of voltage-gated sodium channels [Sherman et al., 2005]. The paranodal axoglial junctions, which flank the node, are formed by an adhesion complex between the glial isoform of neurofascin, NF155, and the axonal proteins Caspr (contactin-associated protein, also known as paranodin) and contactin-1 (reviewed in [Charles et al., 2002]).

In the juxtaparanodes potassium channels are clustered and bound to Caspr-2. In addition, there is contactin-2 present on the axonal as well as the glial side and interacts with itself ([Traka et al., 2002]; [Traka et al., 2003]), (Figure 1.2 on page 8).

Although this architecture is basically similar in the CNS and the peripheral nervous system (PNS), slight differences have been described (reviewed in [Poliak and Peles, 2003]). For example, contactin-1 was found independently of Caspr in the central, but not peripheral nodes of Ranvier, where gliomedin serves as a glial ligand for NF186 instead ([Rios et al., 2000]; [Eshed et al., 2005]). In addition, NrCAM (neuronal cell adhesion molecule) has been reported to be only present in the nodes in the PNS, but not in the CNS [Zonta et al., 2008].

Alterations of the nodal architecture have been observed in MS. In chronic active demyelinating lesions, NF155 was also expressed in elongated stretches at the paranodes concomitant with a disrupted expression of the NF186 clusters at the node itself [Howell et al., 2006]. This pattern could be correlated to axonal damage. Accompanying demyelination, those changes also resulted in the decrease of the conduction velocity that in parts turned into a complete blockage [Trapp and Stys, 2009]. In addition to these morphological changes of the node in MS, there is *in vitro* evidence that the inhibition of NF155 by antibodies blocked myelination or remyelination [Charles et al., 2002].

As already mentioned, recently, two novel axo-glial autoantigens have been identified that are located at and around the node of Ranvier. Briefly, in an unbiased proteomic screen using highly enriched human myelin glycoproteins and a combination of two-dimensional gel electrophoresis, Western blotting and mass spectrometry, NF and contactin-2 ([Mathey et al., 2007]; [Derfuss et al., 2009]) could be identified as potential novel autoimmune targets in MS. This finding makes this unmyelinated and therefore easily accessible area an interesting region for further studies on auto-antibody mediated damage.

1.3.2 Damage mediated by antibodies targeting the node in an animal model of multiple sclerosis

1.3.2.1 Experimental Autoimmune Encephalomyelitis as an animal model for MS

EAE is the most common animal model used in MS research and established in different animal species, e.g. mice, rats or primates. Closely mimicking the histopathologic and some immunologic features of the human disease, EAE is considered a well-suited

model. However, classical EAE models display shortcomings for example in regard to B cell involvement. So far, no model is able to represent all features of the very complex pathology present in humans [Lassmann, 2007]. EAE is elicited either by immunization with CNS antigen or the passive transfer of encephalitogenic T cells. The choice of the protocol used as well as the animal's genetic background may influence the clinical outcome and the underlying CNS pathology. Due to a preferential attack of the spinal cord, animals classically develop an ascending flaccid paralysis that depends on disease severity and is measured with a clinical score ranging from 1 to 5. It initially affects the tail (score 1), and can later involve the hind limbs (score 2/3) as well as the fore limbs (score 4) and also lead to the animal's death (score 5) (reviewed in [Batoulis et al., 2011]).

1.3.2.2 Targeting the node of Ranvier with monoclonal antibodies

In this study two models based on MBP (myelin basic protein) or MOG specific encephalitogenic T cells transferred to either Lewis or DA rats were used, respectively. This transfer EAE has a monophasic course with disease exacerbation starting at around day 3 after the injection of T cells, reflected by the aforementioned signs of paralysis and a loss of body weight.

According to the „two-hit model" one can investigate the pathological relevance of antibodies that are injected at disease onset, as they are now able to enter the brain through the leaky BBB. For example, the adoptive transfer of an NF-specific mAb into animals with EAE induced a severe antibody mediated exacerbation of clinical disease associated with increased axonal injury caused by the antibody's binding to the nodes of Ranvier [Mathey et al., 2007]. In contrast, the adoptive transfer of contactin-2 specific mAbs did not initiate any significant pathological effect in animals with EAE [Derfuss et al., 2009]. This might be explained by the sequestration of contactin-2 within the juxtaparanodal domain of myelinated fibers, which might render it inaccessible to antibodies in the extracellular milieu. However, T cells specific for contactin-2 were found to induce inflammation preferentially in the grey matter of the brain and spinal cord, and paved the way for cortical demyelination mediated by MOG-specific antibodies [Derfuss et al., 2009].

The results of these two studies extend the repertoire of immune responses that might contribute to lesion formation and disease progression. Specifically, autoaggression to axoglial, rather than myelin-specific autoantigens might contribute to the development of cortical lesions (contactin-2) and axonal injury (NF) in MS.

1.4 Immunohistochemistry as a first step towards the identification of autoantibodies

1.4.1 Rationale for the approach

One possible tool in order to identify autoantigens in their naturally occurring conformation is the use of an immunohistochemical approach where patient sera are applied to CNS tissue sections in order to identify IgG staining patterns. Those can later be attributed to specific molecules by means of colocalization experiments and/or mass spectrometry experiments following immunoprecipitation. Two prominent and successful disease examples are given in the following section.

1.4.2 Neuromyelitis optica

Neuromyelitis optica (NMO)-Ig produces a characteristic pattern of immunostaining of cerebral microvessels, pia mater and Virchow-Robin spaces in the CNS, but also stains structures in the kidney and gastric mucosa [Lennon et al., 2004]. This staining pattern identified the water channel protein aquaporin-4 (AQP4) as a potential target for NMO-Ig [Lennon et al., 2005]. AQP4 is the main water channel in the CNS where it is expressed predominantly in astrocytes surrounding blood vessels.

The identification of autoantibodies against AQP4 in NMO can be considered one of the most important findings in neuroinflammatory research within the last decade [Jarius and Wildemann, 2010]. The presence of this highly specific serum autoantibody marker (NMO-IgG) now allows the differentiation of NMO from MS. Not only do those antibodies serve as a biomarker, AQP4-specific autoantibodies participate actively in the disease process [Bennett et al., 2009]. The pathogenic activity of NMO-Ig could also be proven by the injection of antibodies derived from AQP4 positive patients into animals with EAE [Kinoshita et al., 2009]. Nevertheless, a pre-existing inflammatory response was found to be a prerequisite for the antibody to cross the BBB and gain access to its target in the CNS, which also goes along with the aforementioned „two-hit model“ [Bradl et al., 2009].

The finding of autoantibodies being the major pathological entity in this disease is already improving the clinical management of NMO, as it has now become apparent that strategies to reduce the circulating NMO-Ig (anti-AQP4-Ig) titer will be beneficial (reviewed in [Meinl et al., 2010]). B cell depletion with Rituximab has a beneficial effect in most patients, although it reduces anti-AQP4-Ig levels only in some of them

[Pellkofer et al., 2011] which is suggestive of an antibody-independent mode of action, potentially directly at the site of inflammation.

1.4.3 Paraneoplastic diseases

Paraneoplastic neurological disorders are defined as non-metastatic neurological complications of cancer lacking a specific etiology such as vascular, infectious, metabolic or treatment related causes [Dalmau and Rosenfeld, 2010]. Patients with paraneoplastic neuropathy were the first ones to be identified to harbor antibodies targeting neuronal epitopes. Target antigens include nuclear or cytoplasmic proteins to which antibodies only have limited access, which might be indicative of a T cell response targeting neurons. But, the number of neuronal antigens recognized by autoantibodies in patients with paraneoplastic diseases is still growing [Lancaster and Dalmau, 2012].

In a second group of antibody related brain disorders antibodies were found to target intracellular synaptic proteins, e.g. amphiphysin, that might have a direct pathogenic effect. This notion is supported by the fact that some patients with a paraneoplastic stiff-person syndrome harboring anti-amphiphysin antibodies benefit from plasmapheresis [Sommer et al., 2005]. Using a co-transfer experiment in rats, the same study also showed that isolated patient IgG to amphiphysin resulted in a dose-dependent stiffness with spasms resembling human stiff-person syndrome.

Recently, a third group of brain disorders emerged in which antibodies associated with encephalitis target cell surface or synaptic proteins, e.g. the N-methyl-D-aspartate receptor (NMDAR) or the γ-aminobutyric acid receptor-B ($GABA_B$ receptor) (reviewed in [Lancaster and Dalmau, 2012]). Another interesting example is the recent finding that patients with limbic encephalitis possess antibodies against the neuronal secreted protein leucine-rich, glioma-inactivated 1 (LG | 1) as identified by a combination of immunohistochemistry, immunoprecipitation and subsequent mass spectrometry. This reactivity had previously been attributed to voltage-gated potassium channels. One patient in this study also showed antibodies against Caspr2 [Lai et al., 2010]. Such autoantibodies provide an important diagnostic tool although many questions about those conditions yet remain unanswered, e.g. what the trigger of the autoimmune response is or how exactly the antibody response gets established in the CNS.

In summary, autoantibody studies like these give a nice example on how immunohistochemistry can serve as a first step towards the identification of autoantibodies in patients suffering from various neuroinflammatory diseases.

2 Objectives and strategy

There is increasing evidence that B cells and autoantibodies are involved in MS pathology in at least a subset of patients. However, autoantibody targets hitherto largely remain elusive.

The identification of two axo-glial proteins, neurofascin [Mathey et al., 2007] and contactin-2 [Derfuss et al., 2009], put the node of Ranvier into the spotlight as the potential „Achilles heel“ of the nerve fiber, mostly due to its accessibility.

Based on this hypothesis, the aims of this thesis were to:

(i) Study the type of damage that is caused by autoantibodies directed against the node of Ranvier, and

(ii) Identify MS patients with autoantibodies against (para)nodal components, axons, or oligodendrocytes.

To address these points, in the first part of the thesis the binding of two mAbs targeting the node was assessed *in vitro* on rat spinal cord sections. This was followed by an *in vivo* analysis of their pathological potential as autoantibodies in an animal model of MS.

In the second part, the study aimed at the identification of MS patients with autoantibodies to nodal or paranodal proteins in their serum via an immunohistochemical approach. A suitable protocol was therefore established using AQP4-positive NMO patient samples until conditions were found that resulted in the characteristic staining pattern on spinal cord cross sections. This method was then applied to longitudinal spinal cord sections. The absolutely necessary sensitivity in order to detect such reactivity was achieved by the double staining with the paranodal marker Caspr. Additionally, the use of longitudinal sections also allowed for the screening of other staining patterns that eventually revealed binding of patient serum to axons or oligodendrocytes.

3 Material and methods

3.1 Material

3.1.1 Chemicals and consumables

Chemicals were, unless specifically mentioned in the corresponding methods section, procured from the companies Merck (Darmstadt, Germany) and Sigma-Aldrich (Munich, Germany). Consumables such as pipette tips and test tubes or centrifuge tubes were purchased from either Eppendorf (Hamburg, Germany) or BD Falcon (Heidelberg, Germany). Cell-culture material was obtained from Corning (Wiesbaden, Germany) and Nunc (Langenselbold, Germany).

3.1.2 Antibodies

Table 3.1 on page 15 and Table 3.2 on page 16 summarize all the primary and secondary antibodies used in the different experiments detailed in the following methods section.

3.1.3 Patient samples

In total, 105 serum samples were analyzed. They were derived from 49 patients with MS and 56 healthy blood donors. The MS patient group consisted of 39 patients with RRMS, three patients with CIS, three patients with PPMS, and four patients with SSMS. The sera were provided by Prof. Frank Weber from the Max Planck Institute of Psychiatry. Stock solutions were stored at -80°C, working aliquots at -20°C.

Antigen	Specificity	Species	Clonality	Source	Catalogue number	Dilution	Pre-treatment
NF (A12/18.1)	rat, human	mouse	monoclonal	hybridoma in-house	n/a	1/100	n/a
Contactin-1 (Neuro-1)	rat, human	mouse	monoclonal	hybridoma ATCC	n/a	1/100	n/a
Caspr	rat	rabbit	polyclonal	kindly provided by Prof. Elior Peles	n/a	1/1000	n/a
APP	rat	mouse	monoclonal	Chemicon	MAB348	1/1000	citrate buffer
CNP	rat	mouse	monoclonal	Covance	SMI-91R	1/2000	EDTA buffer
CD68 (ED1)	rat	mouse	monoclonal	AbD Serotec	MCA341R	1/100	EDTA buffer
GFAP	rat	rabbit	polyclonal	Dako	Z0334	1/3000	EDTA buffer
Neurofilament-heavy	rat	rabbit	polyclonal	Chemicon	AB9568	1/1000	citrate buffer
Neurofilament-heavy	rat	mouse	monoclonal	Covance	SMI-310R	1/100	n/a
MOG (8.18/C5)	rat	mouse	monoclonal	hybridoma in-house	n/a	1/1000	n/a
Olig2	rat	rabbit	polyclonal	IBL	18953	1/200	n/a

Table 3.1: Summary of primary antibodies

APP: Amyloid precursor protein
ATCC: American type culture collection
Caspr: Contactin-associated protein
CD68: Cluster of differentiation 68
CNP: 2'3'-Cyclic-nucleotide 3'-phosphodiesterase
EDTA: Ethylenediaminetetraacetid acid
GFAP: Glial fibrillary acidic protein
MOG: Myelin oligodendrocyte glycoprotein
n/a: not applicable
NF: Neurofascin

Specificity	Species	Label	Source	Catalogue number	Dilution
mouse IgG (F(ab′)$_2$fragment)	goat	HRP	Jackson	115-036-072	1/1000
mouse IgG (H+L)	sheep	Biotin	GE Healthcare	RPN1001	1/200
mouse IgG (H+L)	donkey	Alexa-488	Invitrogen	A21202	1/1000
mouse IgG (H+L)	goat	Alexa-594	Invitrogen	A11015	1/1000
rabbit IgG (H+L)	donkey	Biotin	GE Healthcare	RPN1004	1/200
rabbit IgG (H+L)	donkey	Alex-594	Invitrogen	A21207	1/1000
human IgG (H+L)	goat	Alexa-488	Invitrogen	A11013	1/200
human IgG (H+L)	goat	Alexa-555	Invitrogen	A21433	1/500
human IgG (F(ab′)$_2$fragment)	goat	DyLight-488	Jackson	109-486-097	1/200

Table 3.2: Summary of secondary antibodies

F(ab′)$_2$	Fragment antigen binding
H+L	Heavy and light chain
HRP	Horseradish peroxidase
IgG	Immunoglobulin G

3.2 Methods

3.2.1 Serum absorption

In order to reduce unspecific binding, serum samples underwent pre-absorption with rabbit liver powder [Coons and Kaplan, 1950]. Sera were diluted 1/10 with 10 % FCS (fetal calf serum)/PBS (phosphate buffered saline) (Gibco, Darmstadt, Germany), which equals 540 μl 10 % FCS/PBS + 60 μL serum, followed by the addition of 40 mg of rabbit liver powder (Sigma-Aldrich, Munich, Germany). The mixture was incubated at room temperature for 1 h on a thermo shaker, followed by centrifugation at 13,000 rpm for 10 min. 25 mg of rabbit liver powder were added to the supernatant, after which the solution was incubated for 1 h and again centrifuged at 13,000 rpm for 10 min. In a last step 20 mg of rabbit liver powder were added and incubated at 4°C overnight on a thermo shaker. This was followed by incubation at room temperature for 1 h the next day. A final centrifugation at 13,000 rpm for 10 min resulted in the supernatant, which was then stored at -20°C.

3.2.2 Analysis of anti-nuclear antibodies

The presence of antinuclear antibodies (ANA) was investigated using standard serological staining methods on Hep2 and primate liver cells (Euroimmun, Lübeck, Germany). The testing was done in the Department of Clinical Chemistry at the University of Munich Medical Center Grosshadern.

3.2.3 Cell culture

The Neuro-1 (ATCC, Wesel, Germany) as well as the A12/18.1-producing mouse hybridoma cell lines were cultured in suspension in the serum-free hybridoma growth medium Hygm 6 (PromoCell, Heidelberg, Germany) at 37°C and 5 % CO_2 (carbon dioxide) after sequential adaption from media containing 10 % FCS (Gibco, Darmstadt, Germany). The media was mixed with 1 % penicillin-streptomycin (Invitrogen, Darmstadt, Germany) to avoid bacterial contamination. Cultures were maintained either by the addition of fresh media or a medium renewal after 2 to 3 days. Therefore the cell suspensions were centrifuged at 125 g for 5 min and subsequently resuspended at a concentration of $\sim 2 \times 10^5$ cells/ml growth medium.

3.2.4 Antibody purification

In order to purify the antibodies produced by the hybridoma cell lines, protein G affinity chromatography was employed according to a standard protocol. In brief, the collected supernatants were run over a protein G column (GE Healthcare, Munich, Germany) via gravity flow overnight at 4°C. After intense washing with PBS, bound IgG was eluted with 0.1 M glycine at pH 3 using fast-protein liquid chromatography (FPLC). Elute fractions of 1 mL were collected and immediately neutralized using 70 μl of 1 M Tris (pH 9).

3.2.5 Glycoprotein preparation

3.2.5.1 Preparation of myelin

Myelin was prepared according to a well-established protocol [Norton and Poduslo, 1973]. Human post mortem white matter was homogenized in 0.32 M sucrose substituted with protease inhibitors (Roche, Mannheim, Germany). The homogenate was centrifuged at 500 g for 10 min at 4°C. The supernatant was further centrifuged at 15,000 g for 30 min at 4°C. The resulting pellet was twice resuspended in 0.2 M sucrose and centrifuged at 15,000 g for 30 min at 4°C . Finally, the pellets dissolved in 0.32 M sucrose were overlaid onto 0.85 M sucrose and centrifuged at 26,000 g for 40 min at 4°C in a swing-out rotor. Myelin accumulating at the resulting interphase was collected, washed in distilled water (dH_2O), and stirred for another 30 min at 4°C. The gradient centrifugation was subsequently repeated. Myelin was again collected and washed three times in dH_2O. The myelin pellets were dissolved in 10 mM Tris (pH 8) and 2 % sodium deoxycholate. The resulting lysate was centrifuged and the supernatant dialyzed against 0.5 % sodium deoxycholate.

3.2.5.2 Preparation of grey matter proteins

Human post mortem gray matter was mixed with 100 ml of 3 % sodium deoxycholate/20 mM Tris (pH 8) containing protease inhibitor (Roche, Mannheim, Germany) and put on a rotator overnight to lyse cells at 4°C. This was followed by centrifugation at 14,000 rpm for 30 min at 4°C. The supernatant was transferred into a fresh tube and dialyzed against 5 L of 0.5 % sodium deoxycholate/20 mM Tris (pH 8) at 4°C overnight, followed by repeated centrifugation under the same conditions.

3.2.5.3 Glycoprotein purification

From the myelin and grey-matter protein preparations glycoproteins were purified separately via lentil-lectin affinity chromatography using a standard protocol and FPLC. In brief, the supernatant (myelin or grey-matter proteins, respectively) was filtered with a 0.45 μm filter (Millipore, Billerica, USA). The resulting solution was run over a lentil-lectin sepharose column (GE Healthcare, Munich, Germany). The column was washed with 0.5 % sodium deoxycholate/20 mM Tris (pH 8) until the absorption had returned to background levels. The bound glycoproteins were eluted with 0.8 M methyl-alpha-D-mannopyranoside. The eluates were dialyzed against 2 % sodium deoxycholate/20mM Tris (pH 8).

3.2.6 Gel electrophoresis, Coomassie staining and Western blot

Proteins were separated in 4 - 12 % polyacrylamide gels (Invitrogen, Darmstadt, Germany) using the NuPAGE SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) system with provided sample- and MOPS-running buffer (Invitrogen, Darmstadt, Germany). Under reducing conditions 10×-reducing buffer (Invitrogen, Darmstadt, Germany) was added to the sample. Except for the recombinant human contactin-1 (R&D Systems, Minneapolis, USA) for which 1 μg of protein was loaded, in all other cases 10 μg portions of protein were used for the separation. Markers used were the Full-Range Rainbow™ molecular weight marker (GE Healthcare, Munich, Germany) and Novex® Sharp Pre-stained protein standard (Invitrogen, Darmstadt, Germany).

For Coomassie staining, gels were incubated for 20 min in the Coomassie staining solution (0.1 % Coomassie Brilliant-Blue, 40 methanol, 10 % acetic acid) under constant shaking at room temperature. The background staining was afterwards eliminated by at least three subsequent 15 min incubations with the destaining solution (50 % methanol, 7 % acetic acid). Gels were eventually stored in 7 % acetic acid.

For Western blotting, gels were electrically transferred to a polyvinylidene difluoride (PVDF) membrane at 150 mA for 90 min using NuPAGE transfer buffer (Invitrogen, Darmstadt, Germany). After blocking in 5 % milk at room temperature for at least 2 h, primary antibodies were diluted in 2 % milk according to the antibody table (Table 3.1 on page 15) overnight at 4°C and detected with appropriate peroxidase-conjugated secondary reagents (Table 3.2 on page 16) and enhanced chemiluminescence (ECL).

3.2.7 Animal experiments

3.2.7.1 Transfer experimental autoimmune encephalomyelitis

EAE was induced by the adoptive transfer of 3×10^6 stimulated antigen-specific T cells against either MBP in Lewis rats or MOG in DA rats, via the intravenous injection into the tail vein. This was followed by the co-transfer of specific amounts of mAbs against either anti-NF (A12/18.1) or contactin-1 (Neuro-1) at disease onset after three and four-and-a-half days respectively, with amounts ranging from 100 to 500 μg, as detailed in the results section. Control animals were treated with an isotype control antibody, here IgG2b for both (eBiosciences, Frankfurt, Germany), at the particular concentration. All animal-transfer experiments were performed in collaboration with Dr. Naoto Kawakami at the Max Planck Institute of Neurobiology.

3.2.7.2 Identification of the injected antibody

To test whether the injected antibodies had bound to their target in the recipient animal, spinal cord was extracted from the sacrificed rats two days after injection of the mAbs and freshly frozen using Tissue-Tek OCT (optimal cutting temperature) mounting medium (Sakura Finetek, Alphen, The Netherlands) on dry ice. Sections of 10 μm were prepared using a Cryostat, dried for at least 2 h, and stored at -20°C until further use. For stainings, sections were fixed in 4 % PFA (paraformaldehyd)/PBS (pH 7.4) on ice for 30 min, followed by a post-fix in ice cold methanol at -20°C for 5 min. Slides were washed four times for 5 min with PBS to remove methanol traces. They were then blocked with 5 % donkey serum and 0.1 % Triton-x100 for 1 h before incubation with an Alexa-488 donkey-anti-mouse IgG secondary antibody diluted in the blocking buffer (1/1000) for 45 min at room temperature. After washing another four times for 5 min each time, the sections were incubated with the primary antibody to Caspr (1/1000) at room temperature for 1 h to localize the paranodes, followed by another washing step and subsequent incubation with the Alexa-594 anti-rabbit IgG secondary antibody mixed with the cell-nucleus marker DAPI (4',6-diamidino-2-phenylindole) (both 1/1000) for 45 min. After a final wash, the sections were mounted using Fluoromount (Dako, Hamburg, Germany).

3.2.8 Staining with hematoxylin-eosin

These histopathological examinations were performed in collaboration with Prof. Hans Lassmann at the Medical University of Vienna. For HE (hematoxylin-eosin) staining

animals were perfused with 4 % PFA/PBS (pH 7.4). The spinal cord was taken out and further fixed in 4 % PFA/PBS overnight in 4°C. Tissue blocks were embedded in paraffin and 4 μm sections were cut using a microtome. Sections underwent dewaxing by decreasing alcohol grades (xylene, 96 %, 70 % and 50 % ethanol, twice each) until a final wash in dH_2O, after which they were incubated in filtered Mayer´s hematoxylin for approximately 5 min. After a rinse in tap water, the staining was differentiated using hydrochloric acid alcohol (0.5 ml 37 % HCl in 100 ml 70 % ethanol) for 5 s in order to decolor the cytoplasm and to avoid non-specific blue background, followed by another tap water rinse and 5 min incubation with Scott´s solution (2 g $KHCO_3$ + 20 g $MgSO_4$ + 1000 ml dH_2O). The sections were rinsed with tap water, incubated in a 1 % eosin solution for 3 min, and re-rinsed. The dehydration took place with increasing alcohol concentrations (50 %, 70 % and three times 96 % ethanol, n-butyl acetate) and the slides were finally mounted in Eukitt (Sigma-Aldrich, Munich, Germany).

3.2.9 Immunohistochemistry

3.2.9.1 Standard protocol for paraffin sections

The experiment was performed in collaboration with Prof. Hans Lassmann at the Medical University of Vienna. For paraffin stainings animals were perfused with 4 % PFA/PBS (pH 7.4). The spinal cord was taken out and further fixed in 4 % PFA/PBS overnight in 4°C. Tissue blocks were embedded in paraffin and 4 μm sections were cut using a microtome. Slides were dewaxed in two portions of xylene for 20 min each, rinsed in 96 % ethanol, followed by endogenous peroxidase blocking in 0.2 % H_2O_2/methanol and another 96 % ethanol rinse. The sections were rehydrated through graded ethanol (70 % and 50 %) and washed with dH_2O. Antigens were retrieved by 1 h incubation in a household food steamer with 10 mM citric acid buffer (pH 6.0) or EDTA (pH 8.5), depending on the primary antibody (Table 3.1 on page 15) and the slides were rinsed with TBS (tris-buffered saline) after 20 min of cooling. Unspecific background reactions were blocked by 20 min incubation with 10 % FCS in a commercial blocking buffer (Dako, Hamburg, Germany). The respective primary antibodies (Table 3.1 on page 15) were diluted in the blocking buffer and applied overnight at 4°C. Slides were washed four to five times in TBS before the appropriate biotinylated secondary antibody (Table 3.2 on page 16) diluted in blocking buffer was applied for 1 h at room temperature, which was subsequently washed off with four to five changes of TBS, followed by the incubation with an avidin-peroxidase complex (Sigma-Aldrich, Munich, Germany) diluted 1/100 in blocking buffer at room tempera-

ture for 1 h. After another washing step with TBS the stain was developed using DAB (3,3′-diaminobenzidine) (Sigma-Aldrich, Munich, Germany), which was washed off with dH_2O. Slides were finally counterstained with hematoxylin, dehydrated through graded ethanol (50 %, 70 %, three times 96 %, n-butyl acetate) and mounted with Eukitt (Sigma-Aldrich, Munich, Germany).

3.2.9.2 Standard protocol for frozen sections

Spinal cord or sciatic nerves were excised from the rats and freshly frozen using Tissue-Tek OCT mounting medium (Sakura Finetek, Alphen, The Netherlands) on dry ice. Rhesus monkey spinal cord was received snap frozen from the Biomedical Primate Research Center in Rijswijk (Netherlands) and later embedded using the same mounting medium. Sections of 10 μm were prepared using a Cryostat, dried for at least 2 h and stored at $-20°C$ until further use. Sections were fixed in 4 % PFA/PBS (pH 7.4) on ice for 30 min, followed by a post-fix in ice-cold methanol at $-20°C$ for 5 min. The slides were washed four times for 5 min with PBS to remove methanol traces. They were then blocked with 5 % serum and 0.1 % Triton-x100 for 1 h. Primary antibodies (Table 3.1 on page 15) were diluted in the same blocking buffer and applied either overnight at 4°C or 1 h at room temperature, followed by four washes with PBS for at least 5 min each. The respective secondary antibodies (Table 3.2 on page 15) were diluted in the blocking buffer and incubated at room temperature for 45 min. After four further 5-min washes, the sections were incubated with the primary antibody (Table 3.1 on page 15) for double staining at room temperature for 1 h, followed by another washing step and subsequent incubation with the secondary antibody (Table 3.2 on page 15) mixed with the cell-nucleus marker DAPI (1/1000) for 45 min. After a final wash, the sections were mounted using Fluoromount (Dako, Hamburg, Germany).

3.2.9.3 Staining with absorbed human serum

For the staining with human sera, cryosections were thawed in cold PBS and fixed in 4 % PFA/PBS (pH 7.4) on ice for 30 min, followed by a post-fix in ice-cold methanol at $-20°C$ for 5 min. The slides were washed four times for 5 min with PBS to remove methanol traces and then blocked in a 40 μg/mL blocking solution of goat IgG serum (Sigma-Aldrich, Munich, Germany) in commercially available antibody diluent (Dako, Hamburg, Germany) at room temperature for at least 2 h. The previously absorbed sera were further diluted 1/20 in 10 % FCS/PBS, resulting in a final dilution of 1/200 and were then incubated overnight at 4°C, followed by a further 1 h incubation at room temperature the following day. Sections were then washed at least four

times for 15 min each time, after which the anti-human secondary antibody (Table 3.2 on page 16) was applied for 2 h, diluted 1/200 in 10 % FCS/PBS, containing 1/1000 DAPI. In case of double staining the appropriate primary and secondary antibodies were applied subsequently after an additional wash according to the standard staining protocol. Tissue sections were mounted in Fluoromount (Dako, Hamburg, Germany).

3.2.9.4 Protocol for F(ab')$_2$ fragments

Preparation of F(ab')$_2$ fragments:

For the generation of F(ab')$_2$ fragments from IgG the Thermo Scientific Pierce F(ab')$_2$ preparation kit (Thermo Scientific, Rockford, IL, USA) was utilized according to the manufacturer's instructions. The kit used immobilized pepsin, a nonspecific endopeptidase that typically produces a F(ab')$_2$ fragment of about 110 kDa by SDS-PAGE (under nonreducing conditions) and numerous small peptides of the Fc portion. The resulting F(ab')$_2$ fragment is composed of a pair of Fab' units connected by two disulfide bonds. The Fc fragment is extensively degraded and subsequently finally separated from F(ab')$_2$ by dialysis.

Staining with F(ab')$_2$ fragments:

The resulting F(ab')$_2$ fragments were used in a staining protocol similar to the one for human serum at a final concentration of 50 μg/mL. The respective secondary antibody was F(ab')$_2$-specific (Table 3.2 on page 16).

3.2.9.5 Staining of tissue from PLP-GFP mice

In order to visualize oligodendrocytes in a distinct manner, PLP-GFP mice were employed which express the green fluorescent protein under the proteolipid protein promoter [Mallon et al., 2002]. This results in the green staining of oligodendrocyte cell bodies and of some of their processes as well as a faint myelin staining. The mice were kindly provided by Prof. Martin Kerschensteiner from the University of Munich. For tissue preparation, animals were perfused with 4 % PFA/PBS (pH 7.4). The spinal cord was taken out and further fixed in 4 % PFA/PBS on ice for at least 30 min. After fixation the tissue was transferred to 30 % sucrose for cryoprotection for 2 days before freezing in Tissue-Tek OCT mounting medium (Sakura Finetek, Alphen, The Netherlands) on dry ice. Cryosections of 10 μm were cut, dried for at least 2 hours, and stored at -20°C until use.

3.2.10 Microscopy

To detect fluorescent antibody staining an Axiovert 200M (Zeiss, Jena, Germany) microscope with an HXP 120 fluorescent lamp (Visitron, Puchheim, Germany) was used that contained 10×, 20×, and 40× high chromatic correction lenses. Image acquisition and analysis was performed by the MetaMorph-Software (V7.7) and ImageJ, respectively.

For further localization analysis confocal microscopy was performed using a Leica TCS SP2 UV system (Leica Microsystems, Wetzlar, Germany). Available excitation lasers were argon, helium-neon, and also ultraviolet. Stacked series of confocal single z-planes were taken with a step size of 0.2 μm to precisely cover the area of interest in the tissue section. Sections were imaged with a pinhole of 1.0 Airy units, 512 $\times$ 512 pixel image format, and three frame averages. To avoid cross-talk between the simultaneously excitable fluorochromes used within one staining protocol, the width of the detection channels and filter settings were carefully controlled, and images were acquired using sequential image recording. For the image acquisition the accompanying LCS Lite confocal software from Leica was used. Images were analyzed using ImageJ.

4 Results

4.1 Features of monoclonal antibodies targeting the node of Ranvier

4.1.1 Nodal staining in the central and the peripheral nervous system

In the first part of the project the features of two different mAbs (one directed to NF, the other one to contactin-1) were compared in order to test the hypothesis whether the node of Ranvier can be considered as an Achilles heel of nerve fibers.

Prior to the stainings, the binding of the two mAbs was verified in a Western blot. Binding of the anti-NF mAb was examined on a human myelin glycoprotein preparation (Figure 4.1 left on page 26). NF was detected using the mAb A12/18.1. Both NF isoforms (155 kDa and 186 kDa) were detected in the highly enriched myelin glycoprotein fraction, as shown by a double band localized just above 150 kDa. This is in harmony with published features of this mAb ([Mathey et al., 2007] ; [Ng et al., 2012]). There was a faint additional band visible over 225 kDa.

The anti-contactin-1 mAb Neuro-1 [Reid et al., 1994], which had not been characterized in all details before, was tested for binding to human recombinant contactin-1 and human grey-matter glycoproteins (Figure 4.1 right on page 26). Contactin-1 has a theoretical molecular weight of 135 kDa. One distinct band at 150 kDa corresponding to contactin-1 is visible in the glycoprotein fraction, as stated by the manufacturer as the predicted molecular mass under reducing conditions. Additional bands at lower and higher molecular weight were recognized in the lane with the recombinant protein that might be based on the monomeric form of the protein, other fragments present in the preparation and/or impurities. In order to increase the resolution of the recombinant contactin-1 lane and further attribute the detected bands, future studies should analyze this protein using lower contactin-1 amounts and, using mass-spectrometry, could provide additional insight into whether, this mAb also recognizes other targets than contactin-1.

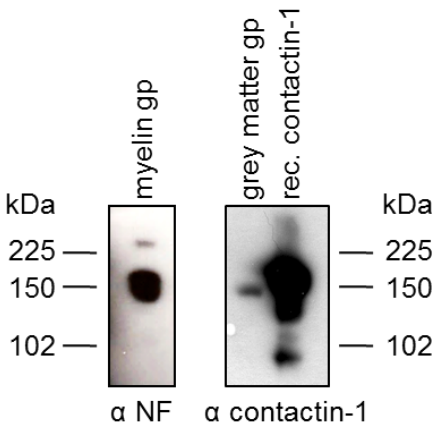

Figure 4.1: Western blot with mAbs against neurofascin and contactin-1

Figure 4.1: *Left: A human myelin glycoprotein (gp) preparation was separated by SDS-PAGE and blotted. NF was detected using the mAb A12/18.1. Both NF isoforms (155 kDa and 186 kDa) are detected in the highly enriched myelin glycoprotein fraction as represented by a double band. These are not completely resolved at the chosen exposure time to also visualize an additional unidentified band above 225 kDa. Right: Human grey-matter glycoproteins (gp) and recombinant (rec.) contactin-1 protein were run on SDS-PAGE and blotted. Contactin-1 with a predicted molecular weight of 135 kDa was detected using the mAb Neuro-1. One distinct band corresponding to contactin-1 is visible in the gp fraction. Additional bands at lower and higher molecular weight are recognized in the lane with the recombinant protein.*

For the immunohistochemical approach, in order to be able to detect reactivity towards the nodes, paranodes or juxtaparanodes, a double staining with Caspr was established. Caspr is localized on the axon in the area adjacent to the node of Ranvier and can therefore serve as a paranodal marker (see introductory Figure 1.2 on page 8). The staining was performed using a polyclonal rabbit anti-Caspr serum [Peles et al., 1997], kindly provided by Prof. Elior Peles from the Weizmann Institute of Science in Israel.

In a first step binding of the anti-NF and the anti-contactin-1 mAbs was compared using CNS as well as PNS tissue, i.e. spinal cord and sciatic nerve. The anti-NF mAb A12/18.1 stained only the node of Ranvier in the CNS and the PNS (Figure 4.2 on page 27) in harmony with already published observations [Mathey et al., 2007]. Although this mAb is known to also bind to the glial isoform NF155 when expressed on the surface of transfected cells [Ng et al., 2012], so far no staining of the paranode has been observed in immunohistochemical approaches.

Staining with the anti-contactin mAb Neuro-1 resulted in a nodal pattern in the CNS and PNS on rat tissue (Figure 4.2 on page 27). Contactin-1 was reported to be present in CNS nodes [Zonta et al., 2008] but absent in the PNS nodes, which sets our finding

in contrast to the aforementioned publication. In addition, staining of fiber structures was seen along the sciatic nerves in the PNS. Contactin-1 is an established component of the paranodal regions in the CNS and PNS, where it interacts with Caspr and NF155 [Charles et al., 2002] (see introductory Figure 1.2 on page 8). This pattern, however, could not be visualized on tissue sections even when using harsher fixation procedures such as e.g. Bouin's fixative (data not shown), which should also render shielded areas more accessible to antibody staining.

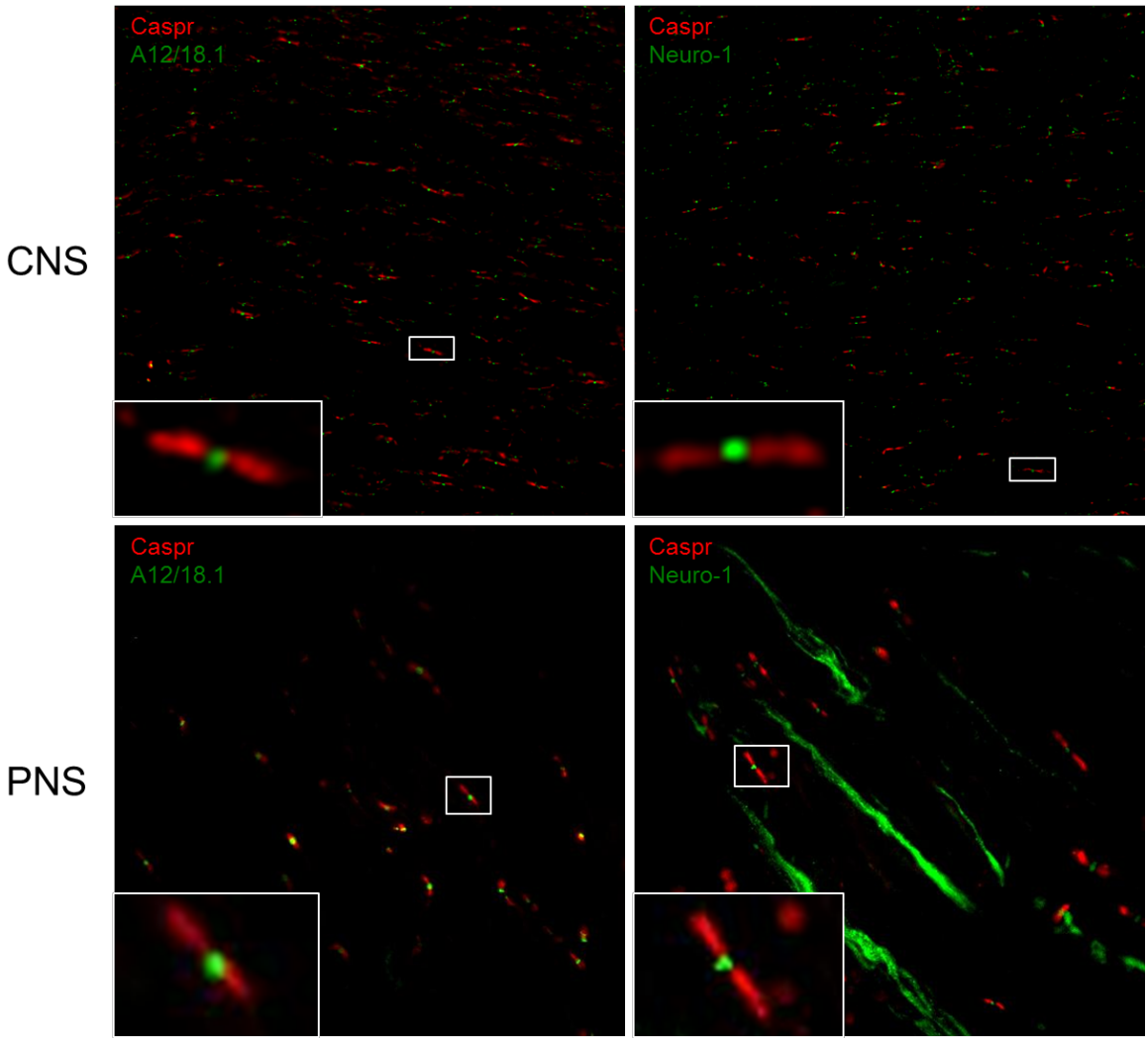

Figure 4.2: Nodal and paranodal staining in the rat CNS and PNS

Figure 4.2: *In the CNS as well as the PNS both mAbs, A12/18.1-recognizing NF and Neuro-1-recognizing contactin-1, bound to the nodes of Ranvier (green). Double staining with a polyclonal antibody against Caspr identified the paranodal domains (red). In the PNS, Neuro-1 additionally stained fibrous structures (Magnification: 10×).*

In order to test these two mAbs in an additional system that is more closely related to humans, the CNS staining was repeated on longitudinal rhesus monkey spinal cord sections (Figure 4.3 on page 28). In this case, the anti-NF mAb A12/18.1 stained neither the node nor any adjacent region, but only indefinable structures. However, a cross-reactivity of this mAb to rhesus monkey has not formally been shown yet. The anti-contactin-1 mAb, on the other hand, additionally stained the paranodes, as visualized by the colocalization with Caspr (Figure 4.3 on page 28).

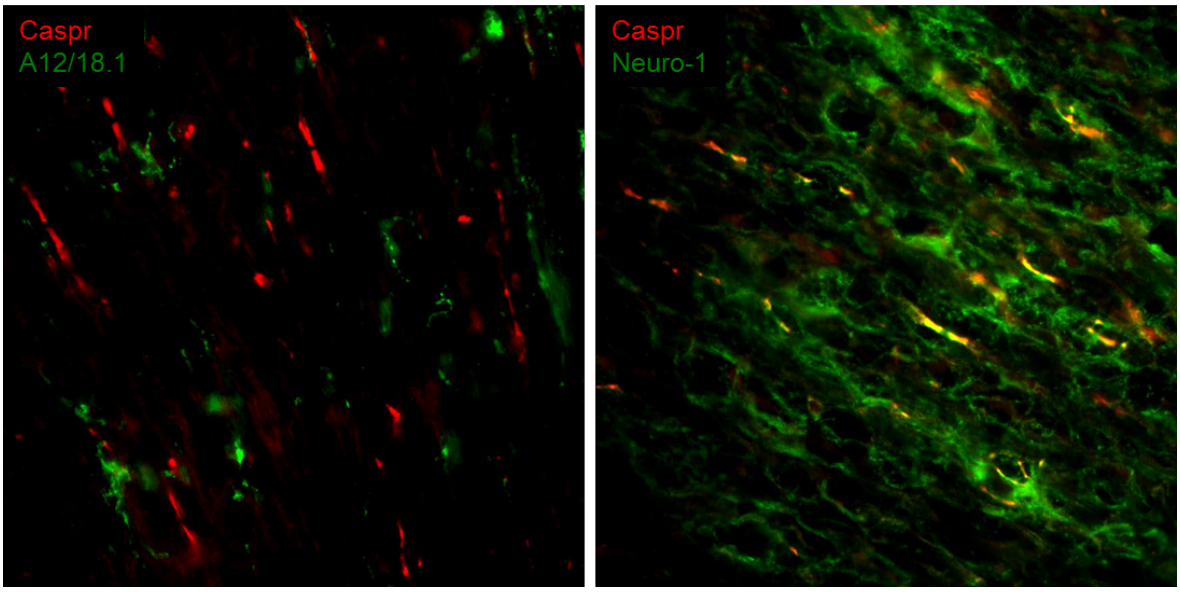

Figure 4.3: Nodal and paranodal staining in the rhesus monkey CNS

Figure 4.3: *NF and contactin-1 mAbs (green) exhibited different staining patterns on rhesus monkey longitudinal spinal cord sections. Double staining with a polyclonal antibody against Caspr identified the paranodal domains (red). In contrast to rat tissue, A12/18.1 did not stain the node of Ranvier any further, whereas the Neuro-1 additionally stained the paranodal regions as visualized by colocalization with Caspr (yellow). (Magnification: 20×)*

4.1.2 Different effects of anti-nodal antibodies on the course of experimental autoimmune encephalomyelitis

Having shown that the mAb against contactin-1 indeed stained the node of Ranvier on rat tissue sections, the second step was to investigate whether this antibody would also have an effect in EAE, an animal model of MS. Previously it had been shown that another mAb that targets the nodes, the anti-NF antibody A12/18.1, exacerbated disease and induced axonal injury [Mathey et al., 2007]. In order to better compare

possible effects of the anti-contactin mAb, the NF mAb was used as a control. Both antibodies were also compared to an isotype control antibody. Neuro-1 and A12/18.1 are both antibodies of the IgG2 isotype, differing only in their subclass of IgG2a and IgG2b, respectively. As both isotypes are considered to possess the same capability to activate the complement system [Waldmann, 1989], only one type (IgG2b) was chosen as a negative control. The EAE transfer experiments were performed in collaboration with Dr. Naoto Kawakami from the Max Planck Institute of Neurobiology. A total of twelve Lewis rats were injected with reactivated MBP-specific T cells, followed by the injection of anti-NF mAb, anti-contactin-1 mAb, or IgG2b-isotype control antibody after three days at a concentration of 4 mg/kg body weight in four animals each. As previously reported, following the injection of anti-NF mAb EAE exacerbated further (Figure 4.4A on page 30). A striking point that had not been observed before was that these animals did not recover even after ten days. In comparison, there was no influence on EAE progression after contactin-1 or control antibody injection, where the animals reached a clinical score of 2.75 and 2.38, respectively, and had completely recovered by day eight (Figure 4.4A on page 30). This finding, however, was not due to the fact that the Neuro-1 mAb did not bind *in vivo*. Both injected antibodies (A12/18.1 and Neuro-1) could be detected after the animals were sacrificed by the staining of spinal cord tissue sections with a fluorescently labeled anti-mouse secondary antibody (Figure 4.5 on page 31). It is noteworthy that at that stage the two mAbs had not been produced under the same conditions. While the anti-NF mAb-producing hybridoma had always been grown under serum-free conditions, there was no such protocol for generating the anti-contactin-1 mAb, whose medium therefore contained FCS. As FCS may contain IgG that then later would be co-purified via the protein G column, the actual amount of mAb cannot be compared directly between the two preparations. To address the possibility whether a lower concentration of Neuro-1 was the reason for the lack of any effect, the contactin-1-specific mAb was also produced under serum-free conditions. The two mAbs, both generated under the same serum-free conditions, were then compared in another EAE transfer experiment (Figure 4.4B on page 30). A total of ten Lewis rats were injected with reactivated MBP-specific T cells followed by injection of either 100 μg anti-NF mAb ($n = 2$) or two different doses of anti-contactin mAb (100 μg and 500 μg; $n = 3$ for each group). The antibody concentration of the anti-contactin-1 mAb was increased five-fold in order to reach a higher sensitivity and better identify an *in vivo* effect of this mAb. Control animals received an injection of 500 μg IgG2b-isotype control antibody. The anti-NF antibody exacerbated disease even at a low dose (100 μg), while there was no change in the disease score with the anti-contactin-1 mAb, even at a high dosage (500 μg) (Figure 4.4B on page 30).

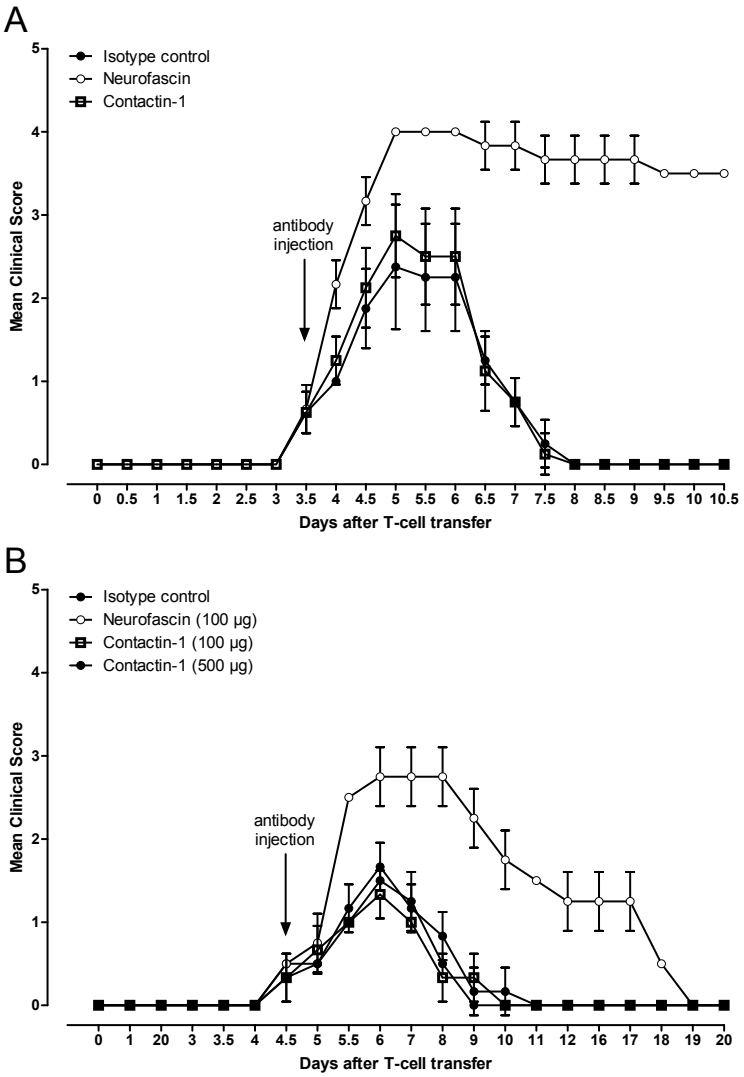

Figure 4.4: Effects of nodal antibodies on the course of EAE in Lewis rats

Figure 4.4: *Passive transfer of anti-NF antibody exacerbates adoptive transfer EAE, whereas anti-contactin-1 antibody does not. A: Lewis rats were injected with 3 × 10^6 reactivated MBP-specific T cells followed by injection of either anti-NF mAb (generated under serum-free conditions), anti-contactin-1 mAb (generated under serum-containing conditions), or IgG2b isotype control antibody on day 3 at a concentration of 4mg/kg body weight. Animals injected with anti-NF mAb reached a maximum clinical score of 4.13 +/- 0.63 and did not recover until 10.5 days after T cell transfer. In contrast, rats injected with either anti-contactin-1 or isotype control antibody reached a clinical score of 2.75 +/- 0.5 or 2.38 +/- 0.75 respectively, and had completely recovered by day 8. Data are reported as mean +/- SD (n=4). Disease exacerbation was also reflected by loss of body weight (data not shown). B: For better comparison, Lewis rats were injected with 3 × 10^6 reactivated MBP-specific T cells followed by injection of either 100 µg anti-NF mAb or 100 µg and 500 µg anti-contactin-1 mAb (both generated under serum-free conditions). Control animals did not receive an injection. Anti-NF mAb exacerbates disease even at a low dose (100 µg), while there is no change in body weight or disease score with the anti-contactin-1 mAb even after increasing the dose five-fold (500 µg).*

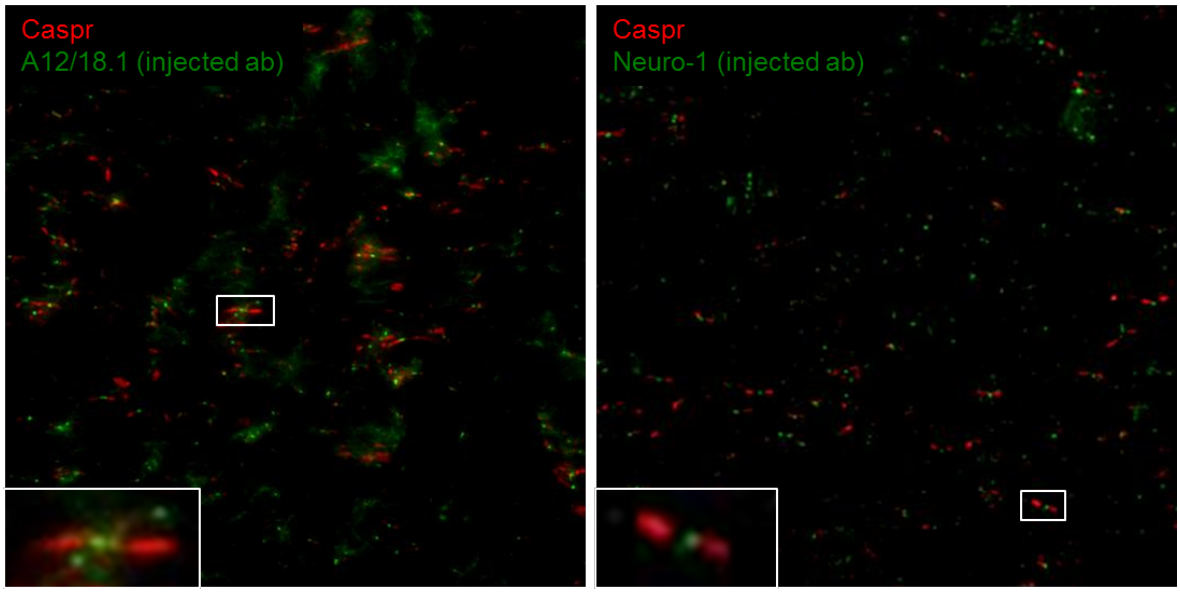

Figure 4.5: Verification of the *in vivo* binding of the injected mAbs

Figure 4.5: *The NF-specific mAb A12.18/1 and the contactin-1 mAb Neuro-1 bind selectively to the node of Ranvier in vivo in Lewis rats with EAE. Fluorescent microscopy of representative longitudinal spinal cord sections 48 h after transfer of the respective mAbs reveals binding to distinct regions of the CNS when visualized with an Alexa Fluor 488-conjugated anti-mouse IgG (green). Double staining with a polyclonal antibody against Caspr identified the paranodal domains (red) and demonstrated binding to the node of Ranvier. There was no deposition of mouse antibody in the CNS of animals injected with IgG2b isotype control antibody (not shown). (Magnification: 20×)*

4.1.3 Analysis of damage occuring with an anti–neurofascin monoclonal antibody

Because of its unusually severe effect on disease progression we wanted to further study the pathological role of the NF mAb after injection. This was done in collaboration with Prof. Hans Lassmann in Vienna. Histopathology of spinal cord seven days after transfer of either anti-NF antibody or anti-contactin-1 antibody was performed. HE staining revealed a vacuolization in the white matter and numerous neurons undergoing chromatolysis in the grey matter in rats injected with A12/18.1, whereas no changes were visible in the Neuro-1 treated animals (Figure 4.6 on page 32). Neurofilament staining revealed a massive accumulation of phosphorylated neurofilament in degenerating neurons in the grey matter as well as empty myelin tubes in the white matter, but only in those rats treated with anti-NF mAb. In addition, in these rats acute axonal damage was detected by APP staining. APP is found in neurons and undergoes transport along the axon which is interrupted in the case of axonal damage. Therefore

APP accumulates in the proximal axonal ends which results in swelling and the formation of APP positive spheroids. The rats treated with anti-NF mAb also harbored extensive infiltration by macrophages (marker ED1)(Figure 4.6 left on page 32).

It is notable that neither structural changes of the nervous system nor immune cell infiltrates were detected in animals treated with the anti-contactin-1 mAb (Figure 4.6 right on page 32).

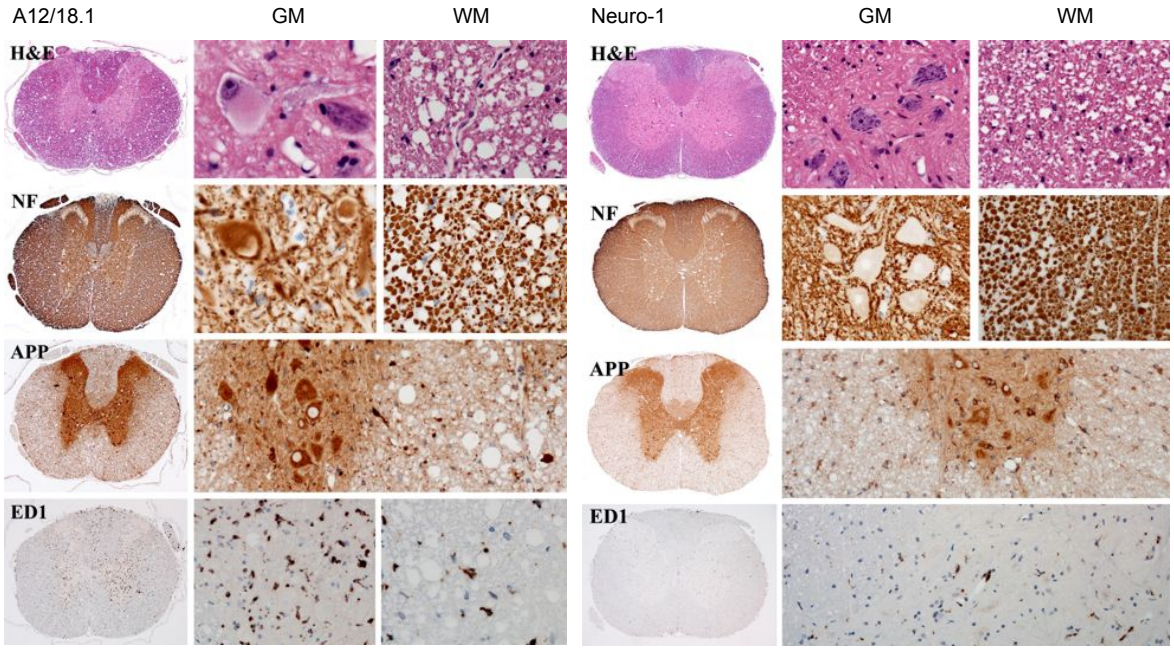

Figure 4.6: Pathology associated with antibody-mediated injury in transfer EAE

Figure 4.6: *Representative histopathology of spinal cord seven days after transfer of either A12/18.1 (left) or Neuro-1 (right). Hematoxylin and eosin staining (H&E) revealed a vacuolization in the white matter (WM) and numerous neurons undergoing chromatolysis in the grey matter (GM) in the rats injected with A12/18.1, whereas no changes were visible in the Neuro-1 treated animal. NF, APP, and ED1 were stained brown by immunohistochemistry, cell nuclei were counterstained blue using hematoxylin. NF staining revealed a massive accumulation of phosphorylated neurofilament in degenerating neurons in GM as well as empty myelin tubes in the WM only in the rats treated with A12/18.1. In addition these rats were afflicted with acute axonal injury or swelling (axonal spheroids) as visualized by APP staining. Also they harbored extensive infiltration by macrophages (ED1). Neither structural changes of the nervous system nor immune cell infiltrates could be detected in animals treated with Neuro-1. (Unseparated pictures contain both WM and GM.)*

A detailed look at the pathology induced by the anti-NF mAb seven days after antibody injection revealed axonal spheroids accumulating in two regions within the posterior funiculus via APP staining: dorsal-subpial and even more in the pyramidal tract (Figure 4.7 top left on page 33). A longitudinal spinal cord section stained for neurofilament nicely represented the nerve-fiber degeneration (Figure 4.7 top right on page 33).

The neurofilament cross-section showed axonal loss at the dorsal surface but to an even greater extent selectively in the pyramidal tract (Figure 4.7 bottom left on page 33).

The staining for CNP confirmed the presence of empty myelin tubes in the white matter, while the GFAP pattern showed an astrocytic gliosis throughout the whole spinal cord (Figure 4.7 bottom middle and right on page 33).

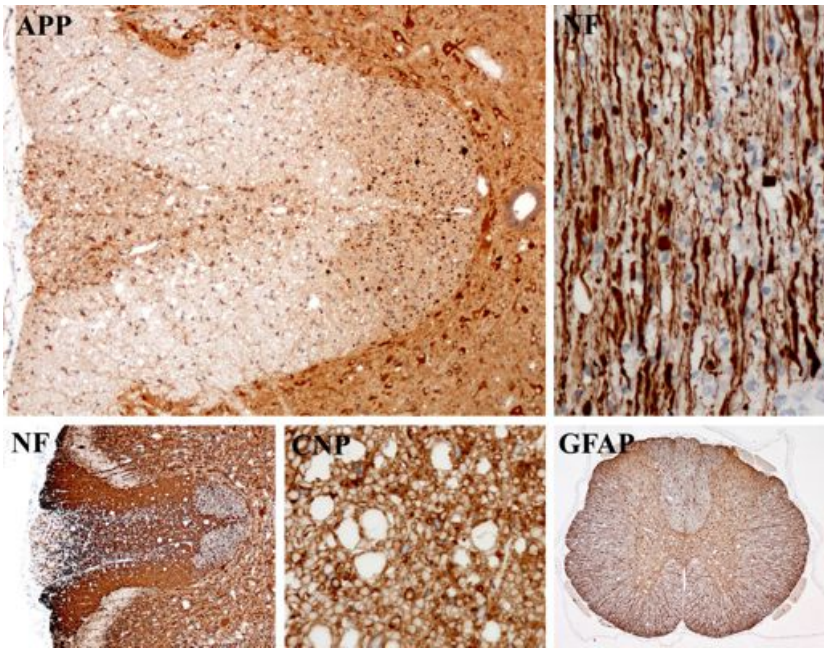

Figure 4.7: Detailed look on the pathology induced by the neurofascin-targeting mAb

Figure 4.7: *Axonal injury (APP), neurofilament (NF), myelin (CNP), and astrocytes (GFAP) were visualized via immunohistochemistry (brown) seven days after the injection of A12/18.1; cell nuclei were counterstained using hematoxylin (blue). The APP stain illustrated axonal spheroids accumulating in two regions within the posterior funiculus: dorsal-subpial and even more in the pyramidal tract. A longitudinal spinal cord section stained for neurofilament nicely represented the nerve-fiber degeneration. The neurofilament cross-section showed axonal loss at the dorsal surface but to an even greater extent selectively in the pyramidal tract. The staining for CNP confirmed the presence of empty myelin tubes. The intense staining of GFAP was indicative of reactive astrocytes/astrocytic gliosis throughout the whole spinal cord.*

Summarizing the distribution of the aforementioned staining patterns, the anti-NF antibody seemed to induce pathology preferentially localized in the corticospinal tract (CST) (compare with corticospinal tract localization shown in Figure 4.8 on page 34).

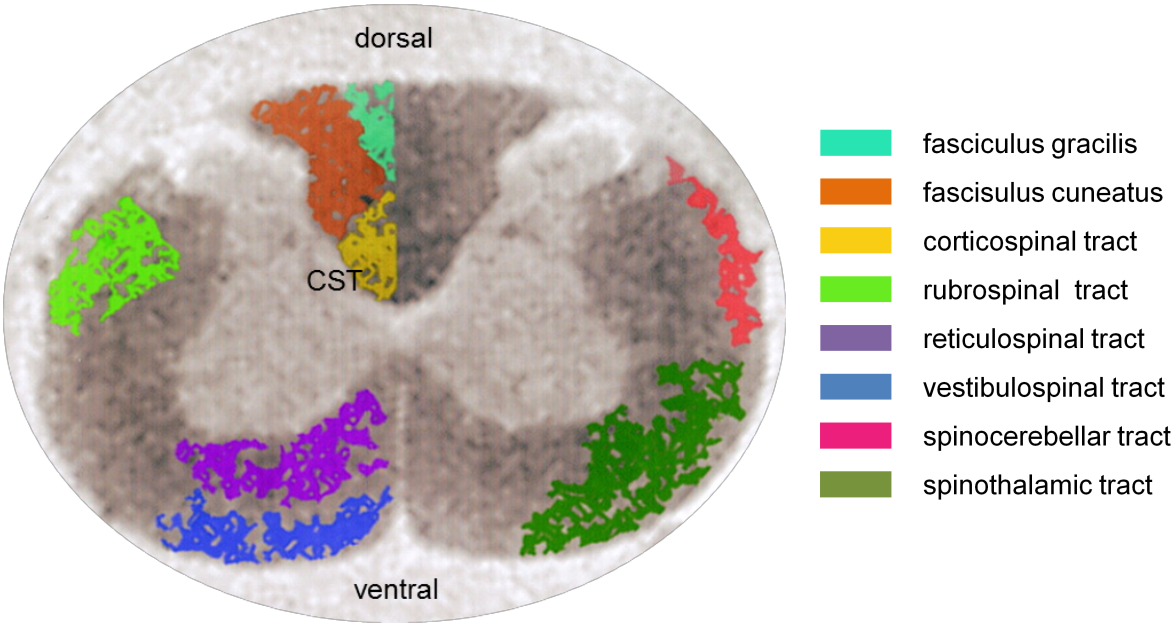

Figure 4.8: Schematic representation of the rat spinal cord cross-section

Figure 4.8: *Schematic diagram of major ascending and descending tracts in the rat spinal cord overlaid on an image of a rat spinal cord cross-section. The location of the region of interest, the corticospinal tract (CST), differs between species. In the human, the CST is located in the lateral white matter, while in the rat it is located in the dorsal white matter (adapted from [Schwartz et al., 2005]).*

To address the question why this effect had not been described in the aforementioned study on NF as a potential target for autoantibodies ([Mathey et al., 2007], the experiment was repeated in the EAE model in the DA rats previously used. Passive transfer of the anti-NF mAb A12/18.1 at day 4.5 exacerbated adoptive transfer EAE up to a maximum clinical score of 3.00 as seen in all three animals. Recovery took place until day 14 after T cell transfer (Figure 4.9A on page 35). The three rats injected with isotype control antibody reached a clinical score of 0.67 and had already completely recovered by day 6 (Figure 4.9A on page 35). Staining for neurofilament (Figure 4.9B on page 35) revealed an accumulation of phosphorylated neurofilament in the anterior horn cells (top) of the anti-NF mAb treated animal, but no changes in the pyramidal tract (bottom). The ED1 pattern showed a slightly higher infiltration by macrophages in anti-NF mAb treated rats. Note that there was no axonal injury in either of the two groups as visualized by an APP staining (Figure 4.9 bottom on page 35).

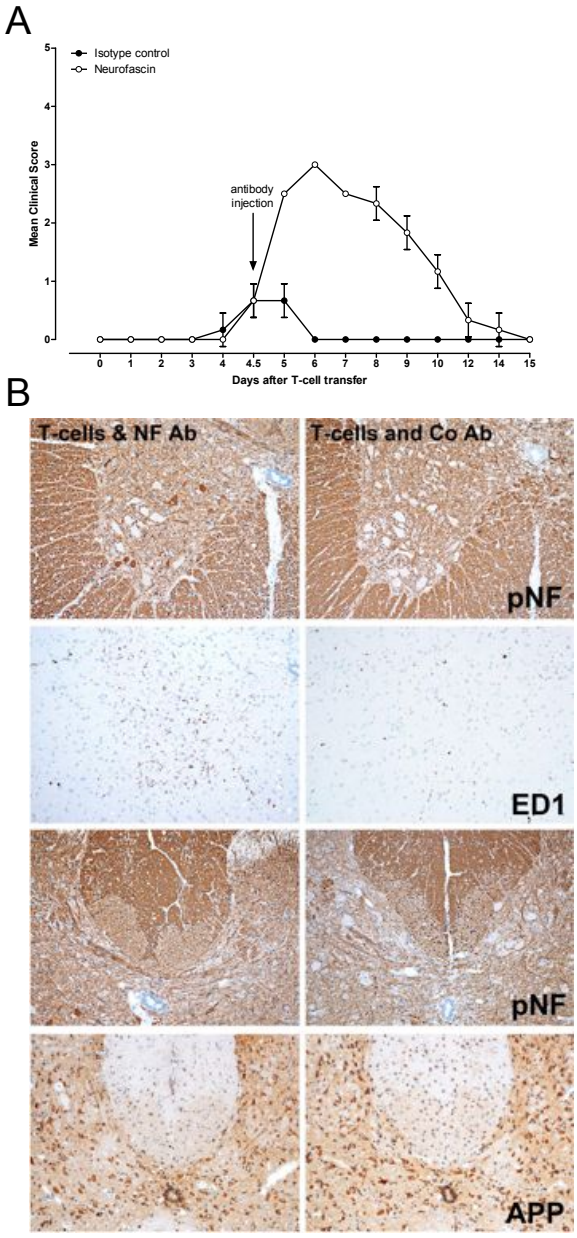

Figure 4.9: Effects of the anti-neurofascin mAb on the course of EAE in DA rats

Figure 4.9: *Passive transfer of A12/18.1 exacerbated adoptive transfer EAE, but did not induce a strikingly different pathology compared to isotype control antibody in this EAE model. A: DA rats were injected with 3 $\times$ 10^6 reactivated MOG-specific T cells, followed by injection of either anti-neurofascin mAb (left) or IgG2b isotype control antibody (right) after 4.5 days at a concentration of 4mg/kg body weight. Animals injected with A12/18.1 reached a maximum clinical score of 3.00 +/- 0.00 and recovered until day 14 after T cell transfer. In contrast, rats injected with isotype control antibody reached a clinical score of 0.67 +/- 0.29 and had already completely recovered by day 6. Data are reported as mean +/- SD (n=3). B: NF, ED1, and APP were stained brown by immunohistochemistry, cell nuclei were counterstained blue using hematoxylin. Staining for NF revealed an accumulation of phosphorylated neurofilament in the anterior horn cells (top) of the anti-neurofascin mAb-treated animal but no changes in the pyramidal tract (bottom). The ED1 pattern showed a slightly higher infiltration by macrophages. Note that there were no differences on axonal injury (APP) between the two groups.*

Summarizing this first part, the anti-NF mAb A12/18.1, injected in an EAE model in the Lewis rat, exacerbated disease and induced a pathology preferentially localized in the corticospinal tract. In contrast, the second mAb, Neuro-1, which also targeted the node *in vitro* and *in vivo* and possesses comparable complement-activating features, had no effect at all. The preferential damage of the motor system with A12/18.1 could not be seen in a second rat strain (DA) that had previously been used in studies into NF's *in vivo* effects.

4.2 Immunohistochemical analysis of multiple sclerosis sera

4.2.1 Establishing a staining protocol for human sera on rat tissue

The goal of the second part of the project was to identify patients that harbor autoantibodies to nodes, paranodes, and other glial or axonal structures. In order to do so a suitable staining procedure for human sera on rat tissue sections needed to be established. To find the best working conditions the protocol was set up using serum samples from AQP4-positive NMO patients that possess a typical staining pattern on spinal cord cross-sections. Figure 4.10 on page 37 shows one exemplary spinal cord cross-section in which the staining of the glia limitans as well as a perivascular staining was achieved by a multistep absorption of patient serum with rabbit liver powder and a final sample dilution of 1/200 (see methods section for details).

4.2.2 Screening of multiple sclerosis sera

4.2.2.1 Recognition of nodes and paranodes

The previously established conditions could then be applied to longitudinal rat spinal cord sections. In order to visualize the potential binding to the nodes of Ranvier, a double staining with Caspr was performed in parallel (Figure 4.11 on page 37). A total of 49 MS sera were tested. For comparison the staining was also performed with seven NMO and 20 healthy control sera, all of them on cross-sections as well as longitudinal spinal cord sections. While the cross-sections stained with NMO samples served as an internal control for the staining procedure, no specific staining pattern could be detected with MS sera on spinal cord cross-sections (data not shown). Using the longitudinal sections, no reactivity against either the node of Ranvier or the adjacent paranode was observed in either sample group.

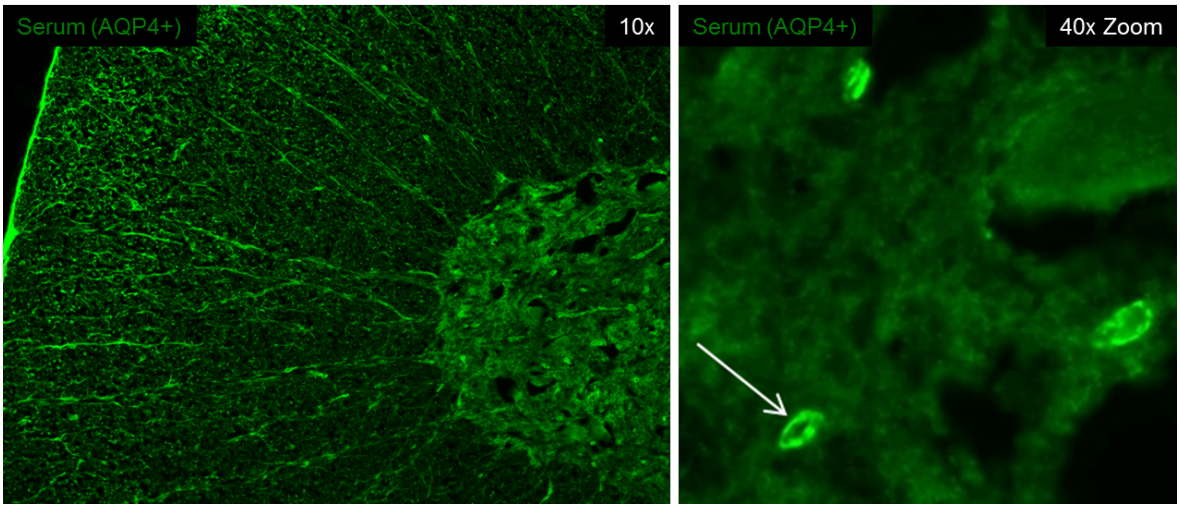

Figure 4.10: Staining of rat spinal cord cross-sections with AQP4-positive NMO sera

Figure 4.10: *Establishing a staining protocol for human sera on rat tissue with serum samples from NMO patients. Rat spinal cord cross-sections (Lewis rat; three months old) were stained with AQP4+ serum samples from NMO patients using an Alexa Fluor 488-conjugated anti-human IgG (green). Note the AQP4 typical pattern of the glia limitans. In the zoom-in of the 40× magnification also the perivascular staining can be appreciated (arrow).*

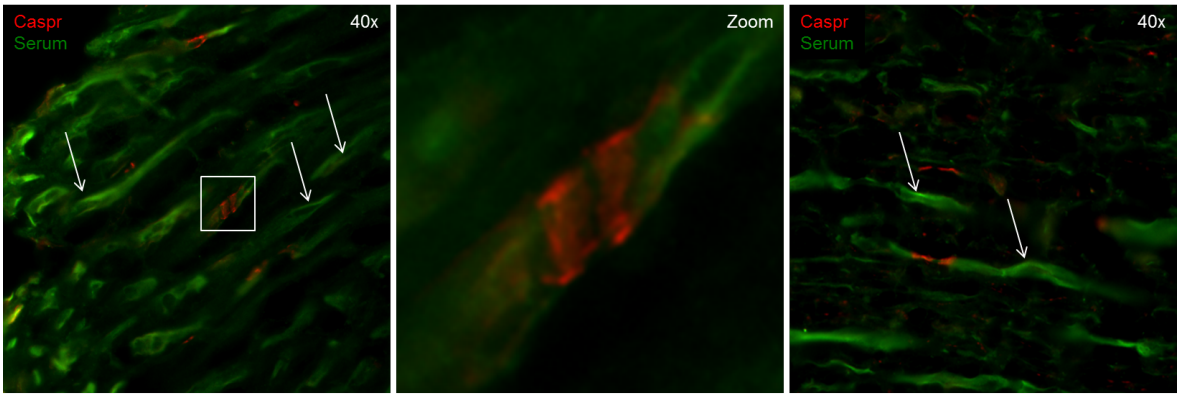

Figure 4.11: Staining of longitudinal rat spinal cord sections with MS sera (I)

Figure 4.11: *A subset of MS patient sera recognize fiber structures, shown are two examples. Rat longitudinal spinal cord sections were stained according to the previously established protocol with serum samples from MS patients using an Alexa Fluor 488-conjugated anti-human IgG (green). Double staining with a polyclonal antibody against Caspr identified the paranodal domains (red) and demonstrated binding adjacent to the paranodes (see zoom). No staining is observed at the node of Ranvier directly. Note the myelin typical structure resembling railroad tracks (arrows).*

4.2.2.2 Recognition of nerve fibers

One pattern was recognized that started adjacent to the Caspr paranodal staining and was evident along the axon (Figure 4.11 on page 37). This pattern was seen in MS samples as well as controls without any apparent varying incidence. Double staining with a myelin marker (MOG) and an axonal marker (neurofilament) was performed to identify the structure that was bound by the patient antibody. The four most intensely staining patient samples were selected for the double-staining experiments and all four colocalized with neurofilament but not with myelin (Figure 4.12 on page 38). This double staining was not performed with control samples. In summary, four out of 49 MS patients provably recognized nerve fibers.

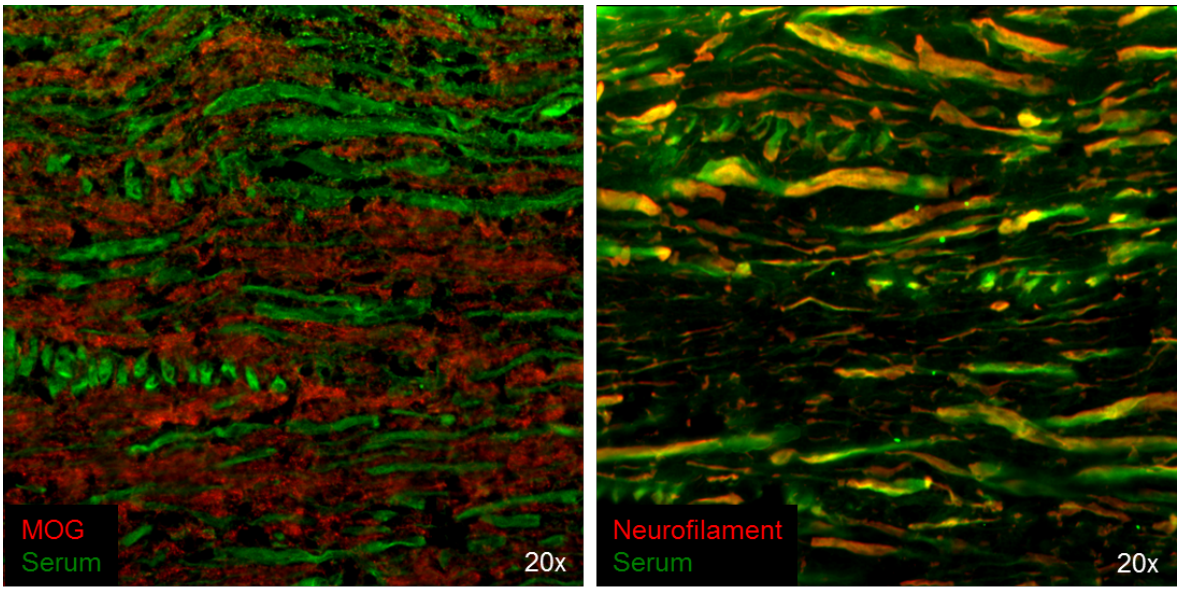

Figure 4.12: Double staining of MS patient sera with markers for axons and myelin

Figure 4.12: *Serum staining of a selected MS patient does not colocalize with myelin but with axons. Rat longitudinal spinal cord sections were stained with serum samples from MS patients using an Alexa Fluor 488-conjugated anti-human IgG (green). Double staining with monoclonal mouse antibodies against either MOG or neurofilament (both in red) show that the pattern does not colocalize with myelin but partially overlaps with the axons.*

4.2.2.3 Recognition of oligodendrocytes

4.2.2.3.1 Verification with double staining A second interesting pattern resembled the staining of oligodendrocyte chains (Figure 4.13 on page 39). A double staining with an oligodendrocyte marker (Olig 2) was performed in order to prove whether the

observed staining was specific for this cell type. The serum staining nicely colocalized with the Olig2 pattern in five out of five selected patient samples. One example is shown in Figure 4.14 on page 39. As expected, staining with human serum also lead to unspecific background binding. Also, the recognition of other cell types cannot be excluded.

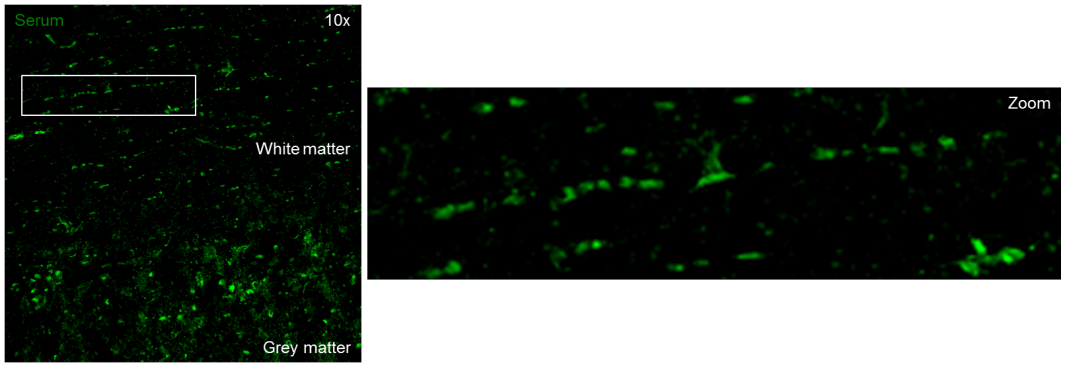

Figure 4.13: Staining of longitudinal rat spinal cord sections with MS sera (II)

Figure 4.13: *A staining pattern reminiscent of oligodendrocyte chains is observed in another subset of MS patients. Rat longitudinal spinal cord sections were stained with serum samples from MS patients using an Alexa Fluor 488-conjugated anti-human IgG (green).*

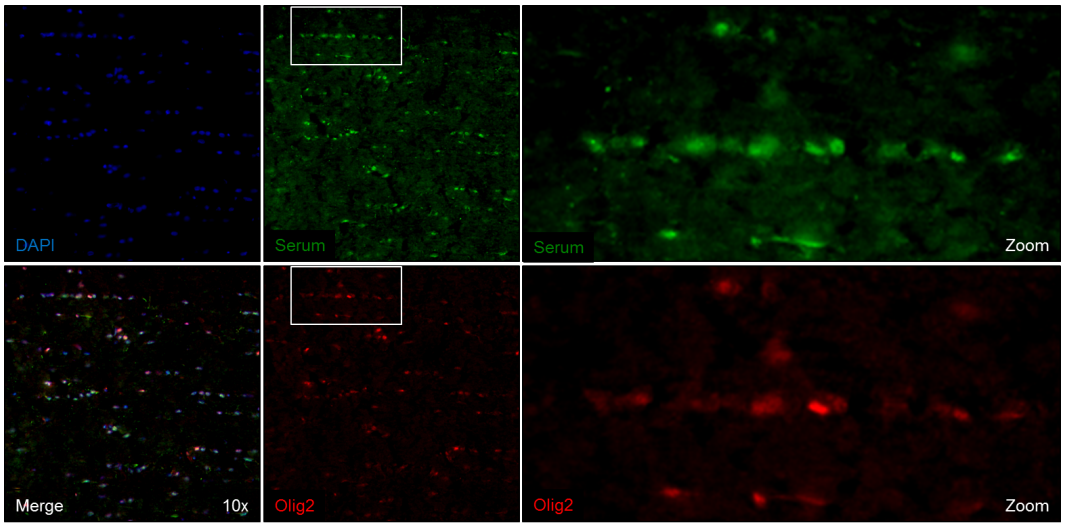

Figure 4.14: Double staining of MS patient sera with an oligodendrocyte marker

Figure 4.14: *Serum samples from MS patients can stain oligodendrocytes. Rat longitudinal spinal cord sections were stained with sera using an Alexa Fluor 488-conjugated anti-human IgG (green). Double staining with the oligodendrocyte marker Olig2 (red) showed colocalization of the two signals. Cell nuclei were visualized via DAPI (blue).*

4.2.2.3.2 Generation of and staining with F(ab')$_2$ fragments Oligodendrocyte staining patterns had already been described in MS serum samples over 30 years ago, but were claimed to be nonspecific [Traugott et al., 1979]. This was based on the observation that F(ab')$_2$ fragments produced from patient antibodies did no longer bind to oligodendrocytes. As the experimental setup in that publication was not properly controlled by a suitable F(ab')$_2$ fragment-specific secondary antibody and far higher serum concentrations were used (undiluted up to 1/128 for one sample), we decided to test patient sera for their oligodendrocyte-binding specificity. Therefore F(ab')$_2$ fragments of five selected sera were generated. The procedure was controlled by SDS gel electrophoresis, in which the molecular weight reduction from 150 kDa for a complete antibody to 110 kDa for a F(ab')$_2$ fragment was observed (Figure 4.15A on page 41). It could then be shown that those F(ab')$_2$ fragments also stained oligodendrocytes when compared to staining with complete IgG (Figure 4.15B on page 41). This means that the staining of oligodendrocytes by patient serum observed in our study is not unspecifically mediated by Fc receptors, but instead is due to a specific antibody-antigen interaction.

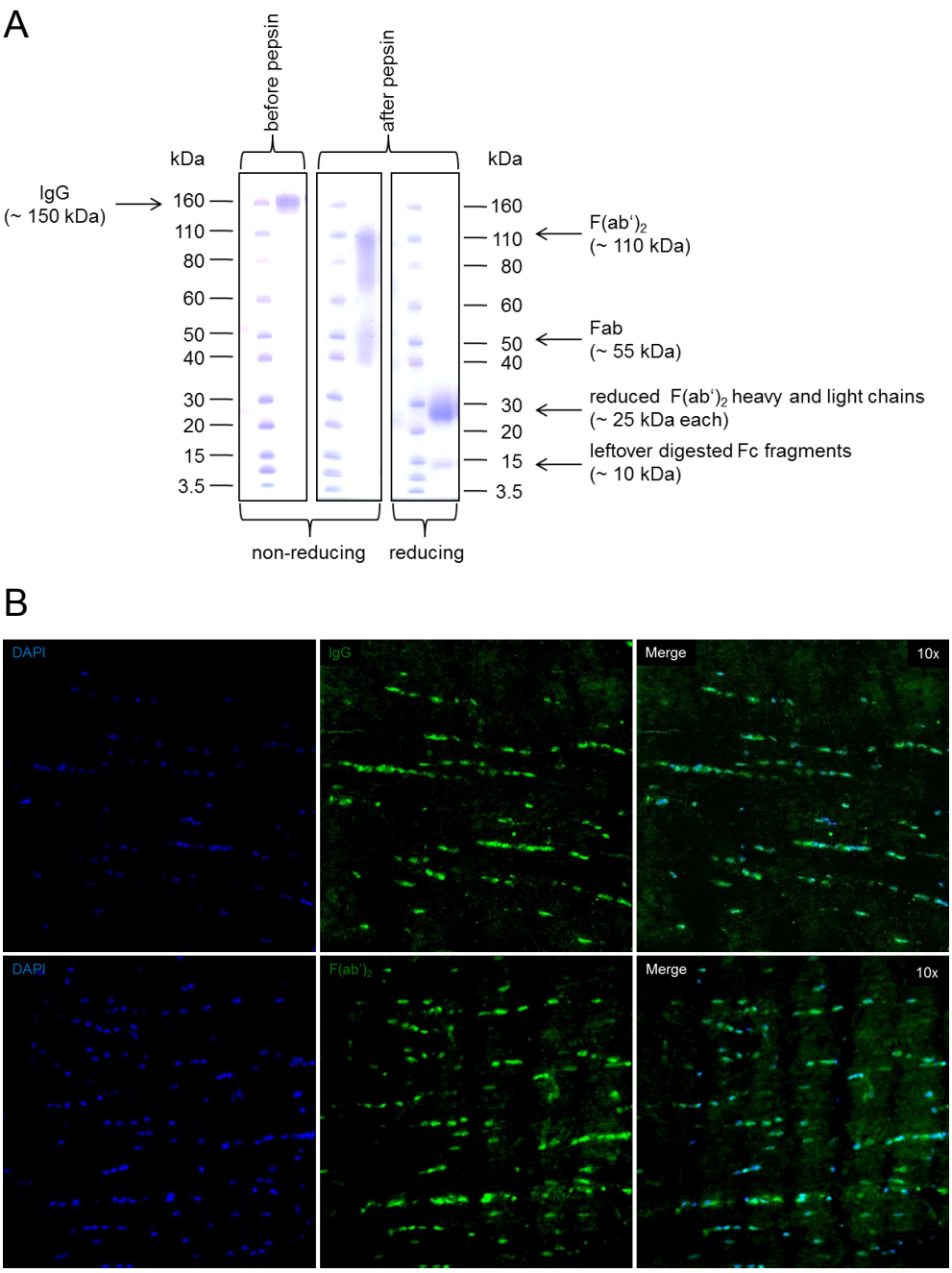

Figure 4.15: Generation of and staining with F(ab')$_2$ fragments from MS sera

Figure 4.15: *F(ab')$_2$ fragments were generated from five selected patients harboring the oligodendrocyte pattern and tested for their staining specificity. A: F(ab')$_2$ fragments generated out of isolated IgG showed a reduced molecular weight of 110 kDa when compared to whole IgG (150 kDa). The purification was complete as there is no band at 150 kDa left. The Fc part could be eliminated, as shown by the lack of a band for heavy chains (∼50 kDa) under reducing conditions. B: F(ab')$_2$ fragments of selected sera also stained oligodendrocytes. Rat longitudinal spinal cord sections were stained using a DyLight 488-conjugated anti-human-F(ab')$_2$ antibody (green). Cell nuclei are depicted in blue (DAPI). All five patients still recognized oligodendrocytes when compared to the pattern resulting with whole IgG (one example shown).*

4.2.2.3.3 Quantitative evaluation We further wanted to investigate whether the finding of oligodendrocyte staining was MS-specific. For that reason a total of 105 MS patient and control sera were tested in a blinded manner. The oligodendrocyte staining was present in 12 out of 56 control and 21 out of 49 MS patient sera ($p<0.05$; two-sided Fisher's exact test) (Figure 4.16 on page 42).

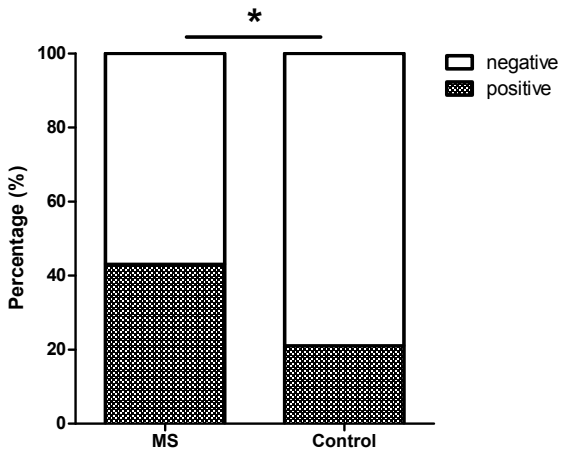

Figure 4.16: Quantitative evaluation of the oligodendrocyte pattern

Figure 4.16: *For quantitative analysis a total of 105 patient and control sera were tested in a blinded manner on rat longitudinal spinal cord sections. The oligodendrocyte staining was present in 12 out of 56 control sera (∼21 %) and 21 out of 49 MS patient sera (∼43 %). This difference was statistically significant by two-sided Fisher's exact test ($p < 0.05$).*

4.2.2.3.4 Subcellular localization In an attempt to distinguish between the control and MS-patient pattern, we intended to look into the subcellular localization of the oligodendrocyte staining. For this analysis by confocal microscopy the eight most intensely stained samples were selected (five patients, three controls). Confocal microscopy revealed a staining of oligodendrocyte nuclei in all eight samples (one example shown in Figure 4.17 on page 43). This might superimpose with the presence of ANAs that were also found to be present with low titers (1/240) in two of three controls and two of five MS patients (data not shown).

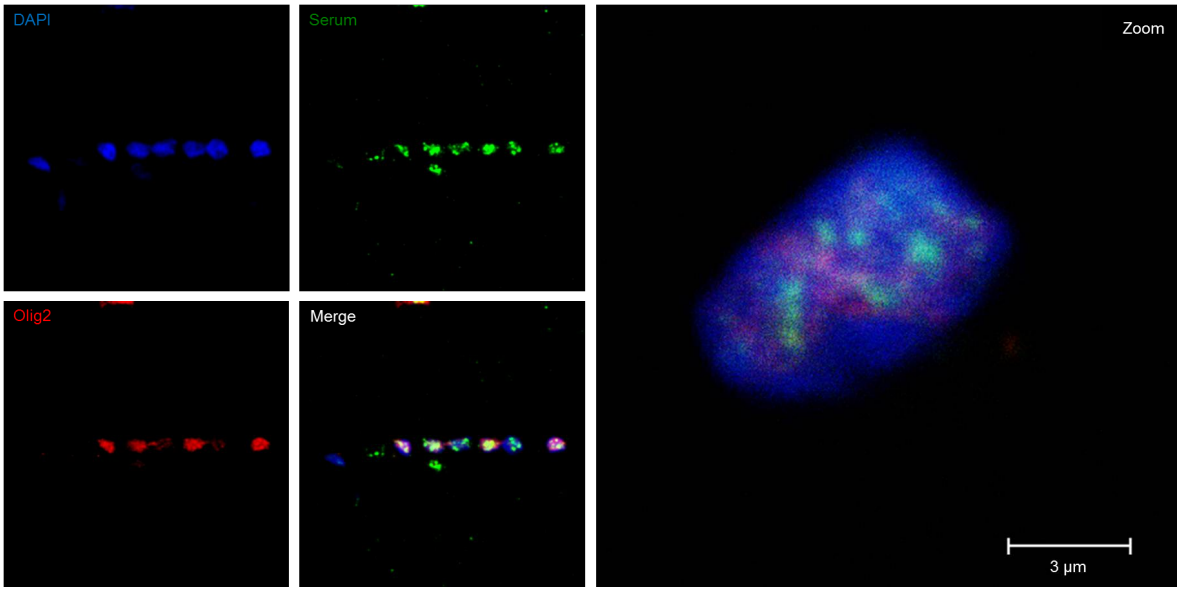

Figure 4.17: Subcellular localization of the oligodendrocyte pattern (I)

Figure 4.17: *Confocal microscopy revealed staining of oligodendrocyte nuclei. Rat longitudinal spinal cord sections were stained with serum samples from eight selected MS patients and controls using an Alexa Fluor 488-conjugated anti-human IgG (green). Oligodendrocytes were visualized via double staining with Olig2 (red), cell nuclei with DAPI (blue). The nuclear pattern was present in all eight selected samples (five MS, three controls).*

Interestingly, a staining of the oligodendrocyte surface and potentially processes could be observed in one out of five investigated patient cases (Figure 4.18 on page 44). This pattern was not seen in any of the three controls, which makes this patient an interesting case for further investigation. The patient sample was therefore also tested on P7 rat tissue to identify any differences due to the developmental stage. Here it also stained oligodendrocytes as well as some processes whose origins further needed to be confirmed (Figure 4.19 on page 45). One possible tool to do this are PLP-GFP mice, in which oligodendrocytes are labeled green directly via the expression of GFP under the PLP promoter [Mallon et al., 2002]. Staining of spinal cord sections derived from those mice with the patient sample also resulted in the staining of oligodendrocyte cell bodies (Figure 4.20 on page 46). Unfortunately oligodendrocyte processes did not appear green in this system, which further complicated the unambiguous attribution of the process staining.

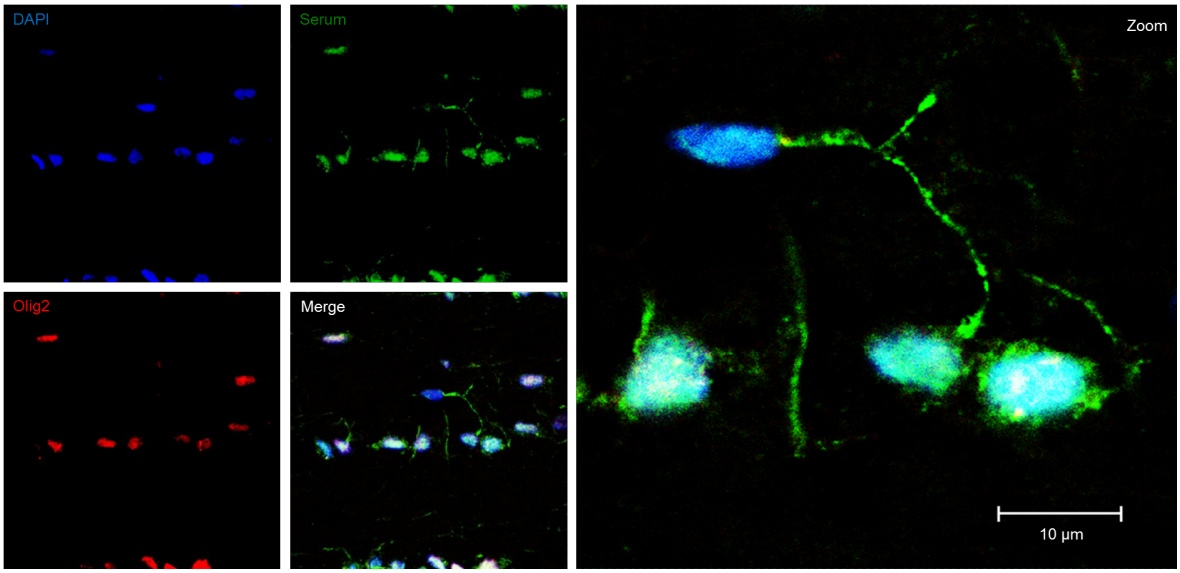

Figure 4.18: Subcellular localization of the oligodendrocyte pattern (II)

Figure 4.18: *Confocal microscopy showed additional staining of oligodendrocyte surface and supposedly processes. Rat longitudinal spinal cord sections were stained with serum samples from eight selected MS patients and controls using an Alexa Fluor 488-conjugated anti-human IgG (green). Oligodendrocytes were visualized via double staining with Olig2 (red), cell nuclei with DAPI (blue). The processes were observed in one out of five investigated patient cases and in none of the controls.*

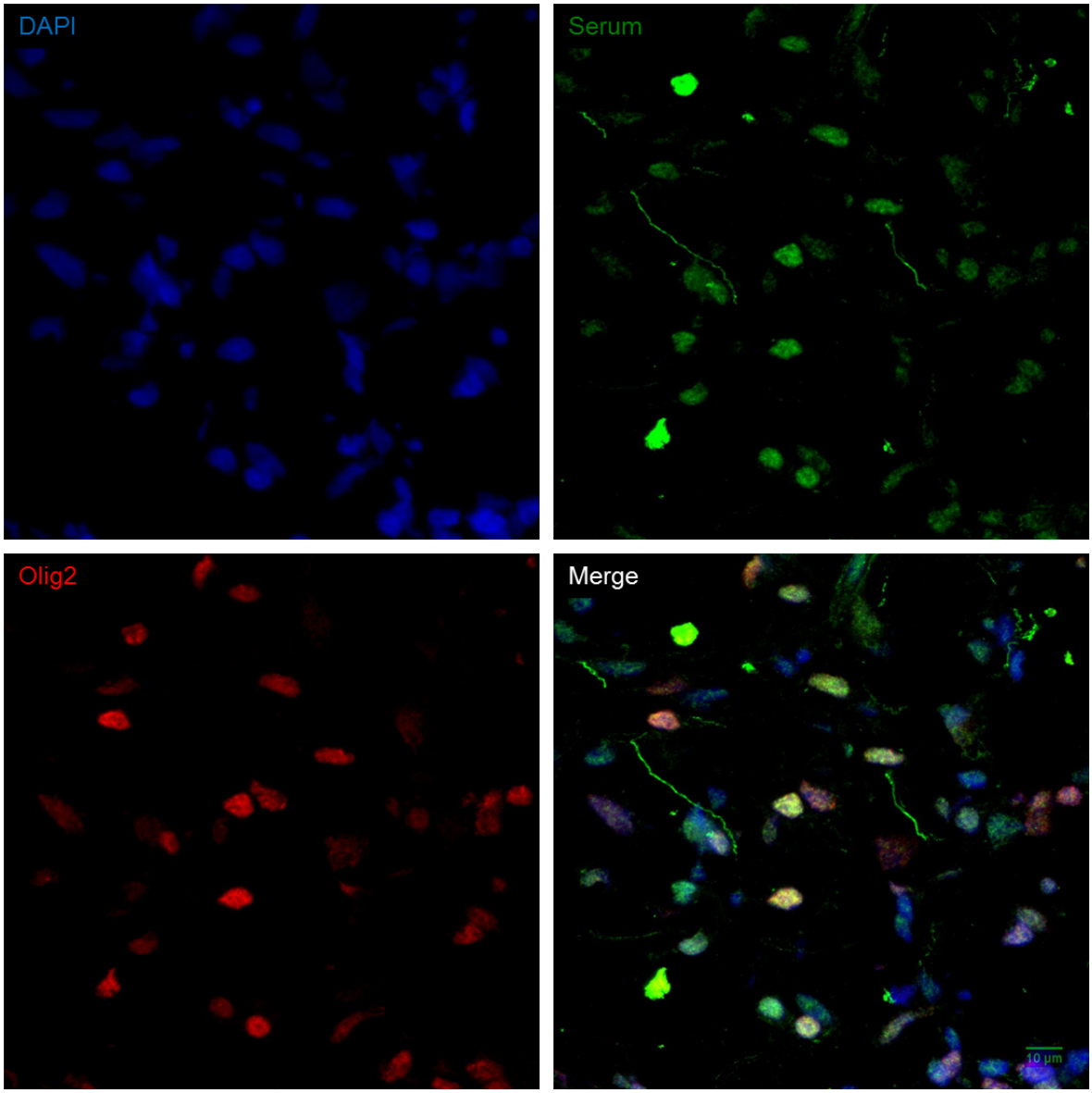

Figure 4.19: Staining of P7 rat tissue with selected MS serum

Figure 4.19: *The selected serum also stained oligodendrocytes and their surfaces on P7 rat tissue. Longitudinal spinal cord sections from seven-day-old Lewis rats were stained using an Alexa Fluor 488-conjugated anti-human IgG (green). Oligodendrocytes were visualized via double staining with Olig2 (red), cell nuclei with DAPI (blue). In addition some processes were also recognized at this developmental stage.*

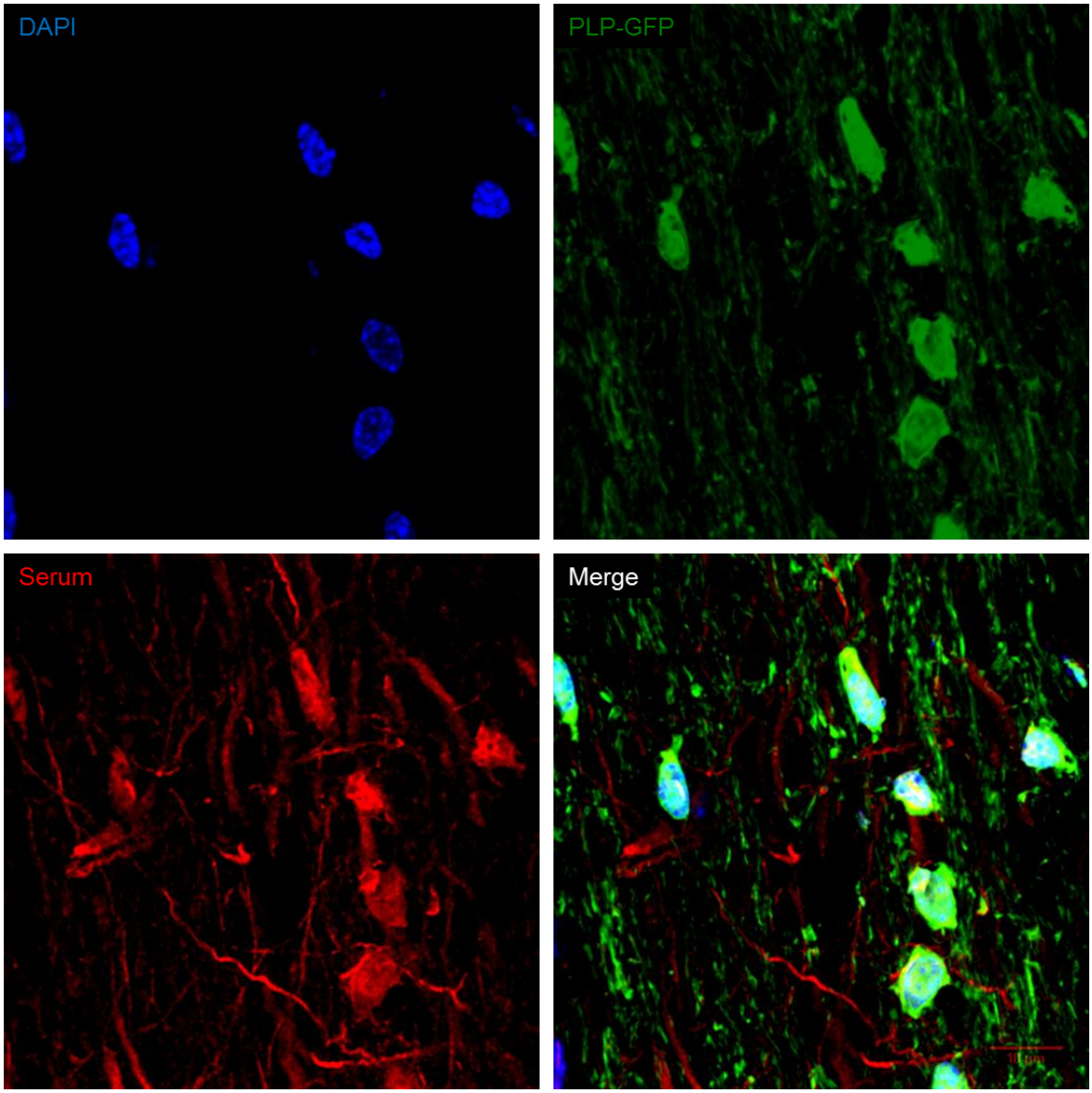

Figure 4.20: Staining of PLP-GFP mouse tissue with selected MS serum

Figure 4.20: *The selected serum also stained oligodendrocytes and additional processes on PLP-GFP mouse tissue. Longitudinal spinal cord sections from six-week-old PLP-GFP mice were stained using an Alexa Fluor 555-conjugated anti-human IgG (red). Oligodendrocytes were visualized via the expression of GFP under the PLP promoter, as was myelin in a less intense manner (green). Cell nuclei are stained by DAPI (blue). Myelin was not stained by this patient sample.*

In summary, one sample out of 49 tested MS patients harbored an interesting staining on the surface of oligodendrocytes, possibly involving processes, which was not detected in any of the 56 control samples tested. This particular patient could therefore be considered a promising candidate for further investigation of the antigen responsible for that reactivity.

5 Discussion

5.1 The node of Ranvier as Achilles heel

In the first part of this thesis, the potential role of the node of Ranvier as an antibody target (or „Achilles heel“) has been assessed. Therefore, the pathogenic features of two different mAbs targeting the node of Ranvier were investigated. The already well characterized pan-NF antibody A12/18.1 binds both isoforms of the protein, NF186 localized directly at the node of Ranvier and NF155 in the paranodal domain, as shown in a cell-based assay [Ng et al., 2012] as well as by Western blot in this work. However, in our staining protocol applied to rat spinal cord sections or sciatic nerve the antibody only binds to the node, as there was no colocalization with the paranodal marker Caspr detectable. This staining pattern is the same in the CNS as well as the PNS.

The second mAb Neuro-1 recognizes contactin-1 [Reid et al., 1994]. Binding of contactin-1 was verified by Western blot with the recombinant protein and a grey matter glycoprotein fraction. Contactin-1 was originally found in the paranodal region ([Menegoz et al., 1997]; [Peles et al., 1997]) but was published to also be a characteristic component of CNS nodes, at the same time being absent in PNS nodes [Zonta et al., 2008]. When applied in our protocol on rat, the Neuro-1 mAb bound the node of Ranvier in CNS and PNS which puts our result in contrast to the aforementioned publication. In the PNS, Neuro-1 additionally stained fiber structures. Interestingly, the staining pattern changed according to the species used. When applied to rhesus monkey spinal cord sections, A12/18.1 did not bind the node of Ranvier anymore and there seemed to be only unspecific binding. Neuro-1, however, in rhesus monkeys now stained the paranode in addition.

Taking all those findings together, they can in part be explained by the fact that axonal antigens are accessible at the node of Ranvier. In contrast, paranodal antigens are covered by the myelin sheath and are therefore harder to target by antibodies. It should be noted that changing tissue fixation conditions by using the harsher Bouin's fixative did not lead to a staining of the paranodes with neither A12/18.1 nor Neuro-1. This altered fixation had been used before by other groups resulting in paranodal staining

with rabbit sera raised against the intracellular domain common to NF155 and NF186 ([Tait et al., 2000]; [Zonta et al., 2008] or contactin-1 fused to the Fc domain of human IgG [Zonta et al., 2008]. Another example for a paranode-recognizing mAb would be the mouse anti pan-NF IgM A4/3.4, which strongly bound NF155 in an ELISA but did not stain paranodes on tissue sections. NF155-specific rabbit antibodies, however, were reactive by ELISA and also led to paranodal staining [Ng et al., 2012]. In summary, recognition of (para)nodal antigens strongly differs depending on the method and the antibodies used. For stainings, different antibodies require different staining procedures, especially during the fixation step and results vary among different species. Nevertheless, two mAbs were characterized that bound the node of Ranvier on rat CNS tissue, one recognizing NF, the other one contactin-1.

These two mAbs were then tested in the EAE animal model of MS, where they were injected in rats after a T cell induced breach of the BBB. When their effects on EAE progression were assessed, surprisingly only the anti-NF mAb A12/18.1 exacerbated disease. This means that two different mAbs, remarkably both with the complement activating isotype IgG2, that were shown to bind the node of Ranvier *in vitro* and *in vivo*, surprisingly had different effects on the course of EAE. Notably, the mAbs only vary in their subtype: Neuro-1 is IgG2a while A12/18.1 is IgG2b. This should, however, not account for the different effect because it had already been shown that the anti-MOG-antibody Z2, which is also IgG2a, has the ability to fix complement and induce demyelination in an EAE model in Lewis rat [Piddlesden et al., 1993].

One possible reason could then be a different accessibility and/or distribution of both molecules at the node of Ranvier. Worse accessibility of the contactin-1 molecules might either hamper the binding of Neuro-1 or account for the lack of proper activation of the complement system. An additional staining for complement activation using an anti-C9 antibody could be helpful to resolve that question. Furthermore, there was an additional fiber staining with Neuro-1 in the PNS which might result in less available antibody binding to the node itself, thereby abolishing any effects.

A more sensitive system to detect *in vivo* effects might be obtained when crosslinking is enhanced. Rats could either be sensitized with mouse serum, so an immune response against mouse is mounted before the actual injection of the mouse antibody targeting contactin-1. Another option would be the simultaneous injection of a rabbit-anti mouse secondary antibody in order to facilitate the activation of the complement system. Both methods are commonly used in animal models studying acquired kidney diseases, e.g. rat models of Membranous Nephropathy (reviewed in [Pippin et al., 2009]).

One can also study pathogenic features of mAbs in a functional approach based on myelinating cell cultures as shown by [Elliott et al., 2012], where the mAb A12/18.1 induced myelin as well as axonal loss in a complement-dependent manner. It would be interesting to assess if the application of Neuro-1 led to a complement-mediated demyelination in this system. Taken together, targeting of the node of Ranvier with a potentially complement-activating antibody alone does not seem to be enough in order to exacerbate EAE. The use of the term „Achilles heel“ to describe the node of Ranvier should therefore be reconsidered.

5.2 Preferential targeting of the motoric system by an anti–neurofascin antibody

Further analysis of the damage induced with the anti-NF mAb led to the observation that in Lewis rats the motor neurons as well as the CST were preferentially targeted. This pathology strongly resembled the one seen in amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by a progressive death of motor neurons resulting in fatal paralysis within a few years. In at least a subset of patients with ALS, evidence for a role of pathogenic antibodies targeting either the presynaptic membrane of motor nerve terminals or voltage-dependent calcium channels has been obtained [Pagani et al., 2011]. Additionally, evidence for a compromised BBB has just recently been identified in ALS patients and in an animal model of ALS [Garbuzova-Davis et al., 2012]. In fact, a total of up to 46 % of ALS patients might present with BBB alterations allowing molecules from the blood (e.g. antibodies) to enter the CSF (reviewed in [Tarasiuk et al., 2012]).

Strain differences were noted as the rat transfer experiment was repeated with the DA rat strain that had initially been used when the NF antibodies were discovered [Mathey et al., 2007]. In this model, the antibody still attacked motor neurons but no preferential damage of the CST could be observed. Apparently the genetic background plays a role on where exactly regions prone to damage are distributed. This is hardly surprising, given the fact that ALS itself is a highly heterogeneous disease with so far very poor understanding of the underlying disease biology. While the majority of ALS cases are of sporadic nature, an estimated 5 to 10 % of cases are linked to hereditary mutations in one of several genes, e.g. the superoxide dismutase 1 gene (SOD1). However, it is not clear how these mutations cause the disease (reviewed in [Tarasiuk et al., 2012]). Indeed, the term ALS is likely to rather describe a constellation of diseases with similar presentation but differing genetic and/or other causes.

The majority of findings on ALS pathology in terms of BBB alterations have been established in mutant or transgenic SOD1 rodent models, identifying a pathway of damage that might similarly occur in patients carrying an SOD1 mutation [Garbuzova-Davis et al., 2012]. Nevertheless, BBB permeability remains a big mystery in most of the sporadic ALS cases and the identification of potential mechanisms of motor neuron degeneration is challenging due to diffuse motor neuron death and the aforementioned complexity of disease manifestation. A non-transgenic animal model, that mimics two important ALS features (breached BBB and motor neuron death) could therefore evolve into a valuable, additional tool for further progress in the study of ALS pathology.

5.3 Autoantibodies to axons and glia in multiple sclerosis patients

5.3.1 Nodes and paranodes

Despite numerous studies highlighting the potential influence of nodal and paranodal components on MS pathology and beyond (reviewed in [Desmazieres et al., 2012]), whether an immune response exists against axonal or glial components expressed at the node or paranode in the sera of MS patients remains controversially discussed and has not been studied in an unbiased approach presenting targets in their naturally occurring conformation before.

Therefore, in the second part of this thesis, 49 MS sera were tested according to a specialized IHC protocol and compared to 30 healthy controls. The protocol was established using AQP4 positive serum samples from NMO patients on rat spinal cord cross sections in order to ensure conditions under which antibody binding could be visualized. This protocol was then adapted for rat longitudinal spinal cord sections to allow a clearer visualization of structures localized along the axon or glial cells, e.g. oligodendrocytes. The unique feature for the following MS screening was the double staining with the paranodal marker Caspr in order to increase the sensitivity even further and detect nodal as well as paranodal binding of patient serum. Unfortunately, in none of the samples binding to either nodes or paranodes could be detected. This was highly astonishing because - as already touched upon in the introduction - several studies have put the node of Ranvier into focus throughout the last years (reviewed in [Desmazieres et al., 2012]).

One example are animal models in which dysmyelinating mutants or animals not synthesizing major myelin components exhibit an abnormal distribution of ion channels at the node of Ranvier as well as structural alterations of the paranode that go along with axonal swelling and degeneration ([Arroyo et al., 2002]; [Boiko et al., 2001] [Griffiths et al., 1998]; [Dupree et al., 1998];). Also, as was shown recently using *in vivo* studies in an EAE model, the node of Ranvier can be the initial point of axonal swelling already prior to demyelination [Nikic et al., 2011]. Moreover, an abnormal distribution of sodium channels was reported on demyelinated axons which led to the disruption of the nodal complex in two experimental settings, in an EAE model [Craner et al., 2004] as well as in MS lesions [Coman et al., 2006]. The nodal component NF186 could also be shown to be altered in the course of demyelination [Howell et al., 2006].

Alterations are not limited to the node of Ranvier itself but also extend to para- and juxtapranodal regions, including for example the redistribution of Caspr and potassium channels, respectively ([Coman et al., 2006]; [Wolswijk and Balesar, 2003]). In MS lesions, a disruption of the paranodal structure was proposed to be an early event because aggregates of NF155 and Caspr could be detected although the node of Ranvier was still intact and demyelination had not begun yet ([Wolswijk and Balesar, 2003]; [Howell et al., 2006]). In summary, studies in animals as well as patients suggest that alterations between the axon-glia-interactions at and around the node of Ranvier can be considered as an initial event in the process of tissue damage that is followed by a redistribution of nodal and paranodal components.

In addition, a potential role of those axo-glial proteins as targets for autoantibodies in the MS immune response seemed highly probable. As already outlined, NF at the node and contactin-2 in the juxtaparanodal area could be identified as autoantibody targets in MS ([Mathey et al., 2007]; [Derfuss et al., 2009]). The further validation of NF as a target in a large cohort of MS patients is currently still ongoing. Regardless of whether that holds true, NF could already be validated as a target in (a small subset of) patients with peripheral neuropathies in two independent studies. Autoantibodies to NF were detected by ELISA in 4 % of the patients suffering from acute inflammatory demyelinating polyneuropathy (AIDP), the most common form of Guillain-Barré syndrome (GBS) as well as 4 % of chronic inflammatory demyelinating polyneuropathy (CIDP) patients, but not in controls [Ng et al., 2012]. In the second study, it was found that 43 % of patients with GBS and 30 % of patients with CIDP showed IgG fixation at nodes or paranodes. The recognized proteins could be identified in a total of eight CIDP and GBS patients as either NF186, gliomedin or contactin [Devaux et al., 2012].

A third aspect of nodal involvement in CNS inflammation is delivered by a study of

CSF samples collected from children during their initial presentation, who may or may not subsequently be diagnosed with MS, in a large-scale proteomics screening. With this approach, molecules localized at and around the node of Ranvier, including but not limited to NF, contactin-1 or-2 and Caspr, were detected to be implicated in those children destined for diagnosis of MS [Dhaunchak et al., 2012].

The outcome of our study, i.e. the absence of (detectable amounts of) autoantibodies against (para)nodal components, could have several reasons. We now appreciate that the percentage of patients harboring such antibodies is relatively small and might only be limited to a certain subgroup of patients. One recent example are the aforementioned antibodies against NF in about 4 % of AIDP/ CIDP patients [Ng et al., 2012]. Another reason for poor detection levels are technical difficulties with nodal and paranodal stainings in general as specified in the first part of the discussion. This implies that the varying needs for specific staining conditions for each target cannot be catered within one standardized protocol and might lead to missed signals, especially when antibody titers in patients are low. Indeed, in the work of [Ng et al., 2012] , IHC indicates reactivities only in the highest-titre patients and there were also neuropathy patients harboring anti-NF antibodies that were not detectable via IHC. Naturally, searching for reactivity with a recombinant antigen is much more sensitive than IHC, but at the same time compromises un-biasedness by only detecting a reactivity against already known and selected proteins.

One could also argue that using rat tissue is not the proper tool in order to detect antibody reactivities with human samples. However, in the aforementioned study of AIDP and CIDP patients, samples with very high NF titers indeed stained the paranodes on rat longitudinal spinal cord sections as visualized by the colocalization with Caspr [Ng et al., 2012]. In a different approach myelinating cultures derived from rat were used to identify patient antibody reactivities [Elliott et al., 2012]. Both studies are examples for the successful use of rat tissue for the evaluation of the binding of autoantibodies.

Independently, one should discuss whether the disruption of the nodal and paranodal domains in MS is a process that is not mediated by autoantibodies. One study, for example, described an association between microglial activation and axon/oligodendrocyte pathology at nodal and paranodal domains in normal-appearing white matter (NAWM) of MS cases and in EAE, where the extent of axoglial disruption correlated with local microglial inflammation and axonal injury but not with the density of infiltrating lymphocytes [Howell et al., 2006]. This would suggest an alternative mechanism for the permanent disruption of axoglial domains.In summary, it so far remains

disputed whether and/or to what extent MS patients harbor antibodies against the node or paranode. At the very least, these reactivities were not detectable by means of IHC in our study.

5.3.2 Axons

The relevance of autoantibodies directed against axonal or neuronal antigens has so far been largely unexplored besides existing evidence that they are present in MS [DeVries, 2004]. Our IHC protocol enabled us to detect antibodies recognizing axons in a subgroup of MS patients (and controls). This was confirmed by an immune double labeling with a marker for neurofilament in four out of four selected MS patient samples.

Neurofilament as a possible axonal target has been discussed recently in the literature where it was detected with a frequency of 25 % in MS patients via an antigen array using matched serum and CSF samples from RRMS patients [Quintana et al., 2012] and not seen in the control group. Neurofilament is localized intracellularly. The pathogenic relevance of antibodies to intracellular antigens remains disputed, because they are considered to be unable to reach their target. However, antibodies could be internalized via endocytosis or antigens, traditionally considered as being intracellular, could be exposed on the surface. Anyhow, autoantibody responses to such antigens are associated with many disorders, including neurodegenerative diseases [Huizinga et al., 2008b]. Prominent examples are ganglioside antibodies in GBS or antibodies to the neurofilament heavy protein in ALS or Alzheimer's disease.

MS is considered a disease with neurodegenerative features, as its pathology is also characterized by apoptotic neurons, neuronal and axonal loss or axonal transection. In addition, clinical features observed in other neurodegenerative diseases, like cognitive impairment, dementia or depression, are present in MS patients (reviewed in [Huizinga et al., 2008a]). The presence of antibodies against neurofilament light protein could be considered a correlate of cerebral atrophy in MS and therefore serve as a biomarker for neuronal damage [Eikelenboom et al., 2003]. The common view is that neuronal damage may be followed by neurofilament in the CSF. For example, the amount of neurofilament heavy chain protein in the CSF was shown to correlate with the relapse rate and the degree of disability in MS [Kuhle et al., 2011]. It might therefore represent a useful surrogate for measuring the rate of neurodegeneration.

Neurofilament is not the only candidate for an axonal antigen. One study suggested that the enzymes triosephosphate isomerase and glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) are targets for axon-reactive B cells that are also clonally expanded in the CSF of MS patients [Kolln et al., 2006]. Interestingly, GAPDH, an enzyme, classically considered to be localized in the cytoplasm was shown to also be present extracellularly on the surface of neurons [Makhina et al., 2009], a fact that endorses its potential role as a target for autoantibodies. Another group studied the pathogenic potential of IgG preparations derived from MS patients in a myelinating cell culture system and identified two out of 37 patient samples with complement-dependent antibody mediated axonal loss [Elliott et al., 2012].

It remains unclear whether autoimmunity against axonal antigens correlates with clinical disease, only representing an immune response after axonal degeneration, or whether it is really part of the disease's pathogenicity. For the latter case, the aforementioned two-hit-model involving a preceding T cell attack could be applicable. It was indeed already shown that MOG-specific T cell lines could be stimulated by MOG as well as neurofilament, representing a concept of autoimmunological cross-reactivity [Krishnamoorthy et al., 2009].

Taken together, our study indicates the presence of antibodies to axons in the serum of MS patients while their specificity remains unknown at present. Further studies will be needed in order to identify respective targets and also clarify the autoantibodies' functional relevance.

5.3.3 Oligodendrocytes

We were able to detect a second staining pattern with our IHC approach. This staining resembled oligodendrocytes, which was verified via the colocalization with the marker Olig-2. An independent and blinded quantification experiment revealed that this pattern was present in about 40 % of the MS patients and only 20 % of controls, which represents a significant difference.

Although this phenomenon had been described in literature before and was claimed to be unspecifically mediated via the Fc part of the patient antibodies (reviewed in [Traugott et al., 1979]), we were now able to show that also F(ab')$_2$ fragments generated by pepsin digestion bound to oligodendrocytes. In addition, in our study patient samples were more diluted and the staining was controlled by a secondary antibody specific for F(ab')$_2$, that does not cross-react with the Fc fraction. We therefore have reason to believe that this oligodendrocyte pattern is due to a real antibody-antigen interaction.

When further analyzing the most intensely stained samples by confocal microscopy, a nuclear staining pattern of oligodendrocytes could be identified in all of the eight chosen sera (five patients and three controls). This might of course be due to the presence of ANAs that have been already reported to be frequently found in MS patients (63.5 %) and also, but to a lesser extent, in control individuals (3.3 %) [Szmyrka-Kaczmarek et al., 2012].

In that particular study the presence of ANAs could be associated with shorter disease duration and lower disability scores. As already discussed in the section on possible axonal antigens, the pathogenic relevance of intracellular (in this case even nuclear) autoantigens remains disputed. However, elevated ANA titers can be indicative of autoimmune diseases and therefore serve as a marker, a prominent example being systemic lupus erythematosus.

Apart from the nuclear pattern, we were able to identify one MS patient sample with staining on the cellular surface and possibly the processes of oligodendrocytes. This was not present in any of the tested controls. Considering that MS features include demyelination and oligodendrocyte damage, a possible target for autoantibodies located directly on the surface of the oligodendrocyte would of course be an interesting candidate for further investigations.

Other studies also put the oligodendrocyte (and glial cells in general) into focus. For example, using a different approach based on myelinating rodent cell cultures, IgG preparations from MS patients with demyelinating activity were shown to bind to the surface of myelinating oligodendrocytes and their contiguous myelin sheaths. This response seemed to be restricted to autoantigens expressed by terminally differentiated myelinating oligodendrocytes [Elliott et al., 2012] and was seen in about 30 % of the MS patients but not in controls. As summarized above, oligodendrocyte reactivities were seen in 40 % of MS patients but also 20 % of controls using our IHC approach, a finding that could be accounted for by different sensitivities between a functional assay and an immunostaining. Both experimental systems work with material derived from rats which is a more practical solution compared to the utilization of human tissue but comes along with the disadvantage of missing potential targets that lack a significant degree of species cross-reactivity [Elliott et al., 2012].

Another group investigated MS patient IgG reactivities via 2D-electrophoresis of normal human white matter and subsequent Western blotting [Lovato et al., 2008] and identified the oligodendroglial molecules transketolase and CNP as putative autoantigens.A very recent finding regarding reactivity to glial cells was the potassium chan-

nel inward rectifier KIR4.1, which was identified as an autoantigen by an unbiased proteomic approach focusing on membrane proteins. This ion channel is expressed on oligodendrocytes but also on astrocytes. KIR4.1 antibodies in serum were quantified by ELISA. Patients with MS or a CIS showed higher values compared to healthy donors and patients with other neurological diseases in the discovery and two subsequent independent confirmation cohorts [Srivastava et al., 2012]. As with our approach no additional astrocyte staining was detected, the implication of yet (an) additional target(s) suggests itself.

In conclusion, antibodies to oligodendrocytes were detected in a proportion of MS patients, but are also present - albeit to a lesser extend - in control individuals. Interestingly, one individual MS patient was identified that possessed antibodies recognizing the surface of oligodendrocytes and possibly their processes. The exact target of the anti-oligodendrocyte antibodies could not be addressed in the realms of this study.

6 Conclusion and outlook

According to Greek mythology, Achilles was one of the greatest Greek warriors, rendered invulnerable because his mother had dipped him in the mythical River Styx. As he was held by his heel, this remained the only vulnerable part of his body. That weakness later led to his downfall during the Trojan War when a poisoned arrow, shot by Apollon, hit his heel and killed him („Achille's Death" from Ovid's Metamorphoses).

Metaphorically speaking, identifying an „Achilles heel" or vulnerable spot in the CNS would be a tremendous step in the unraveling of the pathogenesis of MS. Molecules at and around the node of Ranvier, as an unmyelinated and therefore exposed area, were - in that regard - highly promising targets for the study of autoantibody-mediated damage.

Notwithstanding this, in the first part of the thesis it was shown that targeting the node of Ranvier with a complement-activating antibody alone does not necessarily do the job, as a mAb directed against the nodal component contactin-1 did not induce any damage. Also, when looking for reactivities against the node or its adjacent paranodes in the sera of MS patients via an IHC approach in the second part of the thesis, the results were disappointing and no (para)nodal staining could be detected. However, we were able to identify patients with evidence for autoantibodies to axons and oligodendrocytes.

„Antigen search is not a trivial thing", could be one conclusion of this thesis. Despite decades of research on autoantibodies playing a role in MS pathology, we are still lacking „the" antigen or precisely characterized autoantigenic patterns. Two factors mainly complicate the unraveling of the role of antibodies, mechanistic heterogeneity of the disease on the one hand and the complexity of the disease associated autoantibody repertoire on the other hand. Numerous autoantibody specificities have already been associated with MS, their significance regarding their contribution to pathology or their eligibility as biomarkers remains uncertain.

The identification of (different) antigens will help to assign patients to subgroups and therefore simplify diagnosis, the estimation of prognosis and, of course, optimize therapeutic interventions. The ultimate goal would naturally be a personalized therapy.

Some strategies available to identify antigens of patient derived antibodies in MS or other demyelinating diseases have already been discussed throughout this thesis. In summary, what we currently have at hand in order to reach that goal are the following approaches:

(i.) Immunostaining,

(ii.) Western blotting and subsequent mass spectrometry,

(iii.) Arrays and multiplex approaches,

(iv.) Expression and peptide libraries,

(v.) Recombinant immunoglobulin generated from single or clonally expanded cells as well as

(vi.) Functional approaches with purified patient IgG in cell culture systems.

As each of the techniques naturally possesses its own strengths and weaknesses, for example regarding biasedness or antigen conformation, a combination of several of them should be employed to eventually identify the autoantigen(s) involved in MS pathology.

Finally, one has to ask the question how this thesis could help with that. We were able to show that IHC can indeed serve as a tool to identify patients with interesting antigen recognition patterns. When interpreting the implications of the presence of autoantibodies, we have to keep in mind that many of them can also be found in control individuals, even though at lower levels. They can also be present a long time before disease onset without any pathological implications as long as the BBB remains intact. Nevertheless, we identified one patient that possesses antibodies against the surface of oligodendrocytes, a pattern that could not be detected in any of the investigated healthy donors.

A future aim of the project would clearly be going one step further towards the potential identification of the responsible auto-antigen. Therefore a combination of immunoprecipitation and mass spectrometry could be employed. This approach is not trivial either, as one has to carefully consider which material to use for the precipitation

step (whole spinal cord versus isolated oligodendrocytes) as well as suitable negative controls (healthy donor versus intravenous Ig preparations) in order to facilitate the interpretation of the mass spectrometry results. Interesting candidate targets could then be confirmed in a classical cell-based approach using the transfected antigen(s) in a large screen with patient and control sera.

I sincerely wish my successive PhD student the best of luck with the prosperous continuation of this project!

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